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THE IMPACT OF CONSERVATIVE INTERVENTIONS ON MORPHOLOGICAL MUSCLE AND TENDON PROPERTIES OF GROWING CHILDREN WITH SPASTIC CEREBRAL PALSY

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Don't listen to the person who has the answers; listen to the person who has the questions.

- Albert Einstein -

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Summary

Spastic cerebral palsy (SCP) is one of the most common causes of childhood disability and is caused by a lesion or malformation in the developing brain. Even though the brain lesion itself is non-progressive, the secondary symptoms at the level of the muscle are progressive and may eventually negatively influence quality of life. This highlights the need for treatment options directed at the muscle. However, the heterogeneous nature of the pathology challenges the clinical decision making, resulting in varying treatment success and highlights the need for individualized treatment.

The general aim of this doctoral thesis was to get a better understanding of the heterogeneous macroscopic alterations in the muscle-tendon unit of children with SCP and investigate the impact of intramuscular Botulinum Neurotoxin-A (BoNT-A) injections on muscle morphology, spasticity and function. To achieve the overall aim, five different studies were performed.

In these five studies, the muscle morphology and intrinsic muscle quality were defined by three-dimensional freehand ultrasound (3DfUS), which is a method that creates three-dimensional reconstructions of a muscle by combining two-dimensional ultrasound and motion tracking. In some studies, these ultrasound assessments were combined with instrumented spasticity and gait assessments.

In the first study (**chapter 2.1**), we highlighted the problem of a lack of standardization and proper description of the measurement setup when performing 3DfUS assessments on lower limb muscles. In particular, adjacent joint angles of the assessed muscles were expected to impact muscle belly lengths, and potentially also other muscle parameters. Therefore, we investigated the impact of the lower limb joint angles on the muscle morphology and composition outcomes defined by 3DfUS on the medial gastrocnemius, semitendinosus and rectus femoris in typically developing (TD) children and children with SCP. We used a previously described measurement setup in which the participants were positioned on their side to assess the semitendinosus and rectus femoris, while the assessed limb was placed on a table that allowed adjustment of the knee and hip angles. The medial gastrocnemius was assessed in prone position while the foot was fixated in a custom-made orthosis. The muscles were assessed in three different positions, a patient-specific resting position, at pre-defined common joint angles (that was similar for all participants of both groups) and in a patient-

specific position with maximum joint angles. We showed that changes in adjacent joint angels influenced the muscle belly length of most muscles, with the longest muscle belly length in the maximum position, as expected. However, surprisingly, also the muscle belly volumes of some muscles were influenced by changes in joint angles, with smaller muscle volumes in the resting condition compared to the common and maximum position. These unexpected differences in muscle volume could have been caused by differences in probe tilting or unequal pressure application during data acquisition or by differences in data processing. A short patient friendliness questionnaire, on the child's experience during the 3DfUS assessments, showed that the children were comfortable during the measurements and that both the side and prone position were well-tolerated. The study results highlighted the need for standardized measurement setups. Furthermore, it was shown that muscle morphology assessments were challenging in side position and therefore potentially less accurate. However, this setup was found to be suitable to assess muscles at different lengths.

For the second study (**chapter 2.2**), we created a large reference database of normative muscle morphology data of four lower limb muscles of TD children, with a wide age range. Such a comprehensive normative database was needed as a reference for muscle morphology in pediatric pathologies and to provide more insight into physiological muscle development. Therefore, we assessed the muscle morphology of the medial gastrocnemius, tibialis anterior, semitendinosus and rectus femoris, using 3DfUS, in 118 TD children aged between 3 and 18 years. The results showed that the development of muscle volume and muscle belly length was muscle-specific and could be described by a simple linear relation between the age of 3 and 11 years. In order to compare children with different body sizes, muscle morphology data had to be normalized to body size. Because the efficiency of different normalization techniques was still unknown and clear recommendations for data normalization were lacking, we compared the efficacy of different data scaling techniques. Normalizing muscle morphology data by allometric scaling was found to be most efficient for muscle volume, whereas normalization by either allometric or ratio scaling was appropriate for normalizing muscle belly length data. We recommended that future studies investigating muscle development in pediatric populations should consider muscle-specific development and the impact of puberty, when comparing data to other populations with different age ranges or pathologies and we advised to use efficient and well-described scaling techniques.

In the third study (**chapter 2.3**), we explored the complex interaction between spasticity and deficits in muscle morphology in children with SCP. More insight in this complex interaction was warranted to set more light on the heterogeneous nature of SCP. We studied the associations between spasticity, assessed by an instrumented spasticity assessment, and muscle morphology, assessed by 3DfUS, in 74 children with SCP. The instrumented spasticity assessment allowed identification of different muscle activation patterns in response to a passive stretch, representing spasticity patterns that showed pure high-velocity dependent muscle activation or a mixture of low- and high-velocity-dependent muscle activation. We compared muscle morphology between muscles that presented with different muscle activation patterns. We found only weak to moderate associations between the level of spasticity and muscle morphology. Additionally, we found that muscles with pure high-velocity-dependent muscle activation were larger compared to muscles with mixed low- and high-velocity-dependent muscle activation. Future research is needed to further explore the underlying pathophysiology of these different muscle activation patterns and differences in muscle morphology. Eventually, treatment should focus on the underlying pathophysiological mechanism of the hyper-resistance against stretch rather than solely on the secondary level of deformity or spasticity level.

In the fourth study (**chapter 2.4**), we investigated the isolated effects of BoNT-A injections and lower leg casting. Those treatments are frequently applied in the treatment of spasticity and contracture prevention in children with SCP. While BoNT-A injections and casting are commonly combined in an integrated treatment approach, the individual effect remained partly unknown. Therefore, we investigated the decomposed single treatment-induced effects on muscle and tendon lengths (defined by 3DfUS), spasticity and joint resistance (defined by the instrumented spasticity assessment) and joint range of motion. Two weeks of lower limb casting resulted in an increased maximum dorsiflexion angle, increased tendon length at maximum dorsiflexion and reduced spasticity in the lateral gastrocnemius and soleus muscles. BoNT-A injections to the medial gastrocnemius led to reduced spasticity in this muscle, increased muscle belly length at rest and reduced ankle joint resistance during fast passive ankle joint rotation. We found only a few associations between the treatment-induced alterations in joint angles, muscle belly or tendon lengths on one hand and measures of ankle joint resistance and spasticity on the other hand. In summary, this study found rather minor changes at the muscle level after either intervention, with limited relationships between the effects at the joint, muscle or tendon lengths and spasticity. We concluded that intramuscular BoNT-A injections reduced spasticity in the medial gastrocnemius

accompanied by an increased resting muscle belly length, whereas casting resulted in increased dorsiflexion without any changes in muscle belly length. This highlighted the need for further investigations on the effect of the combined treatments and the development of treatments that could more effectively lengthen the muscle.

In the final study (**chapter 2.5**), we investigated the effect of BoNT-A as part of an integrated approach (combined with stretching casts if necessary, intensive physical therapy and orthosis) on spasticity, on muscle morphology and on gait, in one patient cohort. This was needed because previous studies suggested that BoNT-A could cause muscle atrophy and could negatively influence muscle quality on top of the already hampered muscle growth in children with SCP. Moreover, some research groups recently questioned the beneficial effects of BoNT-A injections on function. However, high quality controlled trials, including objective assessments considering both the effects at the muscle and functional level in one patient group were lacking. Therefore, this final study used a combined set of objective instrumented assessments to assess the impact of BoNT-A on spasticity, muscle morphology and gait, within a controlled design. On one hand, the results first confirmed some beneficial effects at 8-10 weeks after the BoNT-A treatment, namely the successful spasticity reduction in the medial gastrocnemius and semitendinosus and improved ankle kinematics during gait, with limited effects on knee kinematics. On the other hand, this efficient spasticity reduction and gait improvements were combined with an unwanted reductions in normalized muscle volume of the injected muscles, compared to the unchanged normalized muscle volume that was observed in the untreated control group. These results highlighted the need for a close follow-up of muscle recovering after BoNT-A treatment. Treatment selection should be based on a well-considered evaluation of the benefits and harms of BoNT-A injections.

In conclusion, this doctoral thesis contributed to a better understanding of the altered muscle properties in children with SCP and the heterogeneous response to treatment. We highlighted the importance of standardization and detailed documentation of lower limb joint angles during muscle morphology assessments using 3DfUS. Additionally, we established a comprehensive normative database on lower limb muscle morphology and contributed to improved data analysis and presentation by comparing different data scaling methods for muscle morphology parameters. This thesis provided more insight into the association between muscle morphological alterations and spasticity, the isolated effects of BoNT-A injections and lower leg casting and the impact of BoNT-A injections as part of an integrated treatment approach.

We believe that the objective assessments that have been applied during this PhD project provided more insight into the natural course of SCP and the impact of treatment. Accurate treatment selection and thereby improving treatment efficacy will preserve gross motor function and can eventually improve social participation and quality of life in children with SCP.

Samenvatting

Spastische cerebrale parese (SCP) is één van de meest voorkomende oorzaken van fysieke beperkingen bij kinderen en wordt veroorzaakt door een beschadiging in het ontwikkelende brein. Deze aandoening wordt daarom ook wel 'hersenverlamming' genoemd. Ondanks dat het hersenletsel niet progressief is, zijn de secundaire problemen, die zich vaak uiten op spierniveau, wel progressief. Deze secundaire problemen kunnen de kwaliteit van leven negatief beïnvloeden en vereisen daarom behandeling. SCP is een zeer heterogene aandoening en helaas zijn de resultaten van behandeling zeer wisselend, hetgeen de nood aan geïndividualiseerde behandeling benadrukt.

Het algemene doel van dit proefschrift was om een beter inzicht te krijgen in de macroscopische veranderingen in het spier-peescomplex van kinderen met SCP en om meer inzicht te krijgen in het effect van intramusculaire Botuline Neurotoxine-A (BoNT-A) injecties op spiermorfologie, spasticiteit en grof-motorische functie. Er werden vijf verschillende studies uitgevoerd om dit algemene doel te bereiken.

In deze vijf studies, beschreven in dit proefschrift, werd de spiermorfologie onderzocht aan de hand van drie-dimensionele (3D) spierechografie. Deze techniek reconstrueert 3D beelden van een spier door meerdere twee-dimensionele echografiebeelden samen te voegen. Daarnaast werd er in sommige studies gebruik gemaakt van geïnstrumenteerde spasticiteitsmetingen en gangbeeldanalyses.

De eerste studie (**hoofdstuk 2.1**) belichtte het probleem van gebrek aan standaardisatie en grondige beschrijving van de meetpositie. Het veranderen van de hoeken in de aangrenzende gewrichten tijdens de 3D spierechografie kan immers de spierbuiklengte, maar mogelijks ook andere spiermorfologie parameters, beïnvloeden. We onderzochten daarom de invloed van verschillende gewrichtshoeken op spiermorfologieparameters (gemeten met 3D spierechografie) van de mediale gastrocnemius, semitendinosus en rectus femoris van typisch ontwikkelende (TD) kinderen en kinderen met SCP. De spiermorfologie van de semitendinosus en rectus femoris werden onderzocht in een meetopstelling waarbij de kinderen op hun zij werden gepositioneerd. Het onderzochte been werd ondersteund door een plaat waarop de gewrichtshoeken van de heup en knie eenvoudig aangepast konden worden. De mediale gastrocnemius werd in buikligging gemeten, terwijl de voet

gefixeerd werd in een orthese. Alle spieren werden onderzocht in drie verschillende condities, namelijk in een individuele rustpositie, een vooraf bepaalde positie die identiek was voor beide groepen en in een individuele positie met maximale gewrichtshoeken. Deze studie toonde aan dat het veranderen van de aangrenzende gewrichtshoeken resulteerde in veranderingen in de spierbuiklengte, waarbij de langste spierbuiklengte werd waargenomen in de maximale gewrichtspositie. De spiervolumes werden ook deels beïnvloed door het veranderen van de gewrichtshoeken, waarbij er meestal een kleiner spiervolume gemeten werd in de rustpositie in vergelijking met de andere meetposities. De verschillen in spiervolume waren onverwacht en werden mogelijk veroorzaakt door verschillen in de manier waarop de echobeelden werden gemaakt of door verschillen in de dataverwerking. De 3D echografiemetingen werden niet als vervelend ervaren en zowel de zijligging als buikligging werden goed verdragen. Dit bleek uit een korte vragenlijst over de ervaringen van de kinderen tijdens de metingen. Deze studie toonde aan dat gestandaardiseerde meetopstellingen vereist zijn. Daarnaast bleek het moeilijk om een accurate meting van het spiervolume uit te voeren in zijligging, terwijl deze positie wel geschikt bleek voor het meten van spierbuiklengtes aan verschillende gewrichtshoeken.

In de tweede studie (**hoofdstuk 2.2**) werd een grote normatieve database opgezet, met spiermorfologiedata van vier verschillende spieren van het onderste lidmaat. Deze uitgebreide database is vereist voor vervolgonderzoek en kan dienen als referentiedata voor kinderen met verschillende aandoeningen. Deze dataset kan daarnaast ook gebruikt worden om meer inzicht te verwerven in de spierontwikkeling van een normaal ontwikkelend kind. We onderzochten de spiermorfologie van 118 TD kinderen tussen de 3 en 18 jaar oud. De resultaten toonden aan dat de ontwikkeling van zowel het spiervolume als de spierbuiklengte spier-specifiek zijn en beschreven kunnen worden door een rechtlijnige lineaire regressievergelijking in de leeftijden van 3 tot 11 jaar. Spiermorfologische uitkomsten worden vaak genormaliseerd naar een maat van lichaamsgrootte (bijvoorbeeld lengte of gewicht), om spiermorfologie te vergelijken tussen kinderen met verschillende lichaamsdimensies. De effectiviteit van verschillende datanormalisatietechnieken was nog onbekend en er waren geen duidelijke richtlijnen en aanbevelingen over het gebruik van deze technieken. We onderzochten daarom de efficiëntie van verschillende datanormalisatietechnieken. Het normaliseren van spiervolumes was het meest succesvol met behulp van allometrische schaling, terwijl spierbuiklengtes door zowel allometrische als ratio-schalingsmethoden genormaliseerd konden worden. Toekomstige studies die spiermorfologie onderzoeken bij kinderen moeten rekening houden met de spier-specifieke ontwikkeling en de invloed van de puberteit. Daarnaast

adviseren we dat deze studies enkel gebruik maken van normalisatietechnieken waarvan bewezen is dat ze geschikt en effectief zijn.

De derde studie (**hoofdstuk 2.3**) onderzocht de complexe relatie tussen spasticiteit en spiermorfologie bij kinderen met SCP. Meer inzicht in deze relatie kan bijdragen aan het verwerven van meer inzicht in de heterogene presentatie van SCP. We hebben de associaties tussen spasticiteit (gemeten tijdens een geïnstrumenteerde spasticiteitsmeting) en spiermorfologie (gemeten met 3D spierechografie) onderzocht bij 74 kinderen met SCP. Met behulp van de geïnstrumenteerde spasticiteitsmetingen werden eerder beschreven spieractivatiepatronen tijdens een passieve stretch van de spier geïdentificeerd. In deze spieractivatiepatronen, ook wel spasticiteitspatronen genoemd, kon een onderscheid gemaakt worden in spasticiteitspatronen met puur hoge-snelheidsafhankelijke spieractivatie of patronen met een gemixt lage- en hoge-snelheidsafhankelijk spieractivatiepatroon. We vergeleken de spiermorfologie tussen spieren met verschillende spasticiteitspatronen. We vonden enkel zwakke tot matige associaties tussen het spasticiteitsniveau en de spiermorfologie. Daarnaast vonden we dat spieren met puur hoge-snelheidsafhankelijke spasticiteitspatronen groter waren in vergelijking met spieren met een gemixt lage- en hoge-snelheidsafhankelijk spasticiteitspatroon. Verder onderzoek is nodig om de onderliggende pathofysiologie van de verschillende spasticiteitspatronen en de veranderingen in spiermorfologie beter te begrijpen. Behandelingen zouden gericht moeten zijn op de onderliggende factoren die bijdragen aan de verhoogde weerstand die gevoeld wordt rond de gewrichten in SCP, in plaats van op de secundaire veranderingen in de spier of het spasticiteitsniveau.

In de vierde studie (**hoofdstuk 2.4**) onderzochten we de individuele effecten van intramusculaire BoNT-A injecties en onderbeengipsen. Deze behandelingen worden vaak toegepast in de behandeling van spasticiteit en het voorkomen van contracturen bij kinderen met SCP. De individuele effecten van BoNT-A zijn grotendeels onbekend, omdat BoNT-A en gipsen vaak worden gecombineerd in de zogeheten geïntegreerde behandeling. We onderzochten daarom de geïsoleerde effecten van deze behandelingen op spier- en peeslengte (gemeten met 3D spierechografie), spasticiteit en gewrichtsweerstand (gemeten met de geïnstrumenteerde spasticiteitsmetingen) en de bewegelijkheid van het enkelgewricht. Twee weken onderbeengipsen resulteerde in een toename van de maximale dorsiflexiehoek en verminderde spasticiteit in de laterale gastrocnemius en de soleus. BoNT-A injecties in de mediale kop van de gastrocnemius leidden tot verminderde spasticiteit en een toename in rustlengte van de geïnjecteerde spier en

verminderde weerstand tegen beweging van het enkelgewricht tijdens een snelle beweging. We vonden slechts enkele associaties tussen de behandelingseffecten op de gewrichtshoeken en spierbuik- en peeslengte aan de ene kant en de veranderingen in weerstand tegen gewrichtsrotatie en spasticiteit aan de andere kant. Samenvattend vonden we slechts beperkte veranderingen op het niveau van de spierbuik na BoNT-A of gipsen, met beperkte associaties tussen het effect rond het gewricht, de spier- en peeslengte en de spasticiteit. We concludeerden dat BoNT-A injecties zorgden voor een reductie in spasticiteit en een toename in de rustlengte van de mediale gastrocnemius. Twee weken onderbeengipsen resulteerde in een toename van de maximale dorsiflexiehoek, zonder bijkomende veranderingen in spierbuiklengte. Deze bevindingen benadrukken de nood aan vervolgonderzoek naar de gecombineerde behandeling. Verder is er nood aan nieuwe of aangepaste behandelingen die meer doelgericht de spierbuik kunnen verlengen.

De laatste studie (**hoofdstuk 2.5**) onderzocht het effect van BoNT-A injecties als onderdeel van een geïntegreerde behandeling (gecombineerd met gipsen indien nodig, intensieve kinesitherapie en orthesen) op het niveau van spasticiteit, spiermorfologie en de gang in één cohort van kinderen met SCP. Deze studie was noodzakelijk omdat voorgaande studies suggereerden dat BoNT-A spieratrofie kan veroorzaken en de spierkwaliteit negatief kan beïnvloeden, bovenop de reeds verstoorde spiergroei bij kinderen met SCP. Daarnaast stelden sommige onderzoeksgroepen de positieve functionele effecten van BoNT-A in vraag. Er was dus nood aan gecontroleerde trials in één cohort van patiënten, die gebruik maken van objectieve meetmethoden op verschillende niveaus, zoals de spier en grof-motorische functie. In deze laatste studie werd daarom gebruik gemaakt van een gecombineerde set van objectieve metingen om het effect van BoNT-A op spasticiteit, spiermorfologie en de gang te onderzoeken, binnen een gecontroleerd design. De eerste resultaten toonden enerzijds positieve effecten, met name een effectieve spasticiteitsreductie en significante verbeteringen in enkelkinematica met beperkte veranderingen in kniekinematica van het gaan, 8-10 weken na de BoNT-A behandeling. Anderzijds werden ook minder gunstige effecten gevonden, namelijk een afname van het spiervolume van de mediale gastrocnemius en de semitendinosus, in tegenstelling tot het onveranderde spiervolume in de niet-behandelde controlegroep. Deze bevindingen benadrukken de essentie van regelmatige opvolging van het spierherstel na BoNT-A injecties. Daarnaast moeten BoNT-A behandelingen alleen geselecteerd worden na een weloverwogen afweging van de potentiële voor- en nadelen.

We kunnen concluderen dat dit proefschrift heeft bijgedragen aan het verkrijgen van meer inzicht in de veranderingen in spiermorfologie bij kinderen met SCP en de heterogene respons op behandeling. Daarnaast hebben we de essentie van het standaardiseren en het nauwkeurig rapporteren van de gebruikte meetopstelling tijdens de spierechografiemetingen aangetoond. De verschillende studies beschreven in dit proefschrift resulteerden in een uitgebreide normatieve database met spiermorfologiedata van vier spieren van het onderste lidmaat en het verbeteren van de data-analyse en datapresentatie door het vergelijken van verschillende datanormalisatietechnieken. We verworven meer inzicht in de complexe relatie tussen spasticiteit en veranderingen in spiermorfologie, de geïsoleerde effecten van BoNT-A injecties en onderbeengipsen en het effect van BoNT-A als onderdeel van een geïntegreerde behandeling. De objectieve meetmethoden, zoals deze zijn toegepast in dit proefschrift, hebben bijgedragen aan het verkrijgen van meer inzicht in het natuurlijke beloop van SCP en de invloed van behandeling. Het selecteren van de juiste behandeling en het vergroten van de doeltreffendheid van deze behandeling zal bijdragen aan het verbeteren en behouden van de grof-motorische functie en kan uiteindelijk leiden tot toegenomen sociale participatie en kwaliteit van leven voor kinderen met SCP.

List of abbreviations

AFO = ankle foot orthoses
ANOVA = analysis of variance
ANCOVA = analysis of covariance
AU = arbitrary units
B-mode = brightness mode
BL = bilateral involvement
BoNT-A = Botulinum Neurotoxin type-A
C = calcaneus
cm = centimeters
CNS = central nervous system
CP = cerebral palsy
COVID-19 = Coronavirus Disease 2019
dB = decibels
Deg = degrees
DF = dorsiflexion
EI = echogenicity intensity
EMG = electromyography
ES = effect size
FWO = fonds wetenschappelijk onderzoek
GC = gait cycle
GMFCS = Gross motor function classification system
GMFM = gross motor function measure
GPS = gait profile score
HVD = pure high-velocity-dependent muscle activation pattern
Hz = hertz
IC = initial contact
IMU = inertial measurement unit
ISA = instrumented spasticity assessment
IQR = interquartile range
J = Joule
Kg = kilograms
KF = knee flexors
LG = lateral gastrocnemius
LMN = lower motor neuron
m = meters
MAS = modified Ashworth score
max = maximum
MCID = minimally clinically important difference
MEH = medial hamstrings
MFC = medial femoral condyle
MG = medial gastrocnemius
ML = muscle belly length
mHz = megahertz
Mixed = mixed low-/high-velocity-dependent muscle activation pattern
ml = milliliters
mm = millimeters
MTJ = muscle-tendon junction
MTS = modified Tardieu scale
MTU = muscle-tendon unit
MTUL = muscle-tendon unit length
MV = muscle volume
MVC = maximum voluntary contraction
n = number
n.a. = not applicable
n.s. = not significantly different
nML = normalized muscle length
nMV = normalized muscle volume
nTL = normalized tendon length
p = p-value
PASTA = Patient-specific treatment
PF = plantar flexors
P-P = predicted probability plot
r = correlation coefficient
R^2= coefficient of determination
RF = rectus femoris
RMS = root mean square
RMS-EMG = root mean square of the electromyography signal
ROM = range of motion
SCP = spastic cerebral palsy
SDR = selective dorsal rhizotomy
SEM = standard error of measurement
sEMG = surface electromyography
SMC = selective motor control
SnPM = statistical non-parametric mapping
SOL = soleus muscle
ST = semitendinosus
TA = tibialis anterior
TAMTA = treatment algorithms based on muscle and tendon architecture
TD = typically developing
TL = tendon length
UL = unilateral involvement
UMN = upper motor neuron
µV = microvolts
2D US = two-dimensional ultrasound
3D = three-dimensional
3DfUS = three-dimensional freehand ultrasound
3DGA = three-dimensional gait analysis

Part I
General Introduction



General Introduction

This general introduction describes cerebral palsy and its clinical presentation, the musculoskeletal problems, the commonly used interventions and the instrumented measures that can be used to evaluate muscle features and function.

The gaps in literature are summarized in the blue text boxes at the end of the different sections. The black text boxes indicate the specific focus and methodological choices of this doctoral thesis.

1. Cerebral palsy

1.1 Definition of cerebral palsy

Cerebral palsy is, with a prevalence of approximately 1.6 per 1,000 live births, one of the most common causes of childhood disability (1). Cerebral palsy is the result of a disturbance e.g. lesion or malformation, in the developing fetal or infant brain. One of the most common used definitions of cerebral palsy is: “a group of permanent disorders of the development of movement and posture causing activity limitation that are attributed to non-progressive disturbances that occurred in the developing fetal or infant brain. The motor disorders of cerebral palsy are often accompanied by disturbances of sensation, perception, cognition, communication, and behavior; by epilepsy; and by secondary musculoskeletal problems” (2).

Three main categories of brain abnormalities potentially causing cerebral palsy are defined. In the first and early stages of the second trimester (6-20 weeks post conception) brain malformations can occur. During the early third semester (20-30 weeks post conception) mainly white matter lesions arise, whereas gray matter lesions usually occur in the late third trimester (30-40 weeks post conception) (3,4). Premature birth, low birth weight and multiple gestations are the most important risk factors of cerebral palsy (5–7).
While most cases of cerebral palsy occur in utero, approximately 10% occur postnatally (8,9). Postnatal cerebrovascular events, cerebral infection or cerebral trauma are the most common

causes of cerebral palsy after birth (8,9). The diagnosis of cerebral palsy can be challenging in very young children. Yet, the combination of early cranial magnetic resonance imaging (MRI) and assessments of general movements, together with the Hammersmith infant neurological examination has a sensitivity of 97% to detect cases at high risk of cerebral palsy before six months of age (10). As some children with cerebral palsy are diagnosed at an older age, i.e. triggered by failure to reach developmental and motor milestones, the prevalence of cerebral palsy can be higher in older age groups (11).

Cerebral palsy is characterized by a heterogonous picture of neuromuscular symptoms. The primary problems, which are a direct result of the brain lesion, are subdivided in two groups as presented in Figure 1. The brain lesion is often referred to as the upper motor neuron syndrome. The positive features of the upper motor neuron syndrome, due to a loss of inhibition, include increased muscle tone, spasticity, and co-contraction. The negative features are the result of a loss of corticospinal tract connections, causing muscle weakness, reduced neuromotor control, and sensory deficits. Besides that, musculoskeletal problems, like contractures and bony deformities, are common in cerebral palsy and considered as secondary problems. As indicated in the above cited cerebral palsy definition, children with cerebral palsy are usually facing many other problems like cognitive impairment, behavioral problems, communication issues (like difficulty in speaking), eating problems, sensory impairments (like visual impairment and hearing loss), epilepsy (which is often difficult to control), emotional problems and chronic pain (12–17).

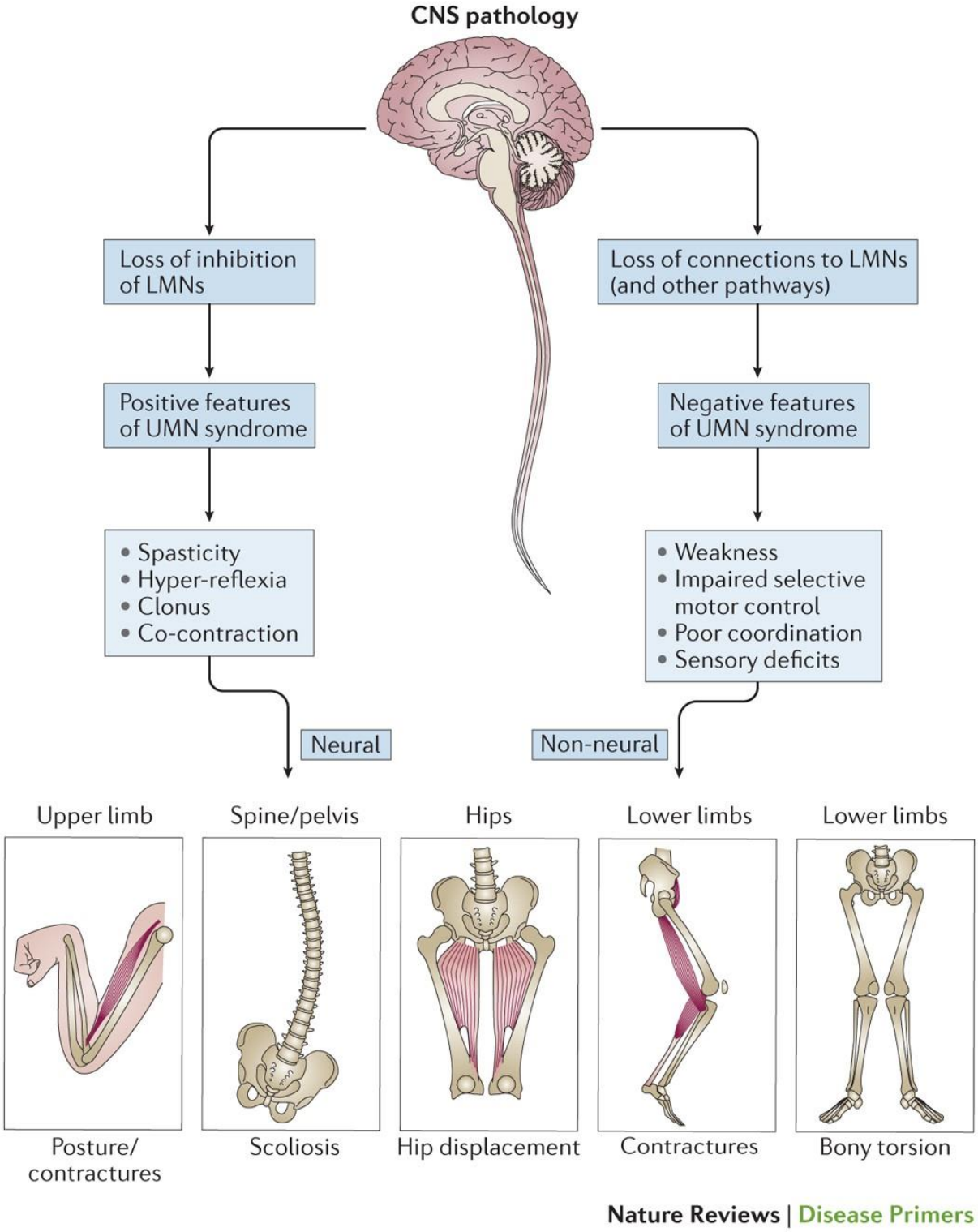

Figure 1. The positive and negative features of cerebral palsy *(From Graham et al. 2016* (18)*, reused with permission)*

CNS = central nervous system, LMN = lower motor neuron, UMN = upper motor neuron

1.2 The heterogeneous nature of cerebral palsy

1.2.1. Classification

Cerebral palsy describes a very heterogeneous pathology. There are several approaches to classify children with cerebral palsy. Most classification systems are based on severity, motor function and topographical distribution.

According to the Surveillance of Cerebral Palsy in Europe, cerebral palsy can be classified into spastic cerebral palsy (SCP), dyskinetic cerebral palsy, and ataxic cerebral palsy (19). Dyskinetic cerebral palsy includes both clinical phenotypes of choreoathetosis and dystonia. Furthermore, mixed patterns are also possible (20,21). As the majority of children with cerebral palsy present with spasticity, SCP is the most common subtype (22,23).
According to the topographic classification, children with cerebral palsy can present with unilateral involvement (hemiplegia) or bilateral involvement. Bilaterally involved children are diplegic in case of predominant involvement of the lower limbs or quadriplegic in case of equal involvement of lower and upper limbs (18,21).

Additionally, there are five commonly used functional classification systems, namely the gross motor function classification system (GMFCS), the classification system for manual ability (MACS), the communication function (CFCS), eating and drinking ability (EDACS) and the visual function classification system (VFCS)(24–28). All those functions are graded on a scale from 1-5, with a higher score indicating more impaired function.

1.2.2. Heterogeneous response to treatment

Due to the complex and multifaceted nature of SCP and accompanied comorbidities, careful treatment selection is challenging. Besides the heterogeneous nature of the disorder itself, the response to treatment shows large variability. Treatment response can vary between patients, but also between repeated treatment sessions within the same patient (29). Additionally, it has been suggested that response to treatment at the muscular level may even be muscle-specific. Unfortunately, treatment outcomes are not always satisfying (18,30–34). Therefore, research should focus on identification of patient characteristics that can predict treatment outcomes, which could support patient-tailored treatment and lead to improved

treatment efficacy. The use of standardized and objective assessments of the muscle, neuromuscular symptoms and function are essential to monitor the heterogeneity of impairments and the response to treatment and eventually contribute to the development of patient-tailored treatment algorithms.

In this doctoral thesis, we primarily used the gross motor function classification system to describe the participants' function. All studies included in this thesis focused on ambulatory children with spastic cerebral palsy, classified as gross motor function levels I till III.

1.3 Neuromuscular symptoms of spastic cerebral palsy

Spastic cerebral palsy, is the most common subtype. In the next section, the most common neuromuscular symptoms in SCP, including spasticity and stiffness, as well as other symptoms including muscle weakness, reduced selective neuromotor control, reduced range of motion and contractures, will be defined and discussed.

1.3.1. Spasticity in spastic cerebral palsy

Definition of spasticity

Spasticity was defined by Lance as "a motor disorder characterized by a velocity-dependent increase in tonic stretch reflexes (muscle tone) with exaggerated tendon jerks, resulting from hyper-excitability of the stretch reflex, as one component of the upper motor neuron syndrome"(35). In 2003, Sanger introduced another definition: "hypertonia in which one or both of the following signs are present: 1) resistance to externally imposed movement increases with increasing speed of stretch and varies with the direction of joint movement, and/or 2) resistance to externally imposed movement rises rapidly above a threshold speed or joint angle" (36). This definition included less details on the pathophysiology, but a better description of the clinical presentation. Even though Lance's definition is still the most frequently used in clinical practice, the definitions by Lance and Sanger are complementary. However, neither of the definitions covers the pathological increase in muscle activation during low-velocity passive stretches, as described by previous research (37–39). In 2005, the European Thematic Network to Develop Standardized Measures of Spasticity, the SPASM

consortium, introduced a new definition of spasticity: "disordered sensorimotor control, resulting from an upper motor neuron lesion, presenting as intermittent or sustained involuntary activation of muscles" (40,41). This definition is more generic, does include muscle activation during low-velocity stretches and includes the full range of symptoms that are described as excess features.

Rather than broadening the definition of spasticity, a better understanding of the underlying pathophysiology may help to make a distinction between spasticity and other positive features as a result of the upper motor neuron lesion. Therefore, we should intent to assess the isolated contributors to the observed increased resistance during muscle stretch.

A recent European consensus study by Van den Noort and colleagues decomposed the different contributors to the clinically observed phenomenon when stretching a muscle at rest, namely the joint hyper-resistance. They clarified the terminology and defined requirements to assess the different aspects (42). The conceptual framework as designed by this European consensus study is summarized in Figure 2.

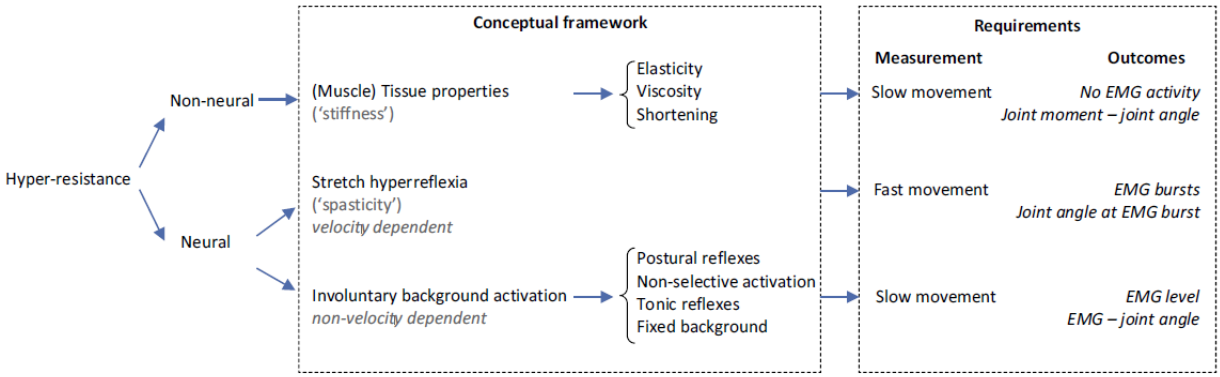

Figure 2. The final conceptual framework of neural and non-neural contributors to hyper-resistance against passive muscle stretch as summarized by the European consensus study by Van den Noort et al. (42) *(From Van den Noort et al. 2017 (42), reused with permission)*

Spasticity, also referred to as stretch hyperreflexia or hyperactive stretch reflex, is the direct result of the upper motor neuron lesion, and included as neural factor in the framework of joint hyper-resistance during stretch. Hypertonia, also called involuntary background activation, is also classified as a neural component and includes the increased muscle tone at

a resting state. Finally, the muscle tissue properties define the non-neural component or stiffness.

Pathophysiology, assessment and prevalence of spasticity

The hyperactive stretch reflex, often referred to as spasticity, which is the primary neural component indicated in the framework by Van den Noort et al. (42) (Figure 2), is presumably caused by supra-spinal disinhibition or dysregulation of the spinal reflex loop as a result of the central nervous lesion or malformation (43,44). This spinal reflex loop, also known as the stretch reflex, is an essential mechanism in motor control as it regulates muscle length in order to maintain balance and control movement. The stretch reflex activity is low in a physiological situation, but can become overactive in case of disinhibition as a result of disturbances in the central nervous system. The hyperactive stretch reflex (or spasticity) causes muscle contraction while elongation of the muscle is desired.

Spasticity is regularly assessed using clinical scales, involving manual tests performed at the joint. The modified Ashworth and modified Tardieu scales are often used to manually classify the levels of spasticity. However, it is not completely clear what is being assessed using these clinical scales, since they cannot distinguish the neural and non-neural contributors to the overall resistance that is felt at the joint (42,45).

Spasticity is the most common clinical symptom in cerebral palsy, affecting 60-80% of the individuals with cerebral palsy (22,23,46). An extensive cohort study by Lindén et al. showed that the degree of plantar flexor spasticity declined after the age of five years (47). Hägglund and Wagner showed that the peak in plantar flexor spasticity is observed between four and five years of age (48). It should be noted that these studies that describe the prevalence of spasticity were mostly based on clinical scales, and may thus not reflect the prevalence of the neural component of hyper-resistance.

1.3.2. Muscle stiffness and contracture development in spastic cerebral palsy

Definition of muscle stiffness and contractures

The term muscle stiffness has often been improperly used to describe different morphological and mechanical muscle properties like elasticity, viscosity or even muscle belly shortening (Figure 2) (42). Additionally, stiffness has often been used interchangeably with the concepts

contractures or muscle shortening (49). Muscle stiffness is the inability of a muscle to move through the full range of motion, even though the muscle belly is not shortened.

Contractures refer to muscle belly shortenings. Contracture development is assumed to be the result of the inability of muscle fibers to reach a length corresponding to bone length. Contractures reduce joint range of motion and may eventually lead to secondary bony deformities (50). A distinction can be made between fixed and dynamic contractures. Fixed contractures include a fixed shortening of the muscle, while dynamic contractures refer to dynamic muscle tightness.

Pathophysiology, assessment and prevalence of muscle stiffness and contractures

Muscle stiffness is considered to be a direct result of alterations in the muscle tissue itself, which can result in higher resistance against stretch, increased joint torque and reduced muscle lengthening.

The etiology of contractures is very complicated and not fully understood. It has long been believed that contracture development was directly related to spasticity. While it is commonly accepted that spasticity contributes to the existence of dynamic contractures, there remains a lot of debate on the role of spasticity in the development of fixed contractures. Previous studies showed that spasticity reduction did not prevent contracture development or improve long term motor functioning (51). Therefore, it has been hypothesized that spasticity is definitely not the only contributing factor (52–56).

Boulard et al. defined a conceptual framework of the potential contributors of reduced range of motion and increased passive stiffness, which is presented in Figure 3 (49). Contracture development is expected to be multifactorial and potentially triggered by increased muscle stiffness due to intrinsic muscle alterations including reduced fascicle length, extracellular matrix alterations and/or increased collagen content (54,57,58). Besides that, reduced satellite cells (muscle stem cells) have been reported in contractured muscles and muscle sarcomeres (contractile elements of the muscle) are overstretched, which might explain the hampered longitudinal muscle growth (56,59). These muscle alterations will be discussed in more detail in the next section (2. Muscle adaptations in spastic cerebral palsy). Besides spasticity and muscle stiffness, muscle weakness might be involved in contracture

development, as it can hinder movement. If the muscle is not actively used over the full range of motion, muscle belly shortening may occur.

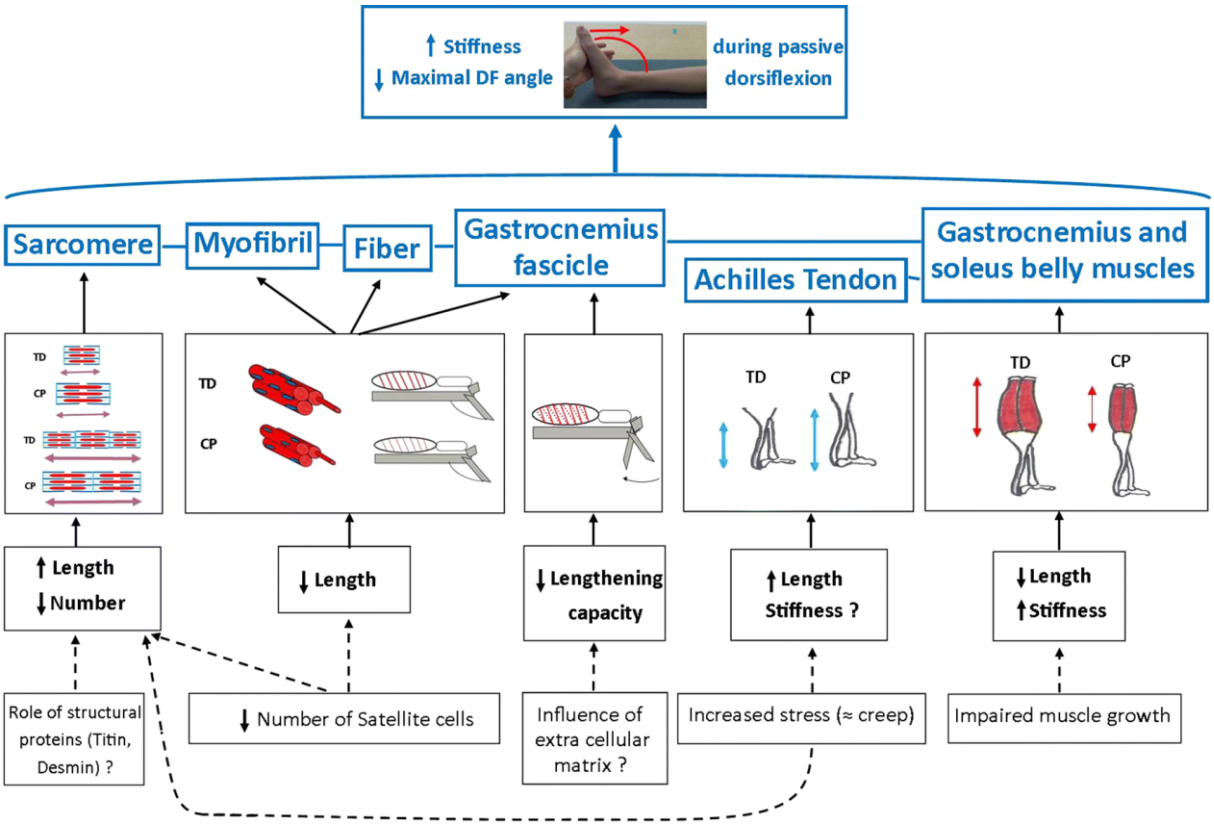

Figure 3. A hypothetical diagram designed by Boulard et al. (49) of the suggested mechanisms and interacting factors that may contribute to increased passive stiffness or reduced range of motion *(From Boulard et al. 2019 (49), reused with permission)*

Differentiation between fixed and dynamic contractures can be challenging, however essential in clinical decision making, as both require different treatments (60,61). Dynamic contractures are often treated by serial casting, Botulinum Neurotoxin-A injections or the use of orthoses, like ankle-foot orthoses (62–64). Fixed contractures on the other hand, require often surgical procedures if previous interventions, including serial casting, were ineffective (61,65–67).

While clinical tests, such as joint range of motion based on goniometry, are often used to detect fixed contractures, their specificity to differentiate between dynamic and fixed contractures is limited (68). It has been suggested that functional measures, like three-dimensional gait analyses, may support the differentiation of contracture types. Indeed, Švehlík et al. showed that ankle kinematics were different between fixed and dynamic equinus

contractures (69). Fixed equinus resulted in persistent equinus throughout the gait cycle, whereas dynamic equinus was characterized by motion towards (more or less) plantar flexion. The discrimination between fixed and dynamic contractures should thus be based on combinations of range of motion estimates, functional assessments and even muscle imaging (70).

Recent studies highlighted that muscle stiffness in SCP is present from an early age on (71). Additionally, the discrepancy between bone and muscle growth has been thought to cause an additional increase in muscle stiffness during childhood (71). Furthermore, Brandenburg et al. found no associations between passive muscle stiffness, level of spasticity, GMFCS level or history of BoNT-A injections (72).

1.3.3. Muscle weakness and selective neuromotor control

Definition of muscle weakness and selective neuromotor control

Muscle weakness is defined as the inability of a muscle to generate strength or maintain an anticipated level of force (73). Muscle weakness is a common problem in SCP and classified as one of the negative features of the upper motor neuron syndrome (18). Previous studies have reported lower limb muscle strength deficits ranging between 40-85% in comparison to typically developing children (74–77).

Selective neuromotor control is defined as “the ability to isolate the muscle activity in a selected pattern in response to the demands of a voluntary motion or posture” and is also one of the negative signs of an upper motor neuron lesion (78).

Pathophysiology and prevalence of muscle weakness and selective neuromotor control

Muscle weakness can be the result of both neural or muscular factors (79). Neural factors include the degree of antagonist co-contraction, reduced neural drive, and incomplete and/or disordered motor unit recruitment patterns resulting in reduced muscle activation. Muscles of children with SCP are smaller in size, contain less contractile tissue, include fewer sarcomeres (contractile elements of the muscle) and have altered structural proteins. These muscular alterations in SCP will be discussed in more detail in the next section (2. Muscle adaptations in spastic cerebral palsy).

Even though deficits in muscle volume and muscle weakness are expected to be interrelated, a recent study by our research group showed larger deficits in muscle strength compared to the deficits in muscle volume (80). This makes sense, since muscle volume represents only one (non-neural) contributing factor to muscle weakness. It was suggested that the other factors contributing to muscle weakness were mainly neural factors, like reduced neuromotor control, disorganized motor unit recruitment and co-contraction (81,82).

Due to dysfunction of the corticospinal tract, children with SCP are less able to control force, speed and timing of muscle contractions, resulting in a disturbance of voluntary movement (78,83). Therefore, reduced selective neuromotor control is considered to be an important neural contributor to muscle weakness, altered muscle use and impaired motor function (83,84). Noble et al. showed that reduced selective neuromotor control is a major factor influencing gross motor function, besides spasticity and muscle volume (85). Additionally, selective motor control plays a role in regular stimulation of the skeletal muscles during infancy. Therefore, reduced selective motor control may be associated with hampered muscle growth and motor skill developmental delays (18).

The clinical presentation of reduced selective motor control includes mirror movements (mainly observed in the upper limbs) and synergistic muscle activation (e.g. co-contraction) or movement patterns (86–88). Coupled hip, knee and ankle movements are present in both term and preterm infants with or without brain lesion. These coupled movements become disassociated over time in the absence of a brain lesion, while the coupled movements often remain present in children with SCP (89,90).

Recent studies showed that muscle weakness and lack of muscle control might be stronger related to impaired daily functioning in comparison to spasticity (74,91–93). Thereby, muscle weakness and reduced muscle control might be an underestimated problem, as it may be dominated by spasticity. Yet, a regular evaluation of muscle strength is important, since it is found to be associated with motor function and walking ability (74,76,94).

This doctoral thesis primarily focused on specific neuromuscular symptoms of spastic cerebral palsy, in particular on the primary symptom of spasticity and de secondary symptom of muscle contractures.

The definitions used in this doctoral thesis are primarily based on this most recent framework of hyper-resistance against stretch, as described by European consensus study of Van den Noort et al. (42) Hyper-resistance includes both neural and non-neural components (Figure 2). The hyperactive stretch reflex represents the neural component. As most clinical studies refer to spasticity instead of the hyperactive stretch reflex, we primarily used the term spasticity to refer to the neural component of joint hyper-resistance.

Spasticity is in this doctoral thesis defined as "the velocity- and muscle length-dependent increase of muscle activity in response to an externally imposed stretch of a muscle at rest" (95).

The heterogeneous nature of spastic cerebral palsy reflects the need for patient-tailored, individualized treatment. The use of standardized and objective assessments is thereby essential to monitor the heterogeneity of impairments and to contribute to the development of patient-tailored treatment algorithms.

2. Muscle adaptations in spastic cerebral palsy

Even though the primary problem in SCP is situated in the central nervous system, or more specifically in the brain, there are several adaptations at the level of the muscles.

Figure 4 provides an overview of commonly observed structural changes in children with SCP in comparison to children with a typical development. The alterations in the skeletal muscle tissue will be discussed in the following sections at both the macroscopic and microscopic level.

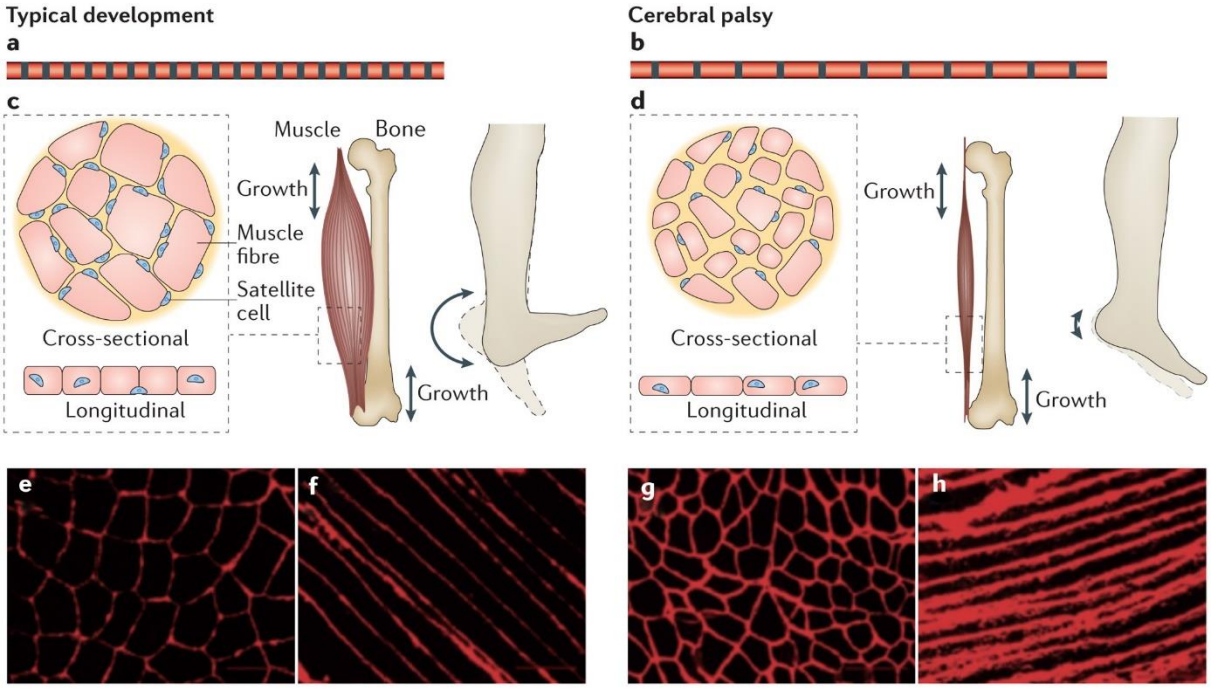

Figure 4. Macroscopic and microscopic alterations in skeletal muscles of children with cerebral palsy, on the right side, compared with typically developing children, on the left side. *(From Graham et al. 2016 (18), reused with permission]*

2.1 Muscle adaptations in spastic cerebral palsy – at a macroscopic level

Muscle imaging techniques (which are described in section 4.1) have substantially improved our understanding of muscle adaptations in SCP by investigating the muscle morphology. Muscle morphology covers the outward appearance of a skeletal muscle, and can be defined

by parameters like muscle volume, anatomical cross-sectional area, muscle thickness, and muscle belly length. The muscles of children with SCP are often smaller i.e. characterized by smaller muscle volume, reduced anatomical cross-sectional area, reduced muscle thickness and shorter muscle belly length (56,96–103). Furthermore, the Achilles tendon is shown to be longer and more compliant compared to children without neurological problems (104–106). Additionally, multiple studies showed that muscle images of children with SCP appear hyperechoic (brighter) (70,107,108). The increased brightness, also referred to as increased echogenicity intensity, can be used as an indirect estimation of muscle composition, which reflects the intrinsic muscle quality (70,96). Muscle imaging studies investigating the muscle pennation angle, fascicle lengths and physiological cross-sectional area are still inconclusive. Some studies found reduced fascicle lengths (58,106,109–112), whereas other studies did not find any differences in fascicle length between children with SCP and typically developing children (98,113).

Handsfield et al. showed that muscle volumes were not uniformly reduced within subjects with SCP, suggesting that muscles are not equally affected (97). Besides that, there was also a large variation between subjects. Recent studies suggested that larger reductions in muscle volume are associated with higher levels of motor impairment (higher gross motor function classification levels) (54,114). Additionally, it was shown that muscle development in SCP is altered from an early age onwards (101). These findings suggest that physical activity and normal neural activity are essential for normal muscle growth during infancy. As the muscle is one of the most plastic tissues in the human body, any disuse or misuse of the muscle can negatively impact its morphology and function. Additionally, it has been shown that children with SCP are less physically active in comparison to typically developing children (115–119). As a result, the mechanical loading is reduced, which inhibits muscle hypertrophy. Furthermore, the already altered muscle morphology may also have biomechanical consequences, potentially resulting in even more altered muscle morphology and muscle growth. Verschuren et al. emphasized that children with SCP are at risk to end up in a vicious cycle of inactivity and muscle disuse and secondary sarcopenia, as seen in elderly (120). Furthermore, Willerslev-Olsen showed that these muscle adaptations were present even before any increased resistance against stretch around the ankle joint was detected (71). Previous studies exploring the relation between spasticity and alterations in muscle

morphology found mainly weak and contradicting associations (121,122). Therefore, the interaction between spasticity and muscle morphology remains largely unknown.

Even though the number of muscle imaging studies is strongly increasing, most studies are limited to the medial gastrocnemius. Due to its superficial orientation, the gastrocnemius is easy to visualize by ultrasonography. Furthermore, the medial gastrocnemius has an essential role in gait and is often targeted by interventions. However, larger studies investigating multiple muscles are required to specify potential muscle-specific alterations. Besides that, there is limited knowledge on physiological muscle development and comprehensive normative datasets of muscle morphology for different lower limb muscles are lacking.

2.2 Muscle adaptations in spastic cerebral palsy – at a microscopic level

Besides the alterations at the macroscopic level, there are several muscular alterations at the microscopic level in SCP.

Previous studies showed that the muscle fiber cross-sectional area was reduced and the fiber type distribution and fiber dominance were altered (123–126). These alteration are believed to be the result of the altered use of muscles and reduced neural stimulation in SCP.

Several histological studies showed an increase in collagen content (54,125,127), fatty infiltration (124,125,127) and other alterations in the extracellular matrix (54,56,123,128). Furthermore, a lower satellite cell count was found (59,127,129–131). These satellite cells are precursors to skeletal muscle cell and can differentiate into skeletal muscle cells or produce more satellite cells and are essential in muscle regeneration (132,133). Moreover, the level of activity of these satellite cells is compromised in muscles of children with SCP (59,127,129–131).

Another important finding is the fact that the number of sarcomeres (the smallest contractile element within the muscle) in series is decreased and the present sarcomeres seem to be overstretched (134–136). The reduced number of serial sarcomeres and the fact that they are over lengthened are believed to cause functional limitations due to limited potential for active muscle excursion and shortening, reduced range of motion and limited force generating

capacity (106,112,135,136). The presence of overstretched sarcomeres and reduced number of satellite cells raise the question whether the shortened muscle bellies are actually able to lengthen.

The reduced muscle regenerative capacity together with extracellular matrix alterations, as well as increased pro-inflammatory gene expression, are considered crucial mechanisms that may contribute to weaker, smaller and stiffer muscles in SCP in comparison to typically developing subjects (127,137–139). Additionally, growth factors are altered, levels of systemic inflammation markers are elevated and the biogenesis of ribosomes is impaired, potentially also contributing to impaired skeletal muscle development during childhood (127,138,139).

> *This doctoral thesis only focused on the macroscopic muscle characteristics in children with spastic cerebral palsy and children with a typical development. The studies in this thesis evaluated the muscle volumes, muscle belly lengths, and estimated muscle composition using echogenicity intensity, of four different lower limb muscles, including the medial gastrocnemius, tibialis anterior, semitendinosus and rectus femoris.*

In order to improve our understanding of the heterogeneous nature and muscle-specific problems in spastic cerebral palsy, there is need for larger muscle morphology database studies and explorations of the associations between the different neuromuscular problems and the muscle morphology. In order to better understand the muscle problems in spastic cerebral palsy, a comprehensive normative database of muscle morphology data of typically developing children is required. Additionally, muscle imaging studies should include multiple lower limb muscles to define potential muscle-specific alterations.

3. Treatment options in spastic cerebral palsy

Unfortunately there is no cure for cerebral palsy. Therefore, patients with cerebral palsy and lower limb spasticity need treatment during their entire life, specifically to manage the neuromuscular symptoms. Due to the important impact on (muscle) growth and crucial steps in motor development, this treatment is specifically important during childhood and adolescence. Given the complexity of the disorder and the heterogeneity of its symptoms, an integrated multi-disciplinary treatment approach is recommended (34,140,141). First line treatment options aim to postpone or prevent the need for more invasive interventions, like orthopedic surgery. Most children with SCP start with regular physical therapy and orthotic wear, like ankle foot orthoses or knee extension braces. In case those interventions are no longer sufficient, other commonly used treatment options that focus on the lower limbs will be considered. These interventions include Botulinum Neurotoxin-A (BoNT-A) injections, casting and strength training, which are further referred to as 'conservative treatment options' in this thesis.

For children with gross motor function classification levels I-III, the main goal of interventions is to preserve the ambulatory function, reduce gait pathology, optimize gross motor function and retain self-independence, self-care and functional transfers. On the other hand, in more severely impaired and non-ambulatory children, interventions are often focused on improving patient's comfort including reducing pain, improve the ease of care by others and improve transfers.

In the current section, tone reduction, stretching and strength training are discussed in more detail, since they have been applied in the subsequent doctoral studies.

3.1 Tone-reduction

Spasticity, or the hyper-active stretch reflex, can limit patients with SCP in their daily functioning and requires therefore treatment (47,50,142). There are several tone reducing treatments available. Oral Baclofen or an intrathecal baclofen pump, intramuscular BoNT-A

injections or selective dorsal rhizotomy are the most common tone-reducing interventions in children with SCP (34,143), and will be discussed in the following sections.

3.1.1. Baclofen

Baclofen, a gamma-aminobutyric acid (GABA) agonist, has been used in oral form to treat spasticity. Oral baclofen has a variable effect on spasticity and the dose is limited to avoid unwanted effects of drowsiness and tiredness. These side effects are the result of high dosages that are required in order to cross the blood brain barrier and to achieve the desired clinical effects of tone reduction (144).

Intrathecal baclofen is directly entered into the cerebrospinal fluid by a catheter, connected to a subcutaneous plump and drug reservoir, running posteriorly into the subarachnoid space. As a result of the intrathecally administration, higher local baclofen concentrations in cerebrospinal fluid are achieved, which is a fraction of the equivalent oral dose, and avoids unwanted side effects of drowsiness and tiredness. Due to the invasiveness, intrathecal baclofen is not often selected is a first-line treatment and commonly used when patients are not eligible or not responding to other, more focal, tone reducing modalities.

3.1.2. Botulinum Neurotoxin-A

Lower limb intramuscular BoNT-A treatment is a commonly used method to primarily reduce the hyperactive stretch reflex (i.e. the neural factor contributing to joint hyper-resistance, also referred to as spasticity), which will potentially result in secondary improvements in joint range of motion, prevention of contracture development and improved gross motor function. Additionally it may contribute to a reduction in patient's discomfort and improve social participation (145–149). BoNT-A temporarily paralyses the muscle by blocking the release of acetylcholine at the neuromuscular junction (145,150,151). The resulting paralysis is temporary and partly reversible, and is believed to last for approximately two to three months (152). Due to the BoNT-A-induced focal paralysis and toxicity, recent concerns have been raised regarding the impact of recurrent BoNT-A on muscle development, muscle quality and morphology in young children with SCP (152–154). Previous investigations in children with SCP showed reduced muscle volumes of the injected muscles (30,108,152,155–159) and neurogenic atrophy (160). Additionally, animal studies showed a loss of contractile tissue,

intramuscular fatty infiltration, and increased connective tissue after BoNT-A injections (57,161–164). Furthermore, animal studies and one study in two healthy adults showed only partial recovery of muscle morphology and function after 12 months (30,162,164,165). As described before, muscle development in SCP is already altered from a very young age onwards and regenerative capacity is potentially limited (56,101,130,166). The potential added impact of BoNT-A treatment on these impaired muscle growth has yet to be defined. Additionally, side effects like including flu-like symptoms, local or generalized weakness, gastrointestinal problems and lower- and upper tract respiratory infections have been reported in 3 - 23% of the children with SCP who are treated with BoNT-A (167–170).

Previous studies by our research group showed beneficial effects of multilevel BoNT-A injections when part of an integrated approach, resulting in reduced gait pathology and postponed need for surgical interventions (141,146,171,172). This integrated approach usually includes additional intensive physical therapy, stretching casts if required and orthotic management. The BoNT-A-induced reduction in hyperactive stretch reflex thereby creates a window of opportunity for adjunct therapies (32,141). More recent studies have confirmed the positive effects of BoNT-A, following this integrated approach, on motor function, including gait (173,174). Nonetheless, these functional benefits have been questioned by other studies, investigating treatment-induced changes in gait by machine learning estimates and both observational and three-dimensional gait analyses (33,175,176). The lack of functional improvement is thought to be the result of BoNT-A-induced muscle weakness, whereas SCP is already characterized by reduced muscle strength, or due to a ceiling effect in gait improvements (149,153). Furthermore, the variety in treatment protocols (such as injection frequency, BoNT-A dosage, the use of casting), lack of active functional training and need for a personalized rehabilitation program may also explain the variable and sometimes unsatisfying results (32,141).

Due to the often applied integrated approach, the isolated effects of BoNT-A remain partially unknown. Furthermore, as most studies use subjective outcomes like clinical scales, including, manual spasticity tests, effects of BoNT-A might be overestimated (141,177,178). Besides that, most studies investigate the impact of BoNT-A on one specific level, for example on spasticity or gait, but do not include a full set of measures, including comprehensive assessments of spasticity, muscle morphology, gait and gross motor function.

3.1.3. Selective dorsal rhizotomy

A permanent method to lower muscle tone is the selective dorsal rhizotomy, which is a neurosurgical procedure during which dorsal sensory nerves in the spine are selectively cut, when they are labelled as probably causing overactive spastic muscles (179–181). By severing the selected spinal sensory nerve rootlets, the sensory input to the muscle is reduced and therefore spasticity is decreased. Besides the fact that this procedure includes a major surgery, with surgical risks and risk for complications, it is always combined with extensive inpatient and outpatient post-operative physical rehabilitation. Intensive therapy post selective dorsal rhizotomy is required in order to rehabilitate motor function and to recover from usually reported post-operative loss in muscle strength (182).

It is believed that selective dorsal rhizotomy minimizes the need for corrective surgical interventions and other tone-reducing therapies and improves motor function and functional independence (182,183). However, determination of patient eligibility is complex and is recommended to be based on multi-model assessments of the patient and family, performed by a multidisciplinary team. The ideal candidate for selective dorsal rhizotomy is usually described as an ambulant child with bilateral SCP, without any dystonic features or truncal weakness, adequate muscle strength and high level of cooperation an motivation of both child and parent or caregiver (182,184).

While most patients and parents are satisfied with the results, the potential risks and the burden of the surgery and long-term rehabilitation should not be underestimated (182).

3.2 Stretching

To limit the risk of contracture development, stretching is commonly proposed as a first-line treatment modality in children with SCP. There is no unambiguous definition of passive and active stretching. Active stretching involves stretching exercises in which either the agonist or antagonist is activated, whereas in passive stretching, stretches can be performed by an external force, for example applied by a therapist. Day and night orthoses, stretching casts, and standing frames are also examples of passive stretching methods.

Ankle foot orthoses are often used to prevent contracture development and provide dynamic stability, resulting in less secondary compensations during gait (185).

As previously mentioned, the muscle bellies are often shortened, whereas the Achilles tendon is longer and more compliant in SCP (53,98,104,105,155,186). Furthermore, sarcomeres are overstretched and the number of satellite cells are reduced, raising the question whether muscles of children with SCP are able to properly lengthen (130,134). Because there is only a limited number of studies investigating the effect of stretching of lower limb muscles in SCP, there is a discrepancy between the widely prescription of stretching and the level of evidence (63,187–195). Moreover, there is much unknown on the isolated effects of stretching exercises and stretching through casting in SCP, partly due to the often applied integrated approach where different stretching techniques are combined.

A review of literature by Kalkman et al. concluded that stretching of the medial gastrocnemius as isolated treatment does not promote lengthening of the muscle (196). Kalkman indicated that it is unlikely that lengthening of the muscle-tendon unit results in functional improvements, as this would require new sarcomeres (contractile material) in-series to be built within the muscle-tendon unit (196,197). Multiple studies showed an increase in maximum dorsiflexion angle after passive stretching sessions in children with SCP (187,188,195,198). However, as the increased dorsiflexion was not always accompanied by alterations at the muscle level, it has been hypothesized that repetitive stretching primarily results in increased tolerance to stretch (198). A possible explanation for the limited alterations in the muscle could be due the more compliant tendon, resulting in less effective transfer of stretch towards the muscle belly. Two different studies, by Zhao et al. and Kalkman et al., showed that Achilles tendon stiffness can be increased by active movement training or strength exercises (190,199). These preparatory exercises are suggested to more directly stimulate the muscle belly during subsequent stretching exercises (190,199). Both studies found indeed an increase in fascicle length after stretching. The study by Kalkman et al. included a control group, receiving stretching exercises only and did not show any alterations in fascicle length. However, the alterations in joint range of motion were similar between the group receiving combined strengthening and stretching exercises and the group receiving stretching exercises only (190). Therefore, it was suggested that the range of motion was again mainly restricted by the tolerance to stretch compared to fascicle length.

In order for a muscle belly to lengthen, it needs to receive the right stimulus (197). The literature review by Kalkman et al. concluded that stretching interventions should focus on lengthening muscle fibers by stimulating serial sarcomerogenesis, as muscles affected by SCP have fewer sarcomeres in series (200). An animal study by Butterfield and Herzog showed that sarcomerogenesis is more efficiently stimulated during muscle fiber strain compared to muscle-tendon unit strain, suggesting that the stretch stimulus has to be directed at the muscle fibers (201). Furthermore, it has been hypothesized that the muscle needs to be activated during stretch, in order to promote sarcomerogenesis (197). The most recent stretching intervention study in SCP by Kruse et al., compared the effects of a single session of proprioceptive neuromuscular facilitation or static stretching on muscle morphology of the medial gastrocnemius and ankle dorsiflexion (195). While stretching following proprioceptive neuromuscular facilitation included activation of the plantar flexor muscles, they did not find an increase in muscle belly length, despite an increase in maximum dorsiflexion angle that was similar between the two stretching methods.

Besides the hypothesized lack of stimulation of sarcomerogenesis during stretching, Gough and Shortland suggested that immobilization, for example by casting, or prolonged passive stretching of the muscle, may result in fiber atrophy, altered metabolic capacity and suppression of muscle protein synthesis (52,202,203). Therefore, all potential negative effects should be carefully considered before applying passive stretch to a muscle that is affected by SCP. However, to the best of our knowledge, it is not known if the above indicated negative effects of passive stretching methods do also occur when using more active stretching methods.

Casting is often applied after BoNT-A injections, not only to maintain the muscle belly length, but also to provide stability. Additionally, it has been shown that functional gains are optimized when combining BoNT-A with lower leg casting (62).

Current evidence suggests that stretching, casting or orthotics as stand-alone treatment are not successful in lengthening the muscle belly. Stretching might be more efficient when combined with other treatments. Future studies should investigate alterations in individual muscle fibers, if feasible at the level of the sarcomeres, to get a better insight into the effects of stretching on the muscle structure of children with SCP (197). Additionally, future studies

should focus on alterations in muscle morphology and associated changes in joint range of motion and whether these changes result in improved muscle and gross motor function.

3.3 Strengthening

In order to improve muscle strength, strengthening exercises and resistance training are recommended. Furthermore, resistance training has been suggested as beneficial adjunct therapy to overcome muscle weakness after intramuscular BoNT-A treatment (32,204).

Previous studies showed that progressive strength training is beneficial in improving muscle strength of the lower limbs in children with SCP (205). Besides that, improvements in lower limb muscle strength are associated with improvements in activity and social participation (92,206). However, the strength training effects at the muscle morphology in children with SCP are inconclusive (154,207).

Our research group performed a progressive strength training intervention in children with SCP (208,209). In this 12-week home-based supervised progressive strength training program, children who performed strength exercises 3-4 times a week were compared to a control group receiving usual care that was matched for age and gross motor function classification level. At baseline and after 12 weeks, muscle volume, gross motor function and both isometric and functional strength were assessed. After 12 weeks of progressive resistance training, both the isometric and functional muscle strength improved and muscle volume increased within the group of children with SCP who participated in the 12-week home-based supervised progressive strength training program. The relative improvements in muscle strength (i.e., changes post strength training relative to the baseline values) were larger compared to the improvements in muscle volume. The improvements in strength were significantly different compared to the control group, however the increases in muscle volume were not. This can be explained by the fact that improvements in muscle strength during childhood are primarily due to motor learning and improved motor neuron recruitment, instead of muscle hypertrophy (210). Nevertheless, this study showed that a home-based supervised progressive strength training program is a promising intervention to improve muscle strength and increase muscle volume, in a cohort of children with SCP that is characterized by muscle

volume deficits. Additionally, the program was shown to be safe, as no serious adverse events were reported.

This doctoral thesis specifically focused on one treatment modality, i.e., tone reduction through Botulinum Neurotoxin-A injections administered following an integrated approach (i.e., combined with serial casting if needed, intensive physical therapy and orthotic management).

Treatment of neuromuscular problems in SCP is often applied as an integrated approach. Therefore, the effect of isolated interventions remains unknown. Treatment response is often variable and not always measured in a valid and objective way. Moreover, assessments are often limited to one level (involving only one muscle group) and to one outcome, whereas muscle-specific measures and measures of function and neuromuscular symptoms should be combined. Instrumented assessments may improve the insight into treatment response variability and allow a critical assessment of the benefits and harms of treatment options, like BoNT-A. Regarding the current concerns on the impact of BoNT-A, well-controlled prospective studies including objective measures at different levels are needed.

4. Instrumented measures of muscle features and function in children with spastic cerebral palsy

As mentioned before, objective instrumented assessments are essential to fine-tune treatment and assess treatment response. In the following section, the assessments of muscle morphology, spasticity and function are described. A selection of these methods were used in the different studies of this doctoral thesis. The specific methodological choices applied in these studies are briefly specified per section (in italic), namely for muscle imaging by ultrasonography, instrumented spasticity assessments and analyses of gait pathology. A more detailed description of the used methods can be found in the separate chapters.

4.1. Muscle imaging

A commonly used method for muscle imaging is magnetic resonance imaging or MRI. Magnetic resonance imaging provides high quality data, but is an expensive technique and children might have to be sedated, due to the long scanning times.

Ultrasonography is a more child-friendly and easier applicable muscle imaging technique. Where conventional brightness mode (B-mode) ultrasound is limited to the recording of two-dimensional muscle images and estimations of parameters like muscle thickness and anatomical cross-sectional area, three-dimensional ultrasound can also provide estimations of the muscle belly volume and muscle belly length.

The method of three-dimensional freehand ultrasound has rather recently been developed and established in our group (211). This method combines conventional two-dimensional brightness mode ultrasound with motion tracking of the ultrasound transducer, thereby creating three dimensional reconstructions of the muscle images (212). This method has been extensively investigated in different reliability studies within our research group and shown to have a good reliability at both the level of assessment, as well as for data processing (213–216).

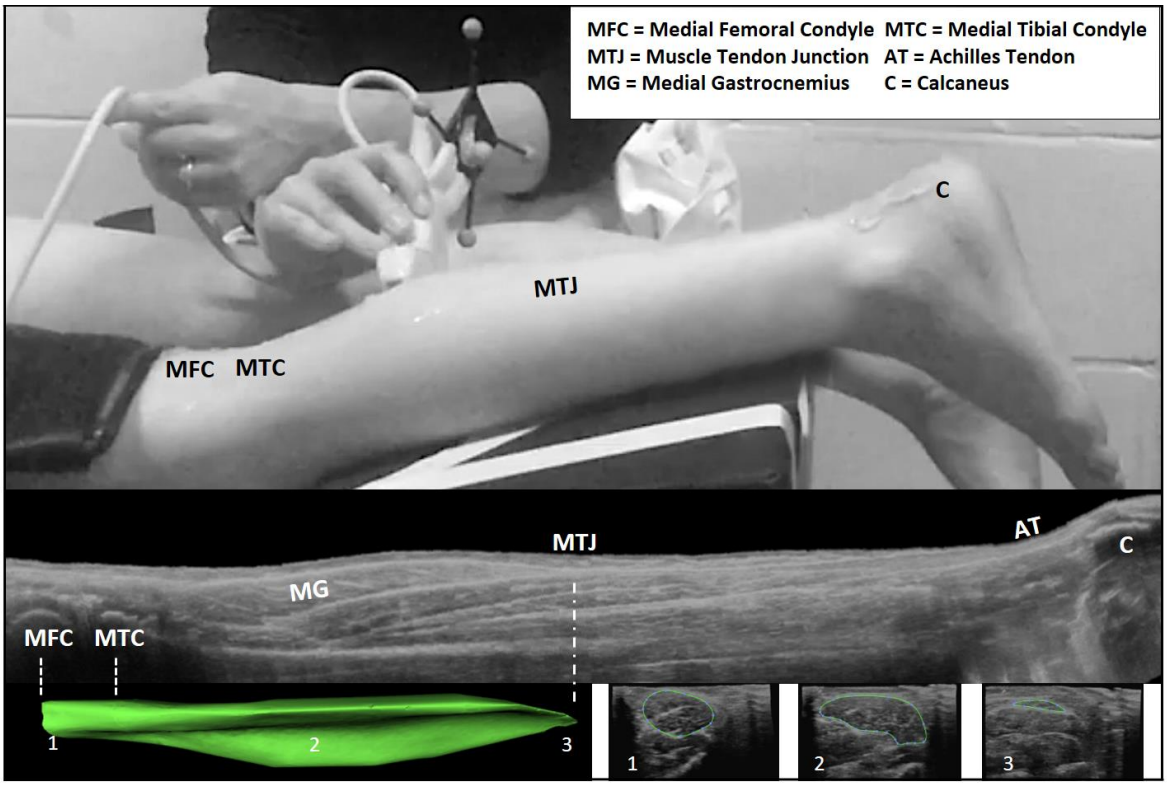

Figure 5. Three-dimensional freehand ultrasound: the top images show an ultrasonography measurement of the medial gastrocnemius. The middle image shows the longitudinal reconstruction of the image followed by a three-dimensional reconstruction of the muscle belly based on manual segmentation in the transverse ultrasonography images. (*Image adapted from PhD thesis Simon-Henri Schless (217), reused with permission)*

Besides the quantification of morphology, ultrasonography can also be used to estimate muscle composition (218). Echogenicity intensity, or the grey value of the transverse ultrasound images, represents as an indirect measure of muscle composition (70,96,219). An increased echogenicity intensity is thought to reflect reduced muscle quality as a result of fibrosis, increased connective tissue, lipid accumulation and loss of contractile elements (52,53,155,156). However, the validity of echogenicity intensity is not completely understood and it cannot differentiate between the various factors that contribute to the reduced muscle quality. Nonetheless, the echogenicity intensity might be a valuable parameter for first explorations and might provide a first estimation of muscle quality without requiring invasive techniques like muscle biopsies or expensive and time-consuming techniques like fat fraction analyses in magnetic resonance imaging scans.

For the current doctoral project, three-dimensional freehand ultrasound data were acquired for the medial gastrocnemius, tibialis anterior, semitendinosus and rectus femoris, through synchronization of a conventional ultrasound device (Telemed-Echoblaster 128 Ext-1 Z system, Lithuania) and a motion tracking system (Optitrack V120:Trio, NaturalPoint, United States of America). Transverse ultrasound images were acquired while the transducer was held perpendicular to the deep aponeurosis of the muscle. A curved custom-made gel pad was attached to the transducer to minimize muscle deformity as a result of the applied pressure during acquisition (220)*. Three-dimensional reconstructions of the muscle and tendon were created using Stradwin software (v6.0, Mechanical Engineering, Cambridge University, UK). Manual transverse segmentations along the inside of the muscle border were drawn, in approximately 5-10 percent of the total images, to estimate the absolute muscle volume by the cubic planimetry technique* (221)*. Muscle and tendon length were estimated by manually indicating anatomical landmarks of the origin, insertion and muscle-tendon junction and by calculating the Euclidean distance between these landmarks. Additionally, echogenicity intensity, which can be used as an indirect estimation of muscle composition, was estimated as the average of the manual segmented ultrasound images used for the interpolated reconstruction of the muscle.*

Three-dimensional freehand ultrasound data acquisition and an impression of data processing are presented in Figure 5. Estimates of muscle volume, muscle belly length, tendon length, muscle-tendon complex length and echogenicity intensity (muscle composition) were included as outcome measures in the different studies of this doctoral thesis.

Despite the increasing use of muscle ultrasonography in pediatric and specifically SCP cohorts, study results are often difficult to compare due to a large variety in measurement and analysis protocols. Moreover, limb position and joint angles are often not reported. While it is expected that alterations in joint angles will result in different muscle lengths, this has never been investigated. In order to compare study results, to pool the data, and to ensure valid follow-up measurements, standardization and clear documentation of the assessment protocols is required.

Another factor that complicates the comparison of studies is the variability in data normalization. Data normalization, also referred to as data scaling, are methods to correct muscle morphology parameters for variability in body dimensions, such that data from children with different anthropometrics can be pooled and/or compared. Moreover, in longitudinal studies, data normalization eliminates the impact of increasing body dimension in the data from growing children with SCP, thereby emphasizing the impact of changes related to the pathology and/or interventions. For example, since muscle volume is strongly related to body mass, some studies divided muscle volume by body mass. However, multiple methods have been used for different muscle morphology parameters. Muscle volume is often normalized to body mass (85,98,226–230,100,122,186,212,222–225), the product of body mass and height (97,107,114,155) or bone length (156,158,204,231). Cross-sectional area is most commonly normalized to body mass (97,122,155,228,232) and muscle lengths have been commonly normalized to bone length (63,97,235,236,98,114,155,186,228,231,233,234) or muscle-tendon unit length (63,224,233,234,236). However, clear recommendations on the use of normalization and a comparison of the previously used data scaling methods are lacking. These afore mentioned data scaling methods are examples of ratio scaling. Recently, allometric scaling, which has been more commonly used to normalize strength measurements to body size, has also been proposed for normalizing muscle morphology data, but not yet for growing children (237,238). Hence, the validity of allometric scaling of muscle morphology parameters in growing children has not yet been established.

> *In this doctoral thesis, different data scaling methods were explored, including both ratio and allometric scaling, to normalize muscle morphology outcomes.*

At the start of the PhD project, reference data of both normative and pathological data were limited and not widely available. In order to compare cohorts or quantify the level of pathology or progression, reference data are essential. Most previous studies focused on a single muscle, in a small cohort with a limited age range. Therefore, large, open access, ultrasonography reference datasets are required. Additionally, insight into muscle-, age-, or gender-specific trends in muscle morphology data are limited and recommendations on inclusion or group-matching criteria are lacking.

4.2. Instrumented spasticity assessment

Assessments of muscle stiffness and spasticity are often based on joint range of motion and manual clinical scales of joint hyper-resistance, such as the Modified Ashworth and Tardieu scale (45). However, these manual scales are primarily based on the resistance felt around the joint and are not able to distinguish between different muscles and the contribution of either tendon and muscle belly. Additionally, those tests are not able to differentiate between the neural and non-neural components contributing to the increased resistance and have poor inter- and intra-rater reliability (239–241). Objective instrumented assessments that integrate multiple signals are considered more sensitive and can distinguish between neural and non-neural components of joint hyper-resistance (42,242,243). An improved characterization of this complex phenomenon of hyper-resistance against stretch may improve clinical decision making and the selection of appropriate therapeutic strategies.

Therefore, the instrumented spasticity assessment was developed (243,244). Through signal integration, joint hyper-resistance could be quantified and decomposed in the hyperactive stretch reflex (the neural component, and also referred to as spasticity) and the non-neural components. This procedure was developed for the plantar flexors, knee flexors, knee extensors and adductor muscles (243).

This procedure of instrumented spasticity assessment involves collection of muscle activity by surface electromyography, joint kinematics, and net joint torque recorded with a six degrees-of-freedom torque load-cell during manually applied passive stretches at different velocities over the full range of motion. These sensors create the opportunity to control for stretch velocity, applied torque, subject relaxation and co-contraction of the antagonist. In Figure 6, the different sensors and starting positions and direction of stretch for the assessment of the knee flexors and plantar flexors are shown.

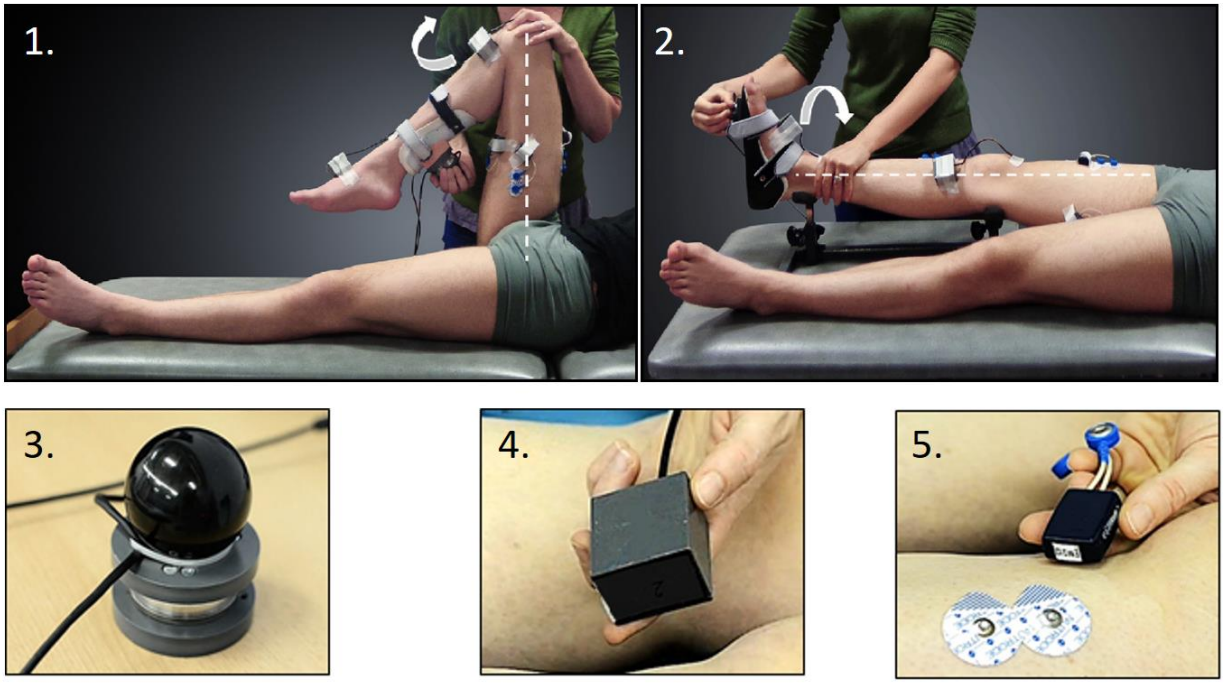

Figure 6. The instrumented spasticity assessment: starting positions and direction of stretch (white arrows) for the knee flexors (image 1) and plantar flexors (image 2). Torque was measured by a force-sensor (image 3) attached to a shank or foot orthosis; joint kinematics (angles) were measured by inertial measurement units (image 4); muscle activity was measured with surface electromyography (image 5) *(From Bar-On et al. 2013 (243), reproduced with permission)*

Outcome parameters that quantify spasticity and stiffness are based on integration of signals and are found to be reliable (245). Additionally, the assessments of muscle activation during passive stretches at different velocities, using surface electromyography, were found to be valid for evaluating treatment response (246,247). Visualization of the surface electromyography signals over the range of motion allows to classify muscle behavior into previously described muscle activation patterns, also referred to as spasticity patterns (37). Furthermore, this objective, instrumented assessment, is able to distinguish between the hyperactive stretch reflex (neural) and passive stiffness (non-neural) and can isolate individual muscles (242,248). Additionally, objective data of range of motion and joint torque can be extracted.

This doctoral thesis included instrumented spasticity assessments of the plantar and knee flexors. The muscle activation was assessed by surface electromyography (Zerowire, Cometa, Milan, Italy), the joint kinematics were defined using inertial

measurement units (Analog Devices, ADIS16354) and net joint torque was recorded with a six degrees-of-freedom torque load-cell (ATI mini45: Industrial Automation). These sensor data were collected during passive stretches at different velocities and used to quantify spasticity and describe different muscle activation patterns during passive stretch. The outcome parameters included the level of muscle activation and work during passive stretches at both low and high velocities. Furthermore, the previously described muscle activation patterns e.g. spasticity patterns were identified (37).

4.3. Analysis of gait pathology

Walking is considered one of the most important activities in daily life as it is essential for social participation and the majority of daily activities. Objective assessments of gait are therefore essential in clinical follow-up of disorders affecting gross motor function. For children with SCP, gait analysis play an important part in clinical management and decision making (249).

There are several visual gait scoring systems (250), which have been used in children with cerebral palsy, including the Edinburgh Visual Gait Score (251), visual gait assessment scale by Dickens et al. (252) and Salford gait tool (253). Due to the subjective nature of these observational approaches, the complexity of gait and the necessity to detect even minor deviations, a marker-based objective three-dimensional gait analysis is preferred over observational analyses. The results of three-dimensional gait analyses are used to identify gait abnormalities, and are commonly discussed by a multidisciplinary team to set patient-specific treatment goals and evaluate natural progression or treatment response.

A three-dimensional gait analysis can objectively assess joint kinematics, kinetics and muscle activation. Kinematic data describe the motion of different segments, whereas kinetics describe the causes of the motion, namely the forces and joint moments that are generated by the child while walking. Kinematic data can be acquired using a marker-based approach (Figure 7). This involves the attachment of reflective markers on specific anatomical landmarks on the skin. The movement of these markers can be tracked by an opto-electronic system

(such as the VICON system as used in the Clinical Motion Analysis Laboratory of the University Hospitals Leuven). Kinetic data are collected using force plates embedded in the runway. These force plates can measure the ground-reaction force, the force exerted by the ground on a body in contact with it, like the foot. When combining the force and kinematic data, joint moments and power can be calculated by inverse dynamics for each individual joint of the lower limbs. Finally, muscle activation is recorded using surface electromyography. Surface electromyography records muscle activity through electrodes attached to the skin, allowing to investigate the amplitude and timing of muscle activity during gait (Figure 7).

All those data are collected while the child is walking barefoot at a self-selected speed over a 10-meter runway. If applicable, the gait analysis can also be performed while the child is wearing shoes with insoles or orthotics. Those additional analyses may contribute to appraisal of ambulation with adapted footwear.

Different parameters can be used to describe and appraise a child's gait pattern based on data collected during the three-dimensional gait analysis. First of all, there are different general scores including the gait profile scores (GPS)(254), gait deviation index (GDI)(255) and gait variable scores (GVS)(254), providing an average estimate of the overall gait pathology (256). Additionally, joint specific kinematic or kinetic parameters can be calculated. Furthermore, spatial-temporal parameters of the gait cycle can be calculated like walking speed, cadence, duration of the stance and swing phase or stride length, stride width or step length.
Recently, multiple studies used statistical parametric mapping to evaluate pathological gait (257,258). This method allows to analyze the full kinematic or kinetic waveforms (over the full gait cycle) and compare pre- and post-treatment assessments or compare full waveforms to for example normative data.

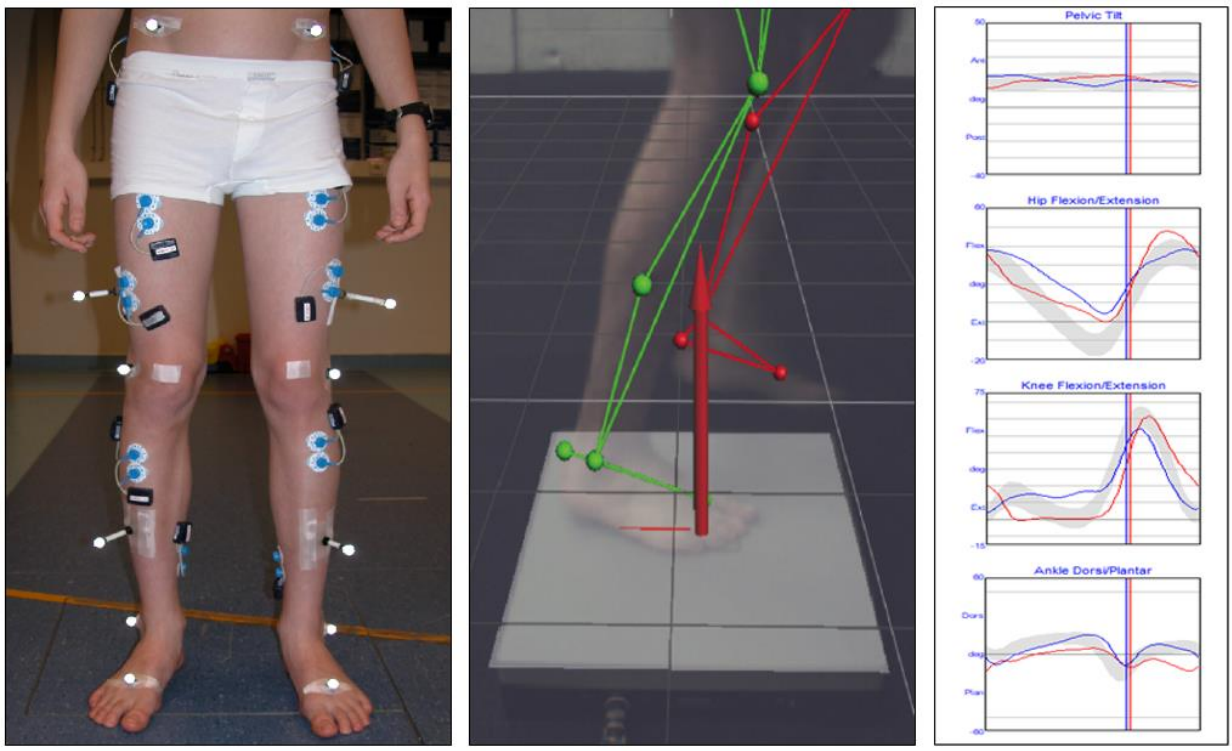

Figure 7. Three-dimensional gait analysis. The left image shows the reflective markers and electrodes of the surface electromyography which are placed on the skin of the lower limbs. The middle image shows the model which is created by the Nexus software and used to calculate the kinematic and kinetic data. The red arrow represents the ground reaction force. The images on the right show the kinematic data per joint after processing the data (*From CMAL database and open access*)

The clinical examination

A three-dimensional gait analyses is commonly combined with a clinical examination of the lower limbs including measures of passive joint range of motion (using goniometry), spasticity assessments using the modified Ashworth scale (259) and modified Tardieu scale (241), manual muscle testing (260) and assessments of selective motor control including the selective control assessment of the lower extremity (SCALE)(261).

This doctoral thesis focused on the ankle and knee kinematics in the sagittal plane, collected during a three-dimensional gait analysis. Kinematic data were captured by the VICON system (Nexus 2.12, Vicon-UK, Oxford, United Kingdom) containing 10 or 12 cameras. The marker placement was standardized and markers were placed relative to anatomical landmarks according to the Vicon plug-in-gait lower limb model (Figure 6). The outcome parameters of the PhD studies included the kinematic waveforms of both

the ankle and knee, isolated kinematic gait parameters of the ankle and knee, the gait profile score (254) *and spatial-temporal parameters.*

Clinical follow-up and research projects are often based on subjective assessments. Objective instrumented assessment are required during clinical follow-up and to reliably assess treatment response. Objective assessments of multiple problems (such as muscle morphology, spasticity, and gait) should be more often combined, in order to assess impairments at the level of the muscle, neuromuscular symptoms, and gross motor function and to explore their interaction.

Ultrasonography is a commonly used method to assess muscle morphology in children with spastic cerebral palsy. However, some remaining challenges have to be tackled. First, comprehensive normative databases are lacking, making it difficult to quantify the level of pathology and monitor muscle development. Secondly, the impact of limb positioning on morphological outcomes is unknown and clear recommendations are lacking. Therefore, there is need for a standardized, well-described, measurement setup to assess muscle morphology in children with spastic cerebral palsy. Thirdly, the efficacy of different scaling techniques to normalize muscle morphology parameters to body dimensions, should be compared to develop effective scaling conventions for muscle morphology for children with spastic cerebral palsy.

Aim and outline of this doctoral thesis

The overall aim of this doctoral thesis was to improve the understanding of the macroscopic alterations in the muscle-tendon unit, gain more insight into the heterogeneous nature of SCP, and to investigate the impact of tone reduction treatment, more specifically intramuscular BoNT-A injections, on muscle morphology, spasticity and function. In order to achieve this overall aim, three objectives were defined, each including different studies, as summarized in Figure 8.

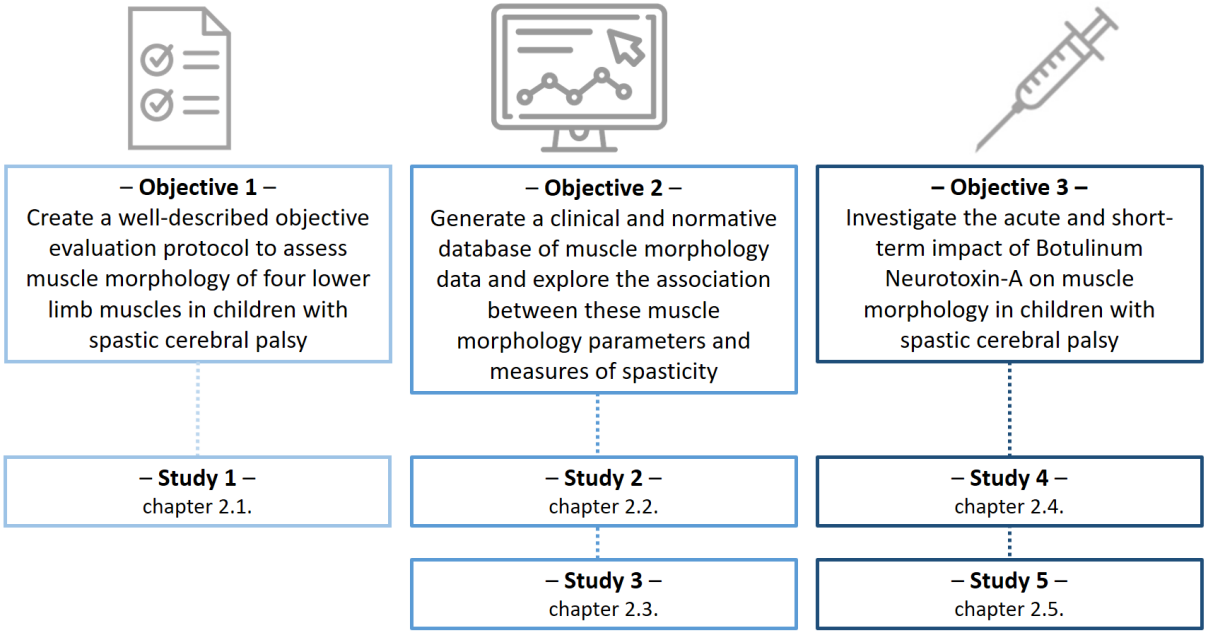

Figure 8. Outline of this doctoral thesis, consisting of three objectives, including different studies

Objective 1. Create a well-described objective evaluation protocol to assess muscle morphology of four lower limb muscles in children with spastic cerebral palsy

Three-dimensional freehand ultrasound is a commonly used and easy applicable technique to investigate lower limb muscle morphology in children. Previous studies used multiple measurement setups, but the positioning of the participant and investigated limbs were not always well-described. Therefore, it is difficult to replicate, compare or pool data of previous studies. Most measurements have been performed at the muscle's resting length. However, any alteration in proximal and distal joint angles are expected to change the tension on the

muscle-tendon unit and may thus consequently influence muscle morphology. Standardized measurement setups are required for reliable follow-up measurements and to compare study results. Yet, the influence of the limb positioning on muscle morphology of different lower limb muscles has never been systematically investigated. Therefore, Study 1 was designed to investigate the influence of limb positioning during a three-dimensional freehand ultrasound protocol on muscle morphology parameters of four lower limb muscles in both typically developing children as well as in children with SCP.

- **Study 1: The impact of joint angles on lower limb muscle morphology outcomes defined by three-dimensional freehand ultrasound in typically developing children and children with spastic cerebral palsy**

  We hypothesized that muscle volume would not be influenced by altered proximal or distal joint angles, while muscle and tendon lengths would.

  This study contributed to the achievement of the PhD goal through the fine-tuning of the three-dimensional freehand ultrasound protocol and pointed towards the relevance of standardized protocols and guidelines to assess muscle properties that should be used for different intervention studies and clinical follow-up of children with SCP.

Objective 2. Generate a clinical and normative database of muscle morphology data and explore the association between these muscle morphology parameters and measures of spasticity

Even though the number of ultrasonography studies investigating muscles in different pediatric populations is rising, comprehensive normative databases are lacking and knowledge on muscle development in typically developing children is limited. Therefore, it remains challenging to quantify the level of pathology or muscle growth deficits of children with SCP. Furthermore, different data scaling methods to normalize muscle morphology parameters have been introduced, however those methods have never been systematically compared. Study 2 aimed to create a comprehensive normative database of muscle morphology parameters and describe typical muscle development of four lower limb muscles

in typically developing children, aged between 3 and 18 years. Additionally, the efficacy of previously proposed data scaling methods was evaluated.

Today, little is known about the functional consequences of altered muscle development in SCP and its relation with the neuromuscular symptoms, like spasticity. Multiple studies showed that the muscle morphology of children with SCP is altered. Spasticity has often been mentioned as an important contributor to altered muscle morphology in children with SCP. However, previous studies indicated only rather low correlations between the degree of spasticity and altered muscle morphology. The lack of correlations may be caused by the used methods to evaluate spasticity or the heterogeneous nature of spasticity. While spasticity is often defined as being velocity-dependent, investigations on muscle activation patterns in lower limb muscles revealed length-dependent features of spasticity and the existence of mixed muscle activation patterns involving both velocity- and length-dependency. Therefore, Study 3 aimed to explore the association between the level of spasticity and alterations in muscle morphology and to investigate differences in muscle morphology between different muscle activation patterns.

- **Study 2: A comprehensive normative reference database of muscle morphology in typically developing children aged 3-18 years – a cross sectional ultrasound study**

  We hypothesized that muscle development could be described by a simple linear equation until the age of 11 years, i.e., the average start of puberty. Furthermore, it was hypothesized that muscle morphology data could be effectively normalized for differences in body size. Proof of concept for these hypotheses was provided by previously described changes in medial gastrocnemius morphology with increasing age by our own as well as other research groups (100,108,155,262). Additionally, ratio and allometric scaling methods have been applied and compared in a previous study investigating the muscle morphology of lumbar multifidus and abdominal muscle (237).

  This study established a unique normative database of four lower limb muscles in typically developing children between the age of 3 and 18 years, which can be used as a normative reference database when studying pathological pediatric populations.

Furthermore, these results contributed to improved insight into physiological muscle development and the application of data scaling methods.

- **Study 3: Associations between muscle morphology and spasticity in children with spastic cerebral palsy**

  We hypothesized that more severely affected muscle morphology, smaller muscle volume and reduced muscle belly length, would be associated to higher levels of spasticity. Furthermore, we expected to find more severely affected muscle morphology in muscles with mixed length-/velocity-dependent muscle activation patterns, compared to muscles with pure high-velocity muscle activation patterns. Proof of concept for these hypotheses was provided by multiple pilot analyses of our research group and the previous study by Bar-On et al. which suggested that muscles with length-dependent features might be more severely affected (37,263).

  The data of this study provided improved insight into the complex interaction between spasticity and alterations in muscle morphology.

Objective 3. Investigate the acute and short-term impact of Botulinum Neurotoxin-A on muscle morphology in children with spastic cerebral palsy

Treatment response is very heterogeneous in SCP and is considered patient-specific, and potentially also muscle-specific. As lower limb BoNT-A injections and lower limb casting have often been combined, their isolated effects remained partly unknown. Study 4 aimed to investigate the isolated effects of medial gastrocnemius BoNT-A injections and lower leg casting.

Additionally, concerns have recently been highlighted on potential harmful effects of repeated BoNT-A injections on muscle growth in young children with SCP. Besides that, due to conflicting findings, there remains a debate on the functional improvements after treatment. There are no previous studies investigating the effects of BoNT-A on both the muscle and function in a controlled design. Therefore, Study 5 aimed to investigate the effects of BoNT-A on muscle morphology of the medial gastrocnemius and semitendinosus, on spasticity and on gait, in one patient cohort using instrumented measures within a prospective controlled design.

- **Study 4: Joint and muscle assessments of the separate effects of Botulinum Neurotoxin-A and lower-leg casting in children with cerebral palsy**

  We hypothesized BoNT-A injections would effectively reduce the hyperactive stretch reflex, whereas casting would result in lengthening of the muscle belly and tendon and would thereby reduce the resistance around the joint. Proof of concept for these hypotheses was provided by previous studies of our research group that confirmed effective reduction of the hyperactive stretch reflex after BoNT-A (246,247) and studies which showed that different passive stretching modalities resulted in improved range of motion (63,188).

  These results contributed to improved insight into the isolated effects of serial casting and Botulinum Neurotoxin-A. This knowledge might support the development of individualized treatment protocols.

- **Study 5: The short-term impact of BoNT-A on muscle morphology and gait in children with spastic cerebral palsy**

  We hypothesized that BoNT-A injections in the medial gastrocnemius and the medial hamstrings would successfully reduce the hyperactive stretch reflex, but would cause reductions in muscle volume and negatively influence muscle quality. Additionally it was expected that ankle and knee kinematics in the sagittal plane would significantly improve after BoNT-A treatment.

  This study provided objective data of the potential benefits and harms of BoNT-A treatment, which may support well-considered treatment selection. Given the heterogeneous response to treatment, the knowledge gained by the objective measures may ultimately help to develop a more individually tailored treatment plan.

This PhD project was embedded in the larger FWO-TBM project TAMTA (acronym for Treatment Algorithms based on Muscle and Tendon Architecture), which aimed to define subject- and muscle characteristics which can predict treatment outcome. These prediction models will eventually be used to develop a clinical decision-making framework based on a comprehensive description of the muscle and tendon morphology in children with SCP.

Details on included participants, assessments and used statistical tests, of the different studies of this doctoral thesis, are summarized in Table 1. More detailed information can be found in the different chapters.

Table 1. Details of the included studies

	Population	Number of participants	Age range*	GMFCS	Assessments	Statistics
Chapter 2.1. Study 1. **The impact of joint angles on lower limb muscle morphology outcomes defined by three-dimensional freehand ultrasound in typically developing children and children with spastic cerebral palsy**	Typically developing children and children with spastic cerebral palsy	TD n=13 SCP n=13	4-16 years SCP: 5-16 years median age 14.27 years 7.35 – 15.13) TD: 4-14 years median age 11.10 years (9.03 – 12.22)	I-III	three-dimensional freehand ultrasound, patient friendliness	• Kolmogorov-Smirnov test • Descriptive statistics • Friedman test • Wilcoxon Signed Ranks test
Chapter 2.2. Study 2. **A comprehensive normative reference database of muscle morphology in typically developing children aged 3-18 years**	Typically developing children	116	3-18 years mean age 10.35 ± 4.49 years	n.a.	three-dimensional freehand ultrasound	• Kolmogorov-Smirnov test • Descriptive statistics • Simple linear regression • Analysis of covariance • Steiger's Z-test
Chapter 2.3. Study 3. **Associations between muscle morphology and spasticity in children with spastic cerebral palsy**	Children with spastic cerebral palsy	74	3-17 years median age 8.2 years (7.3 – 10.3)	I-III	three-dimensional freehand ultrasound , instrumented spasticity assessments, clinical examination	• Kolmogorov-Smirnov test • Descriptive statistics • Spearman correlation • Mann-Whitney U test
Chapter 2.4. Study 4. **Joint and muscle assessments of the separate effects of Botulinum Neurotoxin-A and lower-leg casting in children with cerebral palsy**	Children with spastic cerebral palsy	31	4-14 years mean age 7.4 ± 2.6 years	I-III	three-dimensional freehand ultrasound , instrumented spasticity assessments	• Kolmogorov-Smirnov test • Descriptive statistics • Wilcoxon signed ranks test • Mann-Whitney U test • Spearman correlation
Chapter 2.5. Study 5. **The short-term impact of Botulinum Neurotoxin-A on muscle morphology and gait in children with spastic cerebral palsy**	Children with spastic cerebral palsy	45	3-11 years median age 7.1 years (5.7 – 9.1)	I-III	three-dimensional freehand ultrasound , instrumented spasticity assessments, three-dimension gait analysis, clinical examination	• Kolmogorov-Smirnov test • Descriptive statistics • Mann-Whitney U test • Wilcoxon signed rank test • Statistical non-parametric mapping

**depending on data distribution, mean and standard deviation or median values with the 25th and 75th percentiles are presented.*

GMFCS = gross motor function classification system; TD = typically developing; SCP = spastic cerebral palsy

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Part II
Investigations



Objective 1.

Create a well-described objective evaluation protocol to assess muscle morphology of four lower limb muscles in children with spastic cerebral palsy

Chapter 2.1.

The Impact of Joint Angles on Lower Limb Muscle Morphology Outcomes Defined by Three-dimensional Freehand Ultrasound in Typically Developing Children and Children with Spastic Cerebral Palsy

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To be submitted

Abbreviations

AU = arbitrary units

B-mode = brightness mode

cm = centimeters

dB = decibels

DF = dorsiflexion

EI = echogenicity intensity

GMFCS = gross motor classification system

kg = kilograms

m = meters

MG = medial gastrocnemius

mHz = megahertz

mm = millimeters

MTJ = muscle-tendon junction

n = number

n.a. = not applicable

p = p-value

RF = rectus femoris

SCP = spastic cerebral palsy

ST = semitendinosus

TD = typically developing

3DfUS = three-dimensional freehand ultrasound

Keywords: ultrasound, muscle morphology, standardization, typically developing children, spastic cerebral palsy

Abstract

Three-dimensional freehand ultrasound (3DfUS) is a commonly used method to assess lower limb muscle morphology in pediatric populations. Unfortunately, the adjacent lower limb joint angles during the ultrasound acquisition of shank and thigh muscles are often not described, making it difficult to compare and reproduce results. Because these joint angles may impact the stretch on the assessed muscle, they may potentially influence the muscle morphology outcomes. The current study aimed to quantify the differences in muscle morphology and composition outcomes between measurement setups that were characterized by different joint angles and to contribute to the development of standardization recommendations for future 3DfUS studies.

Lower limb muscle morphology of 13 typically developing (TD) children (median age: 11.10 years (9.03 – 12.22) and 13 children with spastic cerebral palsy (SCP)(median age: 14.27 years (7.35 – 15.13), GMFCS-level I/II/III: 7/5/1) was assessed by 3DfUS in three conditions, namely at a resting length, at common joint angles (i.e., joint angles that were similar for all participants), and at maximum joint excursion (individually defined). Estimations of muscle volume, muscle belly length and echogenicity intensity of the medial gastrocnemius (MG), rectus femoris (RF) and distal compartment of the semitendinosus (ST) in the three conditions were compared by a repeated measures Friedman test and corresponding post-hoc test.

The muscle volume estimations for the MG and RF of the TD children were significantly different between the measurement conditions (p = 0.004, p = 0.007, respectively), with smaller muscle volumes in the resting condition in comparison to the common and maximum joint angles. All muscle belly lengths changed significantly between the three conditions, with the longest muscle belly lengths in the maximum joint positon, with exception of the ST muscle belly length in the TD cohort, which did not change and the MG muscle belly length in the SCP cohort, which showed a clear tendency towards change.

This study showed that limb positioning significantly influenced muscle morphology outcomes. These findings highlighted the need for standardized measurement setups. Measurements of muscle volume were challenging while the participants were positioned on their side, whereas this side position was suitable for assessments of muscle lengths at different joint angles for most muscles.

Introduction

There is a growing number of studies investigating muscle morphology alterations in children with spastic cerebral palsy (SCP) using three-dimensional freehand ultrasound (3DfUS) (1–7). The 3DfUS technique combines B-mode ultrasound with three-dimensional motion tracking of the transducer, to create a three-dimensional reconstruction of the muscle (8). The 3DfUS technique is an easily applicable and well-tolerated method even in very young children. Therefore, it is a promising technique for clinical follow-up or for monitoring the impact of muscle-directed treatment modalities.

However, results on lower limb muscle morphology from different studies are difficult to compare or to be pooled, because the position of the assessed limb, along with the proximal and distal joint angles during data collection, often differs between studies. Moreover, it is remarkable that the limb position and involved joint angles are not always well-described or even reported, making it challenging to replicate results. For example, the ankle joint was commonly positioned at a resting angle while investigating the medial gastrocnemius (MG). This resting angle was not always properly defined (7,9–12). In case ankle positions were specified during muscle imaging, they varied between a neutral ankle angle (0 degrees dorsiflexion)(12), maximum dorsiflexion (1,11–13), 10, 20 or 30 degrees plantarflexion (14) or maximum plantarflexion (12). Thereby, the knee was in full extension, or in flexion with angles varying between 20 and 90 degrees of flexion (1,5,9,14).

Furthermore, clear recommendations on measurement setups, with specifications of the involved joint angles per lower limb muscle are lacking (15). In order to reliably compare results from different studies or to replicate or merge data, standardization and clear documentation of the measurement setup is required. This is especially relevant when investigating length parameters, since the proximal and distal joint angles are expected to influence the muscle belly and tendon lengths due to altered stretch on the muscle-tendon unit. Furthermore, in the perspective of using 3DfUS for clinical follow-up, it is important to standardize the measurement setup within the same patient over time.

So far, the impact of the adjacent joint angles of the investigated muscle on morphology parameters defined by ultrasound has never been systemically examined. Therefore, the first aim of the current study was to quantify the impact of these joint angles on three macroscopic muscle morphology and composition outcomes, including muscle volume and belly length, and echogenicity intensity. The echogenicity intensity is the grey value of the ultrasound images, and is used as an indirect estimation of intrinsic muscle quality. These outcomes were defined in typically developing (TD) children as well as in children with SCP. Unique for the current investigation was the assessment of three different lower limb muscles, namely the MG, semitendinosus (ST) and rectus femoris (RF), in both a SCP and TD cohort.

It was hypothesized that the muscle belly length would be different between different ankle or knee angles when investigating the shank and thigh muscles, because of the altered stretch on the muscle-tendon unit. On the other hand, muscle volume and echogenicity intensity were expected to remain similar for the different measurement positions.

Methods

This observational study was approved by the local ethics committee, Commissie Medische Ethiek UZ/KU Leuven (s59945). Written informed consent was obtained from parents or legal guardian. Children from the age of 12 years, signed an additional assent form.

Participants

The TD children were recruited by graduate students or acquaintances of the assessors. The children with SCP were recruited via the Clinical Motion Analysis Laboratory of the University Hospitals Leuven, Belgium. Exclusion criteria for both cohorts were previous orthopedic surgery in one of the investigated muscles. TD children could not have a history of neurological problems. Specific exclusion criteria for the SCP cohort included, GMFCS levels IV or V, previous selective dorsal rhizotomy, bone realignment surgery within two years before assessment, or intramuscular botulinum neurotoxin-A injections within six months before assessment. Children aged between three and 18 years were eligible for participation. Children had to be cooperative and able to lay still for longer periods of time.

Only one leg was assessed. For the TD children, alternating the dominant and non-dominant leg, whereby leg dominance was determined by kicking a ball, was assessed. For the children with SCP, the most affected leg was assessed based on the most recent clinical examination. Measures of body mass and height were taken. Additionally, the total leg length (between the lower edge of the spina iliac anterior superior and the lower edge of the medial malleolus) was taken using a tape measure.

Three-dimensional freehand ultrasound acquisition

Muscle morphology and composition, including estimates of muscle volume and muscle belly length, as well as echogenicity intensity of the MG, ST and RF were assessed by 3DfUS (2,8). Due to the deep location of the proximal compartment of the ST and the different structures (i.e. subcutaneous tissue, gluteus maximus) covering it, it was not always possible to clearly visualize the full ST muscle (16). Therefore, only the distal compartment of the ST was assessed in this study.

Images were captured using a 5.9 cm 10 MHz linear ultrasound transducer (HL9.0/60/128Z) with a Telemed-Echoblaster 128 Ext-1Z ultrasound device. Four reflective markers were attached to the ultrasound transducer of which the movement was tracked by a motion tracking system (OptiTrack V120: Trio, NaturalPoint, Corvallis, OR, USA) with three fixed optical cameras and a spatial resolution of 1mm. In order to limit deformation of the muscle, a custom-made curved gel pad, named the Portico, was attached to the ultrasound transducer (17). The ultrasound acquisition settings (frequency: 8 MHz; depth: 50 mm; focus: 30 mm; gain: 64%; dynamic range: 56 dB; power: 100%; dynamic focus: on; line density: low and time-gain compensation: neutral (50%)) remained constant during the study.

One experienced assessor performed all data acquisitions. Images were recorded and three-dimensional reconstructions were generated by Stradwin software (version 6.0, Medical Imaging Group, Cambridge University Engineering department, UK). During the acquisition, the ultrasound transducer was slowly moved from the muscle origin until the tendon insertion. A second assessor performed a real-time check on bony landmark visibility and checked if the muscle borders were visible on the images. In case the muscle's width exceeded the width of the transducer, two parallel ultrasound sweeps were performed, further referred to as the multiple sweep technique. Those two parallel sweeps were combined in a single three-dimensional reconstruction of the muscle belly, allowing to visualize the full width of the muscle.
In case of subject movement, muscle contraction or bad quality data, the acquisition was repeated.

General measurement setup and three measurement conditions

The MG, RF and ST were assessed at a resting position (i.e. the spontaneous joint angles in the prone or supine position, representing the resting muscle-tendon unit length), at a common position (i.e., proximal and distal joint angles that were similar for all participants) and at a maximum position (i.e. at the maximal distal joint excursion while keeping the proximal joint in a fixed position, representing the maximal muscle-tendon unit length). These positions were accomplished by adjusting the proximal and distal joint angles. The three conditions are illustrated in Figure 1 and Figure 2 and all joint angles are described in Table 2.

The MG was assessed in prone position, while the foot was fixated in a custom-made orthosis, allowing slight knee flexion. The RF and ST were assessed in a setup that was developed by Haberfehlner and colleagues (18), in which the participant was positioned on his/her side, while the trunk was supported by two pillows that were fixated to the side of the bed. The assessed limb was place on a custom-made low-friction movable table, with the hip in neutral position (no ab-or adduction), while the knee and foot were supported by padded supports. The supports were adjusted to the leg length if necessary. Joint angles could be easily adjusted by sliding the limb over the low-friction surface and were measured and checked by goniometry.

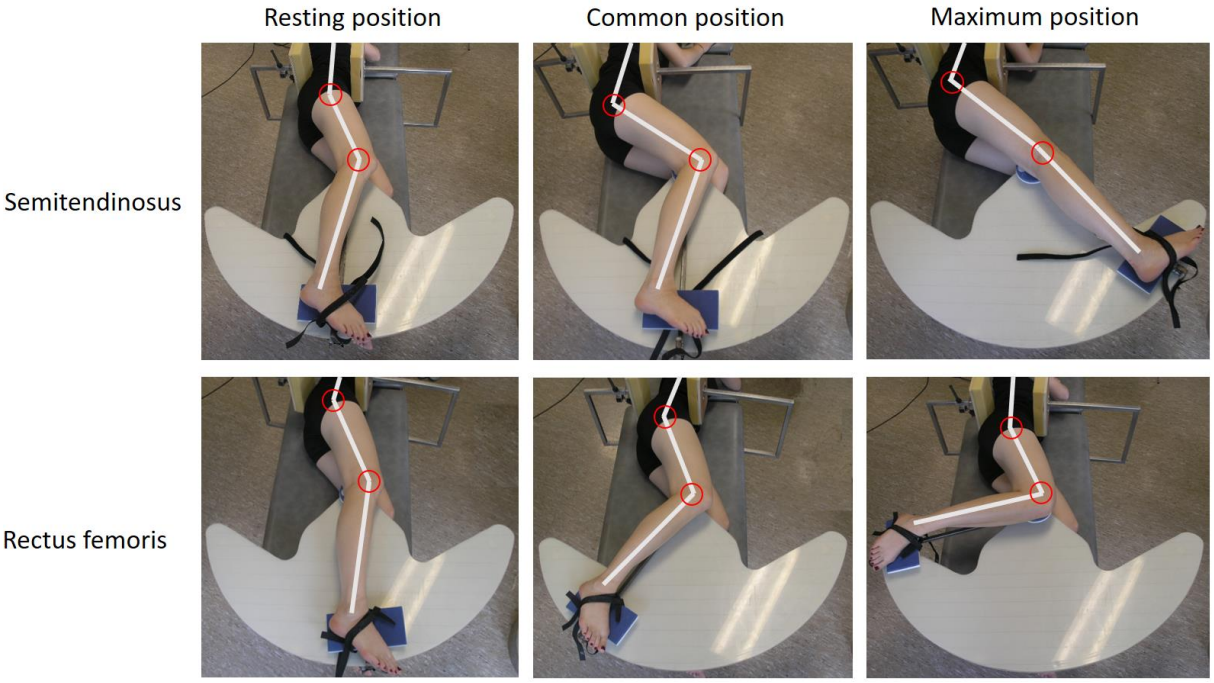

Figure 1. Different limb position during assessments of the semitendinosus and rectus femoris

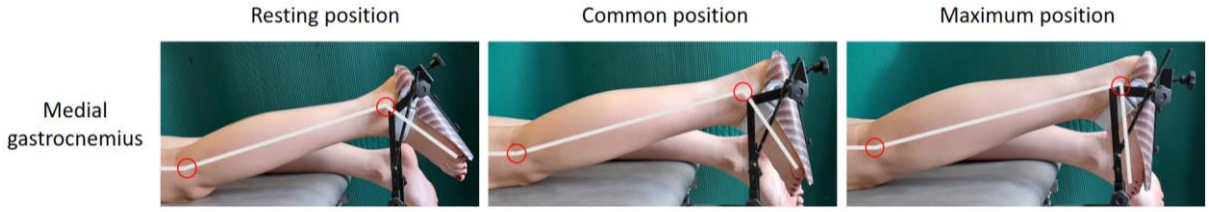

Figure 2. Different limb position during assessment of the medial gastrocnemius

To ensure a similar definition of the resting position as reported in previous studies of our research group (1,2,4–7,9,19–22), each participant was first positioned in prone or supine position, while the lower limb was supported by a triangular cushion in order to avoid bi-

articular stretch on the muscle belly. These joint angles were measured and simulated in the side position. This position represented the spontaneous joint angles in the starting position, and was thus subject specific.

The common position consisted of predefined joint angles that were feasible for both the SCP and TD cohort. For the MG, the common joint angles was 25° knee flexion and 10° ankle plantar flexion. For the RF and ST, the common joint angles were 20° and 70° hip flexion, respectively, and 70° knee flexion for both muscles. These angles were defined based on literature and input of the clinical experts.

The maximum position, which was subject-specific, represented the maximal stretch on the muscle-tendon unit that was tolerated by the participant.

Data processing

All files were coded before being analyzed. Therefore, the condition was unknown for the processors.

Data was processed by two trained assessors (each assessor analyzed a full dataset of all assessments for one muscle) using Stradwin software (version 6.0, Medical Imaging Group, Cambridge University Engineering department, UK). The primary outcomes were muscle volume, muscle belly length and echogenicity intensity. Muscle volume was estimated based on manual segmentations in approximately 5-10% of the ultrasound images using the cubic planimetry technique (23). Additionally, the echogenicity intensity was estimated based on the average of the segmented images used for the three-dimensional reconstruction of the full muscle belly (24). The echogenicity intensity, or gray value of the muscle image, was expressed on an 8-bit grayscale (256 values), of which black corresponded to a value of zero, and white corresponded to a value of 255. Muscle belly length was estimated as the distance between the muscle origin and muscle-tendon junction (MTJ).

For participant descriptive data (Table 1), muscle volume, assessed in the resting condition, was normalized to body mass*height and resting muscle belly length was expressed as a percentage of the total leg length.

Patient friendliness

A short questionnaire, including ten questions, was completed after the data collection, to verify how the patients experienced the ultrasound assessments. Questions were answered on a five point Likert scale, ranging from “strongly agree” to “strongly disagree”, supported by smiley and frowny faces.

The questions and participants’ responses are presented in Table 4.

Statistical analyses

Statistical analyses were performed using SPSS software (version 28.0, International Business Machines Corporation, Armonk, NY). Normality of data distribution was evaluated using a Kolmogorov-Smirnov test. As some parameters were not normally distributed, only non-parametric statistical tests were used and all values were presented as median with corresponding 25th and 75th percentiles.

The difference in muscle parameters between the three conditions (resting, common and maximum) were compared by a Friedman test for the MG, ST and RF. Alpha levels were set at 0.008 after application of the Bonferroni correction within each muscle for three outcomes (muscle volume, muscle belly length and echogenicity intensity) and two groups (TD and SCP) (alpha 0.05/6 = 0.008). Any differences with a p-value lower than 0.05, but not exceeding the 0.008, were labeled as trends or tendencies towards significance. If the outcome of the Friedman test was significant, a post hoc Wilcoxon Signed Ranks test was performed to individually compare the different conditions. The alpha levels of the post-hoc test were set at alpha 0.05.

The results of the patient friendliness questionnaire were described descriptively.

Results

In total, 14 TD and 13 children with SCP were included in this study. One TD child was excluded, because the muscles were difficult to visualize due to their size and the thickness of the subcutaneous tissue.

In total, four ultrasound files had to be excluded due to bad quality data following the multiple sweep technique, as the two parallel sweeps could not be aligned in the three-dimensional reconstruction. These excluded files were two MG files from the TD cohort and one MG and one RF file from the SCP cohort.

Data on the participant characteristics and descriptive data of muscle morphology in the resting condition are summarized in Table 1.

Table 1. Participant characteristics and muscle morphology outcomes and echogenicity intensity as assessed in a resting condition

	TD (n=13)	**SCP (n=13)**
Age (years)	11.10 (9.03 – 12.22)	14.27 (7.35 – 15.13)
Body mass (kg)	34.10 (27.05 – 43.95)	44.20 (22.65 – 55.00)
Height (cm)	147.25 (134.73 -159.88)	152.50 (123.00 – 164.75)
Leg length (cm)	75.80 (64.95 – 83.70)	78.00 (60.90 – 88.35)
GMFCS level (n)	n.a.	I = 7, II = 5, III = 1
Paralysis (n)	n.a.	unilateral = 3, bilateral = 10
Normalized muscle volume MG (ml/kg*m)	1.83 (1.61 – 1.99)	1.10 (0.95 – 1.56)
Normalized muscle volume ST (ml/kg*m)	0.81 (0.63 – 0.95)	0.72 (0.64 – 0.76)
Normalized muscle volume RF (ml/kg*m)	2.28 (1.89 – 2.54)	1.65 (1.34 – 2.04)
Normalized muscle belly length MG (%)	13.25 (12.38 – 13.92)	12.36 (11.37 – 13.01)
Normalized muscle belly length ST (%)	13.21 (11.91 – 13.84)	11.90 (10.75 – 12.31)
Normalized muscle belly length RF (%)	18.09 (17.53 – 19.14)	17.57 (16.68 – 19.26)
Echogenicity intensity MG (AU)	143.14 (132.37 – 148.88)	136.60 (128.15 – 145.40)
Echogenicity intensity ST (AU)	132.80 (113.60 – 145.30)	134.60 (117.30 – 150.10)
Echogenicity intensity RF (AU)	117.43 (96.70 – 130.52)	115.07 (104.06 – 124.40)

AU = arbitrary units, cm = centimeters, GMFCS = gross motor function classification level, kg = kilograms, m = meters, MG = medial gastrocnemius, ml = millimeters, n.a. = not applicable, RF = rectus femoris, SCP = spastic cerebral palsy, TD = typically developing

Joint angles

An overview of the joint angles in the different measurement positions is included in Table 2.

The majority of joint angles was significantly different between the different conditions.

Table 2. Joint angles at the different measurement positions

				Angles (◦)			p-value		
				p25	median	p75	resting - common	resting - maximum	common - maximum
MG	Knee (flexion)	Resting	TD	20	20	22.5	0.033*	0.033*	1.000
			SCP	20	20	25	0.013*	0.007*	1.000
		Common	TD		25				
			SCP						
		Maximum	TD		25				
			SCP	25	25	25			
	Ankle (DF)	Resting	TD	-35	-30	-30	0.001*	0.001*	0.001*
			SCP	-35	-30	-17.5	0.003*	0.002*	0.007*
		Common	TD		-10				
			SCP						
		Maximum	TD	15	20	25			
			SCP	-7.5	10	15			
ST	Hip (flexion)	Resting	TD	20	30	37.5	0.001*	0.001*	1.000
			SCP	35	35	40	0.001*	0.001*	1.000
		Common	TD		70				
			SCP						
		Maximum	TD		70				
			SCP						
	Knee (flexion)	Resting	TD	25	30	40	0.002*	0.002*	0.001*
			SCP	32.5	40	42.5	0.001*	0.001*	0.001*
		Common	TD		70				
			SCP						
		Maximum	TD	0	0	15			
			SCP	0	10	20			
RF	Hip (flexion)	Resting	TD	20	20	25	0.107	0.107	1.000
			SCP	17.5	20	25	0.527	0.527	1.000
		Common	TD		20				
			SCP						
		Maximum	TD		20				
			SCP						
	Knee (flexion)	Resting	TD	25	30	32.5	0.001*	0.001*	0.001*
			SCP	30	30	35	<0.001*	0.001*	0.001*
		Common	TD		70				
			SCP						
		Maximum	TD	95	100	100			
			SCP	95	100	107.5			

*Angle angles are presented as degrees of dorsiflexion. *alpha<0.05*

DF = dorsiflexion, MG = medial gastrocnemius, RF = rectus femoris, SCP = spastic cerebral palsy, TD = typically developing

The results of the repeated measures Friedman test are included in Figure 3 for the TD children and Figure 4 for the SCP cohort. The descriptive data of the different outcomes per condition and the results of the post-hoc test are summarized in Table S1 that is enclosed in the supplementary material.

Muscle volume

Within the TD cohort, the muscle volume of the MG was significantly different between the three conditions (p=0.004). The post-hoc test revealed that the muscle volume in the resting condition was significant smaller compared to the common position (p = 0.010) and maximum position (p=0.003). Furthermore, the muscle volume of the RF significantly differed between the three positions (p=0.007). The post-hoc test showed that the muscle volume estimation in the resting condition was significantly smaller than the maximum position (p = 0.003). Finally, there was a tendency towards a significant larger muscle volume of the distal compartment of the ST for common compared to the resting (Friedman p = 0.050, post-hoc p = 0.021).

In the SCP cohort, there was a tendency towards significant differences in muscle volume of the MG (p = 0.018), pointing towards a slightly higher volume in the maximum position compared to both other positions (resting – maximum p = 0.013, common – maximum p = 0.008).

Muscle belly length

With exception of the distal compartment of the ST (p = 0.223) in the TD cohort and the MG in the SCP cohort (p = 0.009), all other muscles showed significant differences in muscle belly length between the conditions. However, there was a strong tendency (p = 0.009) towards lengthening in the MG muscle belly of the SCP cohort. The longest muscle belly lengths were always reported in the maximum position.

Echogenicity intensity

The echogenicity intensity values showed a tendency towards significant differences between the three measurement positions in the MG (p = 0.020) of the TD cohort and the RF (p = 0.028) of the SCP cohort. However, these tendencies did not show a consistent direction of changes.

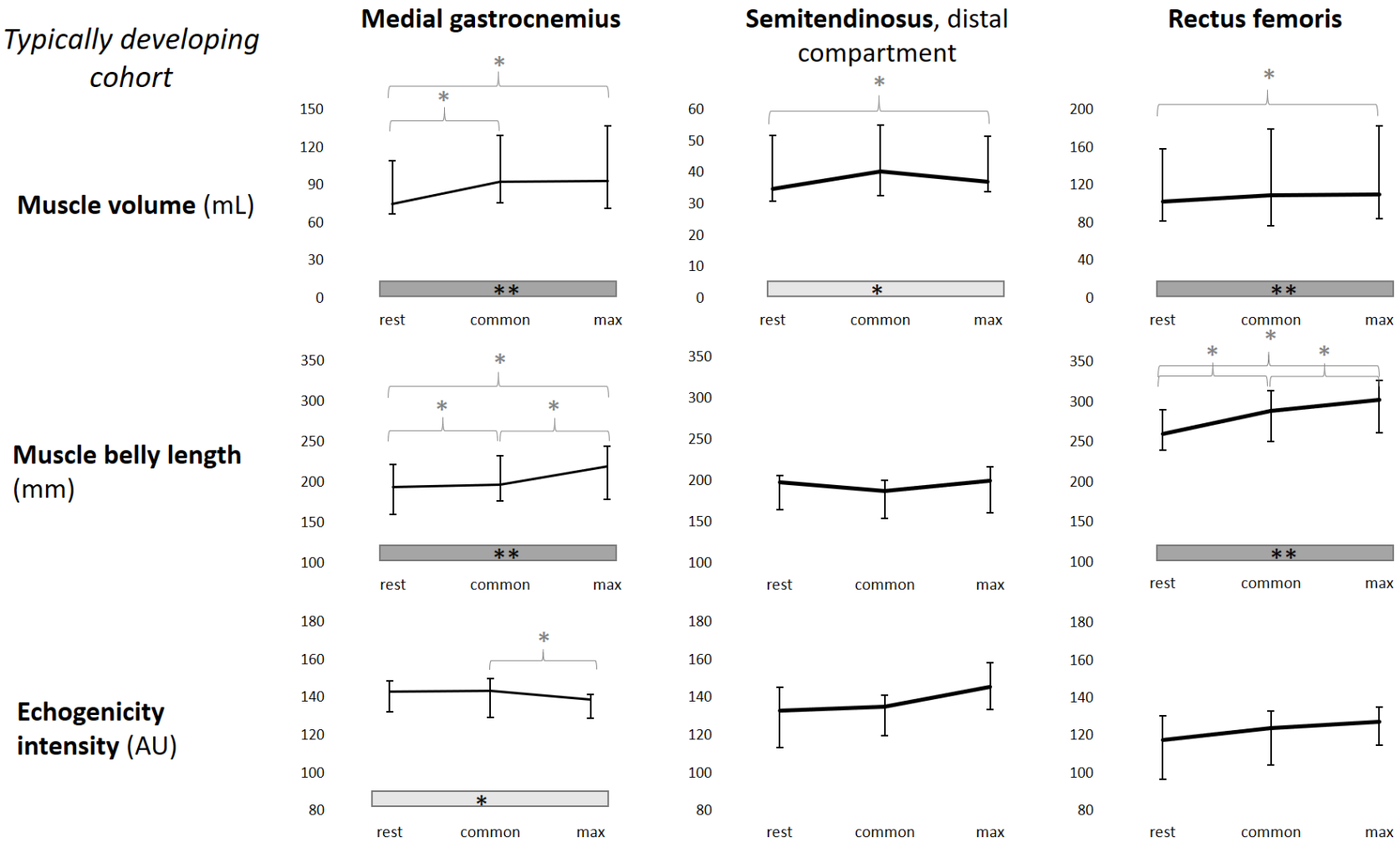

Figure 3. Results of the Friedman test and corresponding post-hoc test of the differences in muscle morphology and composition outcomes between the three conditions, for the three different muscles of the typically developing cohort. Values are presented as median values with corresponding interquartile ranges.

The dark gray bars indicate significant differences (alpha<0.008). The light gray bars indicate trends (alpha<0.05). The braces at the top of the images reflect the results of the post-hoc tests.

***alpha<0.008, *alpha<0.05* *AU = arbitrary units, mL = milliliters, mm =*

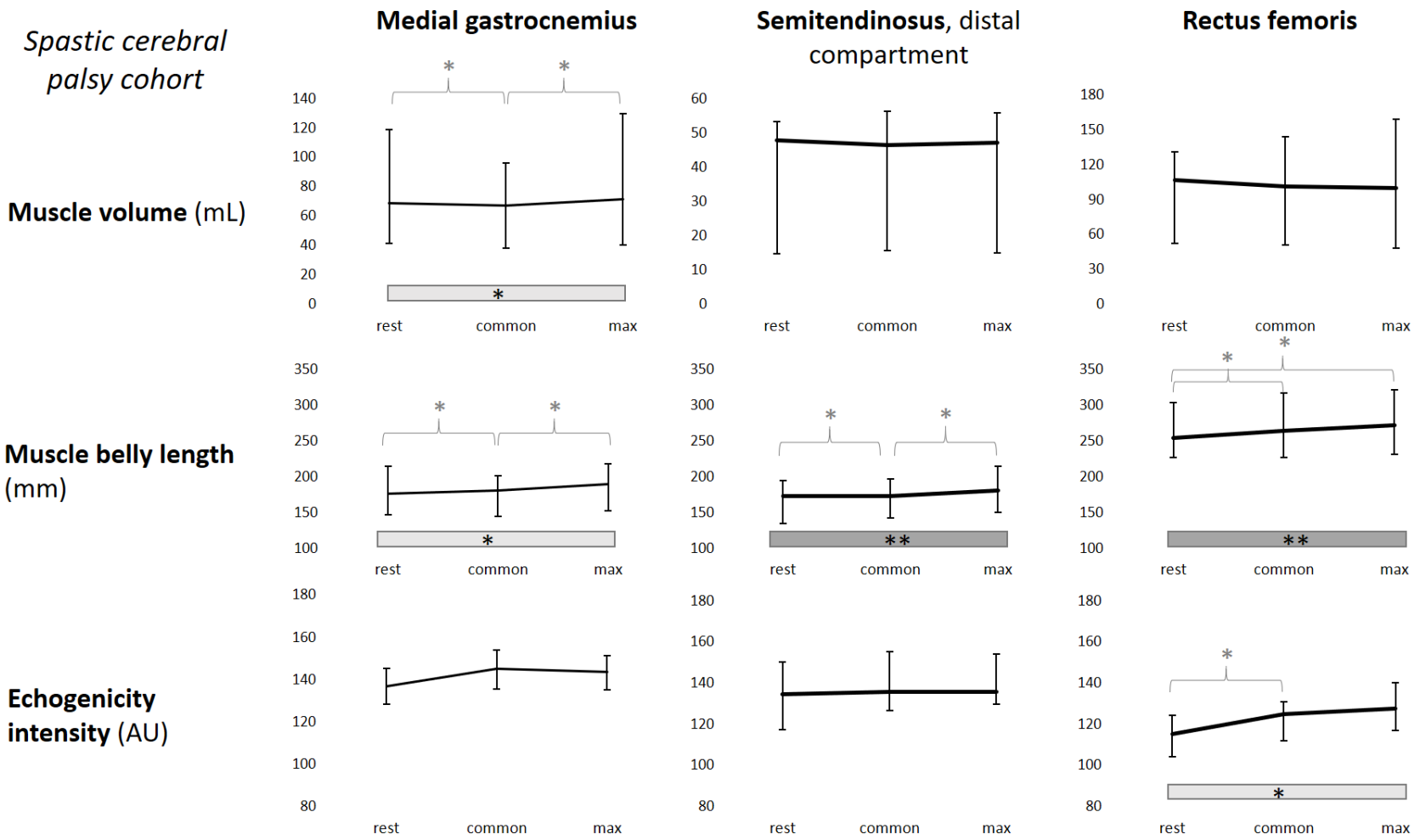

Figure 4. Results of the Friedman test and corresponding post-hoc test of the differences in muscle morphology and composition outcomes between the three conditions, for the three different muscles of the spastic cerebral palsy cohort. Values are presented as median values with corresponding interquartile ranges.

The dark gray bars indicate significant differences (alpha<0.008). The light gray bars indicate trends (alpha<0.05). The braces at the top of the images reflect the results of the post-hoc tests.

***alpha<0.008, *alpha<0.05*

AU = arbitrary units, mL = milliliters, mm = millimeters

Patient friendliness

A subset of eight SCP children (median age 7.46 years) filled in a patient friendliness questionnaire. The results per question are summarized in Table 4.

Table 4. Results of the patient friendliness questionnaire

	strongly agree	agree	neutral	disagree	strongly disagree
1. I did not find the measurement unpleasant	25%	37.5%	0%	37.5%	0%
2. The ultrasound assessment did not hurt	50%	37.5%	0%	0%	12.5%
3. I was not afraid of the ultrasound	87.5%	0%	0%	0%	12.5%
4. It was easy to lay in the right position for the measurement					
A. It was comfortable to lay on my stomach	75%	12.5%	12.5%	0%	0%
4. It was easy to lay in the right position for the measurement					
B. It was comfortable to lay on my side	50%	12.5%	25%	12.5%	0%
5. I felt safe during the ultrasound measurements	62.5%	12.5%	12.5%	12.5%	0%
6. The ultrasound measurements went quick	50%	0%	12.5%	37.5%	0%
7. It was easy to lay still	75%	0%	12.5%	0%	12.5%
8. I did not find the ultrasound gel unpleasant	50%	37.5%	0%	0%	12.5%
9. The researcher explained very well what he/she was going to do	87.5%	12.5%	0%	0%	0%
10. I could ask questions to the researcher	75%	25%	0%	0%	0%

Discussion

The aim of this investigation was to gain more insight into the influence of joint angles on muscle morphology parameters assessed by 3DfUS. The hypothesis, stating that muscle belly volume and echogenicity intensity would not be influenced by changing joint angles, whereas muscle belly length would, was only partly confirmed. The muscle volumes of the MG and RF in the TD cohort were significantly higher in the maximum or common position compared to the resting position. The majority of muscle belly lengths indeed significantly increased when increasing ankle dorsiflexion (for the MG) or knee angles (towards more extension for the ST and towards more flexion for the RF), except for the length of the ST's distal compartment in the TD cohort and the MG in the SCP cohort, which did not differ between the conditions.

Muscle volume

The alterations in muscle volume were unexpected. All significant differences were compared to previous defined intra-acquirer, intra-session standard error of measurement (SEM) estimations, and all reported differences in muscle volume exceeded those SEM values (19). An overview of the SEM values is enclosed in Table S1 of the supplementary material.

The observed increased muscle volume in the TD cohort in the common (MG: +5.9%, RF: +3%, Table S1. supplementary materials) or maximum position (MG: +11.5%, RF: +7.3%, Table S1. supplementary materials) compared to the resting position could potentially be explained by differences in performance of the measurements or data processing. Indeed, the quality of the images can be influenced by the degree of stretch on the muscle belly. From our own experience, we learned that when more stretch is applied to the muscle belly, the muscle borders are easier to recognize on the ultrasound images and the muscle is less likely to deform due to pressure application during the acquisition. The current results thus suggest that muscle volume estimations based on ultrasound images of a resting muscle may potentially be less accurate. It should be noted that the TD dataset of MG and RF resting data included several multiple sweeps. In case of unequal pressure between the two separate sweeps, the overlap of the sweeps in the reconstruction may not always have been optimal. This could be a likely explanation for the differences in muscle volume. Additionally, it was found to be more challenging to perform the multiple sweep technique and control the

amount of applied pressure while the participant was positioned on his/her side compared to supine or prone (where the muscle belly is more easily accessible). Besides that, Barber et al. showed that the use of the ultrasound multiple sweep technique resulted in inconsistent muscle volume estimations in comparison to magnetic resonance imaging (MRI) (25). A validation study of the multiple sweep technique is thus needed.

To the best of our knowledge, there is only one study that compared three-dimensional MG muscle architecture defined by MRI at different lengths (26). They reported a small increase in muscle volume (1.6%) in a stretched, maximum position, in comparison to the volumes in a resting condition. They hypothesized that these alterations could be due to a change of capillary blood flow during stretch or errors in the segmentation process. We assume that the potential changes in muscle volume due to potential alterations in blood flow are within the measurement errors of the 3DfUS method. Therefore alterations in muscle volume are most likely due to data acquisition errors or differences in data processing due to varying degrees of muscle border visibility between the conditions.

Muscle length

As expected, most muscle belly lengths were influenced by changing the adjacent joint angles. The longest muscle belly lengths were observed in the maximum ankle dorsiflexion (MG length TD cohort: +13.4%) or knee extension (ST length SCP cohort: +6.0%) or knee flexion angles (RF length SCP cohort: 7.0%), confirming that the muscles were effectively stretched in both groups. For children with SCP, previous research indicated that it may be challenging to achieve a proper stretch on the gastrocnemius through increasing dorsiflexion angles, because the Achilles tendon was found to be longer and more compliant, which might result in less stretch transfer to the muscle belly (27). There was only a tendency towards MG muscle belly lengthening and not all changes exceeded the pre-defined SEM (Table S1. Supplementary material)(19). The distal compart of the ST did not show any change in the TD cohort and only limited alterations in the SCP cohort (ranging between 2.0% and 6.0% in comparison to the resting length) when the knee extension angles were changed. As only the distal compartment of the ST was analyzed, no conclusion can be drawn on the lengthening behavior of the total ST muscle belly. A previous study by Haberfehlner et al. showed only minor differences in the total ST muscle belly length between ultrasound assessments at 65 degrees knee flexion, or

at the knee angle that represented 0 or 4 Newton meters joint torque (18). The hip remained at 70 degrees of flexion during the acquisition, which was similar to our study in the common and maximum position. These findings might suggest that the used setup and fixed hip angle were not suitable to sufficiently stretch the ST. Indeed, most TD children were able to reach full knee extension while the hip was fixated at 70 degrees. Therefore, larger hip flexion angles might be required to sufficiently stretch the ST in TD children.

Echogenicity intensity

The unexpected small (tendencies) differences in echogenicity intensity between the three positions were potentially due to the performance of the assessor (24). The fact that these changes were small and not characterized by a common direction for different muscles and for increasing joint angles per muscle suggested that these observations may be primarily allocated to the reliability of this outcome. Indeed, Dankel et al. showed that tilting of the ultrasound transducer strongly influences the echogenicity intensity values and negatively affected test-retest reliability (28). The tilting of the transducer was quite difficult to control in side-lying. Two previous studies (by Tomko et al. and by Varanoske et al.) showed that participant position could also influence the echogenicity intensity of the muscle images (29,30). Additionally, Obst et al. showed that echogenicity intensity is related to muscle thickness and recommended to normalize the echogenicity intensity value to muscle thickness, when comparing populations with different muscle geometries (31).

Patient friendliness

Previous studies showed that ultrasound and more specifically 3DfUS is an easy applicable and child-friendly method (8,32). The patient friendliness questionnaire that was designed for this study showed that children were comfortable during the measurements and that both, the side and prone position were well tolerated. The children indicated that the measurements were pleasant and painless. The only negative experience was the duration of the assessments, which was often considered too long. This was potentially due to the fact that the assessments of the current study were part of a larger project involving additional assessments in other conditions. Under normal circumstances one ultrasound sweep takes approximately 30 seconds. A full assessment of one lower leg muscle, including explaining the procedure to the participant, preparing the participant and the setup, and taking at least two

repeated ultrasound sweeps, takes around 15 minutes and is usually well tolerated by the participants.

Recommendations for future muscle ultrasound studies

Based on our experiences during data collection, we can conclude that assessments of the ST and RF morphology in side lying were well tolerated by both TD children and children with SCP. The joint angles were easy to adjust when the participant was positioned on the side, allowing measurements in different limb positions. However, the multiple sweep technique was more challenging to perform while the participant was positioned on his/her side. This was due to less visual control of the transducer, reduced control on transducer tilting, and because it was more challenging to maintain good skin contact with equal distribution of pressure. Despite the use of the portico, which was developed to limit muscle deformity, the 3D reconstructions with overlay of the two parallel sweeps was still not optimal (17). Therefore, assessments of large muscles requiring multiple sweeps to cover the muscle's width are preferably not performed while the participant is lying on his/her side.

Besides the more challenging multiple sweeps, the tendon lengths of the ST and RF were also more difficult to be defined in side lying compared to prone or supine position. Therefore, no data on tendon lengths were included. The padded supports for the knee and foot often limited the visualization of the tendinous insertion and also the most distal part of the ST, close to the MTJ. Due to the high knee flexion angles during the assessments of the RF, the patella and patella tendon were difficult to visualize, because the markers on the transducer could not always be well-tracked when moving over the flexed knee, when using a motion capture system that was configured with only three camera's at a fixed position. Therefore, future imaging studies aiming to investigate tendons or curved structures (large flexion angles) should consider the use of a more advanced three-dimensional motion tracking systems consisting of more cameras. However, the assessments of muscle lengths were of good quality and the side position is shown to be suitable for assessment of muscle belly lengths in different positions.

The measurement setup for the MG has been used in previous studies and shown to be well tolerated and useful to assess planar flexion muscle at different levels of stretch (33–37).

In conclusion, we recommend to perform assessments of muscle volume or muscle composition in prone or supine position, as the assessments in side position were more challenging and potentially less accurate. The side position is useful to assess muscles at different lengths and different levels of stretch. However, future studies should consider the use of joint torque assessments (as will be discussed in the limitations) to further standardize the joint positions. Additionally, joint and limb fixation should be improved and proximal joint angles reconsidered in order to optimally stretch the muscle bellies during ultrasound assessments.

Limitations

Some limitations have to be acknowledged. First, the maximum position was the maximum stretch that was tolerated by the participant. Because this is a subjective feature, it is difficult to define if the actual maximal position was reached and if this condition was similar for all children. Secondly, it should be noted that some TD children had a larger joint ROM, such that their muscle was not really stretched when achieving the maximal joint position the setup could be adjusted to. This was specifically the case for the maximum knee extension and maximum knee flexion angles in order to stretch the ST and RF, respectively. In those cases, the assessment was thus an approximation of the maximum position.

Furthermore, two pillows that were attached to the side of the bed supported the trunk while the participants were positioned on their side. However, the pelvis was not fixated in this measurement setup. Therefore it might have been possible that the pelvic tilt was slightly different between the different conditions in which the ST was assessed. Specifically in the maximum position, in which the knee was moved towards maximum tolerated extension, posterior pelvic tilt might have occurred. Future studies investigating proximal lower limb muscles and aiming to standardize their measurement setup should consider a better fixation of the pelvis.

Additionally, the repeatability of 3DfUS in these standardized positions should be further explored.

In the current study, the common position was defined by common hip, knee and ankle angles. These predefined positions were based on joint angles that were tolerated by both the TD and SCP cohort. The current results confirmed that those joint angles were feasible for both the

TD and SCP cohort. However, muscles from some children might have been barely stretched, whereas muscles from other children were nearly maximally stretched at these common angles. Therefore, future studies could add additional assessments at a common torque, for example 4 Newton meters, like previous studies did (18,37). This common torque condition might be easier to standardize and more relevant to be linked to the muscle's behavior during functional tasks, such as during gait. Standardization by torque is considered specifically interesting for studies that are primarily investigating muscle extensibility, like stretching interventions. Additionally, the resting conditions could be further standardized and tendon slack reduced by a minimal torque, for example 0.5 Newton meters. Furthermore, torque measurements can be used to quantify and compare the level of stretch in a maximum position.

Unfortunately, no data on tendon lengths and the full ST muscle were included. Therefore, no conclusions can be drawn on the behavior of tendon of the three muscles and the ST as a whole when adjusting joint angles.

Conclusion

This study showed that adjacent joint angles could influence lower limb muscle morphology outcomes defined by 3DfUS. Most muscle belly lengths changed when changing the joint angles, as expected. However, unexpected alterations in muscle volume were reported in the TD cohort. The results highlighted the need for standardized measurement setups and clear documentation of adjacent joint angles of the assessed muscle. Muscle morphology assessments while the participants were positioned on their side were well-tolerated by all participants. However, assessments of muscle volume were challenging in this side position, whereas it was suitable for examination of muscle lengths at different joint angles for most muscles. These recommendations are essential for further clinical implementation of muscle imaging techniques, as they will facilitate comparison of data within the same patient over time.

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Supplementary Material

Table S1. Comparison of muscle morphology outcomes assessed in different positions

Muscle volume (ml)		**resting position** median, p25-p75	**common position** median, p25-p75	**maximum position** median, p25-p75	**Friedman test**	**Post-hoc test**	**percentage change**[i]	**SEM**
Medial gastrocnemius	**TD**	74.79 (66.68 – 109.31)	92.23 (75.94 – 129.55)	93.12 (71.54 – 136.81)	0.004**	resting – common p = 0.010* resting – maximum p = 0.003* common – maximum p = 0.209	5.9 % 11.5 % 5.9 %	5.12
	SCP	68.90 (41.30 – 118.85)	67.05 (38.17 – 95.98)	71.60 (40.25 – 129.99)	0.018*	resting – common p = 0.638 resting – maximum p = 0.013* common – maximum p = 0.008*	1.0 % 4.6 % 5.0 %	5.43
Semitendinosus, distal compartment	**TD**	34.60 (30.90 – 51.80)	40.20 (32.60 – 55.00)	36.90 (33.80 – 51.50)	0.050*	resting – common p = 0.483 resting – maximum p = 0.021* common – maximum p = 0.889	6.7 % 7.1 % 1.0 %	1.81
	SCP	47.70 (14.60 – 53.20)	46.35 (15.68 – 56.18)	47.00 (14.90 – 55.80)	0.905	n.a.	1.2 % -1.4 % -1.6 %	2.10
Rectus femoris	**TD**	102.24 (81.83 – 158.07)	109.19 (76.49 – 179.37)	109.89 (84.20 – 182.64)	0.007**	resting – common p = 0.087 resting – maximum p = 0.003* common – maximum p = 0.116	3.0 % 7.3 % 8.9 %	3.31
	SCP	106.43 (51.95 – 131.28)	100.99 (50.82 – 144.23)	100.01 (48.35 – 158.84)	0.472	n.a.	0.9 % 2.5 % -0.6 %	2.59
Muscle belly length (mm)		**resting position** median, p25-p75	**common position** median, p25-p75	**maximum position** median, p25-p75	**Friedman test**	**Post-hoc test**		**SEM**
Medial gastrocnemius	**TD**	193.91 (159.79 – 221.83)	196.63 (177.07 – 233.06)	219.82 (179.05 – 244.29)	<0.001**	resting – common p = 0.001* resting – maximum p = 0.001* common – maximum p = 0.003*	6.1 % 13.4 % 5.4 %	2.06
	SCP	176.50 (146.45 – 214.35)	181.40 (145.18 – 201.41)	189.70 (152.25 – 218.30)	0.009*	resting – common p = 0.814 resting – maximum p = 0.005* common – maximum p = 0.006*	0.6 % 5.9 % 5.8 %	3.63

Semitendinosus, distal compartment	**TD**	**198.30 (165.30 – 206.10)**	**187.70 (154.35 – 199.95)**	**199.70 (161.10 – 216.30)**	**0.223**	**n.a.**	**-6.5 % 3.9 % 6.7 %**	**3.04**
	SCP	173.60 (134.10 – 194.90)	173.20 (141.90 – 196.65)	180.70 (149.70 – 214.80)	0.004**	resting – common p = 0.953 resting – maximum p = 0.004* common – maximum p = 0.008*	2.0 % 6.0 % 4.8 %	6.42
Rectus femoris	**TD**	259.72 (239.15 – 290.37)	289.04 (250.80 – 314.36)	302.08 (261.65 – 326.56)	<0.001**	resting – common p = 0.001* resting – maximum p = 0.001* common – maximum p = 0.002*	6.3 % 11.0 % 4.4 %	5.10
	SCP	253.90 (226.90 – 303.96)	264.87 (226.51 – 316.83)	272.00 (231.74 – 321.56)	<0.001**	resting – common p = 0.007* resting – maximum p = 0.004* common – maximum p = 0.064	6.6 % 7.0 % 2.1 %	8.42
Echogenicity intensity (AU)		**resting position** median, p25-p75	**common position** median, p25-p75	**maximum position** median, p25-p75	**Friedman test**	**Post-hoc test**		**SEM**
Medial gastrocnemius	**TD**	143.14 (132.37 – 148.88)	143.32 (129.49 – 150.13)	138.74 (128.97 – 141.73)	0.020*	resting – common p = 0.272 resting – maximum p = 0.075 common – maximum p = 0.034*	2.1 % -4.1 % -4.3 %	2.44
	SCP	136.60 (128.15 – 145.40)	145.10 (135.30 – 153.78)	143.50 (135.20 – 151.45)	0.205	n.a.	2.3 % 3.7 % 1.0 %	2.88
Semitendinosus, distal compartment	**TD**	132.80 (113.60 – 145.30)	135.10 (119.55 – 141.15)	145.50 (133.40 – 158.60)	0.066	n.a.	0.3 % 4.7 % 4.1 %	5.01
	SCP	134.60 (117.30 – 150.10)	135.85 (126.38 – 155.20)	135.50 (129.80 – 154.20)	0.273	n.a.	1.7 % 2.6 % 1.2 %	5.79
Rectus femoris	**TD**	117.43 (96.70 – 130.52)	123.79 (104.21 – 133.00)	127.21 (114.61 – 134.99)	0.092	n.a.	4.3 % 8.2 % 1.4 %	5.53
	SCP	115.07 (104.06 – 124.40)	124.80 (111.70 – 130.95)	127.60 (116.79 – 140.02)	0.028*	resting – common p = 0.015* resting – maximum p = 0.055 common – maximum p = 0.136	4.2 % 9.6 % 2.6 %	3.01

AU = arbitrary units, ml = milliliters, mm = millimeters, n.a. = not applicable, SCP = spastic cerebral palsy, SEM = intra-acquirer, intra-session standard error of measurement TD = typically developing. [i]*The median percentage change. Changes are expressed as a median percentage of the resting values. In case of a comparison between the common and maximum position, the change was expressed as a percentage of the values from the common position.* ***alpha-level after Bonferroni correction, alpha<0.05/6 = 0.008* **alpha<0.05*

Objective 2.

Generate a clinical and normative database of muscle morphology data and explore the association between these muscle morphology parameters and measures of spasticity

Chapter 2.2.

A Comprehensive Normative Reference Database of Muscle Morphology in Typically Developing Children Aged 3-18 years – A Cross Sectional Ultrasound Study

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Abbreviations

ANCOVA = analysis of covariance

B-mode = brightness mode

cm = centimeters

dB = decibels

kg = kilograms

MG = medial gastrocnemius

mHz = megahertz

mm = millimeter

n = number

n.a. = not applicable

p = p-value

P-P = predicted probability plot

r = correlation coefficient

R^2= coefficient of determination

RF = rectus femoris

ST = semitendinosus

TA = tibialis anterior

3DfUS = three-dimensional freehand ultrasound

Keywords: Typically developing children, ultrasound, muscle morphology, muscle belly length, muscle volume

Abstract

During childhood, muscle growth is stimulated by gradual increase in bone length and body mass, as well as by other factors, such as physical activity, nutrition, metabolic, hormonal and genetic factors. Muscle characteristics, such as muscle volume, anatomical cross-sectional area and muscle belly length, need to continuously adapt to meet the daily functional demands. Pediatric neurological and neuromuscular disorders, like cerebral palsy and Duchenne muscular dystrophy, are characterized by hampered muscle development, which requires treatment and close follow-up. Nowadays ultrasonography is a commonly used technique to evaluate muscle morphology in both pediatric pathologies and typically developing children, as it is a quick, easy applicable and painless method. However, large normative datasets including different muscles and a large age range are lacking, making it challenging to monitor muscle over time and estimate the level of pathology. Moreover, in order to compare individuals with different body sizes as a result of age differences or pathology, muscle morphology data are often normalized to body size. Yet, the efficiency of different normalization techniques is still unknown, and clear recommendations for normalization are lacking.

In this cross-sectional cohort study, muscle morphology of four lower limb muscles (medial gastrocnemius, tibialis anterior, the distal compartment of the semitendinosus, rectus femoris) was assessed by three-dimensional freehand ultrasound in 118 typically developing children (mean age 10.35 ± 4.49 years) between 3 and 18 years of age. The development of muscle morphology was studied over the full age range, as well as separately for the pre-pubertal (3-10 years) and pubertal (11-18 years) cohorts. The assumptions of a simple linear regression were checked. If these assumptions were fulfilled, the cross-sectional growth curves were described by a simple linear regression equation. Additional ANCOVA analyses were performed to evaluate muscle- or gender-specific differences in muscle development. Furthermore, different scaling methods, to normalize muscle morphology parameters, were explored. The most appropriate scaling method was selected based on the smallest slope of the morphology parameter with respect to age, with a non-significant correlation coefficient. Additionally correlation coefficients were compared by a Steiger's Z-test to identify the most efficient scaling technique.

The current results revealed that it is valid to describe muscle volume (with exception of the rectus femoris muscle) and muscle belly length alterations over age by a simple linear regression equation till the age of 11 years. Normalizing muscle morphology data by allometric scaling was found to be most efficient for comparing muscle volumes of different pediatric populations. For muscle lengths, normalization can be achieved by both allometric and ratio scaling.

This study provides a unique normative database of four lower limb muscles in typically developing children between the age of 3 and 18 years. These data can be used as a reference database for pediatric populations and may also serve as a reference frame to better understand both physiological and pathological muscle development.

Introduction

During childhood, the gradual increase in bone length and body mass will stimulate muscle growth (1–4). Muscle characteristics such as muscle volume, anatomical cross-sectional area and muscle belly length need to continuously adapt to meet the daily functional demands (5–10). Muscle growth is related to changes in age, body mass and height (4,11,12) and is also influenced by physical activity, metabolic and hormonal factors, genetic factors and nutrition (13,14). Muscle volume is often seen as a measure of muscular fitness and is related to muscle power and force generation (12,15–17). Impaired muscle development will consequently result in limited functioning and participation. Pediatric neurological and neuromuscular disorders, like cerebral palsy and Duchenne muscular dystrophy, are characterized by hampered muscle development and muscle degeneration, which requires treatment (18–24). Unfortunately, treatment planning is challenging due to the heterogeneous nature of symptoms and natural course of the disorder. Hence, close follow-up is needed to improve the insight in muscle development and muscle-specific problems in different pediatric neurological conditions. Proper quantification of the level of pathology and irregularity in muscle growth requires a comprehensive normative reference database of muscle morphology (25,26).

Nowadays ultrasonography is a commonly used technique to evaluate muscle morphology in both pediatric pathologies and typically developing children, as it is a quick, easy applicable and painless method. Three-dimensional freehand ultrasound (3DfUS), combining conventional B-mode ultrasonography with three-dimensional motion tracking, is frequently used to estimate morphological parameters like muscle volume, muscle belly length and tendon length (27–29). Due to the accessibility, validity and reliability, ultrasonography and more specifically 3DfUS, seems a promising tool to use for follow-up of muscle development and potential treatment selection (26,28,30–34).

In order to compare individuals with different body sizes as a result of age differences or pathology, muscle morphology data is commonly normalized to body size (35). Normalizing muscle morphology data can be done by ratio or allometric scaling (36). Ratio scaling, or

isometric scaling, is applied by dividing the muscle morphology parameter by a measure of body size, like body mass. Therefore, ratio scaling assumes a simple linear relationship between muscle morphology and measure of body size (37). Allometric scaling, on the other hand, assumes a curvilinear relationship between muscle morphology and body size (i.e. the ratio of change between the two variables does not need to be constant) and is based on the theory of geometric symmetry, stating that all humans have the same architecture and shape, but differ in size (37). Multiple studies in cerebral palsy have reported different ratio scaling methods. Muscle volume is often normalized to body mass (20,27,31,33,38–48), the product of body mass and height (26,49–51) or bone length (52–55), whereas, muscle and or tendon lengths are often normalized to bone length (26,39,43,45,49,51,55–60) or muscle-tendon unit length (20,56–59). The different scaling methods make it difficult to compare results and the most efficient scaling technique has yet to be defined. Furthermore, clear recommendations are lacking (35). To the best of our knowledge, allometric scaling has not yet been used to normalize lower limb muscle morphology in pediatric populations.

Today, only limited studies have investigated muscle morphology of typically developing children. Most previous studies only included normative data as a control group or investigated muscle morphology by two-dimensional ultrasound only (25,26,31,33,61–66). Additionally, some studies calculated or predicted growth rates and growth deficits, by assuming that muscle growth can be described by a simple linear regression (11,25,26,42). Furthermore, most ultrasound studies have only investigated one muscle, most often the medial gastrocnemius and in limited age ranges. There is limited knowledge on the change in lower limb muscle morphology during puberty and whether boys and girls show differences before and during puberty. In summary, there is need for an accessible comprehensive normative database of muscle morphology data of multiple lower limb muscles in a large cohort of typically developing children with a wide age range, including pubertal ages. Additionally, the success of normalization and whether the selection of scaling method should be muscle-specific needs to be investigated.

In this cross-sectional study, 3DfUS data of four lower limb muscles have been collected for an extended group of children with a typical development and these data have been made available online (link will be provided after publication). The primary aim of this cross-sectional

study was to cross-sectionally describe muscle morphology development of the medial gastrocnemius (MG), tibialis anterior (TA), semitendinosus (ST) and rectus femoris (RF) of typically developing children, between the ages of 3 and 18 years. Considering puberty, we expected that muscle development would be more heterogeneous after the age of 11, since the average onset of puberty in girls is 11 years of age, while for boys the average age is 12 years (67–69). Therefore, it was hypothesized that the relation between muscle volume and age and muscle belly length and age can be described by a simple linear equation until the age of 11 years.

The second study aim was to investigate whether there were differences in muscle development (change in muscle volume and muscle belly length) between the four muscles or between boys and girls. It was hypothesized that muscle development is similar between boys and girls before the age of 11 years, due to the limited impact of hormonal changes at these pre-pubertal ages. Additionally, it was expected that muscle development is muscle-specific, due to differences in biomechanical loading, function, muscle stretch and cellular processes between muscles (70,71).

Finally, an exploration of ratio and allometric scaling techniques to normalize muscle volume and muscle belly length was performed. It was hypothesized that muscle morphology data can be successfully normalized for differences in body dimensions.

Methods

Participants

This study was approved by the local ethics committees of the University Hospitals Leuven (s59945 TAMTA project, s62187 and s62645 3D-MMAP project) and the University of Ghent (EC/2017/0526) (Ethische Commissie Onderzoek KU Leuven/UZ Leuven, Commissie voor Medische Ethiek - Universiteit Gent). Written informed consent was given by parents or legal guardian or participants of 18 years old. Children above the age of 12 signed an additional informed assent form.

All children who received muscle morphology assessments as part of the ongoing studies (s59945, s62187 and s62645) were enrolled in the database. The study target was to include good-quality 3DfUS images of approximately 100 children for each of the four lower limb muscle. Eventually, a total of 118 typically developing children were recruited through acquaintances and graduate students of the Clinical Motion Analysis Laboratory of the University Hospitals Leuven, KU Leuven and the University of Ghent. All children were aged between 3 and 18 years and had to be fully cooperative and understand the instructions. Children with neurological, neuromuscular or orthopedic disorders, previous lower limb surgery or performing organized sports at an intensive level (over 6 hours a week) were excluded. All measurements were performed at the Clinical Motion Analysis Laboratory of the University Hospitals Leuven and University of Ghent.

Data acquisition

Measures of body mass, height and lower-limb segment lengths were taken. Leg length was measured between the lower edge of the anterior superior iliac spine and the lower border of the medial malleolus. Lower leg length was measured from the medial side of the knee cleft till the lower border of the medial malleolus and for the upper leg length between the greater trochanter and the medial side of the knee cleft. Additionally, the duration of sport participation was registered.

Only one leg was assessed, alternately dominant and non-dominant legs were assessed. Leg dominance was determined by kicking a ball.

Muscle morphology of the MG, TA, distal compartment of the ST and RF was assessed by a previously published 3D-freehand ultrasound (3DfUS) technique (27,28,30). Due to the deep origin of the ST, it was difficult to reliably visualize the muscle borders of proximal compartment by ultrasonography for all recruited children. Therefore, to define ST muscle volume, only the distal compartment of the ST was included in the analyses. Ultrasound images were acquired by a 59 mm 10 MHz linear transducer connected to a conventional B-mode US machine (Telemed Echoblaster 128 Ext-1Z, Telemed Ltd., Vilnius, Lithuania). With four reflective markers attached to the transducer, position and orientation of the ultrasound images were tracked by a portable motion tracking device with three fixed optical cameras (Optitrack, V120:Trio, Naturalpoint, Corvallis, OR, USA). Ultrasound settings were kept constant for all acquisitions at a frequency of 8 MHz, a focus of 3 cm, a gain of 64%, a dynamic range of 56 dB and unaltered time-gain compensation. Default settings of image depth was 5 cm. However, image depth was adjusted up to 7 cm in order to visualize the deep muscle border of larger muscles (i.e. the MG and RF in older children). A custom made gel pad, named Portico, was used to limit muscle deformation during acquisition (72).

The 3DfUS images were acquired by an experienced assessor using the Stradwin software package (version 6.0; Mechanical Engineering, Cambridge University, Cambridge, UK). The muscles were assessed in a resting condition, while the participants were positioned prone or supine. A triangular cushion supported the lower limb to avoid bi-articular stretch on the investigated muscles (images of measurement setup can be found in Schless et al. 2019 (31)). A second assessor, controlling the data acquisition computer, performed a quick real-time quality check including visibility of all bony landmarks and muscle borders throughout the full recording. Ultrasound recordings were repeated in case of bad quality data, movement of the subject or observed muscle contraction. In case the muscle size exceeded the transducer's width, a multiple sweep technique was applied, capturing two parallel sweeps to visualize the full width of the muscle. The 3DfUS technique is proven to be reliable to quantify the size and length of all four muscles (Hanssen et al. unpublished data – under review)(73). A more detailed description of the data acquisition can be found in this recent reliability study.

Data analyses

The 3DfUS data were analyzed with the above specified Stradwin software. Extensive quality checks were performed before processing the files. Quality was judged based on the

smoothness of the 3D reconstruction, potential gaps in the data due to limited visibility of the reflective markers or losing contact with the skin, visibility of muscle borders, and visibility of bony landmarks. In case of bad-quality, the data were excluded from further analyses.

Muscle belly and tendon lengths were calculated as the Euclidean distance between anatomical landmarks, which are summarized in Table 1. The muscle belly length was defined between the origin (origin of the distal compartment for the ST) and muscle-tendon junction, while the tendon length was estimated between muscle-tendon junction and tendon insertion. No tendon length was defined for the TA and ST, as the used technique and position of the subject made it difficult to visualize the osteotendinous junction. Tendon lengths were included in the normative database, but were not further discussed in this manuscript as this manuscript focused on the muscle belly only.

Estimates of muscle volume were created based on manual transverse segmentations along the inside of the muscle border in approximately 5% of the collected transverse B-mode ultrasound images following the cubic planimetry technique (28,74).

Table 1. Anatomical landmarks for calculating muscle and tendon lengths

Muscle	Origin	Insertion
Tibialis anterior	Fronto-lateral surface of tibia	n.a.
Medial gastrocnemius	Medial femoral condyle	Most proximal part of the calcaneus
Rectus femoris	Spina iliaca anterior inferior	Insertion to the proximal edge of the patella
Semitendinosus, distal compartment	Origin distal compartment	n.a.

n.a. = not applicable

Statistical analyses

Statistical analyses were performed in a SPSS software package (version 28; IBM). Data distribution was explored by a Kolmogorov–Smirnov test and visual inspection of the histograms. As all participant characteristics were normally distributed, descriptive statistics were presented by means and accompanying standard deviations.

To address the first hypothesis, the presence of a simple linear relationship was explored in the entire cohort (including all ages) and in two age subgroups referred to as the pre-pubertal (<11 years of age) and pubertal cohort (≥11 years). Additionally, sub-analyses in other age-

subgroups were explored to evaluate the age of 10 and 12 years as potential breakpoint caused by puberty onset, by checking the assumptions of a simple linear regression in the age groups up to and after 10 and 12 years.

The assumptions of a simple linear relationship, i.e. normality, linearity, and homoscedasticity were tested by SPSS. The distribution of the residuals was explored by visually inspecting the Predicted Probability (P-P) plots. Homoscedasticity of the residuals was checked by plotting the predicted values and residuals on a scatterplot. The distribution of residuals had to be random, i.e. no presence of clusters or trends. The P-P and scatter plots were independently judged by two assessors. If the residuals were both normally distributed and homoscedastic, the relation was considered linear.

To address the second hypothesis, slopes per gender or per muscle were compared. In case of a confirmed simple linear relationship between muscle volume or muscle belly length and age in the total, pre-pubertal (<11 years) or pubertal cohort (≥11 years), differences in slope between muscles and between gender were explored by an analysis of covariance (ANCOVA). An interaction variable describing the relationship between the dependent variable (muscle volume or muscle belly length) and the categorical variable (muscle or gender) was created and applied in the ANCOVA to define any significant differences between the slopes of the equations.

To address the third hypothesis, allometric scaling and different previously applied ratio scaling techniques were explored (35). Allometric scaling was done by using the equation:

$a = \frac{S}{m^b}$ (36,37) , more specifically: $a = \frac{muscle\ volume\ (mL)}{body\ mass(kg)^b}$ or $a = \frac{muscle\ belly\ length\ (mm)}{body\ mass(kg)^b}$

a = the allometric scaled muscle morphology parameter, S = the muscle morphology parameter, m = measure of body size (in this stud body mass or height) and b = the derived allometric parameter

The allometric parameter was the slope of a linear regression between the log-transformed body size measure and log-transformed muscle-morphology parameter. Data on these allometric parameters for muscle volume and muscle belly length are enclosed in the supplementary appendix (Table S7).

In agreement with previous studies that applied ratio-scaling, muscle volume was normalized to body mass (20,27,44–48,31,33,38–43), height*body mass (26,49–51) and height^3(52–55). Muscle belly length was normalized to height, leg length or segment length (26,39,59,60,43,45,49,51,55–58). Allometric scaling was applied to normalize muscle volume to body mass and muscle belly length to height. Following the approach of previous studies, normalization techniques were explored for all four muscles over the full age-range.

The best normalization technique was selected based on the smallest slope of the relation (simple linear regression line) between the normalized muscle morphology parameter (volume or muscle belly length) and age, since a perfect normalization was expected to exclude growth, resulting in a horizontal linear regression line (slope = 0). Additionally, the correlation between age and scaled muscle morphology parameter had to be non-significant. All non-significant correlation coefficients were compared by a Steiger's Z-test, in order to compare the different ratio and allometric scaling methods (36,75).

Results

The flow chart in Figure 1 summarizes all included, missing and excluded data. The missing data were defined by the quality check. Specifications of these quality problems are described in Figure 1. Missing data only occurred in the MG (3.6% of the parameters, mainly for muscle volume) and the RF (1.7% of the parameters, mainly for tendon length). Due to these missing data, the sample sizes differed between muscles and between outcomes.

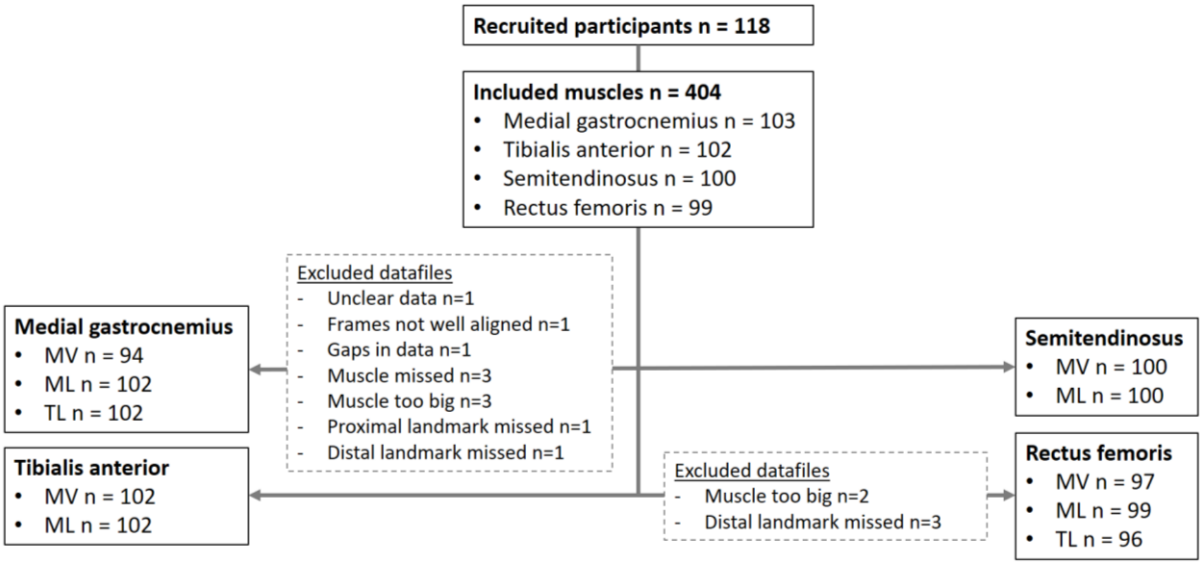

Figure 1. Flowchart of included muscle morphology data
ML = muscle length, MV = muscle volume, n = number, TL = tendon length

Participant characteristics are summarized in Table 2 for the total group of participants and for the four investigated muscles separately.

Table 2. Participant characteristics

	Total n = 118	MG n = 103	TA n = 102	ST n = 100	RF n = 99
Age (years)	10.4 ± 4.5	10.2 ± 4.4	10.7 ± 4.3	10.7 ± 4.3	10.6 ± 4.2
Gender	Male n=54 Female n=64	Male n=48 Female n=55	Male n=44 Female n=58	Male n=44 Female n=56	Male n=42 Female n=57
Ethnicity	Caucasian n=108 Asian n=3 African n=2 Mixed n=3[i] Missing n=2	Caucasian n=94 Asian n=3 African n=1 Mixed n=3 Missing n=2	Caucasian n=94 Asian n=3 African n=1 Mixed n=2 Missing n=2	Caucasian n=92 Asian n=3 African n=1 Mixed n=2 Missing n=2	Caucasian n=91 Asian n=2 African n=1 Mixed n=3 Missing n=2
Body mass (kg)	35.8 ± 17.2[ii]	35.4 ± 17.2	36.7 ± 17.2	36.6 ± 17.1	36.4 ± 16.5
Height (cm)	139.9 ± 25.2[iii]	139.3 ± 24.7	141.8 ± 24.2	141.8 ± 24.0	141.7 ± 23.5

cm = centimeter, kg = kilogram, MG = medial gastrocnemius, n = number, RF = rectus femoris, ST = semitendinosus, TA = tibialis anterior [i]Caucasian/Asian, Caucasian/African, Caucasian/Indian, [ii] missing values n=3, [iii] missing values n=1

The cross-sectional data on muscle volume and muscle belly length of the MG, TA, distal compartment of the ST and RF with respect to age, body mass and body height are presented in Figure 2 and Figure 3.

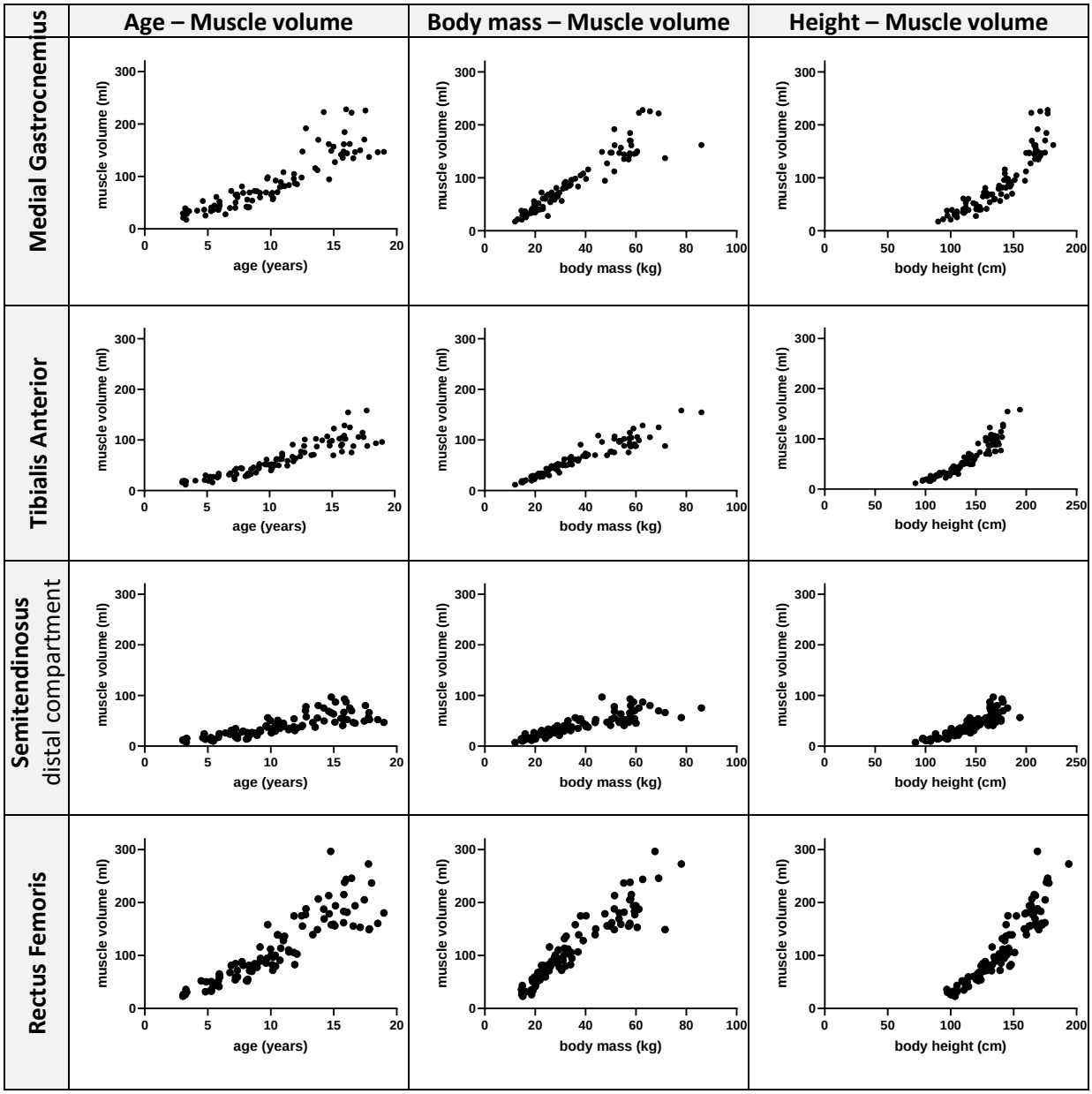

Figure 2. Scatter plots of cross-sectional data of the muscle volume with respect to age, body mass and body height of all four lower limb muscles for the total participant population

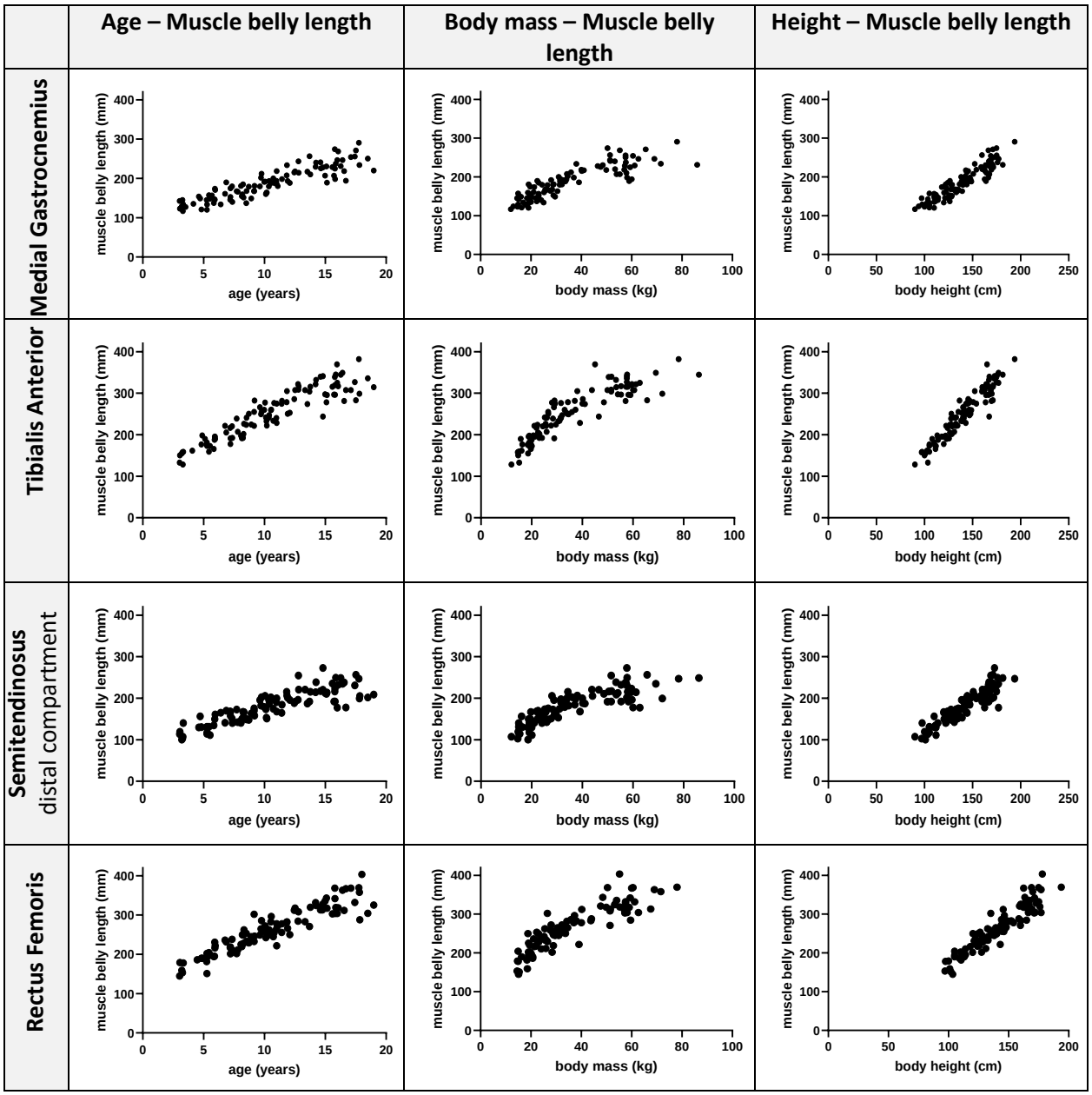

Figure 3. Scatter plots of cross-sectional data of the muscle belly length with respect to age, body mass and body height of all four lower limb muscles for the total participant population

Examination of assumptions simple linear relation

The assumptions of a simple linear regression were checked by visual inspection of the P-P and scatter plots in the total population as in both the pre-pubertal (<11 years of age) and pubertal cohort (≥11 years). All findings are summarized in Table 3. An overview of P-P and scatter plots is enclosed in the supplementary data (Table S1-S6).

Table 3. Assumptions of linear regression for muscle volume

		Medial Gastrocnemius		Tibialis Anterior		Semitendinosus distal compartment		Rectus Femoris	
		Normal distribution of residuals	Homo-scedasticity of residuals	Normal distribution of residuals	Homo-scedasticity of residuals	Normal distribution of residuals	Homo-scedasticity of residuals	Normal distribution of residuals	Homo-scedasticity of residuals
Muscle volume	Total	X	X	X	X	X	X	X	X
	Pre-pubertal (<11 years)	√	√	√	√	√	√	X	X
	Pubertal (≥11 years)	X	X	√	√	X	√	X	√
Muscle belly length	Total	√	√	√	X	√	√	√	X
	Pre-pubertal (<11 years)	√	√	√	√	√	√	√	√
	Pubertal (≥11 years)	√	√	√	√	√	√	X	X

The green checkmark indicates that the assumption was met, whereas a red cross indicates that the assumption was not fulfilled. If both assumptions were met, the muscle- and cohort-specific box are highlighted in green.

Additionally, we analyzed subpopulations before and after the age of 10 and 12 years, respectively. For subgroups based on 12 years of age as cut-off, the assumptions for simple linear relations were not fulfilled in the group below the age of 12 years. These data confirmed that 11 years of age was the most valid estimation of onset of increased data variability. Linearity was confirmed in the pre-pubertal cohort for the muscle volumes of the MG, TA and ST and muscle belly length of all muscles, but not for RF volume, where increased variability was observed from approximately 9 years of age onwards. Additional analyses revealed that RF volume increase was linear till the age of 9 years. The simple linear regression equations (except for RF volume) are summarized in Table 4.

Table 4. Simple linear regression equations describing muscle development of the four muscles

Muscle volume					
	cohort	**Simple linear regression equation**	**r**	**R^2**	**p-value**
Medial gastrocnemius	pre-pubertal	5.497 + 6.788 * age in years	0.819	0.671	<0.001
Tibialis anterior	pre-pubertal	-2.432 + 5.172 * age in years	0.890	0.792	<0.001
	pubertal	-6.261 + 6.713 * age in years	0.625	0.391	<0.001
Semitendinosus, distal compartment	pre-pubertal	-2.685 + 3.754 * age in years	0.794	0.630	<0.001
Muscle belly length					
	cohort	**Simple linear regression equation**	**r**	**R^2**	**p-value**
Medial gastrocnemius	pre-pubertal	104.766 + 7.666 * age in years	0.792	0.628	<0.001
	pubertal	132.180 + 6.416 * age in years	0.547	0.299	<0.001
Tibialis anterior	pre-pubertal	101.659 + 7.704 * age in years	0.892	0.796	<0.001
	pubertal	192.315 + 7.760 * age in years	0.525	0.276	<0.001
Semitendinosus, distal compartment	pre-pubertal	82.181 + 9.709 * age in years	0.840	0.706	<0.001
	pubertal	151.679 + 4.165 * age in years	0.369	0.136	0.014
Rectus femoris	pre-pubertal	119.667 + 14.104 * age in years	0.887	0.788	<0.001

r=correlation coefficient, R^2= Coefficient of Determination; proportion of the variance for a dependent variable that is explained by an independent variable. Pre-pubertal (0-10 years), pubertal (11-18 years)

Comparison of slopes

As linearity was confirmed in the pre-pubertal cohort for the muscle volumes of the MG, TA and ST and muscle belly length of all muscles, an ANCOVA was performed to detect differences in muscle morphology slopes between muscles and gender. The ANCOVA confirmed different slopes ($p < 0.001$) for muscle volume between the MG, TA and ST, indicating muscle-specific volume profiles. Additionally, the slopes of muscle belly length in the pre-pubertal age were significantly different between the four muscles ($p < 0.001$).

Muscle volume and belly length growth rates for all four individual muscles are enclosed in the supplementary data in Table S8.

No differences were found between boys and girls in the pre-pubertal cohort (<11 years) for the muscle volume development of the MG ($p = 0.231$), the TA ($p = 0.446$) and ST ($p = 0.098$). Additionally, no differences between gender for muscle belly length development were reported for all four muscles in the pre-pubertal cohort (MG $p = 0.940$, TA $p = 0.593$, ST $p = 0.577$, RF $p = 0.446$). Differences in muscle volume and muscle belly length between gender

and muscles could not be investigated in the pubertal cohort as the relation of this age group and muscle morphology parameters could not be described by a simple linear regression.

Exploration of normalization techniques

Different normalization techniques based on ratio and allometric scaling were explored in the total cohort. The slopes of the normalized data are presented in Table 5. A table with an overview of all individual normalization techniques and scatter plot overlays of all explorations are enclosed in the supplementary data (Figure S1-S2).

Table 5. Slopes of different normalization techniques for muscle volume and muscle belly length in the total cohort

Normalization of muscle volume				
	ratio scaling			**allometric scaling**
	body mass	**body mass*height**	**height^3**	**body mass**
Medial Gastrocnemius	0.061 *(n=91, r=0.545, p=<0.001)*	-0.023 *(n=90, r=0.336, p=0.001)*	0.033 *(n=92, r=0.244, p=0.019)*	0.001 *(n=91, r=0.016, p=0.880*
Tibialis Anterior	0.040 *(n=99, r=0.612, p=<0.001)*	-0.014 *(n=98, r=0.381, p=<0.001)*	0.027 *(n=101, r=0.396, p=<0.001)*	0.002 *(n=99, r=0.073, p=0.472)*
Semitendinosus, distal compartment	0.014 *(n=97, r=0.227, p=0.025)*	-0.017 *(n=96, r=0.415, p=<0.001)*	0.003 *(n=99, r=0.046, p=0.648)*	0.000 *(n=97, r=0.007, p=0.943)*
Rectus Femoris	0.081 *(n=95, r= 0.512 p=<0.001)*	-0.019 *(n=94, r=0.201, p=0.052)*	0.057 *(n=96, r=0.344, p=<0.001)*	0.002 *(n=95, r=0.042, p=0.689)*
Normalization of muscle belly length				
	ratio scaling			**allometric scaling**
	height	**leg length**	**lower/ upper leg length**	**height**
Medial Gastrocnemius	0.0008 *(n=101, r=0.302, p=0.002)*	-0.0003 *(n=86, r=0.057, p=0.603)*	0.0003 *(n=70, r=0.026, p=0.832)*	0.0004 *(n=101, r=0.028, p=0.782)*
Tibialis Anterior	0.002 *(n=101, r=0.644, p=<0.001)*	0.002 *(n=86, r=0.441, p=<0.001)*	0.005 *(n=64, r=0.342, p=0.006)*	$-5.136*10^{-6}$ *(n=101, r=0.001, p=0.991)*
Semitendinosus, distal compartment	0.001 *(n=99, r=0.300, p=0.003)*	$9.349*10^{-5}$ *(n=83, r=0.019, p=0.862)*	0.0005 *(n=64, r=0.032, p=0.800)*	-0.0004 *(n=99, r=0.041, p=0.686)*
Rectus Femoris	0.002 *(n=98, r=0.503, p=<0.001)*	0.002 *(n=84, r=0.235, p=0.032)*	0.004 *(n=67, r=0.203, p=0.099)*	0.001 *(n=98, r=0.061, p=0.551)*

The most appropriate scaling method per muscle are highlighted in blue.

n = number of cases, n.a. = not applicable, r = correlation coefficient, p = p-value

Overall, allometric scaling was found to be the most efficient technique in normalizing muscle volume, based on smallest slope. For MG and TA, the allometric scaling was the only scaling technique resulting in a non-significant correlation over age. No significant differences were found between ratio scaling to height^3 and allometric scaling for volume of the ST distal compartment (p = 0.509). However, the Steiger's Z-test revealed that the correlation coefficient of the allometric scaled RF volume was significantly smaller compared to the ratio scaling to body mass*height ($p < 0.001$).

Muscle belly length was most thoroughly normalized by either allometric scaling (TA and RF) or ratio scaling to total leg length (ST, distal compartment) or lower leg length (MG). The Steiger's Z-test did not reveal any significant differences between the different scaling methods for muscle belly lengths.

Discussion

This investigation provided normative muscle morphology data, which can serve as a reference of typical muscle development in studies on pediatric pathological conditions like cerebral palsy and Duchenne muscular dystrophy.

The current study showed that cross-sectional changes in muscle volume of the MG, TA and distal compartment of the ST can be described by a simple linear regression equation until the age of 11 years. From the age of 11 years onwards, the variation in volume of these three lower limb muscles increased, most likely as a result of puberty onset and accompanying hormonal and/or metabolic changes. The variability in growth of the RF seemed to increase already from a younger age onwards, i.e. from approximately 9 years of age (supplementary data (Table S8). Muscle belly lengths of all four muscles could be described by a simple linear regression in the pre-pubertal cohort (<11 years), as the distribution of variances remained equal for all muscles.

The results further suggest that cross-sectional observations in growth are muscle-specific. The MG showed the largest monthly growth rate in muscle volume and the distal compartment of the ST the smallest. The TA showed the largest monthly increase in muscle belly length, whereas the distal compartment of the ST had the smallest growth rate. Furthermore, muscle development of all four muscles was found to be similar for boys and girls until the age of 11 years. No muscle- or gender-specific differences were investigated in the pubertal cohort (≥11 years) as those growth curves could not be described by simple linear regressions.

The preliminary exploration of different normalization techniques by means of allometric and ratio scaling proved that muscle volume and muscle belly length can be corrected for differences in body dimensions, resulting in slopes near zero. We verified that normalized muscle morphology parameters became independent of age. This first exploration indicated that allometric scaling was the most efficient scaling method to normalize muscle volume over the full age range. For muscle belly length, allometric scaling or expressing muscle belly length as a ratio of total leg or lower leg length were the most promising methods in correcting for anthropometric variability in typically developing children.

Growth rates

To the best of our knowledge, this is the first cross-sectional 3DfUS study describing muscle morphology in typically developing children in a wide age range and for four different lower limb muscles. As muscle volume is an important determinant of muscular fitness and related to muscle strength and power, there is a growing interest in investigating muscle morphology and specifically muscle size measures. Unfortunately, previous studies included small samples that were insufficient to create a valid normative databases as reference for pathological data and these studies focused mainly on the MG (11,25,26,31,33,61).

Barber et al. (2013) and De Beukelaer at al. (2022) calculated cross-sectional growth rates as muscle volume divided by age (expressed in months), in order to express growth deficits in a population of children with spastic cerebral palsy, assuming muscle growth is linear (25,26). The current study confirmed this underlying assumption for children of 3-11 years old and indicated that linear growth rates for muscle volume of the MG, TA and ST can be defined until the age of 11 years.

Previous investigations showed that estimations of growth rates can be a valuable tool to evaluate the degree of growth deficit or level of pathology in children with cerebral palsy (25,26,42). This method was also applied to the current data and muscle-specific growth rates are enclosed in the supplementary data, Table S8. The MG volume growth rate calculated in this current study, i.e. 0.64 ml/month expressed as muscle volume per age, was in line with previously reported growth rates ranging between 0.52-0.71 ml/month (11,18,25,26,42,76). Additionally, the slope of the regression equation of MG muscle volume with respect to age (Table 4), which takes into account the intercept, was approximately 0.57 ml/month. However, in longitudinal studies, where the growth rate is expressed as the ratio of change in muscle volume over the time of follow-up between repeated assessments, higher growth rates up to 1.14 ml/month have been reported (11,25), suggesting that cross-sectional investigations underestimate muscle growth at certain ages. Therefore, the growth rate calculations seem most suitable for longitudinal designs with a least two assessments. For cross-sectional studies, the slope of the regression equation might serve as a better growth estimate, due to consideration of the intercept. However, the current data suggest that these slopes may still underestimate the amount of muscle growth at specific ages (Table 4). Obst et al. (2020) (42) reported a muscle volume growth rate of the TA of 0.48 millimeters per month, which is in line with a growth rate of 0.41 millimeters per month in the current study.

The current study reported an average muscle belly length growth of the MG of 1.75 millimeters per month over the full age range, which is much higher compared to studies in adolescents (10-19 years, 0.42 millimeters per month) and 5-12 year old children (0.83 millimeters per month)(77,78). However, like muscle volume, a longitudinal study by Obst and colleagues (2022) showed that MG muscle belly length increases up to 2.53 millimeters per month (11). Interestingly, the same study showed that age-predicted growth (based on age–volume or age–length simple linear regression equations), of the MG volume and muscle belly length were significantly underestimated compared to actual (longitudinal) growth over a 12-month period. These findings indicate that caution is needed when predicting muscle volume based solely on age. Therefore, the current study provided additional graphs (Figures 1 and 2) of muscle development over body mass and height, for which the assumptions of a simple linear regression and possible regression equations can also be defined on the available data. It is expected that growth cannot be explained by a change in just one parameter, but represents a combination of parameters, along with additional environmental, genetic and muscle-specific factors.

Increased variation in muscle development during pubertal phase

The fact that muscle volume development over the full age-range (3-18 years) cannot be described by one simple linear regression indicates that changes in muscle volume do not change in direct proportion to changes in age (79). Therefore these parameters need a more refined prediction model than a simple linear regression. Visual inspection of the muscle morphology data with respect to age highlighted increasing heterogeneity in the muscle growth data after the onset of puberty. This was confirmed by lower R^2 values in the pubertal cohort suggesting that age explains less of the variation in muscle volume and length in children aged over 12 years and highlights that other factors co-influence this relation (Table 4). During the pubertal phase, children experience a growth spurt with gender-specific onset, influencing muscle development (80). Our data suggested that muscle development in boys and girls is similar till the age of 11 years. Bénard et al. confirmed that the MG development in children between 5-12 years of age is not gender-specific (78). However, gender-specific muscle development may be expected from puberty onset onwards, due to hormonal changes, like increasing testosterone levels, that are associated with increased muscle mass and force and altered bone-muscle interaction (70,81–83). Therefore, muscle morphology

data of boys and girls can be pooled up until the age of 11 years only, but should be presented separately from puberty onset onwards. Additionally, it is likely that children in the pubertal stage become more active and have different energy demands, resulting in increased muscle growth, compared to children in pre-pubertal stages (14,84). In contrast to the findings in this current study, De Ste Croix et al. showed that muscle strength and body size, which are assumed to be strongly related to muscle size, were similar between boys and girls up until the age of 14 years in a four-year follow-up (85). However, Apibantaweesakul et al. showed that, during childhood, muscles first increase in size, before gaining strength (4). This might explain why there are gender-specific differences in muscle morphology before differences in strength. It should be noted that no strength evaluation was performed in the current study and these findings could therefore not be confirmed.

Alterations in muscle volume in comparison to changes in muscle belly length

Compared to volume, muscle belly length was found to grow more constant with age, indicated by higher R^2 values. Muscle belly length can increase by addition of sarcomeres in series. As some muscles are not parallel fibered, an addition of sarcomeres in parallel and increased fiber cross-sectional area can also result in an increase in muscle belly length (1). The complex combination of muscle growth in parallel and in series may explain the higher heterogeneity in muscle volume growth compared to increase in muscle belly length. However, in disorders affecting muscle belly length, like cerebral palsy, this relation is expected to be altered (46).

Muscle-specific development

The differences in muscle development between different lower limb muscles may be caused by differences in biomechanical loading, mechanical stress, pennation angle, levels of stretch on the muscle belly and development of the nervous system like motor unit recruitment (4,70,71). Additionally, density and activity of satellite cell populations, precursors to skeletal muscle cells, is proven to be muscle-specific (86). Furthermore, Yin and colleagues summarized evidence according heterogeneity in potential of stem cell differentiation between muscles (86). The diversity in function, architecture and satellite cell characteristics could possibly explain the enlarged variation in development of the RF volume from a young age onward. Additionally, Mogi et al. showed that RF muscle thickness was significantly

correlated to maturity index, which was based on peak height velocity, in a cohort of 6-18 year old boys (66). Even though the study by Mogi et al. was limited to boys and investigated muscle thickness by two-dimensional ultrasound, these results suggest that RF growth is closely related to alterations in height, and RF development may thus ideally be described relative to height rather than age (66). Furthermore, it should be taken into consideration that the RF is only one part of the quadriceps complex. Therefore, no conclusions can be drawn on the potential influence of the neighboring and synergistic muscles on RF muscle development. This should also be taken into consideration when investigating the isolated MG and ST. The fact that muscle development is muscle-specific might not be surprising due to the above mentioned muscle-specific differences including loading, stress and satellite cell populations. However, since most 3D-ultrasound study are limited to the MG, it is important to note that most previously reported results cannot be generalized to all muscles.

Scaling methods

The exploration of scaling methods showed that muscle volume can be most effectively normalized by allometric scaling, whereas the preferred normalization technique for muscle belly length seemed to be more muscle-specific. However, it should be mentioned that this was a first exploration based on visual inspection of scatter plots against age and description of slopes. Nuzzo and colleagues (2013) confirmed that allometric scaling was more appropriate for normalizing morphology (cross-sectional area and thickness) of the abdominal muscles and lumbar multifidus compared to ratio scaling (36). They concluded that ratio scaling was an inappropriate technique for normalizing muscle morphology parameters and allometric scaling is recommended in future studies.

Our aim was to normalize muscle growth over age, to eliminate growth, in order to compare children with different body sizes due to age differences or pathologies. Our results suggest that muscle-specific normalization techniques might be needed for muscle and tendon lengths. Furthermore, different scaling methods might be required to normalize pathological muscle morphology parameters. The fact that slopes are near but not equal to zero suggest that multiple factors relate to increases in muscle volume and belly length over age. Additionally, the fact that some correlation coefficients are still quite high or significant indicate that not all scaling techniques were sufficient to exclude age-induced alterations in muscle morphology.

Recommendations for future studies

In order to further investigate muscle development after the age of 11 years, the datasets for these pubertal (and potentially post-pubertal) ages should be expanded with extra cross-sectional and preferably longitudinal data, to allow explorations stratified per gender and based on different relationships, such as non-linear relations with break points. More complex statistical models and more detailed documentation of pubertal stage, body dimensions and skeletal growth might be required in order to describe the changes in muscle morphology during puberty. Thereby, confounding factors like physical fitness and activity, nutritional factors, genetics, endocrine and metabolic factors should be considered.

Since this study only included healthy participants without any neurological disorders, no conclusions can be drawn on muscle development in pediatric pathologies, and caution is warranted when comparing pathological data with this normative database. Assumptions of simple linear regression equations should also be explored in pathological muscles before performing predictions or calculating growth rates. Especially in disorders where the endocrine or metabolic system are disrupted, skeletal and muscle growth development can be altered (87). For example, Bajaj et al. confirmed that muscle volume is related to trabecular and cortical bone architecture in typically developing children, whereas it is shown that disorders affecting the central nervous system, like cerebral palsy, are associated with impaired trabecular bone architecture (88,89). This highlights once more that the findings in this normative dataset cannot be generalized to pathological muscles.

Limitations

Some limitations have to be acknowledged. First, this study did not include any assessments or data on puberty stage. Therefore, the assumption that the increase in variation of muscle development is due to puberty onset needs to be confirmed. Future studies should consider assessments of Tanner stages, hormone levels or estimations of the peak height velocity as marker for puberty onset (90,91). Additionally, the pubertal cohort might also include post-pubertal participants. Future studies that specifically investigate muscle development in the years preceding puberty onset and during pubertal growth spurt along with objective assessments of the onset of the pubertal phase are therefore recommended.

Additionally, participants who participated in more than six hours in sports per week were excluded. The limit of no more than six hours of sports performance a week was set arbitrarily. Yet, physical activity and the balance between active and sedentary time is an important aspect influencing muscle and skeletal growth (92–96). No detailed documentation on sport participation or physical activity was collected in the included children. However, it should be noted that the majority of participants were physically active and might therefore not be a valid representation of all children between 3 and 18 years. Since we recruited healthy volunteers, a bias towards more active children might have been introduced.

The majority of participants were Caucasian. No inclusion criteria based on ethnicity were applied. However, some studies suggested that muscle morphology can be different between ethnicities (97). We visualized the data of the non-Caucasian children and confirmed that their data were similar to the Caucasian participants (supplementary data Figure S3 and S4). Therefore, we assumed that the inclusion of different ethnicities did not influence the conclusions of this study.

In order to perform a first exploration of the different normalization techniques, it was assumed that the assumptions of a linear regression were met over the full age range and slopes were calculated following a simple linear regression model. Additionally, the allometric parameters were derived from a simple linear regression equation. While a log-transformation of the muscle morphology and body size parameters was performed to estimate the allometric parameter, the assumptions of a linear regression were assumed to be fulfilled for ratio scaling. If those techniques will be investigated in more detail in future studies, these assumptions should be checked.

Finally, in order to investigate muscle growth, a longitudinal design would be more appropriate, but would require a long follow-up period. A future longitudinal study would be able to detect within-individual changes in muscle morphology during growth and study the alterations in muscle morphology throughout the pubertal stages in more detail.

Conclusion

This study provides a unique normative database of four lower limb muscles in typically developing children between the age of 3 and 18 years. These data can be used as a reference database for pediatric populations and may also serve as a reference frame to better understand both physiological and pathological muscle development. Future studies investigating muscle development should consider muscle-specific development, the impact of puberty and potential pubertal differences in gender when investigating muscle morphology and comparing data to other cohorts with different age ranges or pathologies. Based on the results of this current investigation, it is considered appropriate to describe muscle volume (and until the age of 9 years for volume of the RF muscle) and muscle belly length alterations over age by a simple linear regression equation up until the age of 11 years in typically developing children. Normalizing muscle morphology data by allometric scaling is a promising tool for comparison muscle volumes of different pediatric populations. For muscle lengths, normalization can be achieved by both allometric and ratio scaling. However, other normalization techniques might be needed in different pathologies and should be carefully considered while matching data or (control) groups.

The full dataset is available for download (link will be provided after publication). It is demonstrated that 3DfUS is a valuable tool to monitor muscle development, which may ultimately support clinical decision making in pathologies that are characterized by altered muscle growth.

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Supplementary Material

Part I. P-P and scatter plot to check the assumptions of a simple linear regression

Table S1. P-P and scatter plots of muscle volume for the total cohort

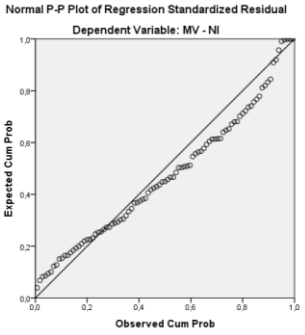
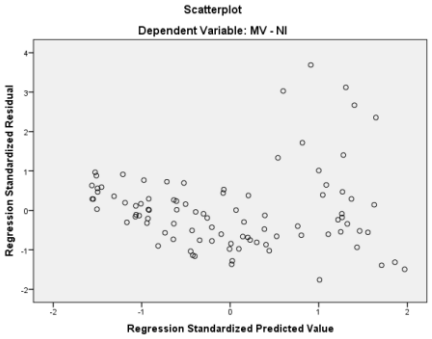
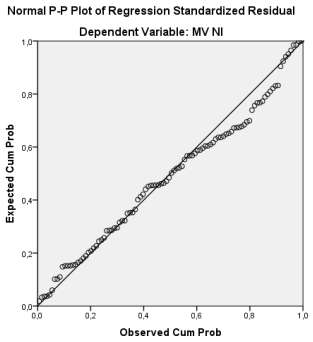
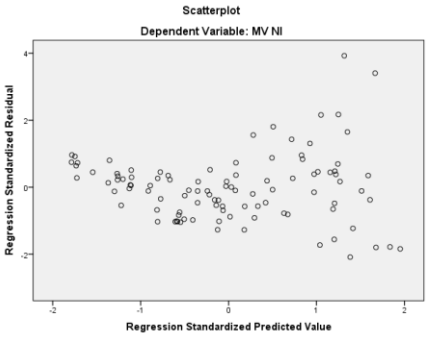
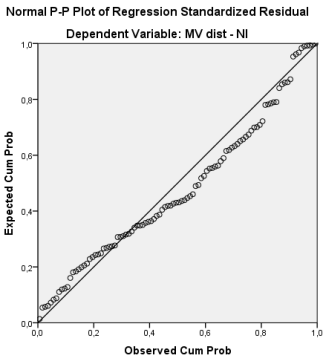
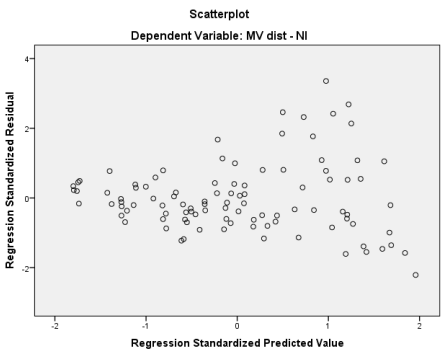
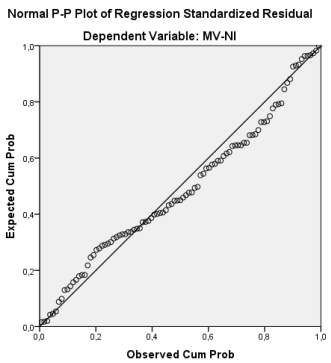
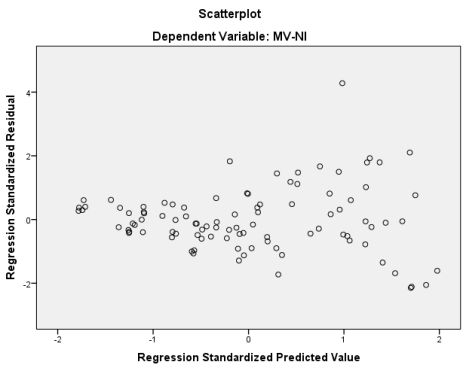
Muscle volume, total cohort	P-P plot	Scatterplot
Medial gastrocnemius		
Tibialis anterior		
Semitendinosus, distal compartment		
Rectus femoris		

Table S2. P-P and scatter plots of muscle volume for the pre-pubertal cohort

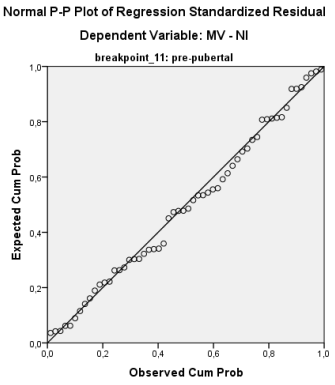
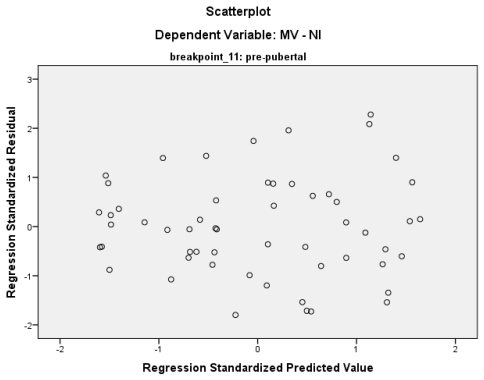
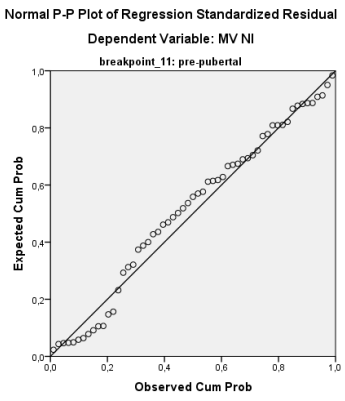
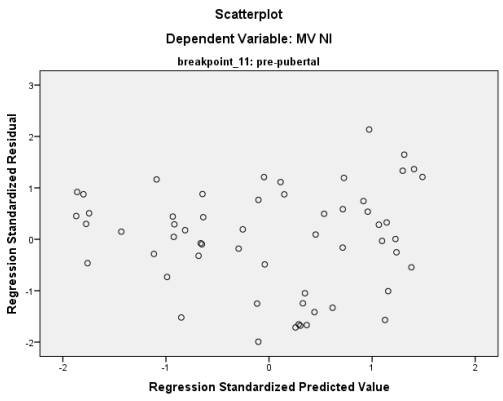
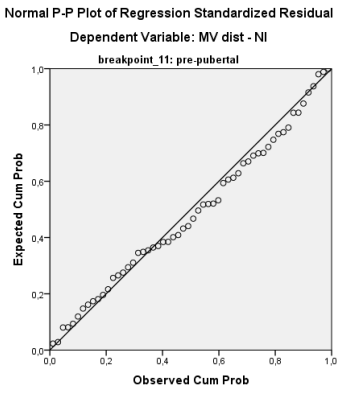
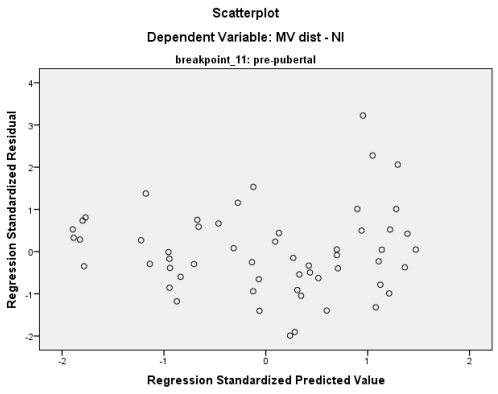
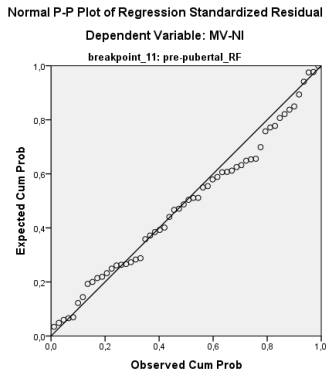
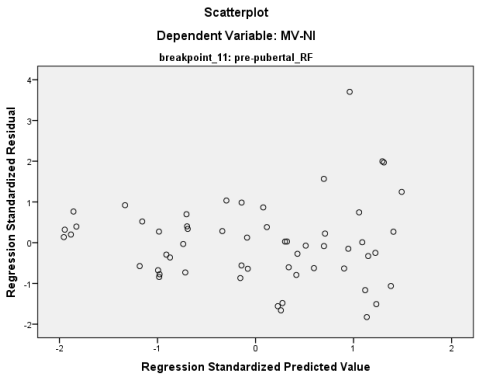
Muscle volume, pre-pubertal	P-P plot	Scatterplot
Medial gastrocnemius		
Tibialis anterior		
Semitendinosus, distal compartment		
Rectus femoris		

Table S3. P-P and scatter plots of muscle volume for the pubertal cohort

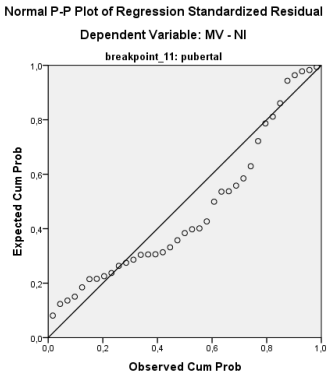
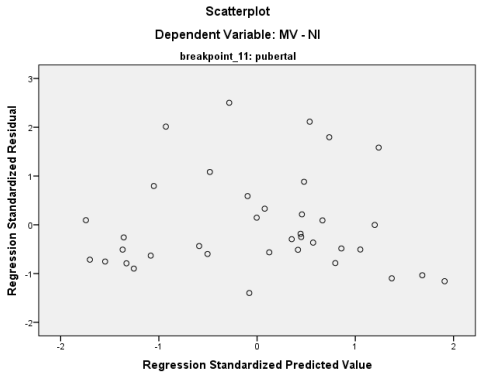
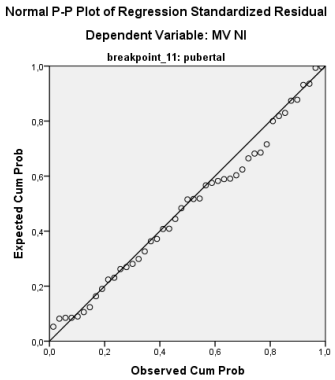
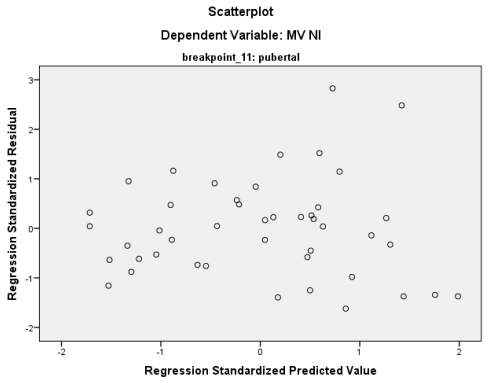
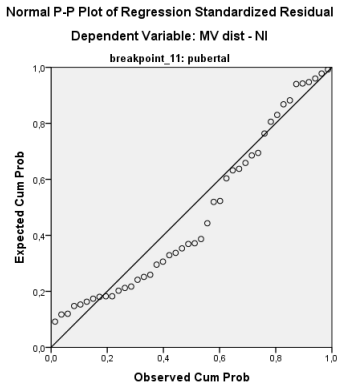
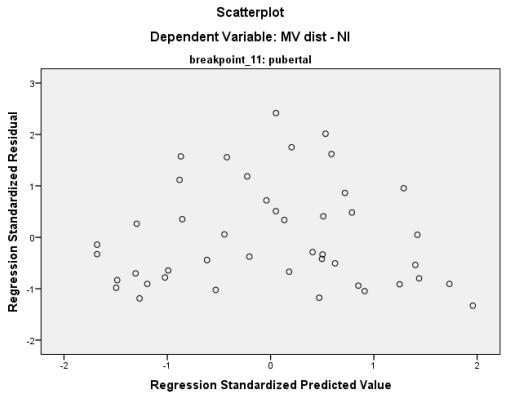
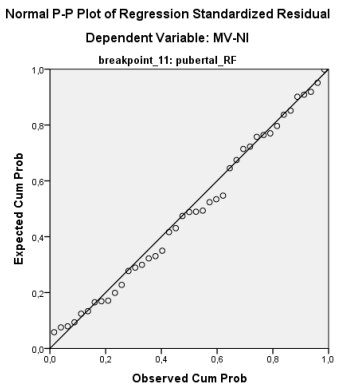
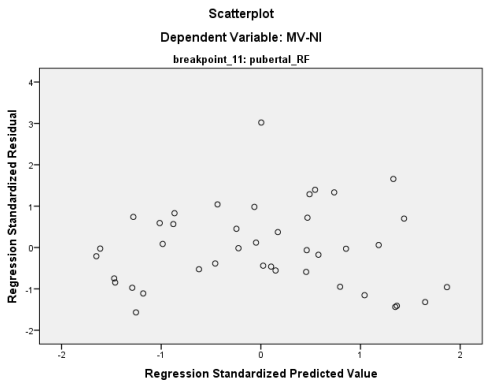
Muscle volume, pubertal	P-P plot	Scatterplot
Medial gastrocnemius		
Tibialis anterior		
Semitendinosus, distal compartment		
Rectus femoris		

Table S4. P-P and scatter plots of muscle belly length for the total cohort

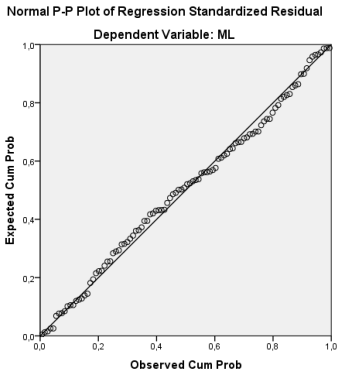
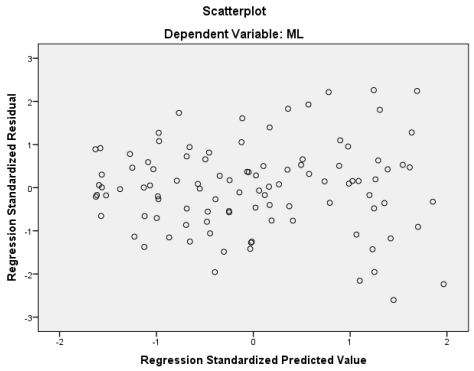
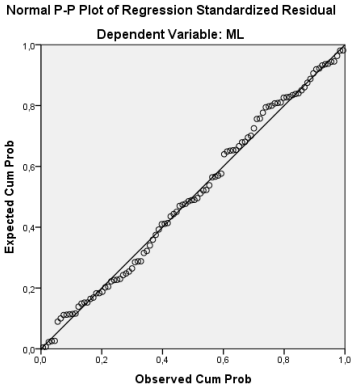
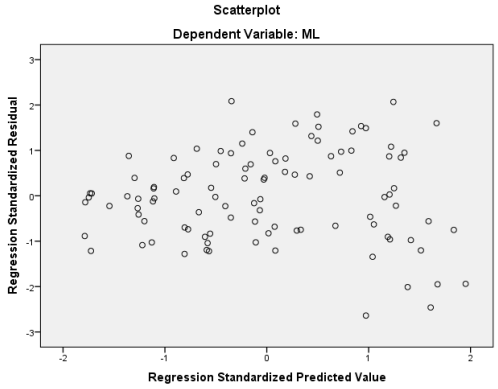
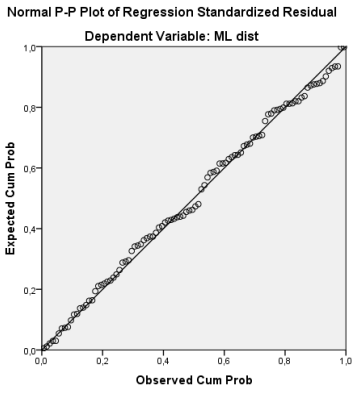
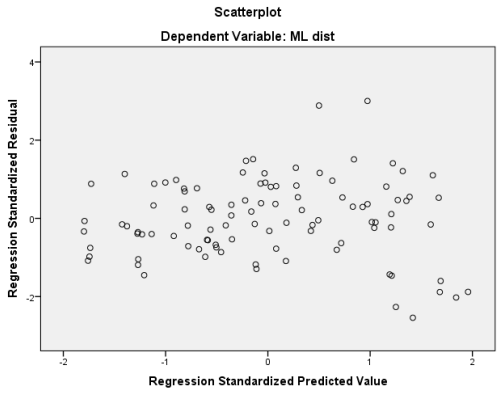
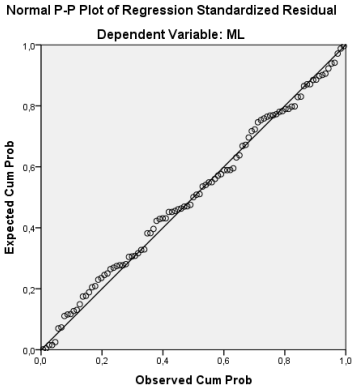
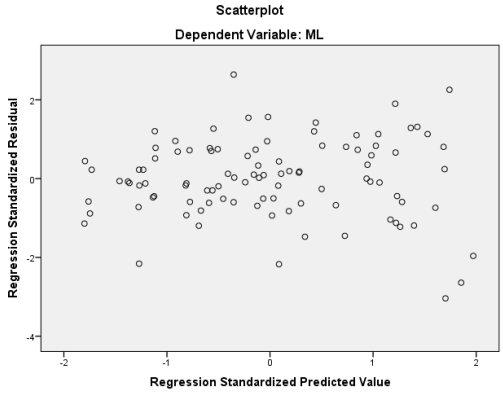
Muscle length, total cohort	P-P plot	Scatterplot
Medial gastrocnemius		
Tibialis anterior		
Semitendinosus, distal compartment		
Rectus femoris		

Table S5. P-P and scatter plots of muscle belly length for the pre-pubertal cohort

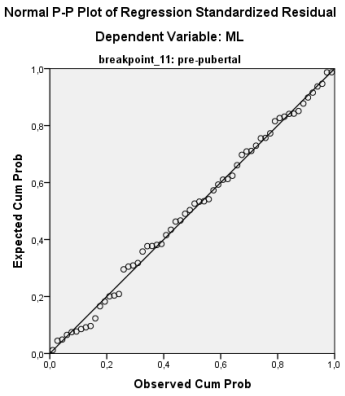
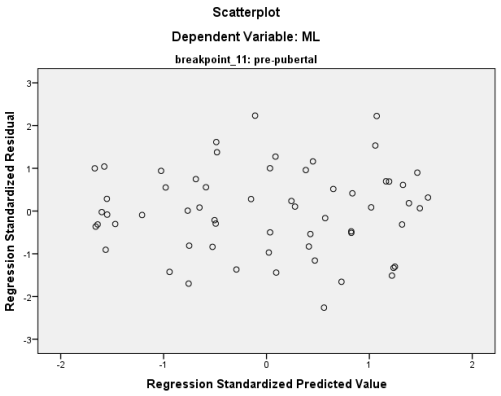
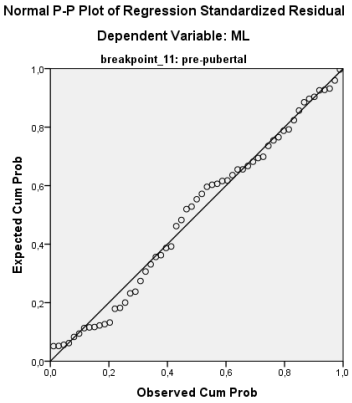
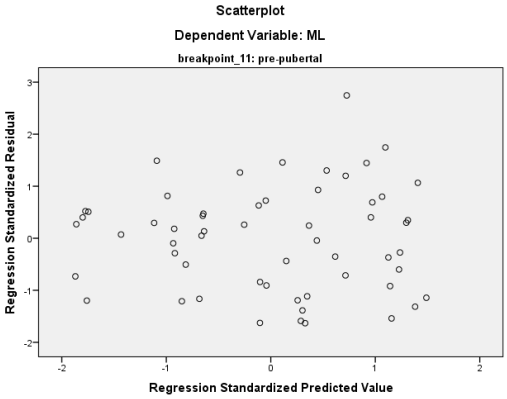
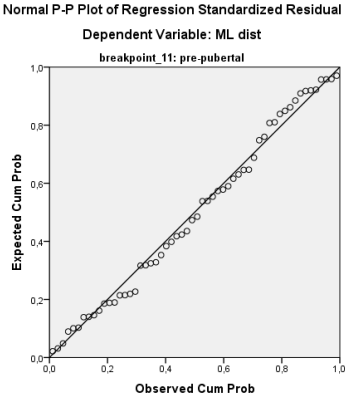
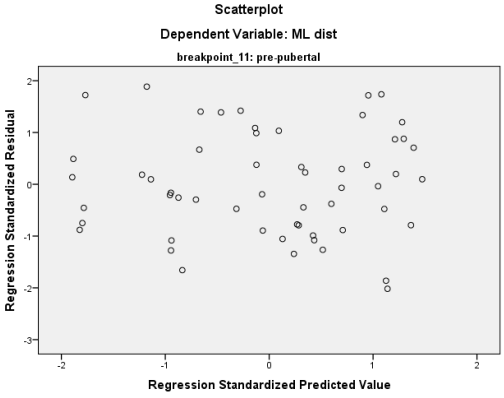
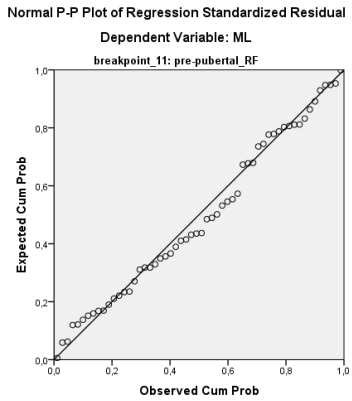
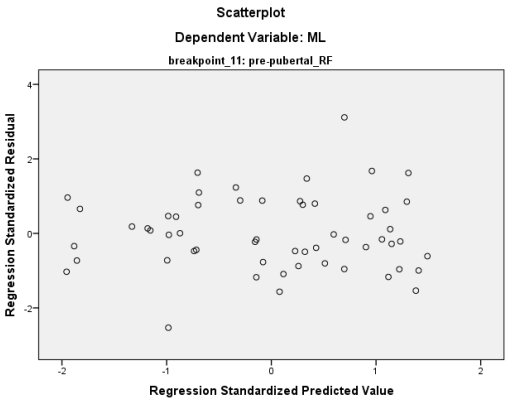
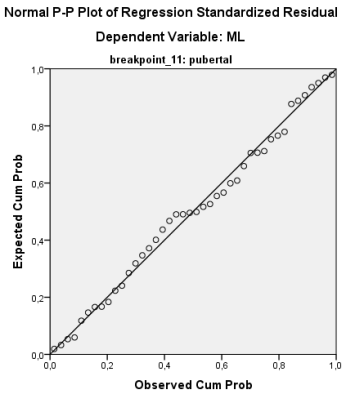
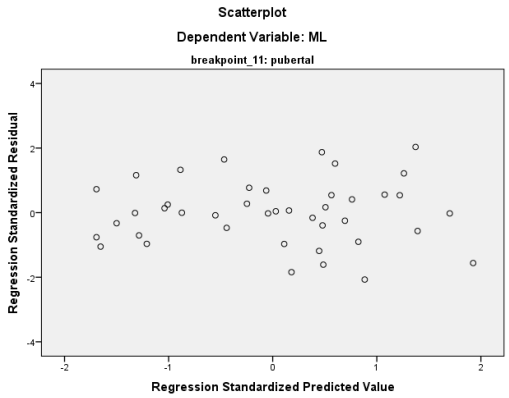
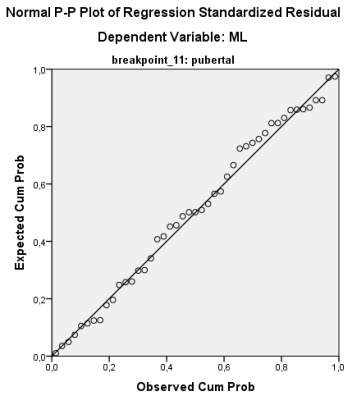
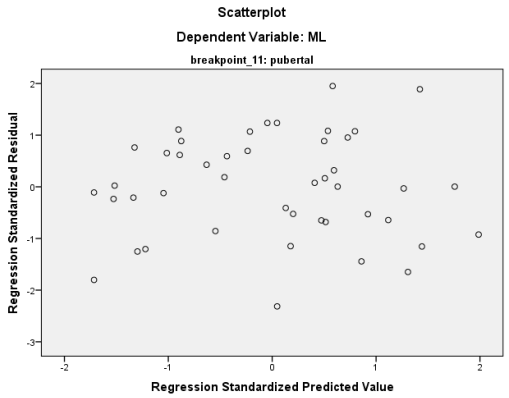
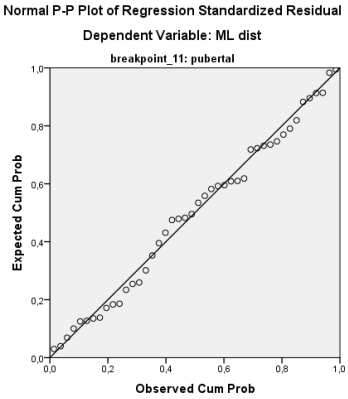
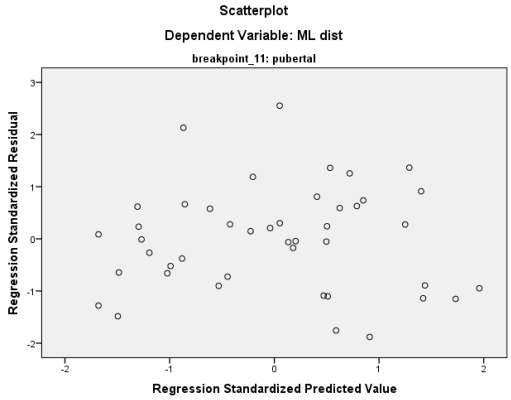
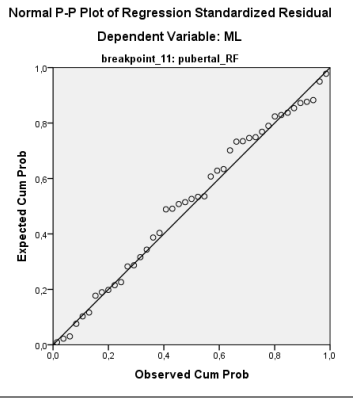
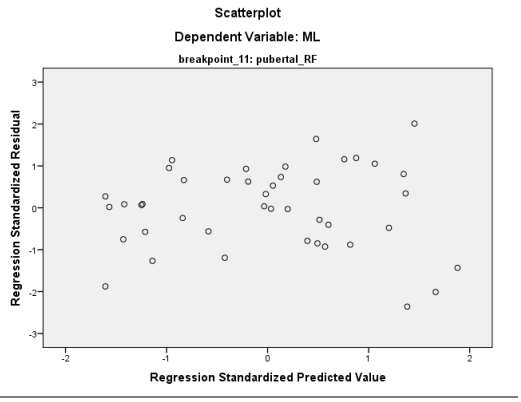
Muscle length, pre-pubertal	P-P plot	Scatterplot
Medial gastrocnemius		
Tibialis anterior		
Semitendinosus, distal compartment		
Rectus femoris		

Table S6. P-P and scatter plots of muscle belly length for the pubertal cohort

Muscle length, pubertal	P-P plot	Scatterplot
Medial gastrocnemius		
Tibialis anterior		
Semitendinosus, distal compartment		
Rectus femoris		

Part II – Allometric scaling parameters

Allometric parameters are the slopes of the regression equations between the log-transformed body size measure and log-transformed muscle morphology parameters. The muscle-specific parameters for both muscle volume and muscle belly length are presented in Table S7.

Table S7. Allometric derived parameters for different muscles and morphological parameters

	Muscle volume	Muscle belly length
Medial gastrocnemius	1.246	1.133
Tibialis anterior	1.229	1.364
Semitendinosus, distal compartment	1.139	1.187
Rectus femoris	1.262	1.233

Part III – muscle-specific growth rates

Muscle-specific growth rates for muscle volume and muscle belly length were calculated by the following formulas following the methods by Barber and De Beukelaer et al.(25,26) :

$$\textbf{growth rate, muscle volume} = \frac{muscle\ volume\ (mL)}{age\ (months)}$$

$$\textbf{growth rate, muscle belly length} = \frac{muscle\ belly\ length\ (mm)}{age\ (months)}$$

Muscle-specific growth rates are presented in Table S8.

Table S8. Muscle-specific growth rates for muscle volume and muscle belly length

Growth rates MV (ml/month)	Medial gastrocnemius	Tibialis anterior	Semitendinosus, distal compartment	Rectus femoris
Total	0.71 ± 0.19	0.46 ± 0.10	0.30 ± 0.08	0.87 ± 0.22
Pre-pubertal (<11 years)	0.64 ± 0.15	0.41 ± 0.07	0.28 ± 0.07	0.78 ± 0.17
				*0.74 ± 0.14
Pubertal (≥11 years)	0.81 ± 0.19	0.53 ± 0.10	0.33 ± 0.09	0.99 ± 0.22

Growth rates ML (mm/month)	Medial gastrocnemius	Tibialis anterior	Semitendinosus, distal compartment	Rectus femoris
Total	1.75 ± 0.64	2.18 ± 0.60	1.58 ± 0.50	2.29 ± 0.66
Pre-pubertal (<11 years)	2.06 ± 0.66	2.50 ± 0.60	1.85 ± 0.50	2.67 ± 0.65
Pubertal (≥11 years)	1.30 ± 0.17	1.76 ± 0.22	1.23 ± 0.18	1.80 ± 0.19

*Growth rates are presented as mean ± standard deviation. Muscle volume growth rates are presented as milliliters per month, whereas muscle belly length growth is expressed as millimeter per month. Highlighted in green = assumptions of a simple linear regression were met. Highlighted in red = assumptions of a simple linear regression were not met and therefore caution is warranted to use the growth rates. *muscle growth rate <9 years of age. ML = muscle belly length, ml = milliliter, mm = millimeter, MV = muscle volume*

Part IV. Comparison of different scaling techniques

Figure S1. Comparison of different scaling techniques for muscle volume of the four muscles

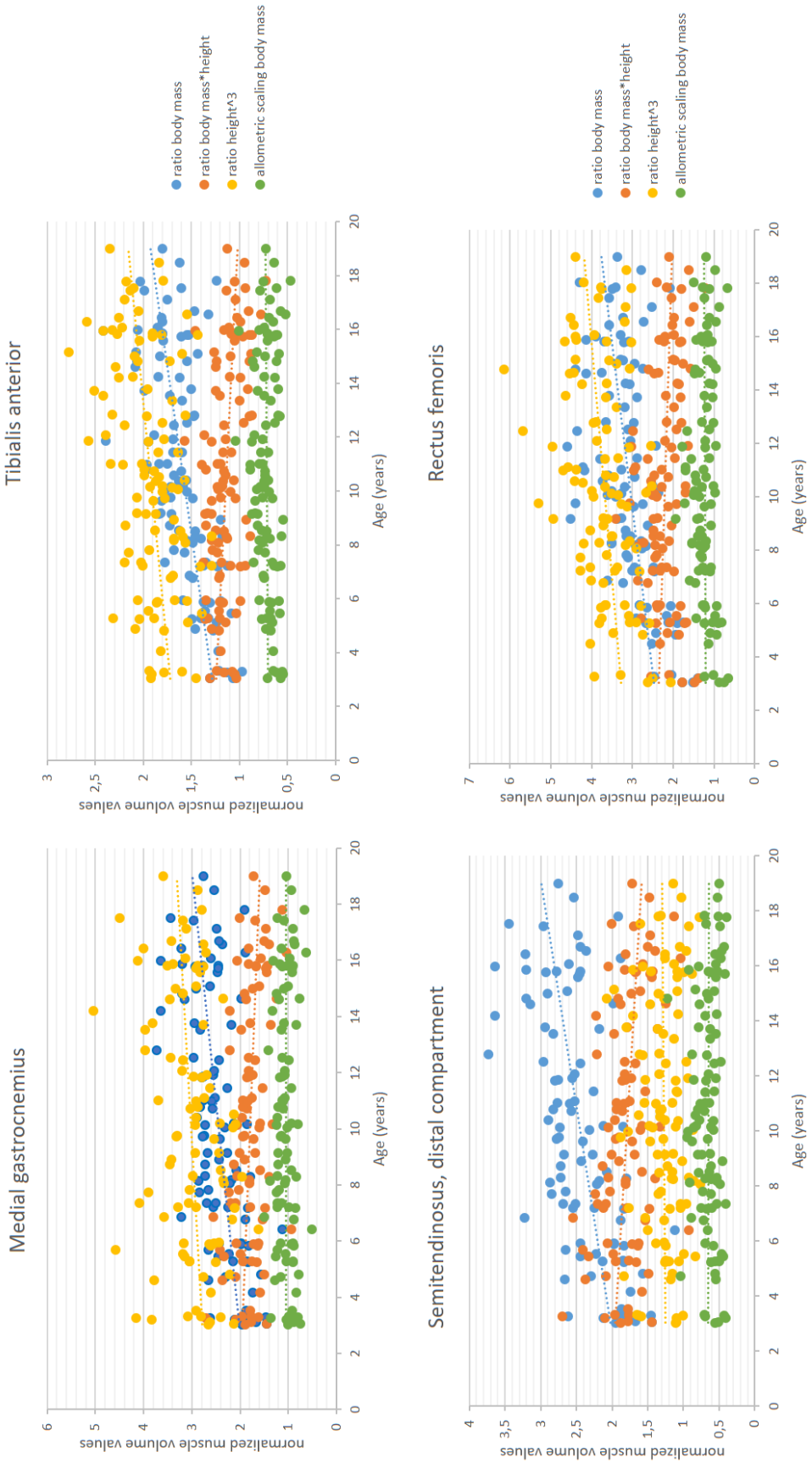
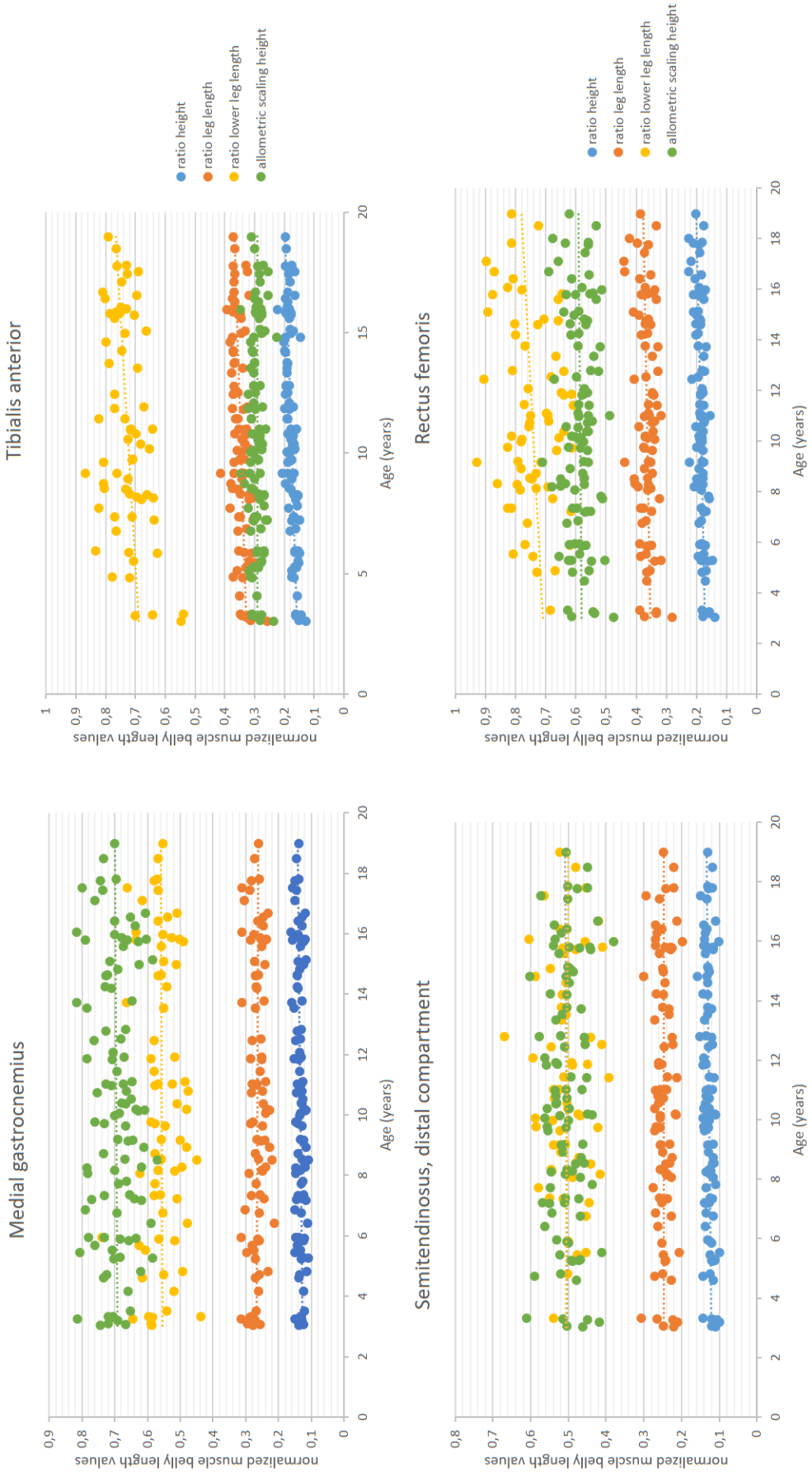
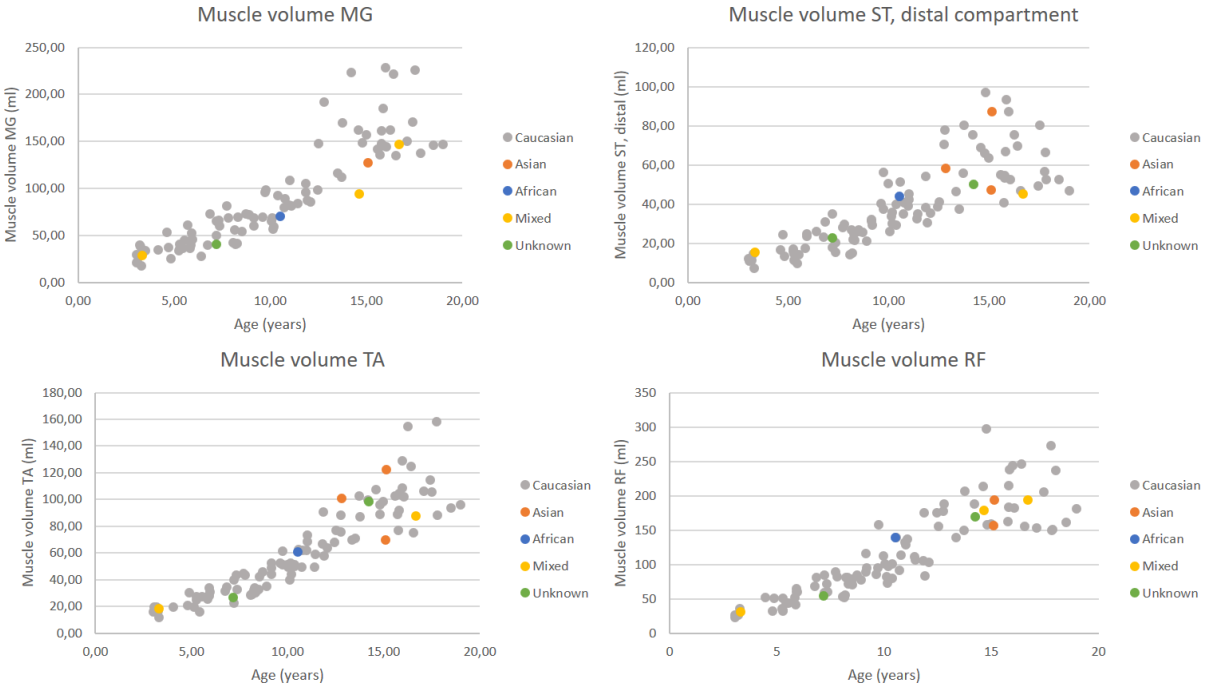


Figure S2. Comparison of different scaling techniques for muscle belly length of the four muscles



Part V. Presentation of different ethnicities

Figure S3. Presentation of different ethnicities within the full cohort for muscle volume data



MG = medial gastrocnemius, ml = milliliters, RF = rectus femoris, ST = semitendinosus, TA = tibialis anterior

Figure S4. Presentation of different ethnicities within the full cohort for muscle belly length data



MG = medial gastrocnemius, mm = millimeters, RF = rectus femoris, ST = semitendinosus, TA = tibialis anterior

Chapter 2.3.

Associations between Muscle Morphology and Spasticity in Children with Spastic Cerebral Palsy

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Abbreviations

AU = arbitrary units

BL = bilateral involvement

ES = effect size

EI = echogenicity intensity

GMFCS = gross motor classification system

HVD = pure high-velocity-dependent muscle activation pattern

KF = knee flexors

MAS = Modified Ashworth scale

MG = medial gastrocnemius

Mixed = mixed length-/velocity-dependent muscle activation pattern

MV = muscle volume

MVC = maximum voluntary contraction

nML = normalized muscle belly length

nMV = normalized muscle volume

nTL = normalized tendon length

PF = plantar flexors

RMS = root mean square

RMS-EMG = root mean square of the electromyography signal

ROM = range of motion

SCP = spastic cerebral palsy

ST = semitendinosus

sEMG = surface electromyography

SMC = selective motor control

ST = semitendinosus

UL = unilateral involvement

3DfUS = three-dimensional freehand ultrasound

Keywords: Cerebral palsy, spasticity, muscle morphology, ultrasound, children

Abstract

Due to the heterogeneous clinical presentation of spastic cerebral palsy (SCP), which makes spasticity treatment challenging, more insight into the complex interaction between spasticity and altered muscle morphology is warranted.

We studied associations between spasticity and muscle morphology and compared muscle morphology between commonly observed spasticity patterns (i.e. different muscle activation patterns during passive stretches).

Spasticity and muscle morphology of the medial gastrocnemius (MG) and semitendinosus (ST) were defined in 74 children with SCP (median age 8 years 2 months, GMFCS I/II/III: 31/25/18, bilateral/unilateral: 46/27). Using an instrumented assessment, spasticity was quantified as the difference in muscle activation recorded during passive stretches at low and high velocities and was classified in mixed length-/velocity-dependent or pure velocity-dependent activation patterns. Three-dimensional freehand ultrasound was used to assess muscle morphology (volume and length) and echogenicity intensity (as a proxy for muscle quality). Spearman correlations and Mann-Whitney-U tests defined associations and group differences, respectively.

A moderate negative association ($r = -0.624$, $p < 0.001$) was found between spasticity and MG muscle volume, while other significant associations between spasticity and muscle morphology parameters were weak. Smaller normalized muscle volume (MG $p = 0.004$, ST $p < 0.001$) and reduced muscle belly length (ST $p = 0.015$) were found in muscles with mixed length-/velocity-dependent patterns compared to muscles with pure velocity-dependent patterns.

Higher spasticity levels were associated with smaller MG and ST volumes and shorter MG muscles. These muscle morphology alterations were more pronounced in muscles that activated during low-velocity stretches compared to muscles that only activated during high-velocity stretches.

Introduction

Cerebral palsy is caused by a non-progressive lesion or malformation in the developing brain, with an incidence of two to three in 1000 living births (1). With a prevalence of approximately 60-80%, spastic cerebral palsy (SCP) is the most common subtype, next to dyskinetic and ataxic cerebral palsy (2,3). Apart from spasticity, children with SCP show muscle weakness and reduced selective motor control (SMC), as a direct result of the upper motor lesion (4). Although the brain lesion itself remains static, further secondary musculoskeletal problems progress with growth. These include increased muscle stiffness, which potentially leads to development of soft tissue contractures, reduced joint range of motion (ROM) and bony deformities (5,6).

The definition of spasticity by Lance stated that spasticity is a velocity-dependent increase in tonic stretch reflex (7). However, multiple previous studies have noted increased muscle activation while stretching the muscle at a low velocity (8–10). Some muscles tended to respond to an alteration in length rather than an alteration in stretch-velocity. We recently confirmed these different muscle activation patterns for different lower limb muscles of children with SCP, by defining muscle activation patterns in response to stretch that were either high-velocity-dependent, low-velocity-dependent or a mixture of both (8).

The heterogeneous clinical presentation of SCP makes effective spasticity treatment planning challenging. The variability in spastic muscle excitation and the different muscle activation patterns observed while stretching muscles of children with SCP may potentially explain the variable response to tone reduction treatment. Indeed, Bar-On et al. recently showed that semitendinosus (ST) muscles with pure high-velocity-dependent muscle activation patterns responded better to treatment with intramuscular botulinum neurotoxin-A compared to muscles with mixed muscle activation patterns (11). Yet, the underlying mechanisms of these different patterns of muscle excitation remained unknown. In addition to spasticity, multiple alterations in muscle morphology as well as reduced muscle quality of lower limb muscles have been described in children with SCP (12–14). These findings raise the questions whether spasticity is related to alterations in muscle morphology and whether muscle morphology

differs between muscle activation patterns. Improved insight in this complex interaction between spasticity and muscle morphology may contribute to the understanding of altered muscle behavior in SCP and may eventually help to explain muscle-specific treatment outcome.

Today, only a limited number of studies have investigated the association between spasticity and muscle morphology in children with SCP. Ohata et al. previously showed a non-significant tendency towards higher maximum cross-sectional area in quadriceps femoris muscles with lower Modified Ashworth scale (MAS) ratings of the knee flexors (15). As there are concerns on the validity and reliability of the MAS (16–19), Pierce et al. performed a similar study using isokinetic dynamometry and surface EMG (sEMG) in eight children with bilateral SCP (20). They found contradicting findings, namely that the volume of the entire quadriceps femoris muscle defined by MRI was larger in muscles with higher levels of spasticity, defined by increased sEMG of the vastus lateralis and increased passive torque. These conflicting results highlight the need for more research on the interaction between spasticity and altered muscle morphology, based on comprehensive instrumented assessments.

Gracies et al. suggested that spasticity may be enhanced in contractured muscles, because the stretching force may be more efficiently transmitted to the muscle spindle in less extensible muscles (10). This could imply that the changes in muscle morphology, such as muscle belly shortening, may be associated with higher levels of spasticity and with length-, rather than velocity-dependent muscle activation patterns observed during stretch. In such cases, muscle length, rather than lengthening velocity, seems to be the primary trigger of muscle excitation response to stretch. This hypothesis needs to be further explored.

The current study was designed to further investigate the complex interaction between spasticity and altered muscle morphology. The first aim was to investigate the association between muscle morphology and spasticity in the medial gastrocnemius (MG) and ST. The second aim was to investigate differences in muscle morphology and quality of the MG and ST between different muscle activation patterns observed during passive muscle stretches during the instrumented spasticity assessment.

Related to the first aim, and following the above specified suggestions made in literature, it was hypothesized that higher levels of spasticity (i.e. higher EMG levels during passive stretch) were associated with smaller muscles (i.e. smaller muscle volume (MV) and reduced belly length) and reduced muscle quality (higher echogenicity intensity (EI))(10). Secondly, based on previous studies (10,11,21), it was hypothesized that muscles with mixed length-/velocity-dependent muscle activation patterns have a more severely altered morphology (reduced MV and shorter muscle belly length) and reduced muscle quality (higher EI) compared to muscles with pure velocity-dependent muscle activation patterns.

Methods

This study was approved by the Ethical Commission UZ/KU Leuven (s59945 and s56041). Participants and parents or legal guardians were informed on the content of the procedure and written informed consent was obtained. Data were collected from June 2017 till July 2021.

Participants

Children with a confirmed diagnosis of SCP aged between 3-18 years, without clinically diagnosed dystonic features in the lower limbs, were recruited via the Cerebral Palsy Reference Centre of the University Hospitals Leuven and the Inkendaal Rehabilitation Hospital. Children with a history of selective dorsal rhizotomy, previous lower limb soft-tissue surgery or bone realignment surgery within two years before assessment, intramuscular botulinum neurotoxin-A within six months before assessment, or an intrathecal baclofen pump, were excluded. The most involved lower limb, according to the most recent clinical examination, was assessed. All children with SCP who received spasticity and muscle morphology assessments as part of two ongoing research projects (s59945 and s56041) were enrolled in the current data analysis. A detailed description of participant characteristics is enclosed in Table S1 and Table S5 of the supplementary material.

For the first aim, data of the total group of children were used to explore the associations between spasticity and morphology parameters. For the second aim, required study samples were determined by power analyses, based on preliminary analyses of an initial dataset (23 children with SCP, median age 8.7 years) and the comparison of MV between two muscle activation patterns (i.e. pure velocity-dependent and mixed muscle activation patterns, as described below), indicating an effect sizes (ES) of 0.80 (average ES of MG and ST) (22). Power analyses based on an ES of 0.80, with 80% power and α-level of 0.05 indicated that 26 participants per group were sufficient to detect differences in MV between the two muscle activation patterns.

Assessments

A full description of each assessment is enclosed in the supplementary material (Part 1. Detailed description of used methods).

Clinical examination

To characterize the participants and investigate associations with muscle morphology, a standardized clinical examination was performed, including the MAS and Modified Tardieu scales to clinically score the level of spasticity, and the manual muscle strength and selectivity scales. Additionally, the passive range of motion was assessed by goniometry.

Instrumented spasticity assessments

A previously established and validated procedure of instrumented spasticity assessment was applied to define the levels of muscle activation of the MG and ST during low- and high-velocity passive stretches over the full ROM, by means of sEMG (Zerowire device, Cometa, Milan, Italy) (23). The sEMG signals were sampled at 2000Hz and filtered offline. Average sEMG collected during three isometric maximum voluntary contractions (MVC) of plantar and knee flexion were used as reference to express raw root-mean square EMG values (RMS-EMG) as a percentage MVC. Three inertial measurement units (IMU; Analog Devices, ADIS16354) placed on the different segments (foot, lower leg, upper leg) were used to calculate joint angles, ROM and angular velocity, after applying a Kalman smoother to the IMU data.

Three-dimensional free-hand ultrasound

The MV of the MG and distal compartment of the ST, as well as muscle and tendon length of the MG and entire belly length of the ST were defined by three-dimensional freehand ultrasound, a method previously described by Cenni et al. (24). Additionally, the EI of the muscle belly was estimated as a proxy for muscle quality (12,25). Ultrasound images were created by moving the transducer from muscle origin to insertion while the participant was in prone position with slight knee flexion (20-30 degrees) to reduce bi-articular stretch on the investigated muscles. Images were recorded by Echowave (study s56041) (UAB, Telemed, Vilnius, LT) or Stradwin version 6.0 software package (study s59945) (Mechanical Engineering, Cambridge University, Cambridge, UK). Ultrasound imaging settings were standardized for all assessments.

Data analyses

Muscle excitation and activation patterns defined by instrumented spasticity assessment

The sEMG data were filtered as previously described (23). Individual stretch repetitions were excluded in case of bad quality sEMG (such as increased overall noise or random obvious signal artefacts), visual detection of antagonist activation (to control for subject relaxation) or inconsistent range of motion or stretch velocities between repetitions (> 20 degrees/second difference). An average trial was created based on at least two stretch repetitions of good quality.

The level of spasticity was defined by the increased muscle excitation during high-velocity minus the level of muscle excitation during low-velocity stretches. Hereto, the average RMS-EMG level during a high-velocity stretch was calculated over an interval of 200ms before maximum velocity up to 90% of the ROM and the average level of RMS-EMG, calculated over a similar interval, during a low-velocity stretch was subtracted. The resulting EMG difference score between the two stretch-velocities, expressed as a percentage of the MVC, reflected the velocity-dependent increase in muscle activation. We previously showed that this spasticity outcome was characterized by high relative (ICC values ranging between 0.98-0.99) and absolute (%SEM ranging between 3.9-14.9%) reliability (26).

Muscle activation patterns were independently classified by two trained researchers and a third expert was consulted in case of disagreement. Muscles were classified following a previously described approach, whereby muscles were labeled as pure high-velocity-dependent (HVD) if there was no muscle activation during low-velocity stretch. If muscle activity exceeded 3% of MVC during the low-velocity stretch, calculated as the average difference between zone 1 (first part of 10-90% ROM) and 3 (last part of 10-90% ROM), the muscle was classified as mixed low-/high-velocity-dependent muscle activation pattern, further labelled as ‘mixed patterns’.

Muscle morphology and quality defined by three-dimensional free-hand ultrasound

Three-dimensional reconstructions of the MG and ST were created by custom-made Python 2.7 (www.python.org) scripts (study s56041) or Stradwin software version 6.0 (study s59945). The MV was estimated following the cubic planimetry technique by drawing manual segmentations along the inside of the muscle border in the transverse images by a trained assessor. EI was expressed in arbitrary units on an 8-bit grey scale (256 values) over the full

interpolated 3D-reconstruction of the muscle. A hyper-echoic images corresponded with a higher EI, which was assumed to be an indication of reduced muscle quality (12).

The MG muscle belly and tendon length were estimated by calculating the Euclidean distance between the muscle origin and muscle-tendon junction and tendon insertion, respectively. Besides the total muscle belly length, the length of the distal compartment of the ST was estimated.

The absolute morphological parameters were normalized to body size for both aims to account for differences in body dimension between participants (27). The MV was normalized to body mass, while lengths of the muscle belly and tendon were expressed as a ratio of the total height (27).

Statistical analyses

Statistical analyses were performed using SPSS statistics version 28.0 (SPSS, Inc., Chicago, IL, USA). Normality of the data distribution was evaluated by the Kolmogorov-Smirnov test ($p<0.05$). As most parameters were not normally distributed, only non-parametric statistics were performed.

Descriptive statistics were used to describe the patient characteristics, clinical examination parameters and the primary outcomes of spasticity (EMG difference score between the two stretch-velocities defined by instrumented spasticity assessment) and morphology (normalized MV, normalized muscle and tendon length, EI).

To achieve the first study aim, the associations between the primary spasticity outcome (EMG difference in between high-velocity and low-velocity stretch) and muscle morphology (normalized values of MV, muscle belly and tendon length, EI) were analyzed by a Spearman correlation (since the data were not normally distributed). Correlation values below 0.5 were considered low, 0.5 – 0.7 as moderate, and correlation coefficients of 0.7 or higher were considered strong (28). A Bonferroni correction was applied to correct for four multiple correlation analyses (i.e. one per morphology outcome), resulting in an alpha level of $0.05/4 = 0.0125$.

To compare results to literature findings, in a secondary analysis involved the associations between MAS and normalized muscle morphology outcomes and quality were assessed by a Spearman correlation analysis.

For the second study aim, the morphology between muscles of children classified with HVD and mixed activity patterns were compared using Mann-Whitney-U tests. A Bonferroni correction for multiple tests was applied per muscle (alpha = 0.05/4=0.0125). Since the groups were defined based on the muscle activation pattern during the passive stretches, there was no guarantee that the groups would be matched on body-sizes. Therefore, morphology parameters were normalized to body size, as described above.

Finally, in a secondary analysis, associations between the level of muscle activation during a low-velocity stretch and the morphological parameters were explored by Spearman correlations. This non-parametric approach was applied because the data were not normally distributed.

Results

Participant characteristics and clinical examination

A total of 74 children with SCP, all receiving regular physical therapy (supplementary material, Table S5), were included in this study. Due to artefacts in the ultrasound images (MG=2, ST=3), insufficient participant relaxation (MG=2, ST=1), absence of hyper-active stretch reflex defined during the instrumented spasticity assessment (MG=3) or processing/performance errors in either one of the assessments (ST=3), 67 datasets for both the MG and ST were included for final analyses.

Participant characteristics and details of the clinical examination are summarized in Table 1, while additional descriptive data are summarized in Table S1 (supplementary material).

Table 1. Participant characteristics

Total group of included participants (n=74)		
Age (years)*	8.2 (7.3 – 10.3)	
Sex (male/female)	38/36	
Body mass (kg)*	25.3 (21.0 – 32.9)	
Height (cm)*	125.3 (117.9 – 137.4)	
Topographic paresis (BL/UL)	47/27	
GMFCS level (I/II/III)	31/25/18	
Participant characteristics, per muscle activation pattern		
Medial gastrocnemius (n=67)	**HVD (n=37)**	**Mixed (n=30)**
Age (years)*	8.8 (7.3 – 10.5)	8.0 (7.1 – 9.3)
Topographic paresis (BL/UL)	21/16	23/7
GMFCS level (I/II/III)	20/13/4	6/11/13
MAS plantar flexors, knee extended	2.5 (2.0 – 3.0)	2.5 (2 – 3)
MTS plantar flexors, knee extended	-10.0 (-20.0 – -5.0)	-10 (-20 – -5)
MAS plantar flexors, knee 90 degrees	2.0 (1.8 – 2.3)	2.0 (2.0 – 2.0)
MTS plantar flexors, knee 90 degrees	-5.0 (-13.8 – 0.0)	0.0 (-10.0 – 3.8)
ROM plantar flexors, average low-velocity stretch (°)*	53.8 (43.5 – 64.0)	55.4 (47.8 – 67.0)
ROM plantar flexors, average high-velocity stretch (°)*	54.4 (45.1 – 62.0)	53.0 (45.9 – 63.7)
Medial hamstrings (n=67)	**HVD (n=35)**	**Mixed (n=32)**
Age (years)*	9.2 (7.5 – 10.8)	7.8 (7.2 – 9.4)
Topographic paresis (BL/UL)	15/20	28/4
GMFCS level (I/II/III)	22/13/0	5/11/16
MAS knee flexors	1.0 (1.0 – 1.5)	2.0 (1.5 – 2.0)
MTS knee flexors	-82.5 (-85.0 – -70.0)	-85.0 (-90.0 – -80.0)
Abs ROM knee flexors, average low-velocity stretch (°)*	66.2 (54.7 – 74.0)	58.8 (47.8 – 71.2)
Abs ROM knee flexors, average high-velocity stretch (°)*	67.7 (55.8 – 79.6)	58.8 (47.2 – 68.8)

Abs = absolute, BL = bilateral involvement, cm= centimeter, GMFCS = Gross Motor Function Classification System, HVD = high-velocity dependent muscle activation pattern, kg = kilogram, Mixed = mixed low-/high-velocity-dependent muscle activation pattern, n = number, ROM = range of motion UL = unilateral involvement **median with 25th and 75th percentile*

Spasticity and morphology parameters for the total group of children with SCP

Clear signs of spasticity and altered morphological for the total cohort and separate per muscle activation pattern can be observed in Table S1 (supplementary material).

Association between spasticity and morphology parameters (study aim 1)

The associations between spasticity and muscle morphology and quality of the MG and ST (primary outcomes) are summarized in Table 2.

Table 2. Correlations between normalized muscle morphology parameters, muscle quality and the average change in RMS-EMG between a low- and high-velocity passive stretch

Medial gastrocnemius	**change in RMS-EMG high-low velocity stretch (%MVC)**	
	r	**p-value**
Normalized muscle volume (ml/kg)	-0.624**	<0.001
Normalized muscle belly length (ratio height)	-0.369**	0.002
Tendon length (ratio height)	0.414**	<0.001
Echogenicity intensity (AU)	0.450**	<0.001
Semitendinosus, distal compartment	**change in RMS-EMG high-low velocity stretch (%MVC)**	
	r	**p-value**
Normalized muscle volume (ml/kg)	-0.297*	0.017
Normalized muscle belly length – total (ratio height)	-0.191	0.167
Normalized muscle belly length – distal (ratio height)	-0.145	0.245
Echogenicity intensity (AU)	0.184	0.183

AU = arbitrary units, kg = kilogram, ml = milliliters, MVC = maximum voluntary contraction, r = correlation coefficient, RMS-EMG = root mean square of the electromyography signal

**alpha<0.05, ** alpha<0.0125*

A significant moderate negative association was found between MV and the EMG difference between low and high-velocity stretch (r= -0.624, p<0.001) in the MG. The EMG differences were significantly but weakly or negligibly correlated with other muscle morphology parameters in the MG and ST. The association between the MAS (secondary outcome) and morphology parameters were also weak (Table S2 and S3, supplementary material).

Difference in muscle morphology between muscle activation patterns (study aim 2)

For the MG, 55.2% of the muscles presented with a HVD activation pattern in comparison to 52.2% of ST muscles. The distribution of muscle activation patterns per GMFCS level is presented in Figure S3 (supplementary material).

Normalized MV of the MG was significantly smaller in the group with mixed muscle activation patterns compared to muscles with HVD (p = 0.004) (Figure 1). Mixed low-/high-velocity-dependent ST muscles showed significantly reduced normalized MV (p < 0.001) as well as shortened total muscle belly length (p = 0.015) than HVD ST muscles (Figure 2).

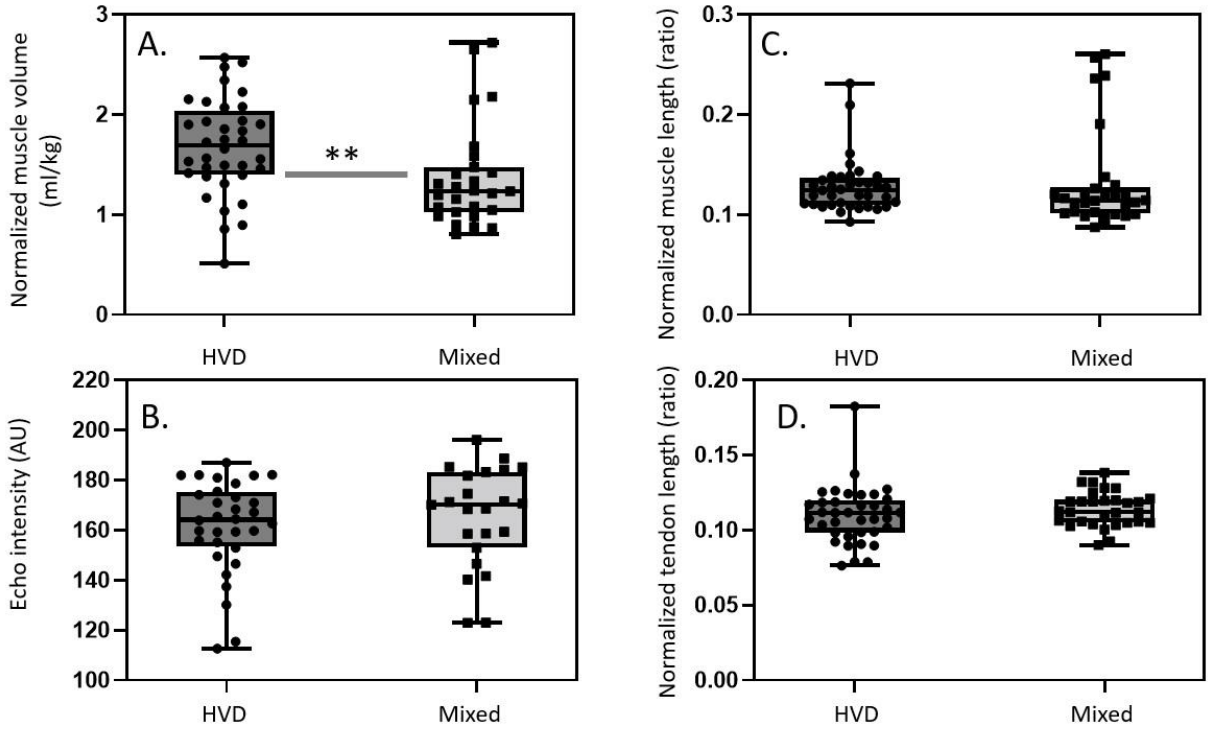

Figure 1. Box plot with individual data points of the muscle morphology and muscle quality comparison between muscle activation patterns of the medial gastrocnemius

A. Comparison of normalized muscle belly volume, **B.** Comparison of echogenicity intensity, **C**. Comparison of normalized muscle length, **D**. Comparison of normalized tendon length

**alpha<0.05 **alpha<0.0125 (corrected p-value after Bonferroni correction)*

AU = arbitrary units, HVD = pure high-velocity-dependent muscle activation pattern, Mixed = mixed low-/high-velocity-dependent muscle activation pattern

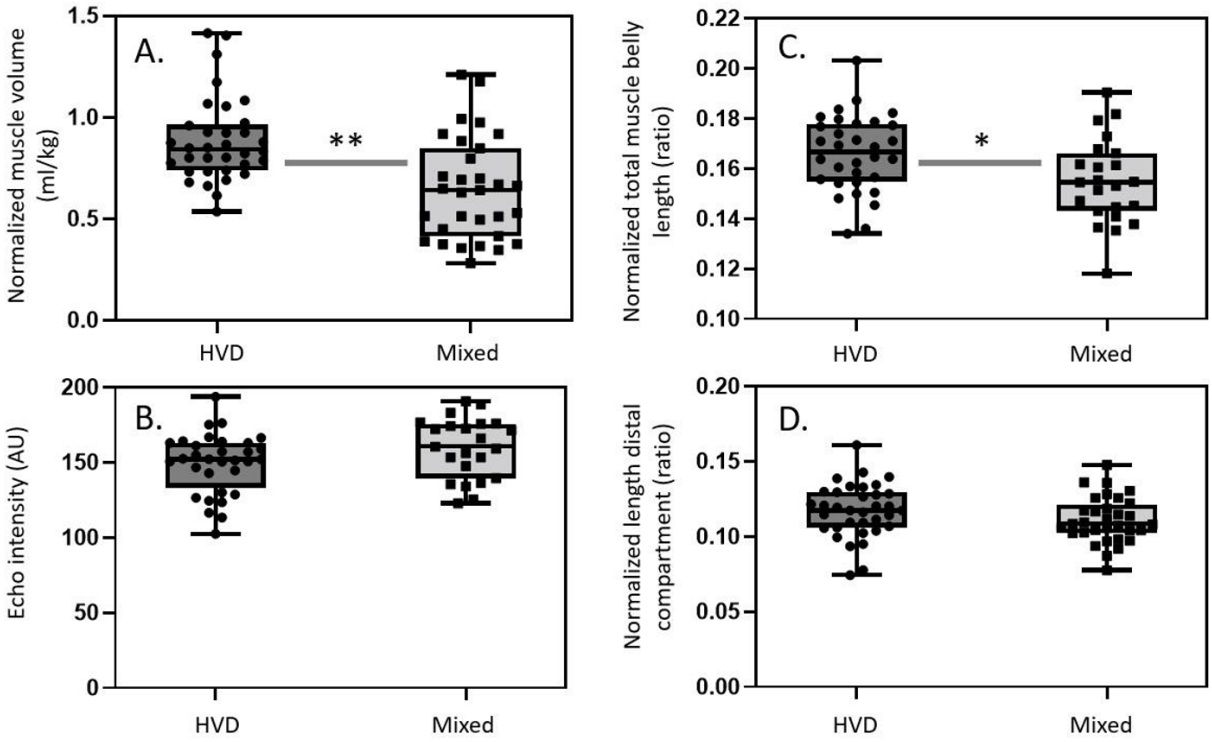

Figure 2. Box plot with individual data points of the muscle morphology and muscle quality comparison between muscle activation patterns of the semitendinosus

A. Comparison of normalized muscle belly volume, **B.** Comparison of echogenicity intensity, **C**. Comparison of normalized total muscle belly length, **D**. Comparison of normalized distal compartment length **alpha<0.05 **alpha<0.0125 (corrected p-value after Bonferroni correction)*

AU = arbitrary units, HVD = pure high-velocity-dependent muscle activation pattern, Mixed = mixed low-/high-velocity-dependent muscle activation pattern

Significant moderate associations between muscle activation levels during a low-velocity stretch and parameters of muscle morphology and quality indicated that muscles with lower normalized MG (r=-0.432, p<0.001) and ST (r=-0.401, p<0.001) volumes had more muscle activity during a low-velocity stretch (Table S7, supplementary material).

Discussion

Summary of findings

The current investigation reported several significant but mainly weak associations between muscle morphology and spasticity levels. Only one moderate correlation was found between MG MV and the EMG difference score between low and high stretch-velocity, showing that smaller MVs were associated with higher levels of spasticity (muscle excitation during passive stretch). Therefore, the first hypothesis could be only partly confirmed for the MG, and not for the ST.

The second hypothesis, which stated that muscles with mixed low-/high-velocity-dependent muscle activation patterns have a more severely altered morphology compared to muscles with HVD activation patterns, was also partly confirmed by the current study results, namely only for morphology (MG MV, ST MV and belly length for ST) and not for muscle quality.

In the following sections, these findings are further discussed and compared to relevant previous findings described in literature.

Aim 1: Association between spasticity and morphology parameters

The analyzed muscles showed a pathological hyper-active stretch reflex (excessive muscle activation during passive stretch) and altered muscle morphology (i.e. reduced MV and shorter muscle belly lengths). These findings were in line with previously presented data (29–31), and indicated that these muscles were characterized by spasticity and were significantly smaller compared to TD muscles, as shown in previous studies (32,33).

The results of the current study consistently indicated that higher levels of spasticity (defined by increased muscle activation during a high-velocity stretch) are associated with smaller MV and shorter muscle belly lengths in the MG and ST, with significant correlations varying between 0.294 and 0.624. Except for the MG MV ($r=0.624$), these correlations were weak.

Pierce et al. found opposite results showing that higher levels of spasticity measured by sEMG ($r=0.831$, $p<0.05$) and peak knee extensor passive torque ($r=0.738$, $p<0.05$) were strongly associated with larger volumes of the entire quadriceps. They hypothesized that over-activation in the muscle, as a result of spasticity, compensates for the disuse induced atrophy. In line with the findings of Pierce et al., previous studies including participants with spinal cord

injuries showed that higher levels of spasticity were associated with larger anatomical cross sectional areas or muscle mass (correlation coefficients ranging between 0.467-0.540) (34–36). The fact that the current study reported contradicting results can be due to the differences in population, timing of spasticity onset, investigated muscle, methodology and/or sample size. Previous studies described that alterations in muscle morphology and muscle activation patterns can vary between lower limb muscles (8,37). The study by Pierce et al. included a very small sample consisting of only eight participants with SCP between seven and 14 years of age, while the current study included a much larger sample aged between 3-18 years. As spasticity stabilizes around the age of five years, and younger children might not have the same degree of physical activity and biomechanical loading in comparison to older children, these small ages differences might have influenced the alterations in muscle morphology (38). Additionally, Pierce analyzed a muscle group, whereas the current study investigated two isolated muscles, making it impossible to compare the results at the level of the individual muscle. Besides that, previous studies showed that both spasticity level and muscle morphology are muscle-specific (8,37). Hence, more research is needed to further outline the potential pathology- and muscle-specific associations between muscle morphology and spasticity.

In the current study, the EI of the MG (EMG: r = 0.450, $p < 0.001$; MAS: r = 0.368, $p = 0.005$) was weak but positively correlated with EMG differences between high and low stretches, as well as with increased MAS scores, suggesting that the reduced intrinsic muscle quality of the MG may weakly contribute to the hyper-active stretch reflex. As only associations were explored, no conclusions can be drawn on causal relations. Moreover, Stecco et al. suggested that the reduced intrinsic muscle quality is potentially both a consequence and a contributor to the spastic reflex, as muscle fibrosis enhances the hyperactive stretch reflex which leads to more fibrosis, resulting in a vicious cycle (39).

Aim 2: Difference in muscle morphology between muscle activation patterns

While the expected differences between spasticity patterns were demonstrated for the normalized MG and ST MV and the ST muscle belly length, no differences in EI between the spasticity patterns were observed. This is in line with the above described weak associations between spasticity and EI. The potential role of intrinsic muscle quality, indirectly estimated through ultrasound imaging, in the phenomenon of spasticity may thus be questioned.

Previous studies have indicated that EI mainly contributes to the non-neural component of increased joint torque, whereas this study focused on the neural component (12,25). However, Gracies et al. showed that stiffer muscles, potentially due to non-neural alterations in the muscle belly, require less force to trigger the muscle spindles (10). Yet, since the EI cannot specify specific components of reduced muscle quality, but provides a rather overall estimation of muscle quality, more research is needed using sensitive assessments of muscle quality.

The smaller MVs or shorter lengths for muscles with mixed muscle activation patterns combined with the above described negative association between MV and the spasticity level suggest that hampered muscle growth is associated to the abnormal muscle activation during all stretches.

The current data showed that the total ST muscle belly length shortened with increasing level of muscle activation during low-velocity stretches. Additionally, the ROM of the knee flexors during high-velocity stretch was significantly less (p = 0.049, supplementary material Table S1) in children with mixed length-/velocity-dependent ST muscles compared to HVD ST muscles, which points towards contractures potentially related to increased stiffness. Besides that, the ROM may be reduced because it is assumed that this increased stiffness might be the cause of an earlier response to stretch, as described by Gracies et al. (10). However, ultrasound assessments were performed in muscles at rest and therefore more advanced techniques like dynamic ultrasound are required to collect data on extensibility or tissue compliance to test this hypothesis.

Potential underlying mechanisms and clinical relevance

Whether altered muscle morphology contributes to spasticity remains a matter of debate. The primary problem in SCP originates in the central nervous system and musculoskeletal problems are therefore often considered as secondary problems. Yet, there are indications that muscle morphology and growth are altered before the observation of the hyper-active stretch reflex. Willerslev-Olsen (40) showed that children with SCP have a reduced MV of the MG before an increase in muscle-tendon complex stiffness of the triceps surae was observed. Additionally, as stated above Stecco et al. (39) highlighted that the hyperactive stretch reflex is mediated by connective tissue alterations. They described that increased muscle stiffness would exacerbate the stretch reflex by affecting the muscle spindle threshold. Consequently,

an increase in fibroses is expected due to collagen deposition, resulting in a vicious cycle (10,39). The current study results partly confirmed this complex interaction between the muscle morphology and hyperactive stretch reflex.

It has been hypothesized that muscle activation during a low-velocity stretch may be due to dystonic features or spastic dystonia (41). Even though dystonia was an exclusion criterion in the current study, the dominant symptom of spasticity may have covered mild dystonia (42). Palisano et al. showed that the coexistence of dystonia in SCP increases with increasing level of gait pathology, which may explain the presence of more diverse muscle activation patterns in GMFCS levels II and III (43). In the current study, there were indications that children with higher GMFCS levels presented more often with mixed muscle activation patterns as well as more severely altered muscle morphology (Figure S3, supplementary material). Yet, the decomposition of dystonia in patients with predominantly spastic but mixed type of tone problems remains a challenge for future research. Furthermore, motor function is associated with both the level of spasticity and muscle morphology (44–46). Based on our data, no conclusions can be drawn on the origin of the differences in muscle morphology, whether this is primarily due to the muscle activation patterns, or GMFCS level, or a combination of both. Due to the small number of participants in the subgroups and unequal distribution of GMFCS I and III over the two muscle activation patterns no subgroup analyses per GMFCS level could be performed. However, a preliminary descriptive sub analyses within GMFCS level II confirmed smaller muscle volumes, shorter muscle belly lengths and increased EI values in the muscles with mixed patterns in comparison to muscles with HVD patterns (Table S6, supplementary information). Future studies should consider investigating muscle morphology and spasticity in relation to gross motor function and gait in more detail.

For efficient tone reduction treatment for spastic muscles, it is essential to distinguish between the neural and non-neural components contributing to the increased resistance at the joint (21) and to understand muscle pathology. Instrumented spasticity assessments may provide more insight into the muscle-specific pathology (e.g. the muscle-specific response to stretch) and thereby potentially contribute to efficient treatment planning. The current study results revealed that the altered muscle morphology should thereby also be considered. The interaction between treatment history and muscle activation patterns and muscle morphology was beyond the scope of this investigation, but should be considered in future studies.

Limitations

While previous research suggested echo intensity as proxy of muscle composition (47), validation studies are lacking in SCP. Therefore, it remains partly unknown to what extent this parameter is sensitive to detecting the severity of muscle fibrosis and other intrinsic alterations in SCP.

While the instrumented spasticity assessment is an objective measure, it is still based on manual performance. Inconsistent performances during the low or high velocity stretch, such as velocity differences amongst repeated trials or between patients, could potentially impact muscle activity patterns. However, no systematic differences in performance of stretches were reported between both muscle activation patterns (Table S1, supplementary material).

Because no distinction could been made between the activity of the ST and semimembranosus using sEMG, the morphology of the ST was correlated to activation of the entire medial hamstrings. Additionally, only the MV of the distal compartment of the ST was analyzed, as the proximal compartment is difficult to visualize by ultrasound, due to its deep origin.

Significant, but mainly weak to moderate correlations between muscle morphology and spasticity levels suggest other co-influencing factors in this relation. For future studies, it is recommended to add assessments of dystonia, to determine the impact of the co-existence of dystonia on the outcomes.

Conclusion

This study showed that alterations in muscle morphology are weak to moderately associated with spasticity and that morphology differs between muscle that are characterized by different activation patterns during passive stretch. Muscles that already activated during a low velocity stretch had smaller muscle volumes and reduced muscle belly lengths. Future studies should further explore the underlying mechanism of muscle activation patterns. Eventually, treatment should focus on the underlying pathophysiological mechanism of the hyper-resistance against stretch rather than solely on the secondary level of deformity or hyper-active stretch reflex.

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Supplementary Material

1. Detailed description of used methods

1.1. Instrumented spasticity assessment

This method has been previously reported by Lynn Bar-On and colleagues. They showed that this method is valid and reliable to assess hyper-resistance/spasticity (23,26).

The participant was positioned in supine. Three types of sensors were placed on the lower limb of the most affected side. Inertial measurement units (IMU) were placed on the three segments (upper-leg, lower-leg, foot) to measure the relative acceleration of the different segments. Muscle activation of the medial gastrocnemius and medial hamstring and the antagonist muscles tibialis anterior and rectus femoris were recorded by surface EMG (Zerowire Cometa, Milan, IT). Circular Ag/AgCl electrodes (1 cm diameter, 2 cm inter-electrode distance) were placed on the mid muscle belly according the SENIAM guidelines. EMG data were sampled at 2000 Hz. Last, a six-degrees freedom load cell (ATI mini45: Industrial Automation) was attached to the lower-leg or foot by a custom-made orthosis, after calibrating the sensor, to calculate joint torque. IMU and torque data were sampled at 200 Hz.

The participant was asked to perform three maximum voluntary contractions (MVC) of the agonist and antagonist muscles. Subsequently, the participant was instructed to relax and calibration trials with predefined movements were performed in order to estimate joint angles. Next, four passive manual stretches of either the plantar flexors (moving the foot towards dorsiflexion, while the knee angle remained constant) or hamstrings (moving the knee towards extension while the hip remained at 90 degrees flexion) were performed at a low velocity. During the stretches of the plantar flexors, the lower-leg was supported by a frame, allowing 30-40 degrees knee flexion. One stretch over the full range of motion starting at plantar flexion or knee flexion was performed in 5 seconds at a constant velocity. In between stretches, at least 10 seconds of rest was applied in order to avoid post-activation depression (44). Next, four passive stretches were performed as fast as possible.

Segment lengths (total leg length, lower-leg length and foot length) and the moment arms (perpendicular distances between joint axis and axis of the load cell) were measured with measuring tape by the assessor.

Data were analyzed using custom-made software (Single-joint software, MATLAB R2020b: MathWorks). The raw surface EMG signals were filtered with a 6th order zero-phase Butterworth bandpass filter (20-500 Hz). The root mean square envelope of the sEMG (RMS-EMG) was extracted by applying a low-pass 30 Hz 6th order zero-phase Butterworth filter on the squared signal. A Kalman smoother was applied on the IMU-data, to estimate joint position and angular/stretch velocity.
No torque data was included in this investigation.

After initial data processing, a quality check was performed. First, the consistency of performance was evaluated. Trials were excluded in case of inconsistent range of motion or stretch velocity (>20 degrees/second). Additionally, the quality of the EMG signals was evaluated. In case any antagonist activation was detected, the trails were excluded. Only the stretch trials of sufficient quality were used during the classification of the muscle activation patterns.

1.2. Three-dimensional freehand ultrasound

This method has been developed and previously reported by Francesco Cenni and proven to be a valid method in assessing both pathological and healthy muscles, with a strong inter acquirer reliability (24,32,45,46).

Muscle morphology was assessed by three-dimensional freehand ultrasound. This is an imaging technique combining conventional B-mode ultrasound with 3-dimensional motion tracking (Figure S1). In this way, two dimensional ultrasound images can be reconstructed into a three dimensional image of the muscle.
A linear ultrasound transduced with a 5.9 cm field of view (HL9.0/60/128Z) was connected to a Telemed-Echoblaster 128 Ext-1Z system to collect images. Four reflective markers were attached to the US transducer. The positions of the reflective markers were tracked by an OptiTrack V120: Trio motion-tracking system (NaturalPoint, Corvallis, OR, USA) with three

fixed optical cameras and a spatial resolution of 1 mm. Additionally, a custom-made curved gel pad named Portico, was placed on the ultrasound transducer to reduce deformation of the muscle by pressure during the acquisition (47).

Images were recorded at 30 Hz. The ultrasound acquisition settings were kept constant between the different acquisitions (frequency, 10 MHz; depth, 5 cm; focus, 1.8-2.8 cm; gain, 46%; dynamic range, 44 dB, and unaltered time-gain compensation).

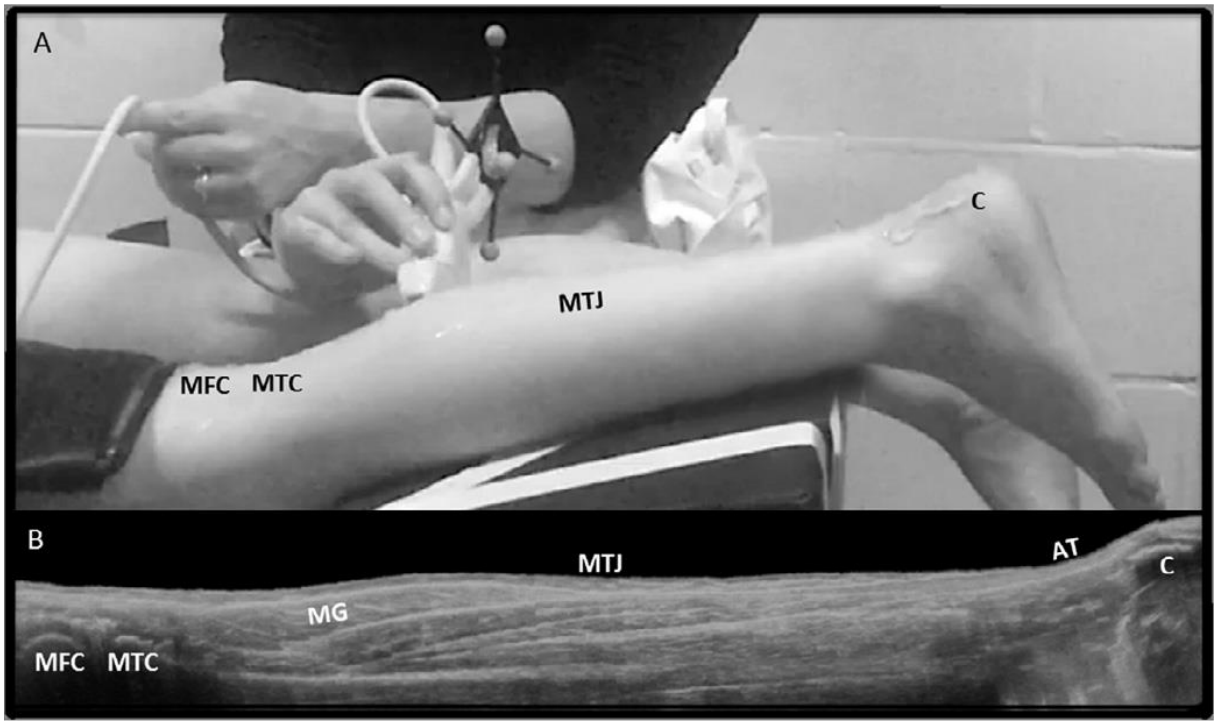

Figure S1. The three-dimensional freehand ultrasound technique. Top image: performance of an ultrasound acquisition (sweep) of the medial head of the gastrocnemius muscle. Bottom image: three-dimensional reconstruction of the medial gastrocnemius with landmarks indicated (*Image from Cenni et al. (2018)*(46)*, reused with permission)*

AT = Achilles tendon, C = calcaneus, MFC = medial femoral condyle, MG = medial gastrocnemius (muscle belly), MTC = medial tibial condyle

The participant was laying prone, while the lower leg was supported by a triangular cushion. As a result, slight knee flexion (approximately 20-30 degrees) was allowed, reducing bi-articular stretch on the muscle-tendon unit. The ankle was in a relaxed, unconstrained condition, during data collection.

Ultrasound sweeps were taken of the medial gastrocnemius and semitendinosus muscle and their corresponding tendons. Ultrasound images were recorded and three-dimensional reconstructions were created by a Stradwin software package (version 6.0) or Echowave (version II 3.5.2.) and custom-made Python software (version 2.7 2.7 www.python.org). After applying acoustic gel to the skin and US transducer, the probe was moved perpendicular to the skin over the muscle while applying as little pressure as possible without losing contact with the skin. The speed of the acquisition was adjusted to aim for a constant frame rate. The participant was instructed to lay still and relaxed. The acquisition was repeated in case of movement of the subject or muscle contraction. For the medial gastrocnemius the transducer was moved from the muscle origin at the medial femur condyle along the tendinous insertion on the calcaneus. For the semitendinosus muscle, the transducer was moved from the muscle insertion at the ischial tuberosity to the medial side of the tibia plateau.

Muscle volume of the medial gastrocnemius and distal compartment of the semitendinosus were estimated by drawing manual segmentations at 5-10% of the transverse ultrasound images. By interpolation according the cubic planimetry technique, the volume was estimated in milliliters (48). The manual segmentations and subsequent three-dimensional reconstructions were also used to estimate the echogenicity intensity, which is an indirect measure for muscle quality(12). The echogenicity intensity was expressed as a grey value in arbitrary units on an 8-bit scale, values between 0-255.

Muscle length was calculated as the Euclidean distance between the origin (MG: femoral condyle, ST: ischial tuberosity, ST distal compartment: opening of the internal aponeurosis) and muscle-tendon junction. The tendon length of the medial gastrocnemius was estimated as the Euclidean distance between the muscle-tendon junction and the insertion at the calcaneus.

To allow between-group comparison, muscle volume and the largest cross-sectional area were normalized to body mass. The muscle belly and tendon length were expressed as a ratio of the total body length.

Due to the deep origin of the semitendinosus and subcutaneous tissue, it was not possible to visualize the proximal compartment of the semitendinosus by ultrasound without deforming

the muscle. Therefore, the muscle volume and echogenicity intensity of the proximal compartment of the semitendinosus were not included in the data analyses.

1.3. Classification of muscle activation patterns

This method was developed and preciously described by Bar-On et al. and is shown to have a good intra-rater reliability (8).

Muscle activation patterns were classified based on the RMS-EMG signals that were expressed as a percentage of MVC. Muscle activation patterns were classified after visual inspection of the level of muscle activation over the range of motion (ROM) and during high- and low-velocity stretches. The ROM, without the first and last 10%, was divided in three zones.

Bar-On et al described five different muscle activation patterns: high velocity-dependent (HVD), mixed high velocity-dependent, mixed, mixed low velocity-dependent, or low velocity-dependent(8). In this study, two groups were created: HVD and mixed low-/high-velocity-dependent. The mixed group contained all patterns of mixed high velocity-dependent, mixed and mixed low velocity-dependent. No pure low velocity-dependent patterns were observed in the current study.
A muscle was classified as mixed if the difference in muscle activation between the first and last zone of the ROM exceeded 3%MVC during the low-velocity stretch. In case the increase in muscle activation over the ROM did not exceed 3%MVC, the muscle was classified as pure high-velocity dependent.

1.4. Clinical examination

All participants underwent a full clinical examination of the lower-limbs. Only the results of the most involved limb were included in this study. First, the passive ROM of the ankle and knee joint was assessed by goniometry. Second, spasticity of the knee flexors and plantar flexors was assessed by the modified Ashworth and a possible catch was defined by the modified Tardieu scale (49–51). Last, muscle strength was assessed by manual muscle testing and selective motor control was assessed by an adjusted selectivity scale (based on the method described by Gage et al.), grading on a 5-point scale between 0 and 2 for knee flexion and plantar flexion (52).

2. Supplementary data

2.1. Descriptive data of participants and data on clinical examination, muscle morphology and instrumented spasticity assessment stratified per muscle activation pattern.

Table S1**.** contains additional data of the participants that were included in this investigation. Only data of the most affected leg is presented.

Table S1. Additional descriptive data of participants and assessments

	Medial gastrocnemius (n=67)			
Participant characteristics	**Total (n=74)**	**HVD (n=37)**	**Mixed (n=30)**	**p-value between groups (HVD & mixed)**
Age (years)*****	8.2 (7.3 – 10.3)	8.8 (7.3 – 10.5)	8.0 (7.1 – 9.3)	0.115
Sex (male/female)(n)	38/36	19/18	16/14	n.a.
Body mass (kg)*****	25.3 (21.0 – 32.9)	27.5 (23.8 – 35.0)	22.9 (19.1 – 27.9)	0.012[i]
Height (cm)*****	125.3 (117.9 – 137.4)	130.0 (124.3 – 145.5)	119.0 (116.6 – 129.9)	0.002[i]
Topographic paresis (BL/UL)(n)	47/27	21/16	23/7	n.a.
GMFCS (I/II/III)(n)	31/25/18	20/13/4	6/11/13	n.a.
Clinical examination	**Total (n=73)**	**HVD (n=37)**	**Mixed (n=30)**	**p-value**
ROM PF knee 90 degrees (°)*****	15 (5 – 20)	15 (5 – 20)	17.5 (8.8 – 25)	0.239
ROM PF knee extended (°)*****	5 (0 – 10)	5 (0 – 10)	7.5 (0 – 15)	0.733
MAS PF knee 90 degrees*	2 (2 – 2)	2 (1.75 – 2.25)	2 (2 – 2)	n.a.
MAS PF knee extended*	3 (2 – 3)	2.5 (2 – 3)	2.5 (2 – 3)	n.a.
Tardieu PF knee 90 degrees (°)*****	-5 (-10 – 0.0)	-5 (-13.75 - 0)	0 (-10 – 3.75)	0.345
Tardieu PF knee extended (°)*****	-10 (-20 - -5)	-10 (-20 – -5)	-10 (-20 – -5)	0.944
MRC plantar flexion*	3.3 (3 – 4)	4 (3 – 4)	3 (2 – 4)	n.a.
Selectivity plantar flexion (scale from 0-2, steps of 0.5)*****	1.5 (1 – 2)	1.5 (1.5 – 2)	1.5 (0.9 – 2)	n.a.
Muscle morphology	**Total (n=67)**	**HVD (n=37)**	**Mixed (n=30)**	**p-value**
nMV (ml/kg)*****	1.49 (1.18 – 1.92)	1.69 (1.40 – 2.04)	1.23 (1.03 – 1.48)	0.004[i]

nML (ratio total height)*	0.12 (0.11 – 0.13)	0.12 (0.11 – 0.14)	0.11 (0.10 – 0.13)	0.101
nTL (ratio total height)*	0.11 (0.10 – 0.12)	0.11 (0.10 – 0.12)	0.11 (0.11 – 0.12)	0.198
EI (AU)*	164.93 (150.36 – 175.26)	164.20 (153.49 – 175.16)	170.18 (152.97 – 183.22)	0.366
Spasticity assessment	**Total (n=67)**	**HVD (n=37)**	**Mixed (n=30)**	**p-value**
ROM PF, average low-velocity stretch (°)*	55.4 (45.6 – 65.8)	53.79 (43.49 – 64.04)	55.44 (47.81 – 66.99)	0.480
ROM PF, average high-velocity stretch (°)*	54.3 (45.3 – 62.2)	54.40 (45.07 – 61.96)	52.97 (45.93 – 63.73)	0.890
Change EMG high (µV)	11.73 (4.32 – 19.84)	7.25 (2.77 – 19.74)	13.18 (8.66 – 18.66)	0.190
Change EMG high (%MVC)*	10.30 (2.51 – 25.15)	3.82 (1.74 – 13.19)	23.16 (9.46 – 31.44)	<0.001[i]
Average EMG low (%MVC)*	2.59 (0.71 – 6.47)	0.78 (0.35 – 1.79)	6.18 (4.14 – 14.19)	<0.001[i]
Average EMG high (%MVC)*	5.14 (1.91 – 12.90)	2.32 (1.12 – 4.54)	12.79 (5.93 – 23.60)	<0.001[i]
Change EMG zone 1-3 (%MVC)*	2.56 (0.22 – 6.82)	0.33 (0.07 – 1.55)	6.97 (4.09 – 17.20)	<0.001[i]
MVC max RMS-EMG (µV)*	0.11 (0.06 – 0.23)	0.20 (0.11 – 0.27)	0.07 (0.04 – 0.12)	0.668
Max average velocity low (°/second)*	17.33 (14.08 – 20.43)	17.46 (14.21 – 20.91)	17.16 (13.66 – 20.07)	0.504
Max average velocity high (°/second)*	138.37 (103.58 – 170.75)	142.35 (105.77 – 169.00)	123.28 (102.95 – 175.56)	<0.001[i]
		Medial hamstrings (n=67)		
Participant characteristics	**Total (n=74)**	**HVD (n=35)**	**Mixed (n=32)**	**p-value**
Age (years)*	8.2 (7.3 – 10.3)	9.2 (7.5 – 10.8)	7.8 (7.2 – 9.4)	0.056
Sex (male/female)(n)	38/36	19/16	14/18	n.a.
Body mass (kg)*	25.3 (21.0 – 32.9)	25.9 (23.2 – 35.9)	23.30 (20.7 – 28.4)	0.027[i]
Height (cm)*	125.3 (117.9 – 137.4)	132.0 (123.4 – 147.5)	119.7 (116.7 – 128.9)	0.002[i]
Topographic paresis (BL/UL)(n)	47/27	15/20	28/4	n.a.
GMFCS (I/II/III)(n)	31/25/18	22/13/0	5/11/16	n.a.
Clinical examination	**Total (n=74)**	**HVD (n=35)**	**Mixed (n=32)**	**p-value**
Popliteal angle unilateral (°)*	-60 (-70 – -55)	-60 (-70 – -50)	-70 (-75 – -60)	0.042[i]
Popliteal angle bilateral (°)*	-60 (-67.5 – -55)	-60 (-66.25 – -50)	-65 (-70 – -55)	0.217
MAS knee flexors*	1.5 (1 – 2)	1 (1 – 1.5)	1.8 (1.5 – 2)	n.a.
Tardieu knee flexors (°)*	-85 (-90 – -80)	-82.5 (-85 – -70)	-85 (-90 – -80)	0.206

MRC knee flexion*	4 (4 – 3)	4 (4 – 4)	3 (3 – 3.1)	n.a.
Selectivity knee flexion (scale from 0-2, steps of 0.5)*	1.5 (1 – 2)	2 (1.5 – 2)	1 (1 – 1.5)	n.a.
Muscle morphology	**Total (n=68)**	**HVD (n=35)**	**Mixed (n=32)**	**p-value**
nMV (ml/(kg)*	0.78 (0.62 – 0.92)	0.84 (0.74 – 0.96)	0.64 (0.41 – 0.85)	<0.001[i]
nML total muscle belly (ratio total height)*	0.16 (0.09 – 0.11)	0.17 (0.15 – 0.18)	0.15 (0.14 – 0.17)	0.015[i]
nML proximal compartment (ratio total height)*	0.10 (0.09 – 0.11)	0.10 (0.10 – 0.11)	0.11 (0.09 – 0.12)	0.331
nML distal compartment (ratio total height)*	0.11 (0.10 – 0.13)	0.12 (0.11 – 0.13)	0.11 (0.10 – 0.12)	0.077
EI (AU)*	153.46 (138.79 – 166.55)	152.15 (133.32 – 163.06)	160.53 (139.59 – 175.83)	0.063
Spasticity assessment	**Total (n=72)**	**HVD (n=35)**	**Mixed (n=32)**	**p-value**
Abs ROM KF, average low-velocity stretch (°)*	61.26 (49.90 – 73.07)	66.22 (54.70 – 74.02)	58.75 (47.75 – 71.15)	0.103
Abs ROM KF, average high-velocity stretch (°)*	61.53 (50.32 – 75.50)	67.68 (55.77 – 79.64)	58.82 (47.18 – 68.80)	0.049[i]
Change EMG high (µV)*	9.64 (4.97 – 13.74)	8.11 (3.92 – 13.29)	11.02 (5.36 – 18.88)	0.369
Change EMG high (%MVC)*	7.89 (3.48 – 14.95)	5.29 (2.01 – 8.56)	12.37 (5.53 – 19.15)	0.003[i]
Average EMG low (%MVC)*	2.11 (0.55 – 7.66)	0.61 (0.27 – 1.57)	7.63 (3.21 – 13.67)	<0.001[i]
Average EMG high (%MVC)*	3.90 (1.53 – 10.44)	1.87 (1.00 – 3.16)	9.50 (4.84 – 14.20)	<0.001[i]
Change EMG zone 1-3 (%MVC)*	2.80 (0.29 – 12.65)	0.30 (0.02 – 1.52)	11.55 (5.30 – 17.71)	<0.001[i]
MVC max RMS-EMG (µV)*	134.00 (76.44 – 199.01)	175.96 (128.28 – 244.32)	89.87 (58.29 – 137.76)	<0.001[i]
Max average velocity low (°/second)*	21.95 (17.75 – 28.80)	22.92 (18.88 – 29.40)	21.15 (16.59 – 27.06)	0.390
Max average velocity high (°/second)*	252.43 (199.16 – 288.09)	268.55 (235.70 – 304.86)	239.90 (184.57 – 261.16)	0.004[i]

Abs = absolute, cm = centimeters, EMG = electromyography, HVD = pure high-velocity dependent, KF = knee flexors, kg = kilograms, m = meter, mixed = mixed length-/velocity dependent, ml = milliliter, MVC = maximum voluntary contraction, n = number, n.a. = not applicable, nML = normalized muscle length, nMV, normalized muscle volume, nTL = normalized tendon length, PF = plantar flexors, RMS = root mean square, ROM = range of motion, µV = micro volts

**median (p25-p75). [i]Alpha<0.05*

2.2 Associations between the Modified Ashworth scale and muscle morphology

The results of the secondary correlation analyses between the MAS, as assessed during the clinical examination, and muscle morphology are presented in Table S2 for the absolute morphology parameters and Table S3 for the normalized morphology parameters.

Table S2. Correlations between normalized muscle morphology parameters and modified Ashworth scale

Medial gastrocnemius	**MAS plantar flexors, knee extended**		**MAS plantar flexors, knee 90°**	
	r	**p-value**	**r**	**p-value**
Normalized muscle volume (ml/kg)	-0.294*	0.018	-0.089	0.479
Normalized muscle belly length (ratio height)	-0.198	0.103	-0.053	0.665
Tendon length (ratio height)	0.080	0.514	-0.058	0.633
Echogenicity intensity (AU)	0.368*	0.005	0.183	0.174
Semitendinosus, distal compartment	**MAS hamstrings**			
	r	**p-value**		
Normalized muscle volume (ml)	-0.312*	0.010		
Normalized muscle belly length - total (ratio height)	-0.212	0.109		
Normalized muscle belly length - proximal (ratio height)	-0.072	0.594		
Normalized muscle belly length - distal (ratio height)	-0.204	0.090		
Echogenicity intensity (AU)	0.301*	0.022		

AU = arbitrary units, kg = kilograms, MAS = Modified Ashworth scale, ml = milliliters, r = correlation coefficient **alpha<0.05*

Table S3. Correlations between absolute muscle morphology parameters and modified Ashworth scale

Medial gastrocnemius	**MAS plantar flexors, knee extended**		**MAS plantar flexors, knee 90°**	
	r	**p-value**	**r**	**p-value**
Muscle volume (ml)	-0.309*	0.012	-0.130	0.300
Muscle belly length (mm)	-0.230	0.057	-0.110	0.366
Tendon length (mm)	0.007	0.957	-0.086	0.483
Echogenicity intensity (AU)	0.368*	0.005	0.183	0.174
Semitendinosus, distal compartment	**MAS hamstrings**			
	r	**p-value**		
Muscle volume (ml)	-0.356*	0.003		
Muscle belly length, total (mm)	-0.320	0.014		
Muscle belly length, proximal (mm)	-0.198	0.136		
Muscle belly length, distal (mm)	-0.307*	0.010		
Echogenicity intensity (AU)	0.301	0.022		

AU = arbitrary units, MAS = Modified Ashworth scale, mm = millimeters, ml = milliliters, r = correlation coefficient **alpha<0.05*

2.3. Associations between absolute muscle morphology parameters and the change in RMS-EMG between a low- and high-velocity passive stretch

The associations presented in the manuscript were between the normalized muscle morphology parameters and the change in EMG expressed as a percentage of the MVC. In Table S4 the correlations between the absolute morphology parameters and the absolute change in raw RMS-EMG are presented.

Table S4. Correlations between absolute muscle morphology parameters and RMS-EMG between a low- and high-velocity passive stretch

Medial gastrocnemius	**Absolute change in RMS-EMG (mV)**	
	r	**p-value**
Muscle volume (ml)	-0.589*	<0.001
Muscle belly length (mm)	-0.371*	0.002
Tendon length (mm)	-0.018	0.886
Echogenicity intensity (AU)	0.418*	0.001
Semitendinosus	**Absolute change in RMS-EMG (mV)**	
	r	**p-value**
Muscle volume, distal compartment (ml)	-0.245	0.051
Muscle belly length, total (mm)	-0.250	0.068
Muscle belly length, proximal compartment (mm)	-0.165	0.233
Muscle belly length, distal compartment (mm)	-0.198	0.112
Echogenicity intensity (AU)	0.249	0.070

AU = arbitrary units, mm = millimeters, ml = milliliters, mV = millivolts, r = correlation coefficient, RMS-EMG = root mean square of the electromyography signal **alpha<0.05*

2.4. Additional information on medical history and current therapy of the included participants

Table S5 presents information on the current therapy and medical history of the participants per muscle activation pattern.

Table S5. Details on current therapy and medical history

Details therapy		
Medial gastrocnemius (n=69)	**HVD (n=37)**	**Mixed (n=30)**
Physical therapy (n)	Yes = 37 No = 0	Yes = 28 No = 0 Unknown = 2
Median number minutes/week PT*	120 (60 – 180)	180 (90 – 240)
AFO's during the day (n)	Yes = 31 No = 6	Yes = 28 No = 1 Unknown = 1
Median frequency AFO*	100 (75 – 100)	100 (100 – 100)
Night AFO's (n)	Yes = 13 No = 23 Unknown = 1	Yes = 16 No = 10 Unknown = 4
Knee extension braces during the night (n)	Yes = 10 No = 26 Unknown = 1	Yes = 13 No = 13 Unknown = 4
Standing frame (n)	Yes = 11 No = 26	Yes = 17 No = 13
Oral baclofen (n)	Yes = 4 No = 28 Unknown = 5	Yes = 11 No = 14 Unknown = 5
Previous botulinum neurotoxin-A injections (n)	Naïve =12 1-3 =15 >3 =9 Unknown = 1	Naïve = 1 1-3 = 13 >3 = 15 Unknown = 1
Previous casting periods (without botulinum neurotoxin-A) (n)	Naïve =27 1-3 = 8 >3 = 2	Naïve = 24 1-3 = 6 >3 = 0
Walking distance, with walking aids if necessary (n)	<100m = 1 100-500m = 5 500-1000m = 5 1000-3000m = 5 >3000m = 20 Unknown = 1	<100m = 3 100-500m = 14 500-1000m = 1 1000-3000m = 4 >3000m = 5 Unknown = 3
Medial hamstrings (n=70)	**HVD (n=35)**	**Mixed (n=32)**
Physical therapy (n)	Yes = 34 Unknown = 1	Yes = 31 Unknown = 1
Median number minutes/week PT*	90 (60 – 180)	180 (90 – 236.25)
AFO's during the day (n)	Yes = 28 No = 5 Unknown = 2	Yes = 30 No = 2

Median frequency AFO*	100 (75 – 100)	100 (100 – 100)
Night AFO's (n)	Yes = 10 No = 23 Unknown = 2	Yes = 18 No = 10 Unknown = 4
Knee extension braces during the night (n)	Yes = 8 No = 24 Unknown = 3	Yes = 14 No = 14 Unknown = 4
Standing frame (n)	Yes = 5 No = 30	Yes = 22 No = 10
Oral baclofen (n)	Yes = 3 No = 26 Unknown = 6	Yes = 11 No = 18 Unknown = 3
Previous botulinum neurotoxin-A injections (n)	Naïve =12 1-3 = 16 >3 = 6 Unknown = 1	Naïve = 1 1-3 = 15 >3 = 15 Unknown = 1
Previous casting periods (without botulinum neurotoxin-A) (n)	Naïve = 25 1-3 = 8 >3 = 2	Naïve = 27 1-3 = 5 >3 = 0
Walking distance, with walking aids if necessary (n)	<100m = 0 100-500m = 4 500-1000m = 4 1000-3000m = 5 >3000m = 20 Unknown = 2	<100m = 5 100-500m = 12 500-1000m = 4 1000-3000m = 4 >3000m = 5 Unknown = 2

AFO = ankle foot orthosis, HVD = pure high-velocity dependent, KF = knee flexors, kg = kilograms, m = meter, mixed = mixed length-/velocity dependent, n = number, PT = physical therapy

**median (p25-p75)*

2.5. Distribution of muscle activation patterns within GMFCS levels and subgroup analyses

The distribution of the muscle activation patterns over the different GMFCS levels is summarized in Figure S3.

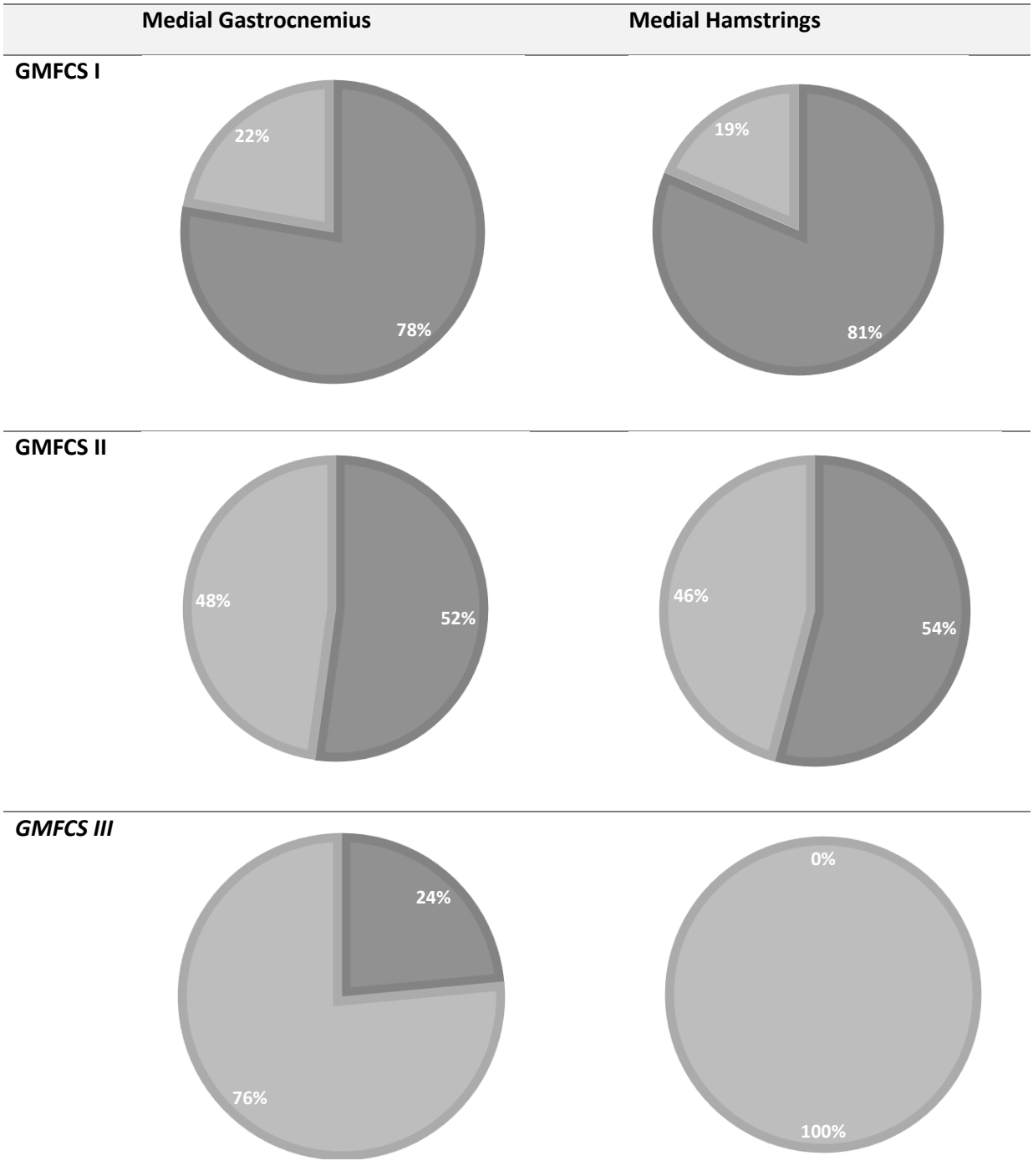

Figure S3. Distribution of muscle activation patterns within GMFCS levels

Dark grey=high-velocity-dependent; light grey=mixed low-/high-velocity-dependent

GMFCS = gross motor function classification system

The origin of the differences in muscle morphology, whether this is primarily due to the muscle activation patterns, or GMFCS level, or a combination of both, remains partly unknown. Therefore, we have performed descriptive analyses per aim, to provide more insight into the data.

For the first aim, scatterplots on the association between level of spasticity and normalized muscle volume, per GMFCS level were created and presented in Figure S3 for the medial gastrocnemius and Figure S4 for the distal compartment of the semitendinosus.

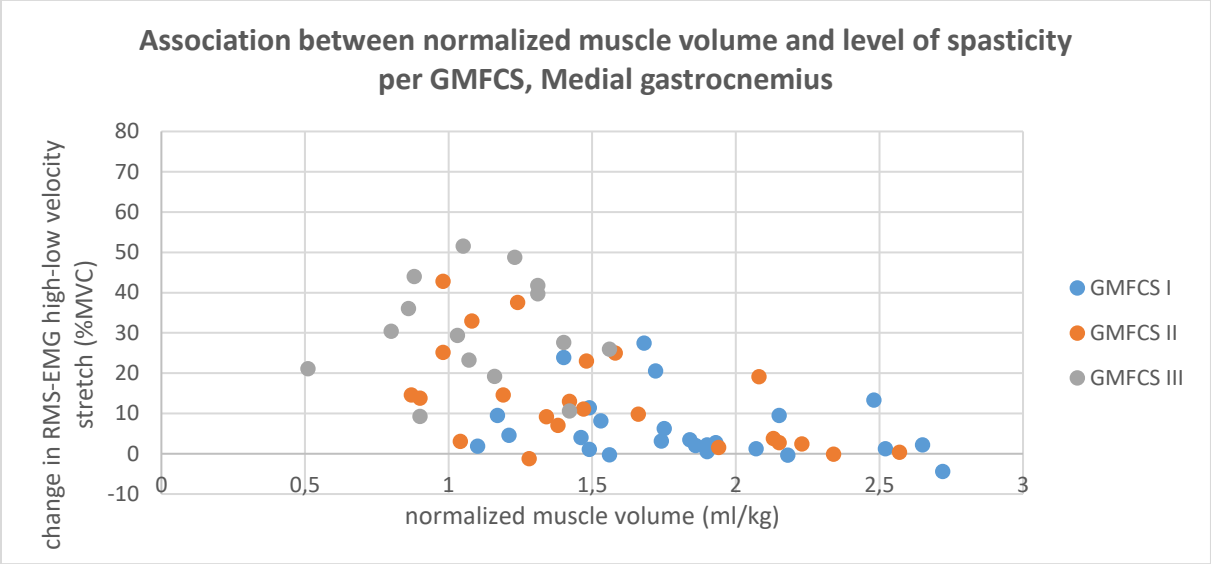

Figure S3. Scatter plot of the association between the level of spasticity and normalized muscle morphology of the medial gastrocnemius

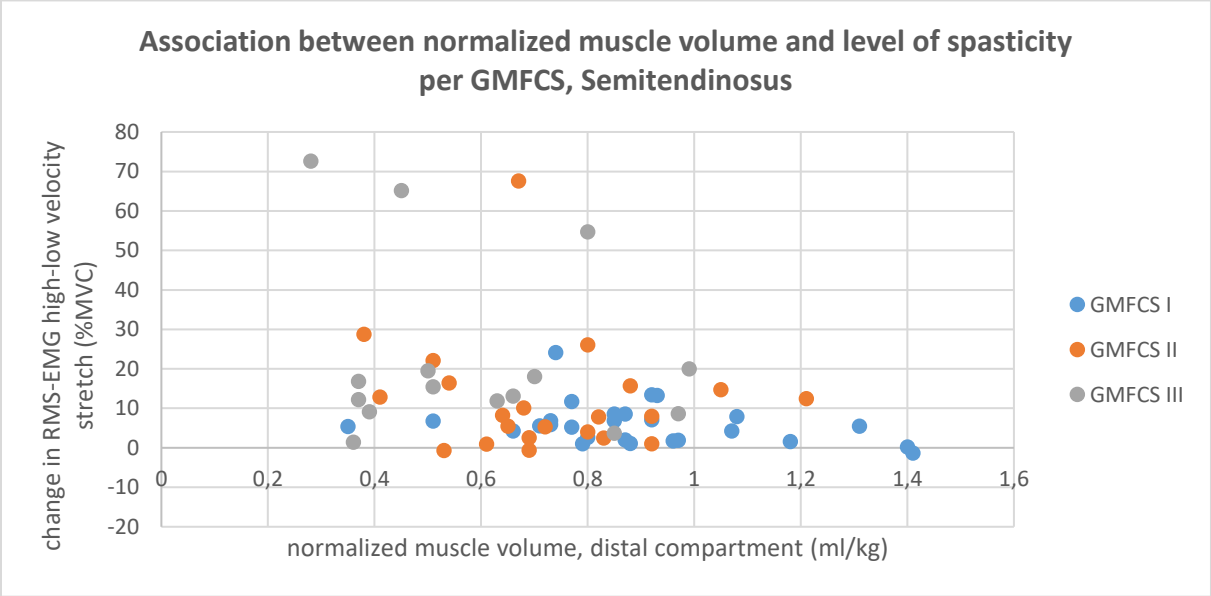

Figure S4. Scatter plot of the association between the level of spasticity and normalized muscle morphology of the distal compartment of the semitendinosus

For the second aim, descriptive analyses of muscle moprhology outcomes were performed within GMFCS level II, as GMFCS level I and III were distributed uneven. A non-parametric Mann Whitney U test was performed to compare the medians between the two patterns with GMFCS level II.

Table S6. Differences in muscle morphology and muscle quality between muscle activation patterns of the medial gastrocnemius and semitendinosus within GMFCS level II

Medial gastrocnemius	**HVD, n=13** *(median, p25-p75)*	**Mixed, n=11** *(median, p25-p75)*	**p-value**
Normalized muscle volume (ml/kg)	1.94 (1.40 – 2.19)	1.22 (0.98 – 1.37)	0.006
Normalized muscle belly length (ratio height)	0.13 (0.12 – 0.14)	0.11 (0.10 – 0.12)	<0.001
Tendon length (ratio height)	0.11 (0.10 – 0.12)	0.11 (0.11 – 0.12)	0.608
Echogenicity intensity (AU)	160.94 (148.75 – 182.00)	171.43 (162.70 – 185.19)	0.280
Semitendinosus	**HVD,** n=13 *(median, p25-p75)*	**Mixed,** n=11 *(median, p25-p75)*	**p-value**
Normalized muscle volume, distal (ml/kg)	0.80 (0.68 – 0.90)	0.65 (0.51 – 0.88)	0.118
Normalized muscle belly length, total (ratio height)	0.17 (0.15 – 0.15)	0.16 (0.15 – 0.18)	0.780
Normalized muscle belly length, distal (ratio height)	0.12 (0.10 – 0.14)	0.11 (0.10 – 0.12)	0.531
Echogenicity intensity, distal (AU)	155.98 (136.31 – 175.50)	172.02 (149.19 – 176.07)	0.481

Outcomes are presented as median values with the 25th and 75th percentiles.

AU = arbitrary units, HVD = high-velocity-dependent, kg = kilograms, Mixed = mixed low-/high-velocity-depedent, ml = milliliters

2.6. Correlations between muscle activation during a low-velocity stretch and parameters of muscle morphology and muscle quality

The second analyses of our second aim includes a correlation analyses between the average level of muscle activation during a low-velocity stretch and the normalized muscle morphology parameters and EI.

Table S7. Correlations between muscle morphology and muscle activation levels during low-velocity stretch

Medial gastrocnemius		**N**	**r**	**p-value**
Average EMG, low-velocity stretch (%MVC)	**nMV**	68	-0.432*	<0.001
Average EMG, low-velocity stretch (%MVC)	**nML**	68	-0.201	0.102
Average EMG, low-velocity stretch (%MVC)	**nTL**	68	0.246	0.045*
Average EMG, low-velocity stretch (%MVC)	**EI**	56	0.259	0.056
Semitendinosus		**N**	**r**	**p-value**
Average EMG, low-velocity stretch (%MVC)	**nMV_distal**	64	-0.401*	<0.001
Average EMG, low-velocity stretch (%MVC)	**nML_total**	54	-0.257	0.061
Average EMG, low-velocity stretch (%MVC)	**nML_proximal**	54	0.238	0.083
Average EMG, low-velocity stretch (%MVC)	**nML_distal**	66	-0.200	0.108
Average EMG, low-velocity stretch (%MVC)	**EI distal**	54	-0.241	0.079

AU = arbitrary units, EI = echogenicity intensity, MVC = maximum voluntary contraction, N = number, nML = muscle length normalized to body length, nMV = muscle volume normalized to body weight, nTL = tendon length normalized to body length, r = correlation coefficient **alpha<0.05*

Objective 3.

Investigate the acute and short-term impact of Botulinum Neurotoxin-A on muscle morphology in children with spastic cerebral palsy

Chapter 2.4.

Joint and Muscle Assessments of the Separate Effects of Botulinum Neurotoxin-A and Lower-Leg Casting in Children With Cerebral Palsy

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Abbreviations

ANOVA = analysis of variance

BoNT-A = Botulinum Neurotoxin-A

C = calcaneus

CP = cerebral palsy

EMG = electromyography

GMFCS = Gross Motor Functional Classification Scale

Hz = hertz

IMU = inertial measurement unit

IQR = interquartile range

J = Joule

LG = lateral gastrocnemius

MFC = medial femoral condyle

MG = medial gastrocnemius; mm, millimeter

ML = muscle belly length

MTJ = muscle tendon junction

MAS = modified Ashworth scale

MTS = modified Tardieu scale

rms = root mean square

rms EMG = root mean square of the electromyographic signal

ROM = range of motion

SDR = selective dorsal rhizotomy

SEM = standard error of measurement

SOL = soleus muscle

TL = tendon length

µV = microvolt

2D US = two-dimensional ultrasound

3D = three-dimensional

3DfUS = three-dimensional freehand ultrasound

Keywords: cerebral palsy, tendon length, muscle length, spasticity, hyper-resistance, casting, Botulinum Neurotoxin, muscle stretch reflex

Abstract

Botulinum Neurotoxin-A (BoNT-A) injections to the medial gastrocnemius (MG) and lower-leg casts are commonly combined to treat ankle equinus in children with spastic cerebral palsy (CP). However, the decomposed treatment effects on muscle or tendon structure, stretch reflexes, and joint are unknown.

In this study, BoNT-A injections to the MG and casting of the lower legs were applied separately to gain insight into the working mechanisms of the isolated treatments on joint, muscle, and tendon levels. Thirty-one children with spastic CP (GMFCS I-III, age 7.4 ± 2.6 years) received either two weeks of lower-leg casts or MG BoNT-A injections. During full range of motion slow and fast passive ankle rotations, joint resistance and MG stretch reflexes were measured. MG muscle and tendon lengths were assessed at resting and at maximum dorsiflexion ankle angles using 3D-freehand ultrasound. Treatment effects were compared using non-parametric statistics. Associations between the effects on joint and muscle or tendon levels were performed using Spearman correlation coefficients ($p < 0.05$).

Increased joint resistance, measured during slow ankle rotations, was not significantly reduced after either treatment. Additional joint resistance assessed during fast rotations only reduced in the BoNT-A group (−37.6%, $p = 0.013$, effect size = 0.47), accompanied by a reduction in MG stretch reflexes (−70.7%, $p = 0.003$, effect size = 0.56). BoNT-A increased the muscle length measured at the resting ankle angle (6.9%, $p = 0.013$, effect size = 0.53). Joint angles shifted toward greater dorsiflexion after casting (32.4%, $p = 0.004$, effect size = 0.56), accompanied by increases in tendon length (5.7%, $p = 0.039$, effect size = 0.57; $r = 0.40$). No associations between the changes in muscle or tendon lengths and the changes in the stretch reflexes were found.

We conclude that intramuscular BoNT-A injections reduced stretch reflexes in the MG accompanied by an increase in resting muscle belly length, whereas casting resulted in increased dorsiflexion without any changes to the muscle length. This supports the need for further investigation on the effect of the combined treatments and the development of treatments that more effectively lengthen the muscle.

Introduction

Cerebral palsy (CP), the most common childhood disability, is caused by an injury to the developing brain that occurs prior to, or shortly after, birth. In case of spastic CP, disturbed muscle innervation and biomechanical loading is combined with progressively worsening muscle contractures and joint hyper-resistance to movement. In the ankle joint, this hyper-resistance impairs the joint's passive range of motion (ROM), resulting in gait impairments such as equinus or excessive knee flexion (1).

Numerous studies have tried to establish the mechanisms that contribute to the development of ankle joint hyper-resistance in spastic CP. Recent literature indicates that hyperactive stretch reflexes, commonly labeled as spasticity, are considered to have less impact (2,3). Instead, alterations such as reduced muscle volume and atrophy, cellular and genetic factors have been related to the increased joint resistance (4–6). Despite the likelihood of multifactorial contributions, the most common non-surgical treatment for joint hyper-resistance is intramuscular injection of Botulinum Neurotoxin-A (BoNT-A) which, mainly targets hyperactive stretch reflexes by causing temporary muscle paralysis. There is no known effect of BoNT-A in treating the non-neural components of joint hyper-resistance. The effectiveness of BoNT-A in correcting gait deviations, and passive joint ROM on the short-term, has been shown to improve when BoNT-A is combined with casting (7–9). The rationale of this treatment combination is that paralysis, followed by passive immobilization in a neutral position, results in tissue elongation through physiological adaptation to prolonged stretch. However, few clinical studies reported the effects of BoNT-A treatment at the muscle level (10–18). Synthesis of existing clinical studies that include post-treatment assessment of muscle morphology is hindered by methodological differences between studies and lack of normalization to account for natural muscle growth. Therefore, results are inconclusive and whether the improved joint ROM occurs due to muscle
remodeling or at the expense of other soft tissues, is not well understood (19). Given the frequent use of BoNT-A and casting in clinical practice, and the current uncertainties about their effect on muscle, more research is required. Knowledge of the working mechanism of the treatments will help understand how to combine them more efficiently. This will support

the development of individually tailored treatments, where BoNT-A and casting are prescribed according to individually assessed causes of joint hyper-resistance. As a first step toward such patient-tailored medicine, careful assessment of the individual effects of BoNT-A and casting on different levels, ranging from joint to muscle and tendon, is required. The aim of the current study is to contribute to the understanding of the individual mechanisms by comprehensively evaluating the separate short-term effects of BoNT-A and lower-leg casting on ankle joint hyper-resistance, plantar flexor stretch reflexes, and muscle-tendon complex lengths. Additionally, the effects between the two treatments will be compared and the relations between treatment effects at the different levels are explored within each treatment group. It is hypothesized that BoNT-A will reduce hyperactive stretch reflexes, whereas casting will reduce the joint hyper-resistance by lengthening the muscle and tendon. Furthermore, any changes that occur at the joint level will be more related to alterations in the muscle and tendon lengths than to a reduction in stretch reflexes.

Methods

Participants

Children with spastic CP, aged between 4 and 17 years, were recruited from the Clinical Motion Laboratory of the University Hospital Pellenberg (Belgium), and included when the multidisciplinary clinical team concluded that there was an indication for BoNT-A treatment of the medial gastrocnemius (MG) and casting of their ankle joint. This decision was based on the results of routine clinical assessments of spasticity by means of Modified Ashworth scale (20) and Tardieu R1 angle (21), ROM (by means of goniometry), strength and selectivity (by means of manual muscle testing and clinical selectivity scores), and a 3D gait analysis. Furthermore, included children had a minimum of 20° ankle ROM in the most involved limb, no behavioral problems that would impede the ability to understand and perform the test procedure, and no dyskinesia, dystonic or ataxic features. Children who received BoNT-A injections in the calf muscles within 6 months prior to the first assessment, a Selective Dorsal Rhizotomy (SDR), or orthopedic surgery on the lower-leg, were excluded. Children with a Modified Ashworth Scale score of two or higher in the hamstrings and psoas muscles, indicating greater proximal compared to distal muscle involvement (and therefore not suited to receive ankle stretching casts without multilevel BoNT-A injections), were also excluded. The local University Hospitals' Ethics Committee (study number s57384) approved this study. All participants were informed on the content of the study and written informed consent for participation was obtained from all parents/legal guardians and children above the age of 12 years.

Interventions

This investigation describes the first part of a larger study including a crossover design whereby BoNT-A (Botox®; Allergan Ltd, Buckinghamshire, UK) and lower-leg casting were applied in two treatment sessions, rather than during the same session. The treatment sessions were separated by a two-week

period. This approach allowed investigation of the individual effects of BoNT-A and casting at a group level, but also ensured that all patients eventually received both of their prescribed clinical treatments.

Patients were randomly allocated by an independent researcher to one of two groups by minimization
such that each allocation minimized imbalance between the groups across multiple factors including Gross Motor Functional Classification Scale (GMFCS) level, topographic classification (unilateral or bilateral involvement), age, and gender. Children allocated to the first group received BoNT-A in the MG as part of their multi-level treatment. BoNT-A dosage and muscle selection was based on patient weight, medical history, findings of a clinical examination, 3D gait analysis, and the clinician's experience. Injection was given under short masked anesthesia, and was ultrasound guided for visual identification of muscles and needle depth.

Children allocated to the second group received two weeks of lower-leg casting. Polyester casts were applied conform clinical guidelines by a specialized nurse, supervised by the treating orthopedic surgeon. In the two weeks following treatment, children in both groups continued with their usual post-treatment physical therapy. Children allocated to the BoNT-A group continued their usual use of their ankle-foot orthoses.

Assessments

Assessments were carried out on the most affected leg, defined by the most recent clinical examination. In the case of equal involvement, the left leg was assessed. Clinical examination of the plantar flexors including the Modified Ashworth scale (20) and Tardieu R1 angle (21) as well as measurements of body weight and height were carried out before the treatment. Assessments of muscle and tendon lengths, ankle joint resistance and stretch reflexes were carried out before, and two weeks after, the individual treatments by the same experienced assessor.

<u>Muscle and tendon lengths</u>

Muscle and tendon lengths were assessed using 3D freehand ultrasound (3DfUS); combining conventional B-mode 2D ultrasound (Telemed EchoBlaster128, Vilnius, Lithuania) with 3D motion analysis (Optitrack NaturalPoint, USA), as previously described (22). The US acquisition parameters were kept constant between the different acquisitions (frequency, 10 MHz; depth, 5 cm; focus, 1.8–2.8 cm; gain, 46%; dynamic range, 44 dB and unaltered time-gain compensation)(23). This method has proven to be valid with a strong inter acquirer reliability

in both healthy and pathological muscles (23,24). Subjects lay prone with the lower-leg supported by a small triangular cushion allowing ~20° of knee flexion and the ankle to rest comfortably over the edge of the cushion, to reduce bi-articular stretch on the plantar flexor muscles. This position is referred to as the resting ankle position. First, the MG was imaged at this resting ankle position. Secondly, imaging was carried out with the ankle at maximum dorsiflexion. Maximum dorsiflexion was achieved by a second examiner who manually fixated the ankle in this position while ensuring that maximal motion occurred at the subtalar joint, avoiding foot add-/abduction or pro-/supination. The US images were recorded by an experienced examiner starting from the medial femoral condyle until the most distal edge of the calcaneus. Two 3DfUS sweeps over the MG were carried out at each ankle position. The knee and ankle joint angles were measured with a goniometer at both positions.

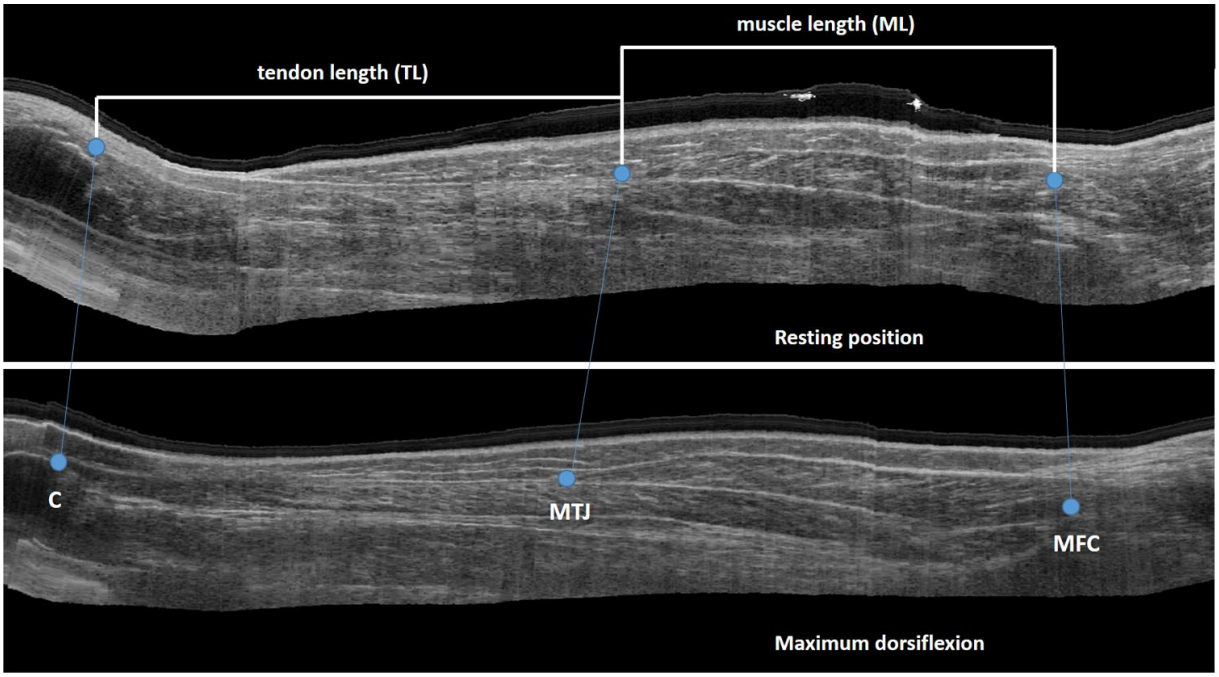

Figure 1. Sagittal US image with landmarks for extraction of muscle and tendon length
C = calcaneus; MTJ = muscle tendon junction; MFC = medial femoral condyle.

Instrumented assessment of ankle joint resistance and stretch reflexes

A previously described assessment was used to distinguish between neural and non-neural components of ankle joint resistance during slow and fast passive joint rotations that stretched the plantar flexors (25). Participants were assessed supine with the lower-limb supported. Three inertial measurement units (IMU's) were used to track the movement of the upper-leg with respect to the lower-leg and the lower-leg with respect to foot. Calibration trials were carried out to define a known angular position and the direction of rotation. A six degrees-of-freedom torque load-cell (ATI mini45: Industrial Automation), attached to a foot orthosis, was used to move the ankle and measure the forces and moments applied to the ankle (25). Surface electromyography (EMG) data (Zerowire, Cometa, Milan, IT) were collected from the MG, lateral gastrocnemius, soleus and tibialis anterior. With the subject fully relaxed, three passive ankle rotations over the full ROM were performed, first at slow velocity (5 seconds to complete full ROM) and then as fast as possible. Between repeated rotations, there was at least a 7s rest interval, to avoid post-activation depression (26).

Data Processing

Muscle and tendon lengths

An open-source software library, developed in Python, was used to create a 3-dimensional view of the MG by integrating 2D US data and motion tracking (22). The 3D reconstruction of the muscle-tendon complex was visualized in a custom-made workflow in Mevislab (www.mevislab.de) (22). Visualization in three planes allowed for accurate identification of the following anatomical landmarks: the most superficial part of the femoral condyle used to define the MG muscle origin; the muscle tendon junction (MTJ), as the muscle insertion and tendon origin; and the most proximal point on the calcaneus as the tendon insertion. The lengths of the MG and corresponding tendon in the two ankle positions were extracted by calculating the Euclidean distances between these landmarks (Figure 1). Muscle-tendon complex length was calculated as the summation of muscle (ML) and tendon length (TL). The same assessor, who was blinded for group allocation and assessment session, extracted all lengths twice. Average lengths were calculated and used for the final statistical analysis. In addition, the standard error of measurement (SEM) associated with extracting the lengths was calculated from the square root of the mean square error from one-way ANOVA (27). The

change in joint angle, ML and TL between the two joint positions was calculated and indicated the degree of the joint (1 ankle angle) and muscle/tendon "extensibility."

Instrumented assessment of ankle joint resistance and stretch reflexes

Data collected during the ankle joint resistance assessment were processed offline using a custom-made Matlab program (Mathworks, R2015a) as previously described (25). Raw EMG signals were filtered with a 6th order zero-phase Butterworth bandpass filter from 20 to 500Hz. The root mean square (rms) envelope of the EMG signal was defined by taking the square root after applying a low-pass 30Hz 6th order zero-phase Butterworth filter on the squared raw signal. Joint angle and angular velocity were calculated from the IMU data by applying a Kalman filter (28). The net ankle joint moment was calculated from the forces and moments applied on the load-cell, the external moment arms, and the predicted torque caused by gravity on the orthotic (25).

ROM and maximum angular velocity were extracted from slow and fast passive ankle rotations. Average rms-EMG was calculated during an interval 200ms before maximum velocity to 90% of the ROM, thereby emphasizing the velocity dependency of the hyperactive stretch reflex and excluding the effects of end ROM. To quantify the hyperactive stretch reflexes, average rms-EMG during slow velocity rotations was subtracted from average rms-EMG during fast rotations.

To quantify joint resistance, work during slow and fast passive rotations was defined as the average area underneath the torque angle graph from maximum velocity to 90% ROM (25). Work during the slow passive rotation represented the non-neural component of joint resistance. The work during slow rotation was subtracted from that during fast rotation to calculate the neural component of joint resistance (25).

Statistical Analysis

An overview of the used statistical analyses is included in Figure 2.

Statistical analyses were performed using SPSS statistics (version 25 IBM). Normality of data distribution was evaluated using the Kolmogorov-Smirnov test. Outlier analysis was performed visually. Within-group treatment effects were evaluated with Wilcoxon signed

ranks tests. Between groups, the treatment-induced changes were compared using Mann-Whitney U-tests. In addition to being statistically significant, treatment-induced changes were only considered meaningful when larger than the SEM.

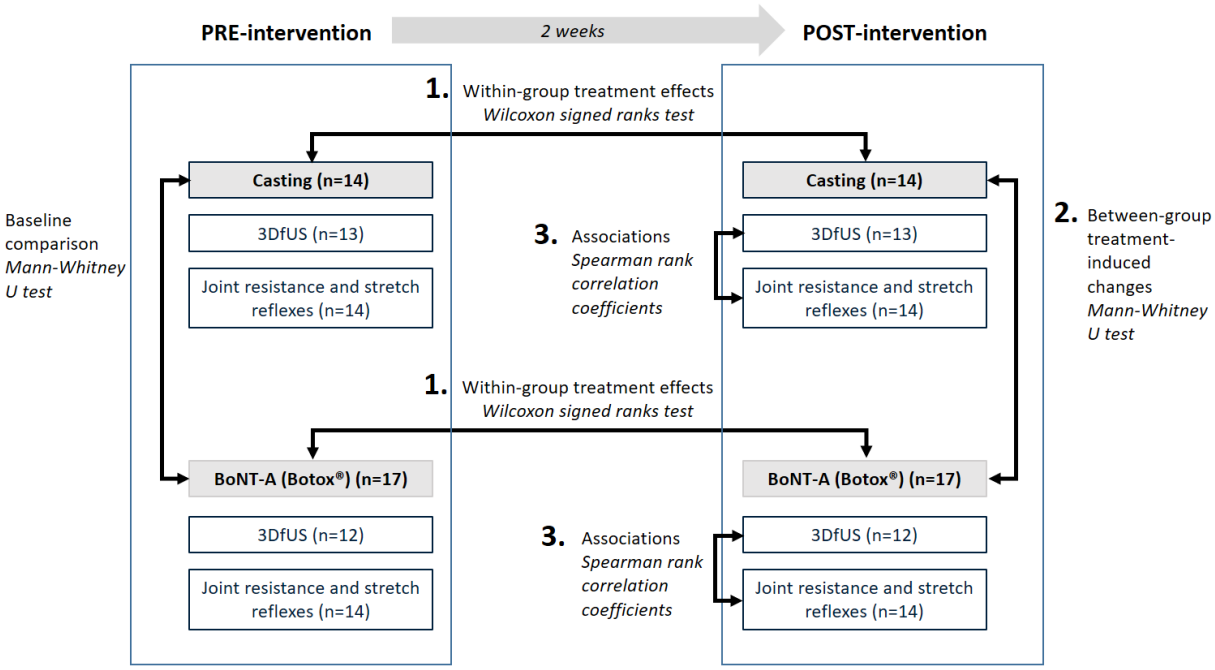

Figure 2. Overview of statistical analyses

3DfUS = 3D freehand ultrasound, BoNT-A = Botulinum Neurotoxin-A

Associations between treatment-induced changes in ankle angles, muscle/tendon lengths, extensibility, ankle joint hyper-resistance and stretch reflex parameters were assessed by Spearman rank correlation coefficients for both treatment groups separately. Correlation values of 0.6 or higher were considered as strong (29).

Effect sizes were calculated according the formula: $r = Z/\sqrt{N}$ (30). The Z-score was extracted from the SPSS output of the Wilcoxon signed rank test. N represents the total number of observations. R-values of 0.5 or higher were considered as large effect sizes, whereas r-values above 0.3 were considered moderate.

Results

Thirty-one children with spastic CP (GMFCS I-III, age 7.4 ± 2.6 years) were included in this investigation. During the course of this study, some data were lost due to dropout and/or technical issues with measurement equipment. The flowchart of the patient enrolment and all available data that were included in the final data analyses, and subdivided per outcome, are presented in Figure 3.

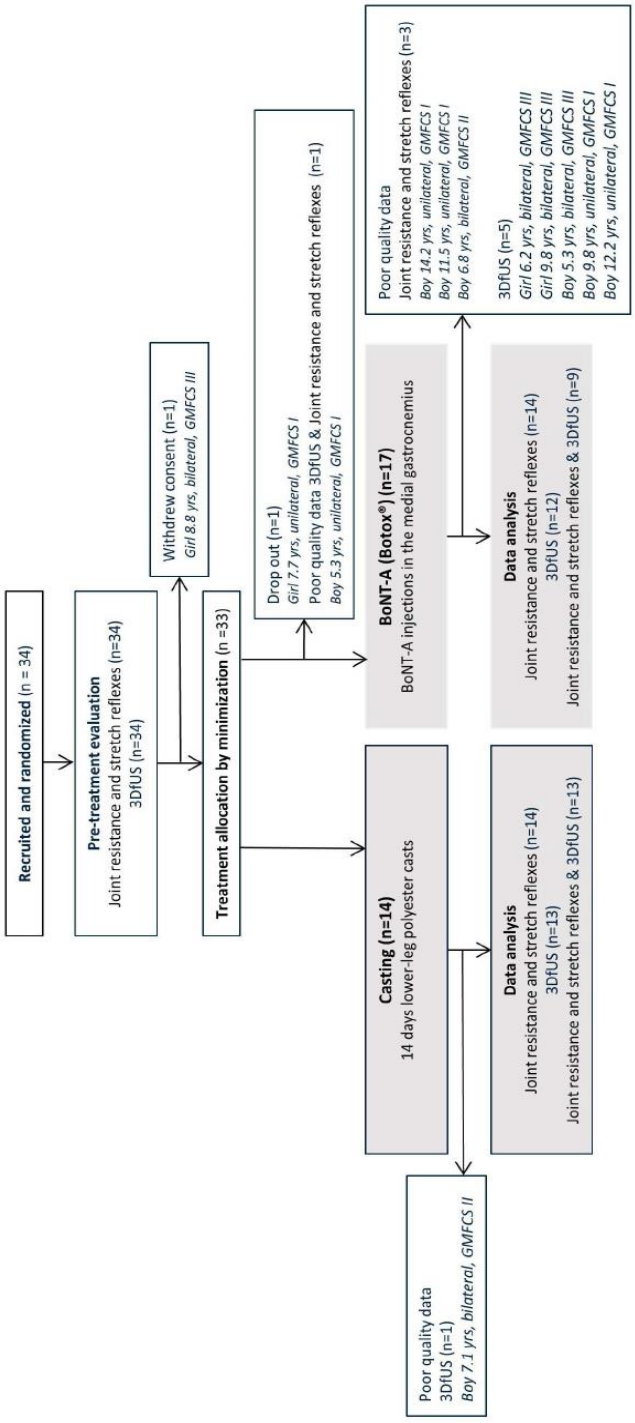

Figure 3. Flowchart of the inclusion of participants with an overview of the available and missing data

3DfUS = 3D freehand ultrasound, GMFCS = gross motor functional classification system, BoNT-A = Botulinum Neurotoxin-A

At baseline (Table 1), the two intervention groups did not differ in age, body weight, height, maximum dorsiflexion ankle angle, Modified Ashworth Scale, Tardieu R1 angle, the difference in ankle angle between resting and maximum dorsiflexion position (Δ ankle angle), extensibility of the muscle or tendon, muscle/tendon lengths, ROM, angular velocity, hyperactive stretch reflexes, and work. The number of
included patients and their characteristics per sub-analysis are included in Supplementary Table 1.

Table 1. Participant characteristics

	Casting (n=14)	**BoNT-A (n=17)**
Age (years)	7.0 (5 – 8.3)	7.0 (5.5 – 10.5)
Body weight (kg)	23.6 (18.4 – 28.7)	25 (19 – 27.6)
GMFCS level (n)	I = 6, II = 6, III = 2	I = 11, II = 4, III = 2
Involvement (n)	Unilateral = 7, Bilateral = 7	Unilateral = 12, Bilateral = 5
Gender (n)	Male = 8, Female = 6	Male = 12, Female = 5
MAS*, knee extended	2 (1.5-3)	2 (1-3)
MTS R1*, knee extended (degrees)	-12.5 (-35 – -5)	-12.5 (-25 – 0)
Treatment details	2 weeks of lower-leg casting: n=14 2 weeks of removable upper- leg casts worn during the night: n= 3	BoNT-A (Botox®) plantar flexors: 4.0 Units/kg (2.33-6.12)* BoNT-A (Botox®) MG: 2.47 Units/kg (1.93-3.66)* BoNT-A (Botox®) LG: 0.37 Units/kg (0.00-0.71)* BoNT-A (Botox®) SOL: 1.49 Units/kg (0.00-2.04)*
Use of day orthoses, prior to treatment	Frequently used (≥50% of the day) n=11 Not frequently used (<50% of the day) n=1 Not used n=2	Frequently used (≥50% of the day) n=14 Not frequently used (<50% of the day) n=1 Not used n=2
Use of night orthoses, prior to treatment	Frequently used (≥50% of the night) n=2 Not frequently used (<50% of the night) n=7 Not used n=5	Frequently used (≥50% of the night) n=2 Not frequently used (<50% of the night) n=4 Not used n=11

*Values are presented as medians with corresponding quartiles (p25-p75), or numbers (n). *median, minimum-maximum values.*

BoNT-A = Botulinum Neurotoxin-A, GMFCS = Gross Motor Functional Classification Scale, MAS = modified Ashworth scale MTS = modified Tardieu scale, MG = medial gastrocnemius muscle, LG = lateral gastrocnemius muscle, SOL = soleus muscle

Muscle and tendon lengths

There was a positive significant treatment effect of casting on the resting (p = 0.004, pre median: −31.5°, inter quartile range (IQR): 20.0◦; post median: −25.0°, IQR: 10.0°, effect size = 0.56) and maximum dorsiflexion (p = 0.026, pre median: 0.0◦, IQR: 3.8°; post median: 10.0°, IQR: 10.0°, effect size = 0.44) angles (Figure 4). Knee angles remained constant between the assessments of muscle/tendon lengths before and after treatment. The SEM values for determining the muscle and

tendon lengths were 2.25 and 2.54 mm, respectively. SEM values were found to be comparable to those previously reported (23). The absolute muscle and tendon lengths pre- and post-treatment are presented in Figure 4 and the treatment-induced change values are presented in Supplementary Table 2. Two weeks of casting caused a significant increase in the TL and muscle-tendon complex length at maximum dorsiflexion (5.6%, p = 0.039, effect size = 0.40, and 4.1%, p = 0.005, effect size = 0.63, respectively). These treatment-induced changes were also significantly larger than those found in the BoNT-A group (p = 0.002 and p = 0.001, respectively).

A significant increase in MG ML at the resting ankle position was found after BoNT-A injections (6.9%, p = 0.013, effect size = 0.53). Additionally, a significant increase in the TL at maximum dorsiflexion occurred, however this increase was within the range of the SEM. At the resting ankle position, ML after BoNT-A injections and the treatment-induced change in ML were significantly larger compared to those assessed following casting (p = 0.023 and p = 0.036, respectively).

Post-treatment, the extensibility of the ML and of the muscle-tendon complex length were significantly reduced after BoNT-A injections (−34.1%, p = 0.010, effect size = 0.55 and −36.3%, p = 0.013, effect size = 0.56, respectively), but unaltered by casting.

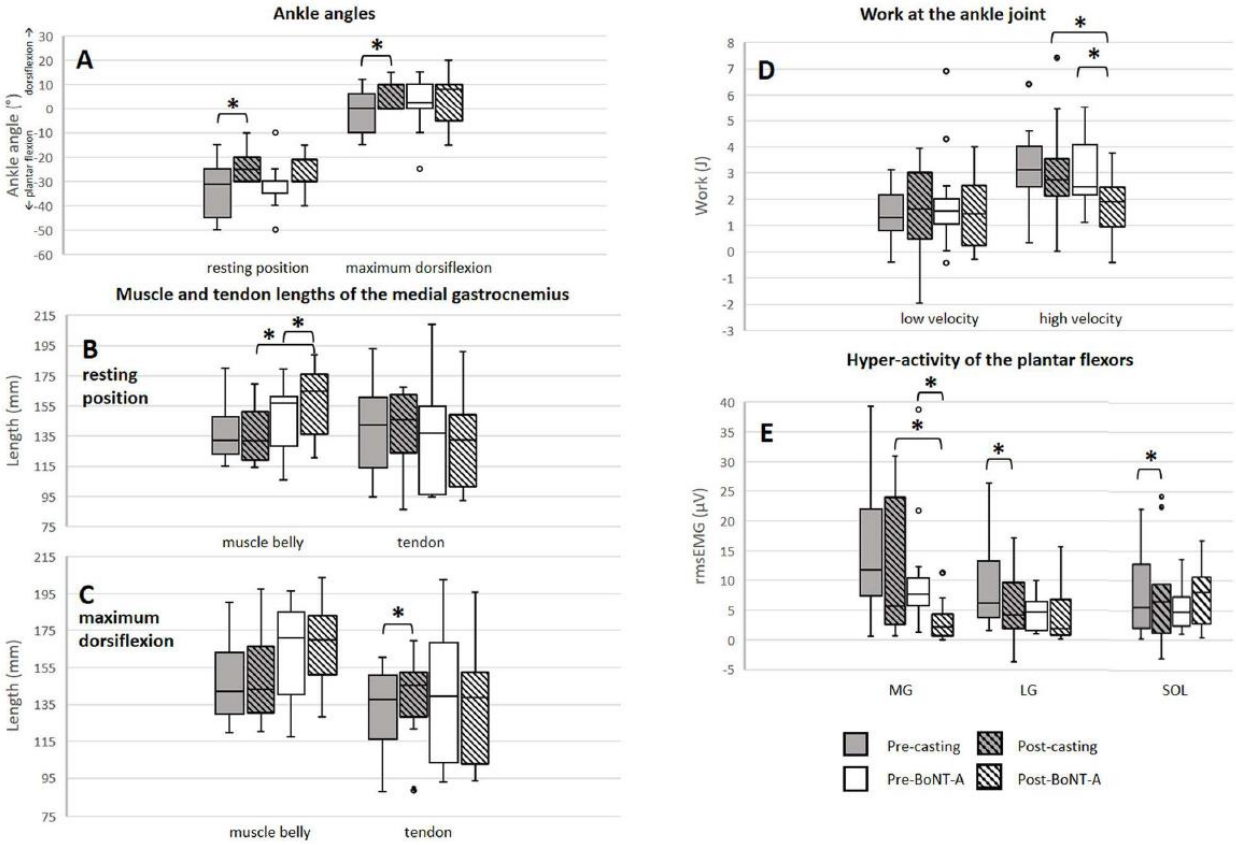

Figure 4. Results of muscle/tendon lengths, ankle joint resistance and stretch reflex assessments. (A) Change in ankle angles. (B) Muscle and tendon lengths at resting position. (C) Muscle and tendon lengths at maximum dorsiflexion. (D) Work at the ankle joint during slow and fast joint rotations. (E) Stretch reflexes of the plantar flexors during fast rotations.

**significant results (p < 0.05, difference > SEM)*

mm = millimeter, J = Joule, rms EMG = root mean square of the electromyographic signal, µV = microvolt, MG = medial gastrocnemius muscle, LG = lateral gastrocnemius muscle, SOL = soleus muscle, BoNT-A = Botulinum Neurotoxin-A

Instrumented assessment of ankle joint resistance and stretch reflexes

The ankle joint ROM and angular velocities applied during passive joint rotation remained unchanged after either treatment. Effects of the treatments on plantar flexor stretch reflexes and joint work are displayed in Figure 4. Two weeks of casting caused a significant reduction in the stretch reflexes of the lateral gastrocnemius (−29.5%, p = 0.047, effect size = 0.44), and soleus (−17.5%, p = 0.034, effect size = 0.43). Post-casting, the average work assessed during slow, and fast minus slow stretch remained unchanged. BoNT-A injections resulted in a

significant reduction in the stretch reflexes of the MG (−72.4%, p = 0.003, effect size = 0.56) together with a reduction in work during fast minus slow stretch
(−22.4%, p = 0.013, effect size = 0.47). There was a trend that this reduction in work was larger in the group receiving BoNT-A compared to casting (p = 0.050).

Associations

Few correlations were found between the changes in joint angles, muscle/tendon lengths and ankle joint resistance. An overview of all correlation values and significance can be found in Supplementary Tables 4, 5. In the casting group, there was a moderate positive correlation (r = 0.56, p = 0.049) between the change in TL at maximum dorsiflexion and the change in ankle angle (Δ ankle angle). Additionally, the increase in TL at maximum dorsiflexion was negatively related (r = −0.67, p = 0.050) to the reduction in stretch reflexes in the lateral gastrocnemius and the amount of work at high velocity (r = −0.60, p = 0.03). Furthermore, the increase in muscle-tendon complex length at rest was associated (r = −0.821, p = 0.02) with the reduction in stretch reflexes in the lateral gastrocnemius.

In the BoNT-A group, the reduction in hyperactive stretch reflexes of the soleus muscle was strongly correlated (r = 0.79, p = 0.036) to the increase in ML of the MG at rest. In addition, the reduction in work at fast minus slow velocity was strongly correlated (r = −0.77, p = 0.016) with the change in ML at the maximum dorsiflexion angle.

Discussion

Summary of findings

The aim of this study was to gain more insight into the working mechanisms of BoNT-A and casting at the joint and muscle levels in children with spastic CP. Our hypotheses that BoNT-A targets the muscles' stretch reflexes, whereas casting targets the muscle belly and tendon length, were partly confirmed. Two weeks of casting increased the dorsiflexion angle, lowered the stretch reflexes in the lateral gastrocnemius and soleus muscles and increased the tendon length measured at maximum dorsiflexion position. BoNT-A reduced the stretch reflexes in the MG, reduced the joint resistance measured during fast passive ankle joint rotation, and increased the muscle length measured at the resting ankle position. Only a few associations between the treatment-induced alterations in joint angles, muscle or tendon lengths and measures of ankle joint resistance, and stretch reflexes were found. In summary, this study found rather minor changes at the muscle level after either intervention, with limited relationships between the effects at the joint, muscle or tendon lengths and stretch reflexes.

Casting

We confirmed previous findings that maximum dorsiflexion increases after lower leg casting (31,32). However, since the joint angle at rest also moved toward greater dorsiflexion, the total joint ROM and change in ankle angle (Δ ankle angle) remained unchanged after treatment. While this result may make it easier to fit a foot into an ankle-foot orthosis configured toward dorsiflexion, it remains unclear to what extent this new biomechanical configuration is functionally useful. In the two subjects whose ROM did increase, this was mainly explained by an increase in their maximum dorsiflexion angle with an accompanied increased tendon length (Supplementary Figure 1). Interestingly, the three children who additionally received removable upper leg casts during the night showed an increase in muscle belly length at maximum dorsiflexion.

Since the plantar flexor muscles of children with spastic CP have been described as stiff and short (33), lengthening of these muscles through treatment is desirable. In clinical practice, it is thereby commonly hypothesized that an increase in dorsiflexion angle following casting is a result of lengthening the muscle. However, our results showed that the increase in maximum

dorsiflexion angle post-casting was related to the increase in tendon length rather than alterations in muscle belly length. Hösl et al. (2015) reported increased tendon length following a period of ankle foot orthotic use (34) and Theis et al. (2013) reported similar results after a manual stretching intervention (35). As far as we are aware, our study is the first to report this effect following two weeks of casts.

Previous studies suggested that children with CP have a more compliant Achilles tendon (36,37). In ambulant children with CP, this adaptation may be beneficial for achieving sufficient dorsiflexion during the stance phase of gait, but may compromise push-off power as the tendon may offer less resilience. Therefore, given that TL is already increased in CP, our findings highlight that caution has to be paid when applying passive stretches due to potentially enhancing the negative effects on the tendon. This is especially important when considering the coherence of the muscle-tendon unit as a complex and the efficiency of the muscle and tendon to interact during function. A recent study by Kalkman et al. (2019) concluded that increased tendon compliance in CP may result in reduced stretch stimulus to the muscle during ankle rotation, thus explaining the minimal effects of stretching interventions on muscle remodeling (38). In a follow-up study, they then confirmed that initially increasing tendon stiffness, by means of strength training, resulted in more efficient muscle stretch (38). Similarly, the group of Zhao et al. (2010) accomplished a reduction in Achilles tendon length and

increased stiffness by combining passive stretching with active movement therapy (39). These findings suggest that stretching should be combined with strengthening exercises in order to optimally target the muscle belly. The reduction in hyperactive stretch reflexes of the lateral gastrocnemius and soleus following casting was unexpected, but might be explained by the alteration in starting ankle angle, as the ankle was spontaneously more dorsiflexed after casting. Meinders et al. (1996) suggested that specifically at the ankle, stretch reflexes are reduced at a longer starting muscle-tendon complex lengths (40). Confirming this, we found that the reduction in hyperactive stretch reflex in the lateral gastrocnemius following casts was associated with an increase in tendon length.

Botulinum Neurotoxin-A

BoNT-A targets the neural component of ankle joint resistance by reducing the hyperactive stretch reflexes. Reduction of hyperactive stretch reflexes is thought to create the opportunity

for the muscle to act over a larger ROM and thereby improve its function (41). Our results confirmed the reduction of MG stretch reflexes, shown by a reduction in rms-EMG of the MG and in the reduced work needed to move the ankle joint during a high-velocity stretch of the plantar flexors. The question remains whether these changes additionally translate to morphological changes at the muscle level. Post BoNT-A, we observed an increase in the resting length of the MG muscle belly. Since spastic muscles have been described with a higher resting muscle tone (42), the increased MG muscle belly length may be a direct result of muscle tone reduction and the ability of the muscle to relax in this position. On the other hand, there were no changes to the muscle length measured at maximum dorsiflexion. Therefore, we found little evidence that the reduction in hyperactive stretch reflex of the MG post BoNT-A induced muscle remodeling. It is known that the muscle needs more time than the current short-term (two weeks) follow-up to adapt and no conclusions can be drawn about the medium- and long-term effect of BoNT-A on muscle length (43). Furthermore, muscle remodeling might take place after combining with casting.

Combined treatment

Answers regarding the combination of the treatments are currently being analyzed as the second part of this research project, which includes a crossover design with all the participants receiving the other treatment. In addition, this second part includes 3D gait analysis before and after receiving the two interventions allowing us to understand effects of the interventions on the functional level. Desloovere et al. (2007) showed that BoNT-A combined with casting was more effective in improving gait, compared to casting alone (44). However, it is unclear how these effects were achieved and additional

insight, which we expect to gain with the second stage of this project, will likely facilitate the understanding of these findings. Following BoNT-A injections only, there were no changes in the ankle angles or joint ROM. Given the effects of casting on these parameters, there is reason to believe that the best results may be achieved when the two treatments are combined.

Ideally, the changes seen on the muscle level following BoNT-A, in combination with static stretch as applied from casting, will result in the desired effect of increasing the joints ROM by promoting muscle length. Such an effect should result in greater ankle mobility that would be transferrable to the child’s gait. However, given the finding that casting only increased the

tendon length, very careful monitoring of how ankle mobility is achieved post-casting is essential, as it may be subject-specific. This highlights that an individualized and fine-tuned combination of the treatments is required. Furthermore, we can question whether a primarily passive stretch applied by casts is beneficial, as evidence shows that muscle activity is required to efficiently stretch the muscle and maintain an optimal configuration of the muscle-tendon complex (39).

Importantly, since neither treatment in isolation affected the increased joint resistance assessed during slow passive stretch, it is unlikely that the combination of the two treatments will affect resistance during slow stretch. If confirmed, this suggests that we need more effective treatments that address hyper-resistance over the full ROM, and not only increases the maximum dorsiflexion. If combined results are still not satisfactory, new treatment modalities should be developed to more efficiently target the muscle. Our data showed a lack of strong responses on the group level. This reflects the large heterogeneity of the children with CP included in this study and therefore the need for larger samples. Given that the sample was taken from the clinical population seeking treatment for ankle joint hyper-resistance, this finding emphasizes the need to generate muscle-specific profiles and establish patient-tailored treatment. Furthermore, we highlight the importance of evaluating the underlying muscle and tendon structures rather than providing treatment based only on conclusions drawn from evaluations at the joint level.

Limitations

The study has limitations that need to be acknowledged.

This study investigated only the very short-term effects of the treatments. This allowed us to evaluate the separate effects without having to compromise on the children's clinical intervention plan. A study design in which the same study sample received both treatments including a wash-out period would have been methodically stronger. However, implementing such an investigation would differ too much from normal clinical treatment and would not be considered ethical (nor would including control groups from whom treatment was denied). In addition, considering the long time span of such a study, other parameters would influence the response.

This clinical study included a small dataset with missing data. As a result, not all participants were included in all analyses. When only including subjects who underwent both assessments

(n = 23, BoNT-A n = 9, casting n = 13), most of our conclusions were still valid (Supplementary Table 3). Nevertheless, more studies need to be conducted to confirm the initial results of this study. Randomization by minimization was applied in order to minimize differences between groups in terms of pathology, age, body weight and height. Even though the groups did not perfectly match, there were no differences reported at baseline. Heterogeneity in treatment history and current treatment adherence are limitations when performing randomized controlled trials in children with CP. It is well-described that muscle morphology of children with CP is different from typically developing peers, although it remains
unclear to what extent treatment history contributes to these alterations. This should be focus for further investigation. In addition, given the wide age range, and the effect of age on the ability of the muscle fiber to adapt, it is possible that the muscle fiber stage influenced our results.

The full data set of this investigation is published online (DOI:https://doi.org/10.6084/m9.figshare.12009375.v1). When more data becomes available, sub analyses according to baseline characteristics may be carried out that will contribute to clinical implementation.

This study only investigated the muscle and tendon lengths of the MG as this is the muscle that is most frequently treated with BoNT-A in daily clinical practice. Future investigations with larger samples investigating more muscles are needed to confirm these initial findings. Given recent validation of the
clinical applicability of the methods used in this study (23,45), such investigations should be feasible in the near future.

We studied the alterations in the length of the entire muscle belly. Yet, this does not provide information on the fascicle behavior, especially since the MG is pennated. More-over, local fascicle remodeling in specific regions of the muscle may have occurred. Furthermore, since neither muscle length nor fascicle length necessarily represent sarcomere number or length, it is important to continue investigations that combine macro- and micro-structure responses to the applied treatments.

Conclusion

This study presents initial findings on treatment response in a heterogeneous group of children with spastic CP. Intramuscular BoNT-A injections reduced the stretch reflex in the MG accompanied by an increase in resting muscle belly length, whereas casting resulted in increased dorsiflexion without any

changes to the muscle length. These results indicate limited treatment-induced MG muscle remodeling. This supports the need for further investigation once the treatments are combined and for the development of treatments that more effectively lengthen the muscle belly. Additionally, future implementation of objective assessments will contribute to a better understanding of treatment effects, which could support clinical decision making and help to identify responders.

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Supplementary Material

Supplementary Table I. Patient characteristic per analysis. Values are medians and interquartile ranges.

	Casting group			Botulinum NeuroToxin-A group		
Performed analyses	**Joint resistance and stretch reflexes (n=14)**	**Ultrasound (n=13)**	**Joint resistance and stretch reflexes + Ultrasound (n=13)**	**Joint resistance and stretch reflexes (n=14)**	**Ultrasound (n=12)**	**Joint resistance and stretch reflexes + Ultrasound (n=9)**
Age (years)	7 (5 - 8.3)	7 (5-8,5)	7 (5-8,5)	7 (5-9.3)	7 (5.3-8.8)	7 (5-8.3)
Body weight (kg)	23.6 (18.4 – 28.7)	22.9 (18.4-28.4)	22.9 (18.4-28.4)	264.1 (18.7-28.2)	20.25 (17.6-26.9)	20.3 (17-27.2)
GMFCS level	I = 6, II = 6, III = 2	I = 6, II = 5, III = 2	I = 6, II = 5, III = 2	I = 9, II = 3, III = 2	I = 9, II = 3, III = 0	I = 8, II = 2, III = 0
Uni- or bilateral involvement	UL = 7 BL = 7	UL = 7 BL = 6	UL = 7 BL = 6	UL = 10 BL = 4	UL = 10 BL = 2	UL = 9 BL = 1
Gender	Male = 8 Female = 6	Male = 7 Female = 6	Male = 7 Female = 6	Male = 9 Female = 5	Male = 9 Female = 3	Male = 7 Female = 3
MAS, knee extended*	2 (1.5-3)	2 (1.5-3)	2 (1.5-3)	2 (1-3)	1.5 (1-3)	1.5 (1-3)
Tardieu R1* (degrees), **knee extended**	-12.5 (-35 - -5)	-10 (-35 - -5)	-10 (-35 - -5)	-15 (-25-0)	-15 (-25-0)	-15 (-25 – 0)

Values are presented as medians with corresponding quartiles (p25-p75), or numbers. *median, min-max values.
BL = bilateral involvement, GMFCS = Gross Motor Functional Classification Scale, MAS = Modified Ashworth scale, MTS = Modified Tardieu Scale, UL = unilateral involvement

Supplementary Table II. Median (and interquartile range) values of all outcome parameters

	Casting (N=14)			Botulinum Neurotoxin-A (N=17)			POST comparison between groups	CHANGE comparison between groups
Parameter	**PRE intervention** *median (p25-p75)*	**POST intervention** *median (p25-p75)*	**p-value**	**PRE intervention** *median (p25-p75)*	**POST intervention** *median (p25-p75)*	**p-value**	**p-value**	**p-value**
Ankle angle - resting (degrees)	-31.50 (-45.00--25.00)	-25.00 (-30.00--20.00)	*0.004*	-30 (-35 --30)	-30 (-30--21.25)	0.221	0.062	*0.015*
Ankle angle - maxDF (degrees)	0.00 (-10.00-6.25)	10.00 (0.00-10.00)	*0.026*	2.5 (0.00-10.00)	8.00 (-5.00-10.00)	0.414	0.413	0.220
Extensibility ankle angles (degrees)	35.00 (27.50-41.25)	30.00 (25.00-31.00)	0.121	35.00 (21.25-40.00)	30.00 (25.00-38.00)	0.782	0.555	0.350
ML - resting (mm)	132.00 (122.84-147.79)	132.18 (118.99-151.56)	0.959	156.50 (127.99-160.96)	164.96 (136.35-176.10)	*0.013*	*0.023*	*0.036*
ML - maxDF (mm)	141.88 (129.41-162.71)	143.16 (130.23-166.42)	0.139	170.67 (139.97-184.77)	170.16 (151.22-183.13)	0.388	0.069	0.821
Extensibility ML (maxDF-rest) (mm)	12.53 (9.40-16.15)	11.36 (5.76-21.21)	0.866	14.59 (7.45-25.02)	9.61 (4.80-14.85)	*0.010*	0.468	0.211
TL - resting (mm)	142.03 (113.54-160.24)	146.16 (123.86-162.45)	0.507	136.94 (95.80-154.26)	132.70 (101.22-149.40)	0.388	0.295	0.270
TL - maxDF (mm)	137.30 (116.09-150.82)	145.42 (128.41-152.40)	*0.039*	139.16 (103.08-168.12)	138.80 (102.98-152.38)	0.041	0.733	*0.002*
Extensibiliy TL (maxDF-rest) (mm)	.-4.35 (-12.30-2.48)	0.33 (-4.50-3.46)	0.196	2.15 (-2.44-15.62)	.-0.98 (-5.54-4.89)	0.213	0.776	0.093
Muscle-tendon complex - resting (mm)	284.33 (238.96-307.93)	278.78 (258.11-305.38)	0.203	290.97 (248.33-312.84)	306.87 (263.57-323.36)	*0.033*	0.651	1.000
Muscle-tendon complex - maxDF (mm)	271.78 (252.36-304.70)	287.66 (266.74-316.36)	*0.005*	302.57 (263.11-325.24)	300.96 (272.87-328.90)	0.110	0.478	*0.001*
Extensibility muscle-tendon complex (mm)	10.31 (2.12-15.54)	8.81 (4.31-13.84)	0.735	16.40 (12.50-22.58)	10.45 (7.86-15.00)	0.013	0.684	0.043
Work - high velocity (J)	3.10 (2.44-4.01)	2.75 (2.12-3.52)	0.510	2.45 (2.16-4.05)	1.90 (0.93-2.46)	*0.013*	0.050	0.077
Work - low velocity (J)	1.30 (0.80-2.14)	1.63 (0.50-3.02)	0.433	1.53 (1.05-1.99)	1.45 (0.22-2.51)	0.875	0.946	1.000

rmsEMG MG - high velocity (μV)	11.70 (7.30-21.90)	5.71 (2.55-23.97)	0.133	7.62 (5.72-10.30)	2.10 (0.63-4.30)	*0.003*	*0.014*	0.650
rmsEMG LG - high velocity (μV)	6.06 (3.64-13.24)	4.27 (1.84-9.63)	*0.047*	4.57 (1.54-6.38)	1.88 (0.78-6.79)	0.859	0.283	0.315
rmsEMG SOL - high velocity (μV)	5.50 (2.02-12.80)	6.46 (1.23-9.46)	0.034	4.83 (2.44-7.34)	8.03 (2.80-10.63)	0.657	0.611	0.169
Angular velocity – low velocity (deg/s)	21.75 (14.38 – 24.86)	17.98 (15.00 – 23.00)	0.507	20.12 (17.31 – 25.91)	19.48 (15.81 – 24.78)	0.875	0.550	0.454
Angular velocity – high velocity (deg/s)	167.46 (149.35 –223.28)	170.33 (147.96 – 199.47)	0.345	174.91 (155.79 – 208.33)	173.94 (155.69 – 193.79)	0.826	0.756	0.603
ROM (degrees)	60.47 (55.45-70.26)	62.82 (52.16-69.08)	0.594	61.02 (45.33-74.42)	55.71 (47.64-70.25)	0.096	0.352	0.571

maxDF = maximum dorsiflexion, ML = muscle length, TL = tendon length, mm = millimeter, J = joule, rmsEMG = root mean square of the electromyographic signal, MG = medial gastrocnemius muscle, LG = lateral gastrocnemius muscle, μV = microvolt, SOL = soleus muscle, ROM = range of motion

Supplementary Table III Results when only including subjects who had both ultrasound and Instrumented assessment of ankle joint resistance and stretch reflexes. Highlighted p-values were found to be significant when analyzing the total groups (n=14 casting group; n=17 Botulinum NeuroToxin-A group).

Parameters that were found to be significant when analyzing the total groups	**Casting N=13**			**Botulinum NeuroToxin-A N=9**			**Between groups – post intervention**
	PRE intervention	**POST intervention**	**p-value**	**PRE intervention**	**POST intervention**	**p-value**	**p-value**
Ankle angle - resting (degrees)	-30.00 (-42.50 – -25.00)	-25.00 (-30.00 – -20.00)	0.004	-30.00 (-42.50 – -25.00)	-30.00 (-42.50 – -25.00)	0.739	
Ankle angle - maxDF (degrees)	0.00 (-7.50 – -7.50)	0.00 (0.00 – 10.00)	0.026	0.00 (-7.50 – 7.50)	0.00 (-7.50 – 7.50)	0.746	
ML - resting (mm)	132.00 (122.84 – 147.79)	132.18 (118.99 – 151.56)	0.959	132.00 (122.8 – 147.79)	132.18 (118.99 – 151.56)	0.069	0.082
TL - maxDF (mm)	137.30 (116.09 – 150.82)	145.42 (128.41 – 152.40)	0.039	137.30 (116.09 – 150.82)	145.42 (128.41 – 152.40)	0.161	
Muscle-tendon complex - maxDF (mm)	271.78 (252.36 – 304.70)	287.66 (266.74 – 316.30)	0.005	271.78 (252.36 – 304.70)	287.66 (266.74 – 316.36)	0.484	
Work - high velocity (J)	3.08 (2.29 – 4.04)	2.92 (1.93 – 3.67)	0.650	3.08 (2.29 – 4.04)	2.92 (1.93 – 3.67)	0.173	
rmsEMG MG - high velocity (μV)	14.03 (7.73 – 23.32)	7.45 (2.15 – 24.86)	0.099	14.03 (7.73 – 23.32)	7.50 (2.15 – 24.86)	0.021	0.049
rmsEMG LG - high velocity (μV)	6.07 (4.08 – 13.25)	4.68 (2.28 – 10.30)	0.066	6.07 (4.08 – 13.25)	4.68 (2.28 – 10.30)	0.144	
rmsEMG SOL - high velocity (μV)	6.84 (2.42 – 13.16)	6.58 (1.71 – 9.46)	0.050	6.84 (2.42 – 13.16)	6.58 (1.71 – 9.46)	1.000	

maxDF = maximum dorsiflexion, ML = muscle length, TL = tendon length, mm = millimeter, J = joule, rmsEMG = root mean square of the electromyographic signal MG = medial gastrocnemius muscle, LG = lateral gastrocnemius muscle, μV = microvolt, SOL = soleus muscle

Supplementary Table IV. Correlations between changes in ultrasound and Instrumented assessment of ankle joint resistance and stretch reflex parameters of the casting group (n=13).

Casting

		Change in resting angle	Change in maxDF angle	Change in Δ ankle angle	Change in muscle-tendon complex length, rest	Change in muscle-tendon complex length, maxDF	Change in ML, rest	Change in ML, maxDF	Change in TL, rest	Change in TL, maxDF	Change MG rmsEMG (µV)	Change LG rmsEMG (µV)	Change SOL rmsEMG (µV)	Change ROM	Change work, low velocity	Change work, high minus low velocity	Change extensibility ML	Change extensibility TL
change in maxDF angle	Correlation Coefficient	0,076																
	Sig. (2-tailed)	0,806																
change in Δ ankle angle	Correlation Coefficient	-,703**	0,511															
	Sig. (2-tailed)	0,007	0,075															
Change in muscle-tendon complex length, rest	Correlation Coefficient	-0,443	-0,35	0,24														
	Sig. (2-tailed)	0,199	0,321	0,504														
Change in muscle-tendon complex length, maxDF	Correlation Coefficient	-0,205	0,558	0,419	-0,214													
	Sig. (2-tailed)	0,57	0,094	0,228	0,645													
Change in ML, rest	Correlation Coefficient	-0,26	0,058	0,409	0,309	-0,679												
	Sig. (2-tailed)	0,469	0,873	0,241	0,385	0,094												
Change in ML, maxDF	Correlation Coefficient	0,466	-0,162	-0,306	-0,5	0,164	-0,393											
	Sig. (2-tailed)	0,175	0,654	0,389	0,253	0,651	0,383											
Change in TL, rest	Correlation Coefficient	-0,188	0,081	0,212	,709*	0,152	-0,212	-,636*										
	Sig. (2-tailed)	0,539	0,791	0,486	0,022	0,676	0,556	0,048										
Change in TL, maxDF	Correlation Coefficient	-0,549	0,172	,555*	0,333	,685*	0,091	-0,588	,599*									
	Sig. (2-tailed)	0,052	0,575	0,049	0,347	0,029	0,803	0,074	0,031									
Change MG rmsEMG (µV)	Correlation Coefficient	0,244	0,519	0,156	0,317	0,079	-0,067	-0,079	0,259	-0,119								
	Sig. (2-tailed)	0,445	0,084	0,628	0,406	0,829	0,865	0,829	0,417	0,713								
Change LG rmsEMG (µV)	Correlation Coefficient	,740*	0,289	-0,235	-,821*	-0,107	-0,357	0,643	-0,583	-,667*	0,048							
	Sig. (2-tailed)	0,023	0,45	0,543	0,023	0,819	0,432	0,119	0,099	0,05	0,911							
Change SOL rmsEMG (µV)	Correlation Coefficient	0,014	-0,096	-0,181	0,467	0,071	-0,167	-0,333	0,473	0,027	0,115	-0,5						
	Sig. (2-tailed)	0,967	0,778	0,594	0,205	0,867	0,668	0,42	0,142	0,937	0,751	0,207						
Change ROM	Correlation Coefficient	0,266	-0,55	-,620*	0,03	-0,321	0,285	0,006	-0,11	0	-0,357	-0,017	-0,073					
	Sig. (2-tailed)	0,379	0,052	0,024	0,934	0,365	0,425	0,987	0,721	1	0,255	0,966	0,832					
Change work, low velocity	Correlation Coefficient	,756**	0,192	n.a.	-0,115	0,176	-0,491	0,273	0,11	-0,28	0,315	0,6	0,445					
	Sig. (2-tailed)	0,003	0,53	n.a.	0,751	0,627	0,15	0,446	0,721	0,354	0,319	0,088	0,17					
Change work, high minus low velocity	Correlation Coefficient	,762**	-0,157	n.a.	-0,503	-0,345	-0,248	0,248	-0,088	-,599*	-0,056	0,55	0,173		,555*			
	Sig. (2-tailed)	0,002	0,608	n.a.	0,138	0,328	0,489	0,489	0,775	0,031	0,863	0,125	0,612		0,049			
Change extensibility ML	Correlation Coefficient	0,501	0,039	-0,335	-0,75	0,607	-,821*	0,714	-0,179	-0,143	-0,357	0,6	-0,314	-0,143	0,393	0,429		
	Sig. (2-tailed)	0,252	0,933	0,463	0,052	0,148	0,023	0,071	0,702	0,76	0,432	0,285	0,544	0,76	0,383	0,337		
Change extensibility TL	Correlation Coefficient	-0,364	-0,099	0,229	-0,297	0,273	0,285	0,503	-,714**	0,044	-0,357	-0,033	-,618*	0,033	-0,451	-0,467	0,214	
	Sig. (2-tailed)	0,221	0,748	0,451	0,405	0,446	0,425	0,138	0,006	0,887	0,255	0,932	0,043	0,915	0,122	0,108	0,645	
Change extensibility muscle-tendon complex	Correlation Coefficient	0,309	0,079	-0,256	-,857*	0,536	-0,607	0,536	-0,429	0,071	-0,679	0,6	-0,771	0,143	0,036	0,25	,857*	0,536
	Sig. (2-tailed)	0,501	0,867	0,579	0,014	0,215	0,148	0,215	0,337	0,879	0,094	0,285	0,072	0,76	0,939	0,589	0,014	0,215

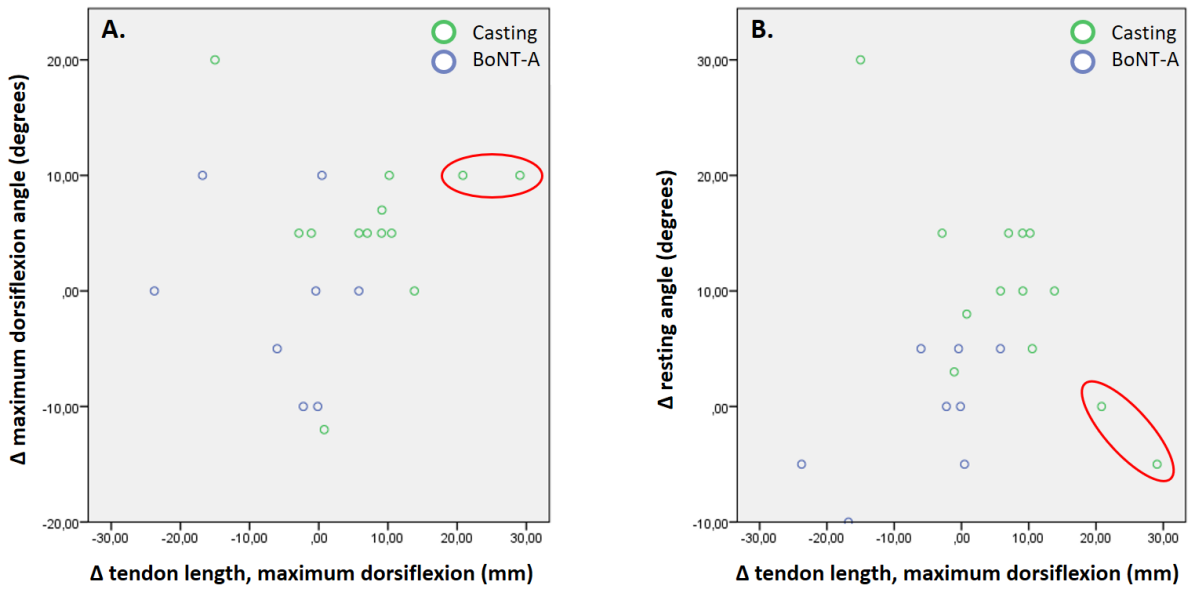
maxDF = maximum dorsiflexion, ML = muscle length, TL = tendon length, mm = millimeter, J = joule, rmsEMG = root mean square of the electromyographic signal MG = medial gastrocnemius muscle, LG = lateral gastrocnemius muscle, µV = microvolt, SOL = soleus muscle, ROM = range of motion. Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed).*

Supplementary Table V. Correlations between changes in ultrasound and Instrumented assessment of ankle joint resistance and stretch reflex parameters of the BoNT-A group (n=9).

BoNT-A

		Change in resting angle	Change in maxDF angle	Change in Δ ankle angle	Change in muscle-tendon complex length, rest	Change in muscle-tendon complex length, maxDF	Change in ML, rest	Change in ML, maxDF	Change in TL, rest	Change in TL, maxDF	Change MG rmsEMG (µV)	Change LG rmsEMG (µV)	Change SOL rmsEMG (µV)	Change ROM	Change work, low velocity	Change work, high minus low velocity	Change extensibility ML	Change extensibility TL
change in maxDF angle	Correlation Coefficient	-0,424																
	Sig. (2-tailed)	0,255	.															
change in Δ ankle angle	Correlation Coefficient	-,673*	,924**															
	Sig. (2-tailed)	0,047	0	.														
Change in muscle-tendon complex length, rest	Correlation Coefficient	0,025	0,037	0,05														
	Sig. (2-tailed)	0,953	0,931	0,906	.													
Change in muscle-tendon complex length, maxDF	Correlation Coefficient	0,025	0,63	0,43	,857*													
	Sig. (2-tailed)	0,954	0,094	0,288	0,014	.												
Change in ML, rest	Correlation Coefficient	0,05	0,482	0,401	,738*	,786*												
	Sig. (2-tailed)	0,906	0,227	0,325	0,037	0,036	.											
Change in ML, maxDF	Correlation Coefficient	-0,477	0,472	0,552	,833*	0,643	,714*											
	Sig. (2-tailed)	0,195	0,199	0,123	0,01	0,086	0,047	.										
Change in TL, rest	Correlation Coefficient	-0,121	-0,232	-0,104	0,143	-0,048	-0,5	0,25										
	Sig. (2-tailed)	0,756	0,548	0,791	0,736	0,911	0,207	0,516	.									
Change in TL, maxDF	Correlation Coefficient	0,42	-0,012	-0,16	0,214	0,357	0,321	-0,429	-0,381									
	Sig. (2-tailed)	0,3	0,977	0,706	0,645	0,385	0,482	0,289	0,352	.								
Change MG rmsEMG (µV)	Correlation Coefficient	-0,191	-0,086	0,155	-0,119	-0,286	-0,333	0,117	0,6	-0,143								
	Sig. (2-tailed)	0,623	0,826	0,69	0,779	0,493	0,42	0,765	0,088	0,736	.							
Change LG rmsEMG (µV)	Correlation Coefficient	0,949	-0,2	-0,316	-0,4	-0,2	-0,4	-0,4	0,8	-0,4	0,8							
	Sig. (2-tailed)	0,051	0,8	0,684	0,6	0,8	0,6	0,6	0,2	0,6	0,2	.						
Change SOL rmsEMG (µV)	Correlation Coefficient	0,113	0,692	0,543	0,357	0,679	,786*	0,286	-0,643	0,607	-0,5	-0,4						
	Sig. (2-tailed)	0,809	0,085	0,208	0,432	0,094	0,036	0,535	0,119	0,148	0,253	0,6	.					
Change ROM	Correlation Coefficient	-0,269	0,129	0,069	-0,119	-0,119	0,095	-0,183	-,667*	0,048	-0,533	-0,8	-0,036					
	Sig. (2-tailed)	0,485	0,741	0,86	0,779	0,779	0,823	0,637	0,05	0,911	0,139	0,2	0,939	.				
Change work, low velocity	Correlation Coefficient	-0,529	0,532	,690*	0,381	0,452	0,143	0,65	0,5	-0,214	0,4	0,2	0,214	-0,483				
	Sig. (2-tailed)	0,143	0,14	0,04	0,352	0,26	0,736	0,058	0,17	0,61	0,286	0,8	0,645	0,187	.			
Change work, high minus low velocity	Correlation Coefficient	0,477	-0,309	-0,38	-0,476	-0,167	-0,619	-,767*	0	0,5	-0,083	0,4	-0,071	-0,167	-0,133			
	Sig. (2-tailed)	0,195	0,418	0,314	0,233	0,693	0,102	0,016	1	0,207	0,831	0,6	0,879	0,668	0,732	.		
Change extensibility ML	Correlation Coefficient	-0,202	-0,469	-0,225	0,143	-0,393	-0,452	0,167	,762*	-0,536	0,595	1,000**	-,857*	-0,19	0,381	0,071		
	Sig. (2-tailed)	0,632	0,241	0,592	0,736	0,383	0,26	0,693	0,028	0,215	0,12	.	0,014	0,651	0,352	0,867	.	
Change extensibility TL	Correlation Coefficient	0,408	0,025	-0,147	0,393	0,333	0,714	-0,238	-0,69	,786*	-0,643	-0,8	,929**	0,19	-0,31	0,286	-,893**	
	Sig. (2-tailed)	0,316	0,954	0,728	0,383	0,42	0,071	0,57	0,058	0,021	0,086	0,2	0,003	0,651	0,456	0,493	0,007	.
Change extensibility muscle-tendon complex	Correlation Coefficient	0,094	0,505	0,393	0,429	0,714	0,5	0,036	-0,321	,929**	-0,107	-0,4	,786*	-0,071	0,321	0,286	-0,571	,821*
	Sig. (2-tailed)	0,84	0,247	0,383	0,337	0,071	0,253	0,939	0,482	0,003	0,819	0,6	0,036	0,879	0,482	0,535	0,18	0,023

*maxDF = maximum dorsiflexion, ML = muscle length, TL = tendon length, mm = millimeter, J = joule, rmsEMG = root mean square of the electromyographic signal MG = medial gastrocnemius muscle, LG = lateral gastrocnemius muscle, µV = microvolt, SOL = soleus muscle, ROM = range of motion. * Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed).*



Supplementary Figure I. Scatterplots of change (post-pre) in tendon length at maximum dorsiflexion and change (post-pre) in (A) maximum dorsiflexion angle and (B) change (post-pre) in resting angle. Highlighting the deviating response of two subjects in the casting group.

Note that in figure A. those two subjects whose maximum dorsiflexion angle increased by 10 degrees without (or minimal) change in the resting angle, there was also a corresponding increase in tendon length.

Chapter 2.5.

The Short-Term Impact of Botulinum Neurotoxin-A on Muscle Morphology and Gait in Children with Spastic Cerebral Palsy

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Abbreviations

AFO = ankle foot orthoses
BoNT-A = Botulinum Neurotoxin type-A
cm = centimeters
COVID-19 = Coronavirus Disease 2019
Deg = degrees; DF = dorsiflexion
EMG = electromyography
GC = gait cycle
GMFCS = Gross motor function classification system
GPS = gait profile score
IC = initial contact
ISA = instrumented spasticity assessment
Kg = kilograms; m = meters
MAS = modified Ashworth score
max = maximum
MCID = minimally clinically important difference
MEH = medial hamstrings
MG = medial gastrocnemius
ml = milliliters; n = number
n.a. = not applicable
n.s. = not significantly different
PF = plantar flexors
RMS = root mean square
ROM = range of motion
SCP = spastic cerebral palsy
SEM = standard error of measurement
SnPM = statistical non-parametric mapping
ST = semitendinosus
µV = microvolts
3DfUS = three-dimensional freehand ultrasound
3DGA = three-dimensional gait analysis

Keywords: botulinum neurotoxin type-A, spastic cerebral palsy, gait, ultrasound, muscle morphology, treatment

Abstract

Children with spastic cerebral palsy (SCP) are often treated with intramuscular Botulinum Neurotoxin type-A (BoNT-A). Recent studies demonstrated BoNT-A-induced muscle atrophy and variable effects on gait pathology.

This group-matched controlled study in children with SCP compared changes in muscle morphology 8–10 weeks post-BoNT-A treatment (n = 25, median age 6.4 years, GMFCS level I/II/III (14/9/2)) to morphological changes of an untreated control group (n = 20, median age 7.6 years, GMFCS level I/II/III (14/5/1)). Additionally, the effects on gait and spasticity were assessed in all treated children and a subgroup (n = 14), respectively. BoNT-A treatment was applied following an established integrated approach. Gastrocnemius and semitendinosus volume and echogenicity intensity were assessed by 3D-freehand ultrasound, spasticity was quantified through electromyography during passive muscle stretches at different velocities. Ankle and knee kinematics were evaluated by 3D-gait analysis.

Medial gastrocnemius (p = 0.018, -5.2%) and semitendinosus muscle volume (p = 0.030, -16.2%) reduced post-BoNT-A, but not in the untreated control group, while echogenicity intensity did not change. Spasticity reduced and ankle gait kinematics significantly improved, combined with limited effects on knee kinematics.

This study demonstrated that BoNT-A reduces spasticity and partly improves pathological gait but reduces muscle volume 8–10 weeks post-injections. Close post-BoNT-A follow-up and well-considered treatment selection is advised before BoNT-A application in SCP.

Introduction

Spasticity occurs in approximately 60–80% of the children with cerebral palsy (1,2). Therefore, spastic cerebral palsy (SCP) is the most common subtype. Spasticity is thought to be a result of imbalanced signals from the central nervous system resulting in impaired gross motor function, such as pathological gait, as well as in discomfort and pain (3,4). Graham et al. described four stages of musculoskeletal pathology in SCP (5).

Botulinum Neurotoxin type-A (BoNT-A) is widely used during the first stage of musculoskeletal SCP pathology, which is characterized by hypertonia, as an effective treatment in reducing the hyperactive stretch reflex by temporarily blocking the release of Acetylcholine at the neuromuscular junction (6–8). By reducing the hyperactive stretch reflex, BoNT-A aims to increase range of motion (ROM), reduce contracture development and improve function and gait (8–11). Additionally, patient comfort, physical activity and social participation may improve (12). Yet, the etiology of contractures is very complicated and not fully understood, and spasticity is definitely not the only contributing factor (13–17). Still, BoNT-A injections remain the first line of treatment to prevent the child from developing to the second stage of contractures, which can only be surgically corrected (5).

As BoNT-A induces a local, temporary paralysis in the muscle, recent concerns have been raised regarding the impact of repeated BoNT-A injections on muscle morphology in young children with SCP (18–20). A review by Multani et al. summarized evidence of muscle atrophy, fatty infiltration, loss of contractile tissue and increased connective tissue after BoNT-A treatment (18). However, it should be noted that the highest levels of muscle atrophy and quality reduction with only partial recovery of muscle morphology and function at 12 months after BoNT-A injection have been reported in animal studies or studies including healthy volunteers (21–23). Yet, knowledge on BoNT-A-impact on muscle morphology in a pediatric SCP population is important, specifically since muscle growth is already impaired in SCP from an early age on (24). Moreover, multiple studies showed that muscle volume and muscle quality can be negatively influenced by BoNT-A (25–30). It has also been shown that SCP muscles have a reduced number of satellite cells, suggesting that the regenerative capacity of the muscle might not be sufficient to ensure efficient muscle recovery, for example to overcome the potential paralysis-induced atrophy following BoNT-A injections (17,31,32).

Furthermore, early retrospective studies in young children with SCP who received one to five BoNT-A treatment sessions following an integrated approach (i.e., BoNT-A injections combined with stretching casts if relevant, intensive physical therapy, and orthotic management) showed beneficial effects on gait pathology at five to ten years of age (defined by three-dimensional gait analysis in 60 children with SCP) (33). Moreover, BoNT-A treatment resulted in the reduced frequency of surgical interventions and postponed the need for surgery (defined in 424 children with SCP) (34). The positive effects of BoNT-A, following this integrated approach, on gross motor function, including gait, have been confirmed by more recent studies (35,36). A cohort study of 28 children with SCP showed that children who received repeated integrated BoNT-A treatment at a young age, improved or maintained their gross motor function at an older age (35). However, these benefits have been recently questioned, in other studies that showed limited reductions in pathological gait (37–39). BoNT-A-induced muscle weakness has been suggested as the potential cause for the lack of functional improvement, whereas SCP is already characterized by reduced muscle strength (19).

Despite the early reported positive effects of BoNT-A on gait and the potential to postpone more invasive treatments, the reported negative effects are concerning. Due to its temporary effect, BoNT-A injections have to be repeated. Different research centers (40–42), as well as previous BoNT-A treatment recommendations, reported repeated injections every six months or even less, while other centers reported intervals between repeated BoNT-A sessions of more than one year. Interestingly, the most recent recommendations advise an interval of at least one year (18,25,43). Previous animal (22) and human (21,26) studies indeed showed that muscles are not recovered after six months. Multiple experts have asserted the need to revise treatment protocols as irreversible muscle atrophy may occur and the detrimental effects might become more severe after multiple injections (44). Clearly, there is an urgent need for a critical reflection on the benefits and harms of BoNT-A in young children with SCP, which should be based on a comprehensive assessment of a multidimensional set of outcomes (18,23). These outcomes should not only include clinical measures but also measures of the muscle morphology and objective measures of spasticity, as well as functional outcomes, such as gait analysis. A quantitative objective approach is important, as previous studies suggested that the benefits of BoNT-A can be overestimated when using subjective outcomes (18,45,46). Even though the number of studies investigating the impact of BoNT-A in SCP has been rising

in the past decade, previous research had some limitations. Small sample sizes, without control groups, were often included, subjective outcomes or isolated gait parameters were used and the focus was limited to only one muscle.

Therefore, the primary aim of the current group-matched controlled study was to investigate the impact of BoNT-A injections administered following an integrated therapeutic approach (combined with stretching casts if relevant, intensive physical therapy, and orthotic management) on the muscle morphology of two lower-limb muscles in children with SCP, aged between 3–11 years. The secondary aim was to confirm the impact of BoNT-A on spasticity and gait. The objective measures of the muscle morphology of the medial gastrocnemius (MG) and the distal compartment of the semitendinosus (ST) by three-dimensional freehand ultrasound (3DfUS), instrumented spasticity assessments and three-dimensional gait analysis (3DGA) were used to quantify the effects of BoNT-A. It was hypothesized that BoNT-A would reduce the muscle volume and negatively affect muscle quality. Furthermore, it was expected that multilevel integrated BoNT-A treatment would reduce the hyperactive stretch reflex and significantly improve gait kinematics of the ankle and knee in the sagittal plane.

Materials and Methods

This study included participants of two different projects. Both projects were approved by the local ethics committee of the University Hospitals Leuven, Belgium (s59945 TAMTA project, s62187 and s62645 3D-MMAP project) (Ethische Commissie Onderzoek KU Leuven/ UZ Leuven). Written informed consent was obtained from the parents or legal guardian. Participants were included between May 2019 and June 2022.
This trial is registered at ClinicalTrials.gov, identifier: NCT05126693.

Participants

Children aged between 3–11 years, GMFCS levels I-III with SCP, scheduled for BoNT-A in the MG and/or ST, as part of a multilevel treatment, were included. Participants were recruited via two parallel prospective studies. Both studies investigated the effect of integrated BoNT-A treatment on muscle morphology and gait, while one study also evaluated the effects on spasticity (s59945 TAMTA project). The selection criteria for the participants were similar in both studies. The exclusion criteria were: any signs of dystonia or ataxia in the lower limbs, severe muscle weakness or poor selectivity in the plantar flexors or knee flexors and cognitive problems that would impede the measurements, intrathecal baclofen pump, previous muscle surgery in the lower limbs or selective dorsal rhizotomy, previous bony corrections in the lower limb within two years before assessments, previous BoNT-A treatment within six months before the baseline assessment or within six months before the pre-BoNT-A 3DGA. Additionally, the physical therapy frequency had to be consistent for at least three months before inclusion. The participants were measured twice, before and 8–10 weeks after BoNT-A treatment to evaluate the short-term effects. All children (of both studies) received 3DfUS assessments and a 3DGA with the clinical examination of the lower limbs. A subset of children (i.e., the participants of one of the two studies) underwent an additional instrumented spasticity assessment in order to evaluate the BoNT-A-induced tone reduction. During the follow-up, all children received usual care, including intensive physical therapy, orthotic management and oral tone reduction if needed.
An age- and functional severity-matched (group-matching based on age and GMFCS level) control group, meeting the same inclusion criteria as the intervention group, but not receiving

any intervention apart from their usual care, was included. The participants of the control group were recruited via Cerebral Palsy Reference Center of the University Hospitals Leuven, Belgium and the Inkendaal Rehabilitation Hospital Vlezenbeek, Belgium. All children eligible for inclusion were measured (n = 30, Figure 1); however, the final data selection was based on accurate matching with the participants in the intervention group. The participants in the control group underwent 3DfUS measurements only, with an interval of 8–10 weeks between the two assessments. Participant characteristics are summarized in Table 1, showing no structural differences, as assessed by a Mann–Whitney U test, in the maximum dorsiflexion angle, popliteal angle and spasticity levels assessed by the modified Ashworth scale.

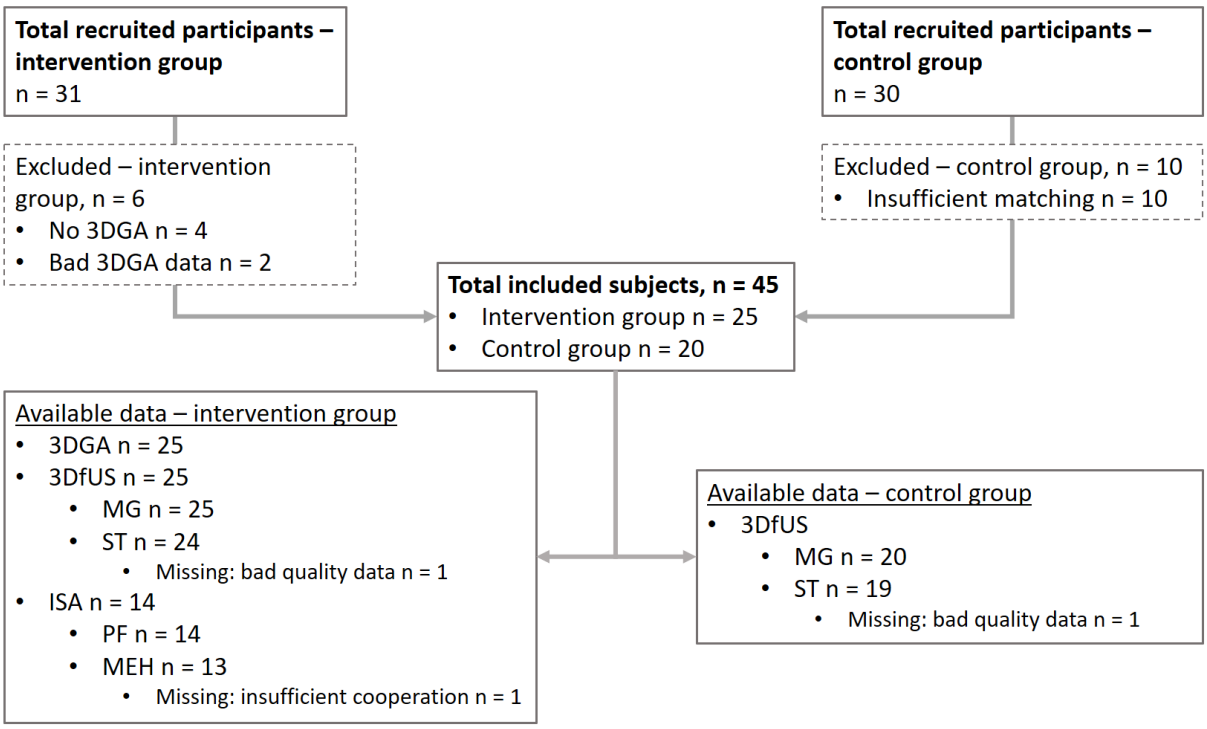

Figure 1. Flow chart of all recruited and finally included subjects in both the intervention and untreated control group, together with a summary of data availability

3DfUS = three-dimensional freehand ultrasound, 3DGA = three-dimensional gait analysis, ISA = instrumented spasticity assessment, MEH = medial hamstrings, MG = medial gastrocnemius, n = number, PF = plantar flexors, ST = semitendinosus.

An overview of the recruited and eventually included subjects and data availability is summarized in Figure 1.

Procedure

The most affected leg, based on the most recent clinical examination (highest modified Ashworth score for the ankle and knee joints), was assessed. The measures of body mass, height, total leg length and lower leg length were taken. A standardized anamnesis was performed to request treatment history and usual care details.

Muscle selection for BoNT-A injection was primarily based on the most recent clinical examination and multidisciplinary evaluation of the 3DGA data. BoNT-A, Onabotulinum toxin A (Botox®, Allergan, Diegem, Belgium), injections were applied by a pediatric orthopedic surgeon under general (mask) anesthesia, guided by ultrasonography or palpation (30,47). A dose of 100 units of BoNT-A was diluted in 5 mL of saline. The BoNT-A dilution was injected at multiple sites, in areas with the largest concentration of motor endplates in the muscles selected for treatment (47). A maximum of 50 units was injected per site.

BoNT-A is suggested to be more effective when part of an integrated approach, i.e., combined with casting, orthotic wear and physical therapy (48). Therefore, lower leg casts and removable upper leg casts were applied as indicated. The majority of children used day and night orthoses (Table 1) and all children received intensive physical therapy in the 8–10 weeks following BoNT-A treatment.

All additional study assessments (3DfUS, clinical examination, instrumented spasticity assessment and 3DGA) were performed by three trained assessors. The pre- and post-assessments were always performed by the same assessor.

Assessments

Muscle morphology

The muscle morphology of the MG and the distal compartment of the ST muscle were assessed by 3DfUS (49). This technique, combining B-mode ultrasonography with three-dimensional motion tracking, is proven to be valid and reliable in muscle morphology assessments in children with SCP in previous studies (50–52).

Ultrasound assessments were performed while the participant was placed in a prone position. The lower leg was supported by a triangular cushion to allow slight knee flexion (approximately 25 degrees) and avoid bi-articular stretch on the MG and ST. The ankle was in resting position without fixation. The joint angles were measured by goniometry.

Large amounts of acoustic transmission gel were used to assure smooth movement of the transducer and optimal contact between the transducer and skin. An additional curved gel pad, the Portico, was used to reduce muscle deformation during the acquisition (53). Muscle images were recorded by Stradwin software (version 6.0; Mechanical Engineering, Cambridge University, Cambridge, UK) while moving the transducer from muscle origin to tendinous insertion. The acquisitions were repeated in case of bad quality images, movement or muscle contraction or when the bony landmarks or muscle borders were missed.

After data collection, the muscle images were processed by a trained assessor using the Stradwin software (version 6.0; Mechanical Engineering, Cambridge University, Cambridge, UK). Muscle volume (in millimeters) was estimated by the cubic planimetry technique based on segmentations that were manually drawn along the inside of the muscle borders in 5–10% of the transverse 2D muscle images (54). Additionally, echogenicity intensity, as a proxy of muscle quality, was based on the average of the interpolated reconstruction of the muscle and expressed as a gray value on an 8-bit scale (values between 0–255), whereby higher values (whiter images) indicated reduced muscle quality (55,56). Muscle belly lengths were calculated as the Euclidean distance between the muscle origin (MG: posterior surface of medial femoral condyle, ST: ischial tuberosity) and the muscle-tendon junction. The tendon length of the MG was calculated between the muscle–tendon junction and tendinous insertion. The MTU length of the MG was calculated by adding the muscle belly to the tendon length.

In order to compare children with different body dimensions, muscle volume was normalized to the product of body mass height and muscle and tendon lengths were expressed as a percentage of total leg length.

The absolute muscle-specific growth rates for muscle volume during the period between the two assessments were calculated by the following formulas (26,40):

$$\textbf{growth rate, muscle volume} = \frac{\text{muscle volume (mL) POST} - \text{ muscle volume (mL) PRE}}{\text{age (months) POST} - \text{ age (months) PRE}}$$

The primary outcomes included the normalized muscle volume and echogenicity intensity of the MG and distal compartment of the ST and the absolute muscle volume growth rates. The

secondary outcomes, muscle, tendon and muscle-tendon unit lengths and normalized muscle volume growth rates are included in the supplementary material (Table S1).

Gait analysis

Gait, overground barefoot walking at a self-selected speed, was assessed by 3DGA in a 10 to 12 camera (Oxford Metrics, Oxford, UK) gait lab (Vicon Motion Systems, Oxford, UK). Twelve reflective markers were placed on anatomical landmarks according the Vicon Plug-In-Gait lower limb model (57). Ground reaction forces were measured with force plates (Advanced Mechanical Technology Inc., Watertown, MA, USA) that were embedded in the walkway. During the 3DGA, kinematic data (joint angles for ankle, knee and hip and the segmental orientation of the pelvis and foot), kinetic data (joint moments and power) and the surface EMG data of the medial gastrocnemius, soleus, tibialis anterior, rectus femoris, vastus lateralis, the medial and lateral hamstrings and gluteus medius were collected for each leg. The kinematic data of the most affected leg (same as for the 3DfUS and instrumented spasticity assessments) and a selection of spatial-temporal parameters were included in this current investigation but the kinetic and EMG data were not included in any of the analyses.

Gait cycles were identified and manually marked using the Vicon Nexus software (Oxford Metrics, Oxford, UK). All trials were exported and imported to a custom-made Matlab script (The MathWorks, Natick, MA, USA, version 2020b). All gait cycles were judged on quality. Trials were excluded in case of artefacts, gaps in data or indications of inaccurate marker placement. Furthermore, the knee varus–valgus angle was evaluated and trials with absolute knee varus–valgus ROM exceeding 15 degrees, a knee valgus angle exceeding -10 degrees, or trials that were not representative of the participants' gait pattern were excluded (58). As many good-quality steps as possible were used to create average trials for the ankle and knee joint kinematics.

The GPS, and specific kinematic gait parameters that were found to be sensitive to detect BoNT-A-induced changes, were extracted per joint (59). Additionally, the spatial-temporal parameters cadence, walking velocity and stride length were calculated. Finally, the full kinematic waveforms were exported for pre–post comparison.

The primary outcomes included the kinematic waveforms, BoNT-A-sensitive kinematic gait parameters (59), GPS and spatial-temporal parameters. The secondary outcomes of elaborate kinematic parameters are summarized in the supplementary material (Table S2).

Spasticity assessment

A subset of participants (n = 14) underwent an instrumented spasticity assessment in order to objectively evaluate the change in the hyperactive stretch reflex during passive stretch (60). This method has been previously described by Bar-On and colleagues (60) and is proven sensitive enough to detect treatment-induced changes (61–63).

Four low-velocity (over 5 seconds) passive stretches of the plantar flexors and medial hamstrings were performed over the full ROM. Next, four full ROM high-velocity passive stretches were performed, whereby the stretch was performed as fast as possible. The interval between the stretch repetitions was at least 7 seconds to avoid post-activation depression. During the stretches, muscle activation was recorded by surface EMG (Zerowire Cometa, Milan, Italy).

The raw EMG signals were filtered by a 6th order zero-phase Butterworth bandpass filter (20–500 Hz). The root mean square envelope of the EMG signal (RMS–EMG) was computed after application of a low-pass 30 Hz 6th order zero-phase Butterworth filter.

All data analyses were carried out with MATLAB Software (The MathWorks, Natick, MA, USA, version 2020b). The quality of the individual stretches were checked using custom-made software. Stretch repetitions were excluded in case of inconsistent stretch velocities, poor surface EMG data, out-of-plane movement or indications of insufficient participant relaxation (constant EMG activity of the antagonist).

In order to quantify the alteration in muscle tone after BoNT-A treatment, the average RMS–EMG expressed in millivolts was calculated as the square root of the area underneath the RMS–EMG time curve, divided by the duration of the time interval, during the high-velocity stretch. The interval started 200 milliseconds prior to the time point where maximum stretch velocity was reached and ended at the time corresponding to 90% of the full ROM. This value was referred to as the muscle activation during a high-velocity stretch. All BoNT-A-induced changes were compared to previous reported SEM or BoNT-A-induced values (60–64).

The average RMS–EMG expressed in millivolts during a high-velocity stretch was used as the primary outcome to assess alterations in muscle tone. Additionally, the increase during the high-velocity stretch was calculated by subtracting the muscle activation during a low-velocity stretch, which was calculated following the same method. This value was called the change in muscle activation and was included as a secondary outcome. This secondary outcome

together with the RMS–EMG during a low-velocity stretch are enclosed in the supplementary material (Table S4).

Clinical examination

A standardized clinical examination including assessments of ROM, spasticity, selectivity and strength was performed during the pre- and post-BoNT-A assessments of the intervention group and during the pre-assessment of the control group. These data were used to describe and compare both groups at baseline. The maximal dorsiflexion angle and popliteal angle (unilateral) were assessed by goniometry. The level of spasticity was assessed by the modified Ashworth scale and modified Tardieu scale (65,66). Muscle strength was assessed by manual muscle testing and selectivity was assessed by the Selective Control Assessment of the Lower Extremity (67,68). The evaluations of strength and selectivity were not included in the current investigation.

Statistical analysis

The normality of data distribution was checked by a Kolmogorov–Smirnov test ($p < 0.05$). Since most parameters were not normally distributed, non-parametric statistical tests were performed.

Participant characteristics, maximal dorsiflexion angle (measured with the knee extended), popliteal angle (unilateral), and normalized muscle morphology parameters of the intervention and control group were compared at baseline by a Mann–Whitney U test (Table 1).

A Wilcoxon signed rank test was used to evaluate (within) treatment effects in muscle morphology, instrumented spasticity assessment outcomes and spatial-temporal parameters of the 3DGAs (alpha = 0.05). A Bonferroni correction was applied for the gait kinematic parameters per joint (ankle: alpha 0.05/4 = 0.0125, knee: alpha 0.05/3 = 0.0167). Gait parameters were considered meaningful if statistically significant changes were noted that were exceeding the SEM or MCID (69,70).

Additionally, median difference scores (xpost - xpre) for muscle morphology parameters were calculated in both the intervention and control group and between-group differences were tested by a Mann–Whitney U test. Additionally, the changes were expressed as a percentage

change of the baseline values for both groups, which are summarized in Table S1 of the supplementary material.

Paired t-tests of the ankle and knee kinematics over the entire gait cycle using SnPM analysis were performed to identify treatment-induced changes in these sagittal plane motions. The averaged ankle and knee kinematic waveforms, consisting of 101 points were imported to the SPM1d software (version 0.4, available for download at http://www.spm1 d.org/, accessed on 15 August 2022) in Matlab (The MathWorks, Natick, MA, USA, version 2020b). Clusters were formed if the critical threshold, based on random field theory, was crossed (58,71,72) (alpha = 0.05). In case of significant clusters, the p-value, details on the location and the duration (percentages of the entire gait cycle) were reported. Clusters were considered meaningful when their duration exceeded 3% of the gait cycle and when the differences between the means of the pre- and post-BoNT-A joint kinematics exceeded the respective SEMs (58,73).

Results

Participants

For the current study, the participants of two sub-studies on the effect of integrated BoNT-A treatment on muscle morphology and gait were pooled. This resulted in a total sample of 45 children (29 boys, 16 girls), with a median age of 7.1 years (5.7–9.1), who all had routine physical therapy and the use of orthoses as usual care. Twenty-five children, with an indication for BoNT-A injections in the ST and/or MG were included in the intervention group, while 20 children without an indication for BoNT-A were included in the untreated control group. The indication for BoNT-A treatment was set a by a multidisciplinary team based on clinical examination including assessments of ROM, spasticity, selectivity and strength, and the longitudinal evaluations of the pathological gait. Participants' baseline characteristics and BoNT-A treatment details are summarized in Table 1.

All children in the intervention group underwent an assessment of muscle morphology by 3DfUS and received 3DGA to assess the level of gait pathology before and 8–10 weeks after the BoNT-A intervention. For one of the two sub-studies, the participants (n = 14, median age of 7.0 years (5.3–9.6), 6 boys and 8 girls, bilateral/unilateral 7/7, GMFCS level I/II/III 9/4/1 received an additional instrumented spasticity assessment at the same time points. The median interval between the baseline assessment (3DfUS + instrumented spasticity assessment) and integrated BoNT-A treatment was 0.2 (0.0–0.6) months. Due to practical reasons and the clinical follow-up, the pre-3DGA could not always be combined with the baseline assessments (3DfUS + instrumented spasticity assessment) required for this study. Therefore, the median interval between the pre-3DGA and the BoNT-A treatment was 1.1 (0.5–2.6) months. The median interval between the BoNT-A treatment and post-treatment evaluation (3DfUS + instrumented spasticity assessment + 3DGA) was 2.1 (1.9–2.4) months.

For the primary aim, 20 children with SCP, receiving no intervention and continuing usual care, were included in the control group. These children underwent 3DfUS assessments at baseline and after a period of 8–10 weeks, with a median interval of 2.7 (1.9–2.8) months but no 3DGA nor instrumented spasticity assessment.

Table 1. Participant characteristics of the included participants of the intervention and control group.

	Intervention group (n = 25)	Control group (n = 20)	Between group p-Value
Age (years)	6.4 (4.6–8.5)	7.6 (6.2–9.4)	0.144
Gender (n)	boys = 13, girls = 12	boys = 16, girls = 4	
GMFCS (n)	I = 14, II = 9, III = 2	I = 14, II = 5, III = 1	
Paralysis (n)	bilateral = 15 unilateral = 10	bilateral = 10 unilateral = 10	
Body height (cm)	114.5 (106.5–130.5)	127.3 (115.7–133.8)	0.021
Body mass (kg)	20.0 (17.0–23.9)	24.4 (21.1–30.7)	0.010
Maximal dorsiflexion angle, knee extended (°)	5.0 (0.0–10.0)	10.0 (0.0–10.0)	0.267
Popliteal angle, unilateral (°)	−55.0 (−42.5–−62.5)	−55.0 (−50.0–−63.8)	0.737
MAS PF, knee extended	2.0 (1.6–3.0)	1.5 (1.4–1.6)	
MAS PF, knee 90 degrees	2.8 (1.5–2.0)	1.5 (1.4–1.5)	
MAS hamstrings	1.5 (1.0–1.9)	1.0 (0.8–1.5)	
BoNT-A MG (units/kg), **n = 24**	2.7 (2.2–2.9)	n.a.	
BoNT-A ST (units/kg), **n = 21**	1.7 (1.4–2.0)	n.a.	
BoNT-A treatment history (n)	BoNT-A naive = 10 1–3 previous BoNT-A = 11 >4 previous BoNT-A = 4	BoNT-A naive = 9 1–3 previous BoNT-A = 10 >4 previous BoNT-A = 1	
Orthotic management, AFO's during the day (n)	Frequently used (>50% of the day) = 20 Not frequently used (≤50% of the day) = 3 Not used = 2	Frequently used (>50% of the day) = 13 Not frequently used (≤50% of the day) = 2 Not used = 2 Unknown = 3	
Oral tone reduction (n)	Yes = 3 No = 22	Yes = 0 No = 20	

Values are presented as medians with 25th and 75th percentiles

AFO = ankle foot orthoses, BoNT-A = Botulinum Neurotoxin type-A, cm = centimeters, GMFCS = Gross motor function classification system, kg = kilograms, MAS = modified Ashworth score, MG = medial gastrocnemius, n = number, n.a. = not applicable, PF = plantar flexors, ST = semitendinosus

The duration of the interval between BoNT-A treatment and the post-assessment in the intervention group and the interval between the baseline and post-assessment of the control group was not significantly different (p = 0.740).

As part of the integrated approach, 20 children received lower-leg casts immediately following the BoNT-A injections. These casts were applied for at least 10 days. Of the 20 children, 15 received additional removable upper-leg casts (as part of the integrated approach). Two children had full leg casts for at least ten days. Three children did not receive casting.

At baseline, there was no difference in age (p = 0.144) between the intervention and control group. Body mass (p = 0.010) and height (p = 0.021) were significantly different. Therefore, the muscle morphology parameters were normalized to body dimension (body mass and height).

Muscle morphology

At baseline, no significant differences in normalized muscle volume (normalized to the product of body mass and height) or echogenicity intensity of the MG and ST were found between the intervention and control group.

The normalized muscle volume and echogenicity intensity before and after BoNT-A treatment are presented in Figure 2, Figure 3 and in the supplementary material, Table S1. The normalized muscle volume of both the MG (p = 0.018) and the distal compartment of the ST (p = 0.030) showed a significant reduction of 5.2% and 16.2% (supplementary material, Table S1) after BoNT-A treatment, respectively (Figure 2). No differences in the echogenicity intensity of the MG (p = 0.407) and ST (p = 0.145) were found within the intervention group (Figure 3).

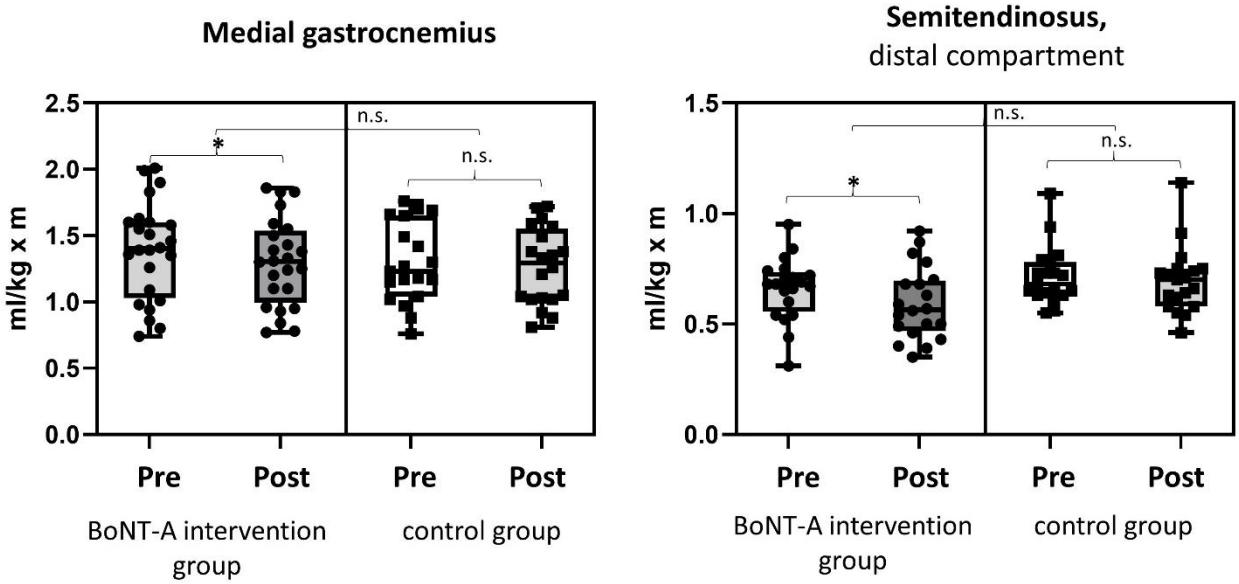

Figure 2. Box plots with individual data points of normalized muscle volume of the medial gastrocnemius and distal compartment of the semitendinosus before and after BoNT-A treatment in the BoNT-A intervention group and at the two assessments in the control untreated group.

** alpha < 0.05, BoNT-A = Botulinum Neurotoxin-A, kg = kilograms, m = meters, ml = milliliters, n.s. = not significantly different.*

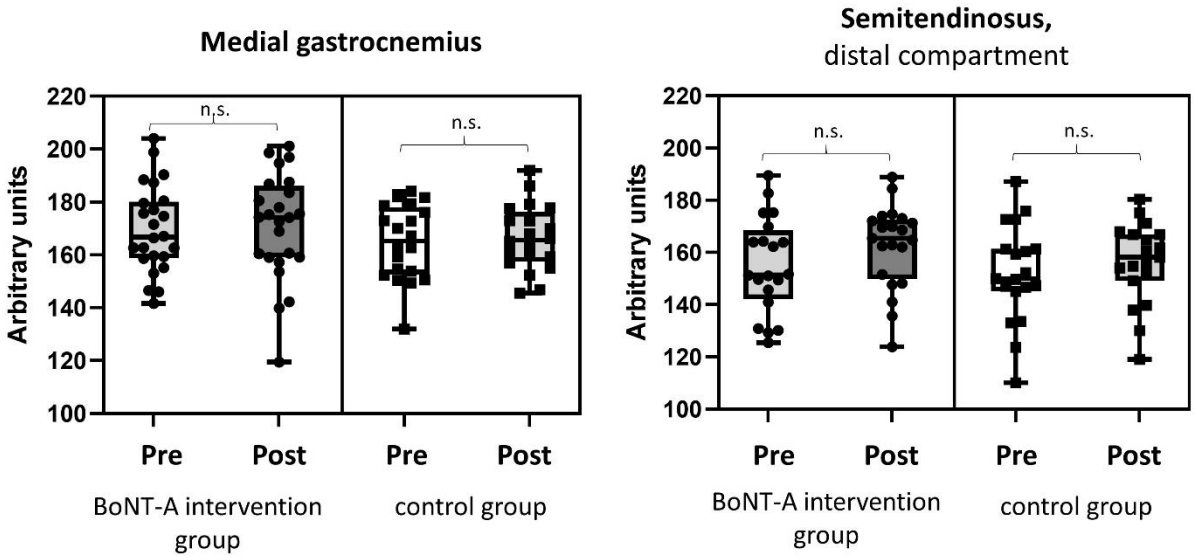

Figure 3. Box plots with individual data points of echogenicity intensity of the medial gastrocnemius and distal compartment of the semitendinosus before and after BoNT-A treatment in the BoNT-A intervention group and at the two assessments in the untreated control group.

BoNT-A = Botulinum Neurotoxin-A, n.s. = not significantly different

No changes in muscle volume or echogenicity intensity were reported in the control group (supplementary material, Table S1). The changes in MG (p = 0.118) and ST (p = 0.069) muscle volume post-BoNT-A were not significantly different from the evolution in muscle volume in the control group.

Absolute muscle growth rates are presented in Table 2. Muscle growth rates calculated over the period between the two assessments were not significantly different between the intervention and control group for both muscles (MG p = 0.346, ST p = 0.226).

Table 2. Muscle-specific growth rates calculated over the period between the two assessments for both the intervention and control group.

	Intervention Group	Control Group	Between-Group *p*-Value
Medial gastrocnemius growth rate (mL/month)	0.00 (−1.34 – 0.97)	0.17 (−0.23 – 0.87)	0.346
Semitendinosus, distal compartment growth rate (mL/month)	−0.49 (−1.45 – 0.58)	0.02 (−0.39 – 0.48)	0.226

Growth rates are presented as medians with corresponding 25th and 75th percentiles. kg = kilograms, mL = milliliters.

All muscle morphology parameters, including differences' scores and between-group comparisons are summarized in Table S1 of the supplementary material.

Gait

The kinematic and spatial-temporal parameters are summarized in Table 3.

Table 3. Sagittal plane gait kinematic parameters, gait profile score and spatial-temporal parameters of the intervention group during the pre- and post-treatment assessments and difference scores.

Gait	**Intervention Group**			
Kinematic Parameters—Ankle[1]	**Pre BoNT-A**	**Post BoNT-A**	**Difference Score**	***p*-Value, Treatment-Induced/within Group**
ankle ROM in sagittal plane (total GC) (degrees)	25.85 (19.83–34.55)	24.18 (21.51–29.13)	−1.74 (−9.04–3.07)	0.143
ankle angle in sagittal plane at IC (degrees)	−3.83 (−11.13–2.14)	−2.25 (−5.25–4.94)	3.56 (0.47–6.36)	0.002**
maximum ankle angle (max DF) in sagittal plane during stance phase (degrees)	9.47 (0.70–14.52)	12.89 (7.37–17.76)	3.99 (−0.32–9.99)	0.004**
maximum ankle angle (max DF) in sagittal plane during swing phase (degrees)	−1.71 (−9.63–3.12)	2.53 (−3.66–7.03)	5.34 (1.16–9.70)	<0.001**
Kinematic parameters—Knee[1]	**pre BoNT-A**	**post BoNT-A**	**difference score**	***p*-value, treatment-induced/within group**
knee ROM in sagittal plane (degrees)	53.50 (45.13–62.03)	54.84 (46.85–60.12)	1.94 (−3.79–8.84)	0.158
knee angle in sagittal plane at IC (degrees)	26.45 (20.93–35.43)	25.53 (17.26–30.53)	−1.69 (−9.19–1.84)	0.048
minimal knee angle in sagittal plane during stance (degrees)	5.06 (0.79–14.05)	6.74 (−1.22–10.09)	−2.44 (−7.61–2.23)	0.065
Gait profile score[1]	**pre BoNT-A**	**post BoNT-A**	**difference score**	***p*-value, treatment-induced/within group**
Gait profile score for angles (degrees)	9.80 (7.41–11.33)	8.30 (6.71–9.41)	−1.76 (−2.51 – −0.15)	0.005*

Spatial-temporal parameters[1]	pre BoNT-A	post BoNT-A	difference score	*p*-value, treatment-induced/within group
Cadence (number of steps/second)	2.25 (1.95–2.43)	2.19 (1.91–2.35)	−0.05 (−0.28–0.10)	0.156
Walking velocity (meter/second)	0.89 (0.67–1.10)	0.85 (0.73–1.05)	0.01 (−0.10–0.10)	0.911
Stride length (meters)	0.81 (0.66–0.96)	0.79 (0.69–1.04)	0.02 (−0.03–0.08)	0.108

*Pre, post and difference scores are presented as median and 25th and 75th percentiles. A Bonferroni correction was applied for the kinematic parameters per joint (ankle: alpha 0.05/4 = 0.0125, knee: alpha 0.05/3 = 0.0167), *alpha<0.05, **significant different after Bonferroni correction: alpha < 0.0125 for the ankle and alpha < 0.0167 for the knee.*

DF = dorsiflexion, GC = gait cycle, IC = initial contact, max = maximum, ROM = range of motion.

The ankle angle at initial contact (p = 0.002), the maximum dorsiflexion angle during both stance (p = 0.004) and swing phase (p < 0.001) significantly improved (while accounting for multiple testing, Bonferroni correction). The maximum dorsiflexion angle during swing exceeded the previously reported standard error of measurement (SEM) of 4.7 degrees (supplementary material, Table S1) (69). No significant improvements were found in knee kinematics in the sagittal plane following BoNT-A treatment. However, there was a tendency towards more knee flexion during stance (p = 0.065), whereas knee flexion at initial contact seemed slightly reduced (p = 0.048).

The gait profile score (GPS) showed a significant decrease (p = 0.005) after BoNT-A treatment, indicating less gait pathology. The median improvement of 1.76 degrees exceeded the minimally clinically important difference (MCID) of 1.6 degrees (70). Cadence, walking velocity and stride length remained unchanged.

The statistical non-parametric mapping (SnPM) analyses of the full kinematic ankle and knee waveforms revealed significant improvements in ankle kinematics, located in two clusters. One large cluster (p < 0.001) reached from 19–100% of the gait cycle and exceeded the SEM (ankle dorsi-plantar flexion, 3.3 degrees) (73). Another small cluster (p = 0.0165, 0–1.99%) at initial contact did not exceed 3% of the gait cycle and was therefore not considered clinically relevant (58). The knee kinematics showed a small cluster at the end of swing from 92–96% of the gait cycle, exceeding 3% of the gait cycle and the SEM (knee flexion-extension, 2.4 degrees) (73).

An overlay of the pre- and post-treatment kinematic waveforms for the ankle and knee is presented in Figure 4.

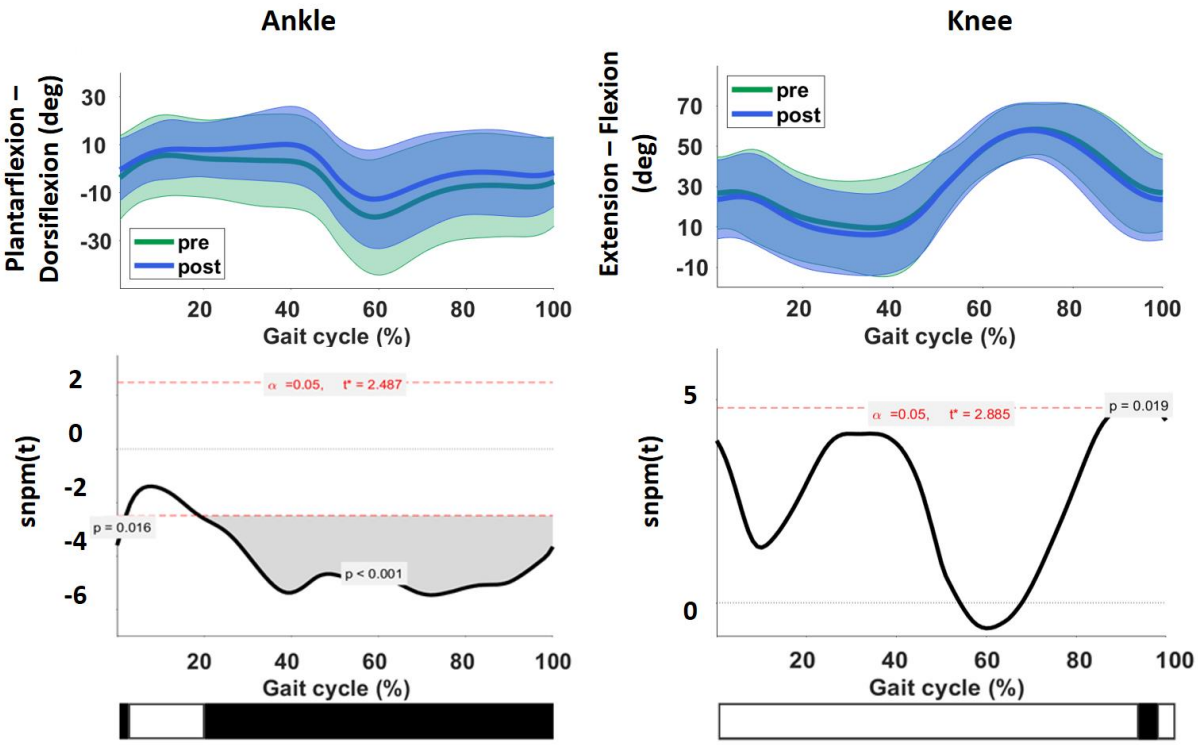

Figure 4. Overlay of the kinematic waveforms of the ankle and knee joint during gait, pre- and post-intervention

The green waveform represents the pre-treatment kinematics, and the blue waveform represents the post-treatment joint kinematics. The black bar at the bottom of the image indicates the location of the significant clusters, exceeding 3% of the gait cycle and exceeding the standard error of measurement. Deg = degrees. t = critical threshold, calculated by the random field theory.

Instrumented Spasticity Assessment

The instrumented spasticity assessment performed in 14 children of the intervention group showed a significant decrease in muscle activation of the MG (p = 0.019) and medial hamstrings (including the ST) (p = 0.041) during the high-velocity passive stretches. All results are summarized in Table 4.

Table 4. Outcomes of the instrumented spasticity assessment for a subgroup (n=14) of the BoNT-A intervention group.

Instrumented Spasticity Assessment—Treated Muscles	**Intervention Group (n = 14)**			
parameter	**pre BoNT-A**	**post BoNT-A**	**difference post-pre**	**p-value, treatment-induced/ within group**
Medial gastrocnemius, EMG—absolute high RMS–EMG (µV)	11.52 (4.87–22.07)	4.91 (3.67–9.45)	−4.73 (−15.74–1.31)	0.019*
Medial hamstrings, EMG—absolute high RMS–EMG (µV)	12.07 (5.77–16.04)	7.98 (3.68–10.58)	−2.82 (7.64–0.21)	0.041*

**alpha<0.05, EMG = electromyography, RMS = root mean square, µV = microvolts.*

The difference in EMG signal between the low- and high-velocity stretches, a secondary outcome, showed a borderline significant decrease after integrated BoNT-A treatment in the ST (p = 0.060), exceeding the SEM (Table S4, supplementary material) (64).

Clinical examination and range of motion

The results of the clinical examination are summarized in the supplementary material, Table S3. Maximal dorsiflexion (p = 0.302) and popliteal angle (unilateral) (p = 0.564) remained unchanged after BoNT-A injections.

Discussion

Summary of findings

The primary aim of this study was to investigate the effect of integrated BoNT-A treatment on muscle morphology of the MG and ST. The second aim was to provide additional evidence regarding the effects on spasticity and gait. To achieve the study aims, an integrated set of objective assessments was used. We hypothesized that BoNT-A injections would result in reduced spasticity and improved gait but would induce muscle atrophy. The current results revealed that integrated BoNT-A treatment successfully reduced the hyperactive stretch reflex but also reduced muscle volume of the MG and distal compartment of the ST 8–10 weeks after BoNT-A injections. No changes in muscle quality were observed. Ankle kinematics during gait improved significantly, whereas effects on knee kinematics were limited. The study hypotheses were thus partly confirmed.

Comparison with Literature

<u>The impact of Botulinum Neurotoxin-A on muscle volume</u>

The current data showed a significant reduction (median change) in the normalized muscle volume of both the MG and ST of approximately 5.2% and 16.2%, 8–10 weeks after BoNT-A injections, which were applied as part of an integrated approach, respectively. Muscle growth rates, calculated over the period between the two assessments, were not significantly different from the control group. Several studies have reported similar rates of muscle atrophy after BoNT-A injections (21–23,25–30).

Alexander et al. reported reductions in MG volume, as absolute value and normalized to bone length at four (5.9%), 13 (9.4%) and 25 (6.8%) weeks, following the first single-level BoNT-a treatment to the MG (27). The greatest reduction, 13 weeks post-BoNT-A (9.4%), was slightly higher compared to the current data (5.2% median change, 8–10 weeks post-BoNT-A). Besides the MG atrophy, the (untreated) soleus muscle became hypertrophic in the sample of Alexander et al., resulting in an unchanged total volume of the plantar flexors. They assumed that the hypertrophy potentially developed to maintain plantar flexor strength and function. The reduction in volume of the MG combined with the hypertrophy of the soleus was also described by Williams et al. after five weeks post-BoNT-A (29). They found an average

reduction of 4.5% in the gastrocnemius (medial and lateral head). Five of those children received injections in the medial hamstrings as well, resulting in a volume reduction of 5.9%. All children in their study had a history of previous injections and received a BoNT-A injection in the MG as part of a multi-level treatment.

A recent study by De Beukelaer et al. was the first to quantify the BoNT-A effect on muscle morphology in an intervention group compared to a control group of children who did not receive BoNT-A treatment. They showed a reduction in anatomical cross-sectional area six months after the first BoNT-A injection, which was not observed in the control group of children, who remained BoNT-A naive during this period (26). They did not find any significant between group differences in muscle volume following BoNT-A.

The decrease in ST volume reported in the current study is higher compared to the MG volume reduction and higher in comparison to previous studies of the distal lower limb muscles. The level of atrophy in the ST is more in line with a previous study by Van Campenhout et al. showing approximately 20.5% (79.5% of the initial volume) reduction in volume of the psoas two months after BoNT-A treatment (30), suggesting that proximal lower limb muscles might be more affected by BoNT-A. However, these findings should be confirmed in future studies including both distal and proximal lower limb muscles.

The current findings are in contrast with the results reported by Barber et al. (40), who showed an increase in absolute MG volume, reflecting an unchanged MG growth rate 12 months after BoNT-A. Williams et al. combined BoNT-A with strength training and also showed a significant increase in muscle volume of the hamstrings and plantar flexors (next to other lower limb muscles) six months post treatment (74). However, it should be noted that improvements in absolute muscle volume observed in the latter studies were most likely a result of natural growth.

The differences in findings between the previously mentioned studies in SCP can be explained by the differences in study population, sample size, used methods, duration of follow-up, treatment protocol and content of usual care.

Besides the immediate impact of BoNT-A on muscle volume as described in the current prospective study, there are indications in the literature that previous BoNT-A injections in children who are not BoNT-A naïve may influence muscle morphology. A retrospective study by Schless et al. compared muscle volumes between children naïve to BoNT-A and children with a history of at least three previous BoNT-A treatments (median of five previous

interventions) and showed that the absolute muscle volume was roughly 29% smaller in the children with a history of previous BoNT-A injections (25). This may also further explain differences between the different studies. Longitudinal studies are required to further evaluate the impact of BoNT-A on muscle morphology and monitor the muscle-specific recovery profiles.

It should also be noted that the described alterations in muscle volume of children with SCP are less extreme compared to animal studies and studies with healthy adults (21,22,75). This is potentially due to the aggressive treatment protocols that were applied in those studies.

The impact of Botulinum Neurotoxin-A on echogenicity intensity

In the current study, no changes in echogenicity intensity were found, suggesting that integrated BoNT-A treatment does not negatively impact muscle quality in the short-term period. This is in contrast with a previous retrospective study by our research group showing that the MG echogenicity intensity of children with a history of BoNT-A was significantly higher compared to children who remained BoNT-A naïve, pointing towards long-lasting alterations in the muscles as a result of BoNT-A (25). A more recent study by De Beukelaer et al. showed a tendency towards higher echogenicity intensity values in children with a history of previous injections compared to BoNT-A naïve children at baseline (26). The fact that no alterations in echogenicity intensity were found in the current study could be explained by the fact that only the short-term effects of a single BoNT-A treatment session or short follow-up period were investigated. The higher echogenicity intensity values, as described in previous studies, suggest a reduced muscle quality, which may be caused by increased connective tissue, loss of contractile elements, fibrosis and fatty infiltration (13,14,26,27). Indeed, multiple animal studies reported fibrosis or collagen deposition, increased lipid accumulation and loss of contractile elements after BoNT-A injections (22,44,76,77) and echogenicity intensity has been linked to these factors, contributing to declined muscle quality and contracture development (55,56,78). Moreover, a causal relation between muscle fibrosis and non-responsiveness to BoNT-A has been suggested (43,79). Yet, since echogenicity intensity represents an indirect measure of muscle quality and cannot decompose the contribution of different components, such as increased fat infiltration and connective tissue, the earlier retrospective results in humans should be interpreted carefully. The validity of echogenicity

intensity is still not fully understood. It has been related to muscle composition in several pathologies; however, validation studies in SCP are lacking (56,80,81).

The impact of Botulinum Neurotoxin-A on muscle/tendon length, range of motion and spasticity

The secondary morphological outcomes (Table S1, supplementary material) showed an increase in muscle belly length (p = 0.009) and total muscle-tendon unit length (p = 0.011) of the MG after BoNT-A injections, while these length data did not change for the ST. However, the maximum ankle dorsiflexion angle defined by goniometry during the clinical examination did not change post-BoNT-A. A previous investigation showed a similar increase in MG muscle belly length combined with unchanged maximum ankle dorsiflexion already at two weeks post-BoNT-A injection (61). Additionally, the resting ankle angle during the 3DfUS shifted towards increased dorsiflexion (median increase of 10 (12.5–0) degrees dorsiflexion, p = 0.003) after integrated BoNT-A treatment. This could explain the increase in muscle belly and total muscle-tendon unit length. Additionally, a previous observational study by Malaiya et al. did not find an association between MG muscle belly length and maximum dorsiflexion angle in a cohort of 16 children with unilateral SCP (82).

For the instrumented spasticity assessments, the average EMG signal while stretching the muscle at high-velocity was significantly reduced post-BoNT-A compared to the baseline condition. This resulted in a (tendency) towards a reduced difference in EMG between high and low stretch velocity (which exceeded the SEM). These results confirmed previous findings that BoNT-A injections in the MG and ST successfully reduces the hyperactive stretch reflex (61–63).

The impact of Botulinum Neurotoxin-A on gait

In contrast to the studies by Hastings-Ison, the current study found significant improvements in the overall gait pathology defined by the GPS (37,38). The SnPM analyses of the full kinematic waveforms showed one large relevant ankle cluster, as well as one small cluster in the knee during swing. These improvements were confirmed by beneficial changes in gait features of the ankle (significant for ankle at initial contact and maximal dorsiflexion in stance and swing) and knee (tendencies for knee angle at initial contact and knee ROM). These findings are in line with a previous study (59). Hastings-Ison et al. did not find any alteration

in maximum ankle dorsiflexion or knee kinematics in the total group. However, they showed improved ankle dorsiflexion and increased knee flexion during mid stance in bilaterally involved children but not in unilaterally involved participants (37). The current data were not separately analyzed for children with uni- and bilateral involvement due to lack of power. We previously performed a systematic review on the BoNT-A effect on gait and compared the BoNT-A effect on gait features with the effect defined by statistical parametric mapping in a group of 51 children with SCP who received multilevel BoNT-A treatment (59). The latter study showed significant improvements at the ankle as well as at the knee joint, and these beneficial effects were also more pronounced at the ankle compared to the knee joint. Overall, we conclude that BoNT-A improved the gait of the children with SCP at 8–10 weeks post-BoNT-A.

Frequency of botulinum neurotoxin-A administration

As highlighted by the study of Multani and colleagues, the BoNT-A injection frequency is an important matter of debate (18,83). Studies by Kanovsky and Hastings-Ison showed that BoNT-A treatment once per year was effective with fewer adverse events compared to multiple injections per year (41,42). These findings were supported by animal studies showing more muscle weakness and atrophy, increased loss of contractile tissue and exacerbated fibrosis after more frequent injections (23,44,76). As the muscle might need more time to recover, even if the clinical effect of tone reduction is dissolved, the duration between injections should be based on assessments of the muscle, preferably at both the microscopic and macroscopic level (23,44,75).

Combined outcome assessments and clinical implication

This is the first study that combined the assessments of muscle morphology and gait, in order to critically review the benefits and potential harm of integrated BoNT-A treatment. The observed clinical benefits included reduced spasticity of the plantar flexors and knee flexors and improved ankle motion during gait but had rather limited effects on knee kinematics. On the other hand, the muscle volume was reduced 8–10 weeks post-BoNT-A treatment. These pros and cons should be weighted before the selection of a (repeated) BoNT-A treatment for young children with SCP. The recovery profile of the individual patient should be objectively monitored. The current results revealed muscle atrophy in the short-term period following BoNT-A injections and other studies highlighted that muscle recovery is still ongoing six

months post-BoNT-A (26,27,40). These findings suggest that muscle atrophy lasts longer than the clinical effects, such as tone reduction (23,75). An animal study by Minamoto et al. hypothesized that the effect and potential negative effects of BoNT-A are much stronger if injected into a muscle which has not completely recovered (44). The current evidence implies that BoNT-A injections should be provided with an interval of at least 12 months. As stated earlier, the first BoNT-A injections seem to be most distinctive in both the beneficial functional effects and the harmful effects on the muscle. Therefore, muscle recovery is especially crucial after the first injection and an interval of at least 12 months before the second injection is advised.

Future perspectives

The observed muscle atrophy, after approximately two months post-BoNT-A in the current study and six months post-BoNT-A in a previous study (26), represents the rather short-term effect of BoNT-A on muscle morphology. Hence, no conclusion can be drawn on the long-term effects. More research is needed to define the time course of muscle recovery. Future studies should investigate the long-term effects and include the microscopic and molecular analyses of intrinsic muscle properties and functional evaluations in order to critically appraise the benefit–harm balance of BoNT-A treatment. Thereby, more research on dosage dependent effects, the frequency of BoNT-A injections and treatment history is relevant. In order to gain more insights into the patient- and or even muscle-specific response to BoNT-A and in order to properly identify responders and non-responders, the objective measures of muscle spasticity as well as morphology and function, such as gait (37,39,48,62), should be combined in future studies and implemented in clinical practice. These findings may improve efficient treatment planning.

Most studies, including the current study, focused on the effects of BoNT-A in ambulatory children. Therefore, future studies should also include participants with less functional capacity in order to evaluate the benefits and potential harms in non-ambulatory children.

Limitations

Some limitations have to be acknowledged. This study included a non-randomized control group. As BoNT-A has been proven to be effective in reducing spasticity and time is running out for growing children with spasticity, as this may lead to impaired musculoskeletal

development, it was considered unethical to temporarily withdraw treatment from patients. Therefore, randomization procedures were not possible. We minimalized bias by group-matching age and GMFCS level. The control group presented with slightly lower spasticity levels compared to the intervention group at baseline, which was not surprising since these children had no indication for BoNT-A treatment. No control data were available for gait and spasticity assessments. However, gait and spasticity parameters were evaluated using previously reported reliability values (64,69,70). In the current study, BoNT-A was applied as part of an integrated approach, whereby BoNT-A injections were combined with casting, orthotic wear and intensive physical therapy. Hence, no conclusions can be drawn on the contribution of the isolated treatments.

Due to the deep origin of the ST, only the distal compartment could be visualized by 3DfUS, even though both compartments were targeted by BoNT-A (47). Muscles were only assessed in resting length, therefore no conclusion on alterations in extensibility can be drawn.

The assessments were performed by three different assessors. However, because all assessors were well-trained (i.e., minimum of two years of experience), regular comparisons of performance to ensure standardization were made and because the pre- and post-assessments were always performed by the same assessor, we do not believe that this could have influenced our results.

This study was ongoing during the onset of the COVID-19 pandemic. As the majority of participants was included after the first COVID-19 outbreak and the application of an additional inclusion criterion stating that the physical therapy had to be stable for at least three months, we believe that the pandemic did not influence the outcomes of the current investigation.

Conclusion

This study including a comprehensive set of objective measures showed that BoNT-A injections, when administered as part of an integrated approach (i.e., BoNT-A injections combined with stretching casts if relevant, intensive physical therapy and orthotic management), results in effective tone reduction, with positive effects on ankle kinematics but limited effects on knee kinematics during gait, combined with muscle atrophy, 8 to 10 weeks post-BoNT-A. There is a need for evidence-based guidelines rather than guidelines based on clinical experience in order to further evaluate the impact of BoNT-A. Close follow-ups with an integrated set of objective measures are required to gain more insight into the patient-specific response and recovery profile. Furthermore, patient-specific goals and expectations should be carefully evaluated and contemplated before BoNT-A treatment is planned in young children with SCP.

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Supplementary Material

Table S1. Muscle morphology parameters of both the intervention and control group.

Part I – Primary outcomes											
	Intervention group					**Control group**					
parameter – treated muscles	**pre**	**post**	**Median difference post-pre**	**Median within group, percent change**	**p-value, within group**	**pre**	**post**	**Median difference post-pre**	**Median within group, percent change**	**p-value, within group**	**p-value between groups**
MG – muscle volume (ml/kg*m)	1.40 (1.03 – 1.60)	1.31 (0.99 – 1.54)	-0.08 (-0.16 – 0.04)	-5.15	0.018	1.23 (1.04 – 1.65)	1.29 (1.02 – 1.55)	0.00 (-0.09 – 0.06)	-0.25	0.841	0.118
MG – echogenicity intensity (AU)	166.75 (158.78 – 180.19)	174.17 (159.08 – 186.04)	2.74 (-5.63 – 11.11)	1.46	0.407	165.28 (152.60 – 178.02)	165.66 (157.43 – 179.37)	-0.08 (-3.77 – 9.33)	-0.50	0.455	0.814
MG Growth rate – absolute (ml/month)	0.00 (-1.34 – 0.97)					0.17 (-0.23 – 0.87)					0.346
ST – muscle volume (ml/kg*m)	0.68 (0.57 – 0.73)	0.57 (0.47 – 0.69)	-0.09 (-0.16 – 0.01)	-16.18	0.030	0.68 (0.63 – 0.78)	0.70 (0.58 – 0.75)	-0.01 (-0.07 – 0.04)	-1.21	0.445	0.069
ST – echogenicity intensity (AU)	151.45 (142.13 – 168.47)	165.42 (149.90 – 172.55)	6.58 (-8.27 – 16.61)	4.92	0.145	149.90 (145.00 – 161.30)	158.11 (149.20 – 166.87)	5.20 (-6.90 – 11.90)	3.54	0.227	0.728
ST Growth rate – absolute (ml/month)	-0.49 (-1.45 – 0.58)					0.02 (-0.39 – 0.48)					0.226

Part II – Secondary outcomes											
	Intervention group					Control group					
parameter – treated muscles	**pre**	**post**	**Median difference post-pre**	**Median within group, percent change**	**p-value, within group**	**pre**	**post**	**Median difference post-pre**	**Median within group, percent change**	**p-value, within group**	**p-value between groups**
MG – MTU (%)	45.54 (44.06 – 45.91)	46.37 (44.61 – 46.96)	0.88 (-0.22 – 1.59)	2.05	0.011	46.23 (45.21 – 47.39)	46.35 (45.40 – 46.91)	-0.35 (-0.86 – 0.52)	-0.74	0.286	0.008
MG – muscle belly length (%)	22.86 (21.49 – 25.17)	23.23 (21.69 – 25.66)	0.44 (0.03 – 1.25)	2.14	0.009	24.04 (22.44 – 26.12)	24.16 (22.47 – 25.83)	-0.13 (-0.92 – 0.71)	-0.50	0.811	0.033
MG – tendon length (%)	21.57 (19.60 – 23.00)	21.79 (19.62 – 24.25)	-0.03 (-0.88 – 1.17)	-0.15	0.568	22.04 (20.93 – 23.59)	21.91 (20.72 – 23.46)	-0.26 (-0.55 – 0.24)	-1.10	0.157	0.2374
MG Growth rate – normalized (ml/kg/month)	-0.02 (-0.07 – 0.03)					0.01 (-0.04 – 0.02)					0.248
ST – muscle belly length, total (%)	34.26 (31.07 – 35.97)	34.63 (31.07 – 35.97)	0.15 (-0.87 – 0.97)	0.43	0.809	33.98 (31.66 – 35.55)	34.33 (31.64 – 36.04)	-0.10 (-1.06 – 1.82)	-0.28	0.865	0.684
ST – muscle belly length, proximal (%)	21.71 (20.16 – 24.04)	22.58 (20.77 – 23.65)	0.76 (-2.19 – 2.46)	3.13	0.778	22.03 (19.71 – 23.59)	21.26 (19.81 – 24.35)	0.14 (-0.86 – 1.27)	0.58	0.820	0.639
ST – muscle belly length, distal (%)	22.74 (21.61 – 24.79)	22.78 (21.34 – 24.54)	-0.32 (-0.90 – 1.47)	4.92	0.681	23.17 (21.78 – 26.21)	23.84 (21.48 – 26.28)	-0.22 (-0.74 – 0.83)	-0.91	0.687	0.675
ST Growth rate – normalized (ml/kg/month)	-0.02 (-0.08 – 0.01)					-0.00 (-0.03 – 0.02)					0.138

Kg = kilograms, m = meter, MG = medial gastrocnemius, ml = milliliters, MTU = muscle tendon unit length, ST = semitendinosus

Table S2. Results of the gait kinematics, gait profile and gait variable score and the spatial-temporal parameters, intervention group.

Part I – Primary outcomes					
Gait	**Intervention group**				
Kinematic parameters - Ankle	**pre**	**post**	**Median difference post-pre**	**p-value, treatment induced/within group**	**SEM** (69)
ankle ROM in sagittal plane (total GC) (degrees)	25.85 (19.83 – 34.55)	24.18 (21.51 – 29.13)	-1.74 (-9.04 – 3.07)	0.143	3.7
ankle angle in sagittal plane at IC (degrees)	-3.83 (-11.13 – 2.14)	-2.25 (-5.25 – 4.94)	3.56 (0.47 – 6.36)	0.002	4.4
max ankle angle (max DF) in sagittal plane during stance phase (degrees)	9.47 (0.70 – 14.52)	12.89 (7.37 – 17.76)	3.99 (-0.32 – 9.99)	0.004	4.7
max ankle angle (max DF) in sagittal plane during swing phase (degrees)	-1.71 (-9.63 – 3.12)	2.53 (-3.66 – 7.03)	5.34 (1.16 – 9.70)	<0.001	4.7
Kinematic parameters - Knee	**pre**	**post**	**Median difference post-pre**	**p-value, treatment induced/within group**	**SEM** (69)
knee ROM in sagittal plane (degrees)	53.50 (45.13 – 62.03)	54.84 (46.85 – 60.12)	1.94 (-3.79 – 8.84)	0.158	5.2
knee angle in sagittal plane at IC (degrees)	26.45 (20.93 – 35.43)	25.53 (17.26 – 30.53)	-1.69 (-9.19 – 1.84)	0.048	3.5
min knee angle in sagittal plane during stance (degrees)	5.06 (0.79 – 14.05)	6.74 (-1.22 – 10.09)	-2.44 (-7.61 – 2.23)	0.065	3.9
Gait profile score	**pre**	**post**	**Median difference post-pre**	**p-value, treatment induced/within group**	**MCID** (70)
Gait profile score for angles (degrees)	9.80 (7.41 – 11.33)	8.30 (6.71 – 9.41)	-1.76 (-2.51 - -0.15)	0.005	1.6
Spatial-temporal parameters	**pre**	**post**	**Median difference post-pre**	**p-value, treatment induced/within group**	**SEM** (69)
Cadence (number of steps/second)	2.25 (1.95 – 2.43)	2.19 (1.91 – 2.35)	-0.05 (-0.28 – 0.10)	0.156	11.7

Walking velocity (meter/second)	0.89 (0.67 – 1.10)	0.85 (0.73 – 1.05)	0.01 (-0.10 – 0.10)	0.911	0.1
Stride length (meters)	0.81 (0.66 – 0.96)	0.79 (0.69 – 1.04)	0.02 (-0.03 – 0.08)	0.108	0.1
Part II – Secondary outcomes					
Gait	**Intervention group**				
Kinematic parameters	**pre**	**post**	**Median difference post-pre**	**p-value, treatment induced/within group**	**SEM** (69)
ankle ROM in sagittal plane during stance phase (degrees)	16.85 (12.89 – 21.38)	15.92 (14.03 – 20.10)	0.31 (-4.38 – 3.74)	0.737	3.7
knee ROM in sagittal plane during stance (degrees)	21.69 (17.75 – 29.74)	26.86 (22.55 – 31.20)	3.68 (-0.43 – 6.91)	0.028	5.2

DF = dorsiflexion, GC = gait cycle, IC = initial contact, max = maximum, MCID = minimally clinically important difference, ROM = range of motion, SEM = standard error of measurement

Table S3. Results of the clinical examination in the intervention group.

Clinical examination – treated muscles	**Intervention group**			
parameter	**pre**	**post**	**Median difference post-pre**	**p-value, treatment induced/within group**
ROM plantar flexors, knee extended (degrees)	5.0 (0.0 – 10.0)	5.0 (0.0 – 10.0)	0.0 (0.0 – 5.0)	0.302
MAS plantar flexors, knee extended	2.0 (2.0 – 3.0)	2.0 (1.5 – 2.0)	0.0 (-1.0 – 0.0)	n.a.
MTS plantar flexors, knee extended	-15.0 (-21.3 - -13.8)	-10.0 (-15.0 - -5.0)	5.0 (0.0 – 15.0)	0.027
MAS plantar flexors, knee 90 degrees	2.0 (1.5 – 2.0)	1.5 (1.5 – 1.5)	0.0 (0.0 – 0.5)	n.a.
MTS plantar flexors, knee 90 degrees	-10.0 (-12.5 - - 5.0)	-5.0 (-10.0 – 0.0)	0.0 (0.0 – 8.8)	0.085
Popliteal angle, unilateral (degrees)	-60.0 (-65.0 - -45.0)	-55.0 (-65.0 - -48.8)	0.0 (-5.0 – 1.3)	0.564
MAS knee flexors	1.5 (1.0 – 1.75)	1.5 (1.0 – 1.5)	0.0 (-0.5 – 0.0)	n.a.
MTS knee flexors	-82.50 (-90 - -70)	-77.50 (-80.00 - -70.00)	5.0 (0.0 – 10.0)	0.048

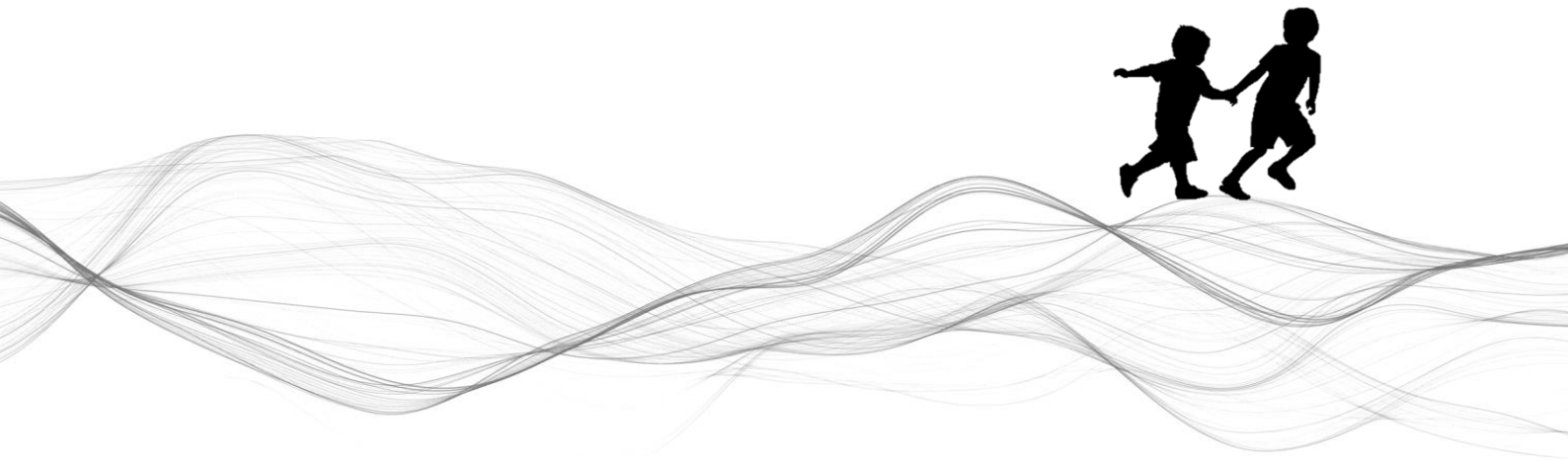
MAS = modified Ashworth score, MTS = modified Tardieu score, n.a. = not applicable, ROM = range of motion

Table S4. Results of the instrumented spasticity assessment of a subgroup (n=14) in the intervention group.

Part I – Primary outcomes					
Instrumented spasticity assessment – treated muscles	**Intervention group (n=14)**				
parameter	**pre**	**post**	**Median difference post-pre**	**p-value, treatment induced/ within group**	**SEM** intra rater
Medial gastrocnemius, EMG – absolute high rms-EMG (µV)	11.52 (4.87 – 22.07)	4.91 (3.67 – 9.45)	-4.73 (-15.74 – 1.31)	0.019	unknown
Medial hamstrings, EMG – absolute high rms-EMG (µV)	12.07 (5.77 – 16.04)	7.98 (3.68 – 10.58)	-2.82 (7.64 – 0.21)	0.041	unknown
Part II – Secondary outcomes					
Instrumented spasticity assessment – treated muscles	**Intervention group (n=14)**				
parameter	**pre**	**post**	**Median difference post-pre**	**p-value, treatment induced/ within group**	**SEM**(63,64) intra rater
Medial gastrocnemius, EMG – change rms-EMG (µV)	5.10 (2.17 – 12.67)	3.52 (3.07 – 6.59)	-2.64 (-8.06 – 2.48)	0.101	2.0
Medial hamstrings, EMG – change rms-EMG (µV)	6.94 (4.47 – 13.19)	4.44 (1.44 – 9.39)	-3.13 (-5.78 – 0.87)	0.060	1.0

EMG = electromyography, rms = root mean square, SEM = standard error of measurement, µV = microvolts

Part III
General Discussion



General Discussion

3.1. Summary of the results

The general aim of this doctoral thesis was to improve the understanding of the macroscopic alterations in the muscle-tendon unit and the heterogeneous nature of spastic cerebral palsy (SCP) and to investigate the impact of intramuscular BoNT-A (BoNT-A) injections on muscle morphology, spasticity and function. Three main objectives were defined to achieve the overall aim.

Objective 1. Create a well-described objective evaluation protocol to assess muscle morphology of four lower limb muscles in children with spastic cerebral palsy

To address this goal, we evaluated the influence of changes in adjacent joint angles on muscle volume, echogenicity intensity and muscle length of the medial gastrocnemius, semitendinosus and rectus femoris in typically developing children and children with SCP (**Chapter 2.1**). We showed that adjusting the joint angles does influence muscle morphology outcomes and thereby highlighted the importance of the well-defined and well-described standardization of limb position when performing follow-up measures or comparing results between different studies.

Objective 2. Generate a clinical and normative database of muscle morphology parameters and explore the association between these muscle morphology parameters and measures of spasticity

The normative database of muscle morphology data of typically developing children with a wide age range was used to explore muscle development curves of four lower limb muscles (**chapter 2.2**). The enrolled children with SCP of the clinical database often received combined assessments of muscle morphology with instrumented spasticity assessments. This allowed to explore the association between spasticity and muscle morphology alterations in two lower limb muscles of children with SCP and to investigate whether muscle morphology is different between different spasticity patterns, i.e. muscle activation patterns during passive stretch (**chapter 2.3).**

Objective 3. Investigate the acute and short-term impact of Botulinum Neurotoxin-A on muscle morphology in children with spastic cerebral palsy

We aimed to gain more insight into the beneficial and harmful effects of BoNT-A injections to lower limb muscles and of lower leg casting. Therefore, we first investigated the isolated effects of medial gastrocnemius BoNT-A injections and lower leg casting on the joint, muscle, and tendon level (**chapter 2.4**). Secondly, we investigated the short-term effects of BoNT-A on muscle morphology of the medial gastrocnemius and semitendinosus, on spasticity, and gait pathology (**chapter 2.5)**.

The **general discussion** of this doctoral thesis starts with a summary of the results, followed by a reflection on the methodological considerations, a reflection on the complex interaction between spasticity and muscle morphology, a critical review on BoNT-A treatment, followed by the clinical relevance and implementation of the study findings. Finally, the overall conclusion will summarize how this doctoral thesis has contributed to improved understanding of the heterogeneous nature of SCP and its muscular alterations.

3.1.1. A well-described objective evaluation protocol for muscle imaging assessments (chapter 2.1)

Three-dimensional freehand ultrasound is a commonly used technique to assess muscle morphology in children (1–7).

However, previous studies that applied the three-dimensional freehand ultrasound technique did not always report and/or properly describe the position of the assessed limb (8). Clear recommendations on overall measurement position and specific joint angles per muscle morphology outcome were lacking (8). In order to reliably compare, replicate or merge data, clear documentation and standardization of the limb position during muscle imaging assessments are necessary.

Therefore, the first study (**chapter 2.1)**, systematically investigated the impact of altering lower limb joint angles on the estimations of muscle volume, muscle belly length and echogenicity intensity, in both typically developing children as children with SCP.

We established a modified standardized setup, such that the lower limb muscles could be assessed at different lengths. For the medial gastrocnemius, this could be achieved in prone

position with the foot fixated in an orthosis, whereas for the rectus femoris and semitendinosus, assessments were performed in side position. In this side position, the limb was supported by a custom-made low-friction movable table, in order to easily adjust the position of the joint and corresponding joint angles. The muscle volume, muscle belly length and echogenicity intensity were estimated in a resting condition (the same joint angles as if muscle morphology would be assessed in prone or supine position), at a common pre-defined joint angle, and at the maximum dorsiflexion angle.

A total of 13 typically developing children and 13 children with SCP participated in this study. Within the typically developing cohort, the muscle volume of the medial gastrocnemius (p = 0.004) and rectus femoris (p = 0.007) were significantly different between the three conditions. Post-hoc tests confirmed differences between measurements positions, which all exceeded the previously defined standard error of measurement values (9).
Furthermore, the muscle belly length was influenced by altering the joint angles, with exception of the distal compartment of the semitendinosus in the typically developing cohort.

The differences in muscle volume were unexpected. As it is unlikely that the muscle volume would change during the assessments, it was expected that those differences could be explained by differences in performance during the data acquisition or data processing (10). Muscle images were easier to process when stretch was applied to the muscle belly. Additionally, the risk of muscle deformation caused by pressure of the probe was lower during the acquisition of a stretched compared to a relaxed muscle. These findings suggested that muscle volume estimations based on ultrasonography images of a relaxed muscle may be less repeatable compared to these measures on a stretched muscle. However, this hypothesis has yet to be tested in future studies. Additionally, it was shown that the overlap of separate ultrasound sweeps within a multiple sweep was not always optimal when the muscle morphology was assessed in side position. This was potentially the results of less visual control on probe position, probe tilting and applied pressure.
Additionally, the participants confirmed that the ultrasonography assessments in side position were well-tolerated. The patient friendliness questionnaire showed that the measurements were pleasant and painless.

Overall, this study showed that limb position and joint angles could be easily adjusted if the participants were positioned on their side. We found that alterations in joint angles could influence lower limb muscle morphology outcomes. The proposed specific setups are recommended to assess muscles at different lengths. This is specifically relevant for intervention studies that target muscle and tendon lengths (such as for stretching studies). However, assessments of muscle volume and echogenicity were more challenging in side-lying position. Therefore, when the main focus of a study is on the muscle volume and composition at rest, assessments in side lying are not recommended.

3.1.2. The clinical need of a normative muscle imaging database (chapter 2.2)

So far, comprehensive normative databases, including muscle morphology data of different lower limb muscles and a large age range were lacking. Therefore, it was challenging to estimate the level of pathology and monitor muscle development over time. Moreover, muscle morphology was often normalized to body size, in order to compare individuals with different body sizes. However, clear recommendations for data normalization were lacking and the efficiency of different normalization techniques was not yet systematically investigated (8). Therefore, the second study of this doctoral thesis (**chapter 2.2)**, provided a large normative database to gain insight into muscle development of typically developing children.

In this extensive cohort study, cross-sectional muscle morphology data of the medial gastrocnemius, tibialis anterior, the distal compartment of the semitendinosus and rectus femoris were collected by three-dimensional freehand ultrasound in 118 typically developing children, aged between 3 and 18 years, with a mean age of 10.4 years (± 4.5 years). The development of muscle morphology was studied over the full age range, as well as separately for the pre-pubertal (3-10 years) and pubertal (11-18 years) cohorts.

The data showed that changes in muscle volume and belly length with increasing age could be described by a simple linear regression equation till the age of 11 years, with exception of the rectus femoris volume. The development of the latter muscle was more heterogeneous from the age of 9 years onwards. We confirmed that muscle development is muscle-specific and more heterogeneous during the pubertal ages. Allometric scaling (11,12) was shown to be the

most effective method to normalize muscle volumes, whereas normalization of muscle belly lengths was muscle-specific and could be accomplished by both ratio and allometric scaling. Yet, the validity of these data scaling techniques needs to be further explored and the impact of different scaling techniques should be investigated in pathological cohorts. This comprehensive normative dataset may serve as reference database for future studies to examine muscle development in children with hampered muscle development.

3.1.3. The complex interaction of spasticity and muscle alterations in SCP (chapter 2.3)

In our clinical database of muscle morphology data of children with SCP, combined with instrumented assessments of spasticity, we further unraveled the complex interaction between spasticity and alterations in muscle morphology.

In **chapter 2.3,** we explored the associations between spasticity and muscle morphology and we compared muscle morphology between commonly observed spasticity patterns, i.e. muscle activation patterns observed during passive stretches.

A sample of 74 children with SCP including both children with unilateral and bilateral involvement, covering gross motor function classification levels I to III (GMFCS I/II/III: 31/25/18). Three-dimensional freehand ultrasound was used to assess muscle morphology and echogenicity intensity of the medial gastrocnemius and distal compartment of the semitendinosus. Levels of spasticity were assessed using the EMG response during low- and high-velocity passive stretches, as part of the instrumented spasticity assessments. The EMG response, or muscle activation, during the passive stretches allowed distinction of different spasticity patterns.

We found a moderate negative association ($r = -0.624$, $p < 0.001$) between the level of spasticity and medial gastrocnemius volume. We observed other additional significant but mostly weak associations between spasticity and muscle morphology parameters, namely for the volume of the distal compartment of the semitendinosus ($r = -0.297$), the echogenicity intensity of the medial gastrocnemius ($r = 0.450$) and the muscle belly ($r = -0.369$) and tendon length ($r = 0.414$) of the medial gastrocnemius.

We further showed that muscles were smaller and shorter when they showed activation during a low-velocity passive stretch, in comparison to muscles that only activated during a high-velocity stretch.

These findings confirmed the complex interaction between spasticity and altered muscle morphology and suggested that other factors contribute to this interaction.

3.1.4. The impact of Botulinum Neurotoxin-A treatment on muscle morphology (chapter 2.4 and 2.5)

BoNT-A injections are often combined with casting, as part of an integrated approach (13). Hence, the isolated effects of the different treatment modalities are therefore difficult to quantify. Additionally, there are recent concerns on the use of BoNT-A in young children with SCP, as it may induce muscle atrophy and negatively influence muscle quality. Moreover, some research groups recently questioned the beneficial effects on function (14). Furthermore, prospective studies including untreated control groups are scarce. In the fourth study (**chapter 2.4)**, we investigated the isolated effects of medial gastrocnemius BoNT-A treatment and lower leg castings during the acute phase, i.e., a period of two weeks, following the BoNT-A injection session. In the fifth study (**chapter 2.5)**, we evaluated the short-term effect of BoNT-A treatment (i.e over 8-10 weeks), whereby BoNT-A was administered following an integrated approach.

In **chapter 2.4**, medial gastrocnemius BoNT-A injections and lower leg casting were applied separately to investigate the decomposed treatment effects on the muscle or tendon structure, the hyperactive stretch reflex, and joint hyper-resistance. A total of 31 children with SCP and a mean age of age 7.4 (± 2.6) years, covering gross motor function classification levels I till III, were included in this investigation. The children received either two weeks of lower-leg casts or BoNT-A injections to the medial gastrocnemius. Muscle and tendon lengths were estimated by three-dimensional freehand ultrasound and instrumented spasticity assessments were used to quantify the hyperactive stretch reflex and joint resistance during low- and high-velocity passive stretches at baseline (pre-intervention) and after two weeks (post -intervention).

In **chapter 2.5**, BoNT-A injections were applied as part of an integrated approach (i.e., BoNT-A injections combined with casting if required, intensive physical therapy, and orthotic

management). Twenty-five children with SCP (median age 6.4 years, GMFCS level I/II/III (14/9/2)), with an indication for BoNT-A treatment of the medial gastrocnemius and/or semitendinosus, were included in this study. Additionally, an untreated age- and severity-matched control group was included and contained 20 children with SCP (median age 7.6 years, GMFCS level I/II/III (14/5/1)) who maintained usual care (i.e. regular physical therapy and state-of-the art orthotic management). Muscle morphology was assessed in both cohorts using three-dimensional freehand ultrasound. Gait pathology was evaluated by three-dimensional gait analysis in the intervention group. Additionally, a subset of treated children underwent an instrumented spasticity assessment in order to confirm the previously reported reduction in hyperactive stretch reflex following BoNT-A injections (15,16).

In **chapter 2.4**, we showed that two weeks of lower leg casting resulted in increased tendon length (5.7%, $p = 0.039$), resting and maximum dorsiflexion joint angles shifted toward greater dorsiflexion (32.4%, $p = 0.004$), and joint resistance remained unchanged, while the hyperactive stretch reflex of the lateral gastrocnemius (−29.5%, $p = 0.047$) and soleus (−17.5%, $p = 0.034$) were significantly reduced. Two weeks after BoNT-A injections, the resting muscle belly length of the medial gastrocnemius lengthened and the hyperactive stretch reflex was significantly reduced (−70.7%, $p = 0.003$) together with a reduction in ankle joint resistance (−37.6%, $p = 0.013$). Only limited associations between the changes in muscle or tendon lengths and the changes in the stretch reflexes were found after both treatments.

In **chapter 2.5**, we described that BoNT-A injections to the medial gastrocnemius and semitendinosus muscles resulted in significantly reduced muscle volumes ($p = 0.018$, −5.2% and $p = 0.030$, −16.2%, respectively) and unchanged echogenicity intensity. No changes in muscle morphology or echogenicity intensity were observed in the untreated control group. We confirmed that the hyperactive stretch reflex was significantly reduced in both muscles, 8-10 weeks after BoNT-A. Furthermore, ankle kinematics during gait significantly improved, together with limited effects in knee kinematics.

These two studies confirmed that BoNT-A successfully reduced the hyperactive stretch reflex and improved gait, but caused unwanted reductions in muscle volume. While this muscle atrophy following BoNT-A injections was expected due to the temporal neuro-denervation, these findings highlighted the essence of sufficient muscle recovery post-BoNT-A injections.

The muscle should thus be closely monitored after BoNT-A treatment and strategies to accelerate muscle recovery might be required. While medial gastrocnemius muscle lengths at a resting ankle angle were significantly increased during the acute and short-term period after BoNT-A injections, no differences in muscle length were observed at maximum dorsiflexion positon. Two weeks of casting was also not sufficient to lengthen the medial gastrocnemius muscle belly. These findings were surprising and suggest that limited muscle remodeling occurred during the acute phase following BoNT-A treatment and/or casting, highlighting the need for interventions that can target the muscle belly more effectively. We further showed that BoNT-A treatment reduced the pathological ankle kinematics over 8-10 weeks.

An overview of the different studies of this doctoral thesis, with a summary of the most important findings and scientific and clinical contributions is enclosed in Table 1.

Table 1. Overview of studies, with interpretation of hypotheses, most important findings and scientific and clinical contributions				
Studies	**Hypothesis**	**Hypothesis confirmation**	**Most important findings**	**Scientific and clinical contribution**
Study 1: Impact of limb positioning on lower limb muscle morphology outcomes in typically developing children and children with spastic cerebral palsy Chapter 2.1. ⇨ PhD objective 1	Muscle volume and echogenicity intensity are not influenced by altered proximal or distal joint angles.	Partly confirmed	• Alterations in joint angles influenced muscle volume of the medial gastrocnemius and rectus of typically developing children and the medial gastrocnemius of children with SCP. • There was a tendency towards changes in echogenicity intensity of the medial gastrocnemius in typically developing children and of the rectus femoris in children with SCP after alterations in joint angles. • Muscle volume and echogenicity intensity were more challenging to asses in larger children when they were positioned on their side and could be more reliable defined in prone or supine position.	Results highlighted the relevance of standardized protocols and well-described guidelines to evaluate muscle morphological in research projects and clinical follow-up of children with SCP. Assessments of muscle morphology while participants were positioned on their side were well tolerated by typically developing children and children with SCP. Joint angels and limb position were easy to adjust when using a foot orthoses to change the length of the medial gastrocnemius and when the participants were positioned on their side to change the length of the rectus femoris and semitendinosus.
	Muscle and tendon lengths are influenced by altered proximal or distal joint angles.	Partly confirmed	• The muscle belly length was influenced by altering the joint angles, with exception of the distal compartment of the semitendinosus in the typically developing cohort.	
Study 2: A comprehensive normative reference database of muscle morphology in typically developing children aged 3-18 years – a cross sectional ultrasound study Chapter 2.2. ⇨ PhD objective 2	Muscle development can be described by a simple linear equation until the age of 11 years, i.e., the average start of puberty.	Partly confirmed	• A unique normative database of muscle morphology data of four lower limb muscles in typically developing children between the age of 3 and 18 years was developed. • Alterations in muscle volume and muscle belly length over age could be described by a simple linear regression equation till the age of 11 years, except for the rectus femoris. • Muscle development over age was muscle-specific. • Muscle development was similar for boys and girls until the age of 11 years.	A unique normative database of muscle morphology outcomes of four lower limb muscles in typically developing children was established. These findings provided more insight into physiological muscle development and the utilization of different data scaling methods. These data will be shared online and can serve as a normative reference database for future studies investigating (pathological) muscle development in pediatric populations.
	Muscle morphology data can be effectively normalized for differences in body size.	Confirmed	• Normalizing muscle volume by allometric scaling was most efficient to exclude the impact of changes in body size due to growth and is thus recommended to be used for comparison between different pediatric populations, while muscle lengths can be normalized by both allometric and ratio scaling.	

<u>Study 3</u>: Associations between muscle morphology and spasticity in children with spastic cerebral palsy **Chapter 2.3.** ⇨ **PhD objective 2**	More severely affected muscle morphology parameters, i.e. smaller muscle volume and reduced muscle belly length, are associated to higher levels of spasticity.	Partly confirmed	• Medial gastrocnemius muscle volume was moderate, but negatively association with the level of spasticity. • Weak negative associations were found between the level of spasticity and muscle volume of the distal compartment of the semitendinosus. • The level of spasticity was weakly associated with tendon length and echogenicity intensity of the medial gastrocnemius.	These data provided more insight into the complicated interaction between muscle morphology alterations and the presence of spasticity in children with SCP.
	More severely affected muscle morphology is found in muscles with mixed low-/high-velocity-dependent muscle activation patterns, compared to muscles with pure high-velocity muscle activation patterns.	Confirmed	• Medial gastrocnemius muscles with mixed low-/high-velocity-dependent patterns had significant smaller normalized muscle volumes compared to muscles with pure velocity-dependent muscle activation patterns. • Semitendinosus muscles with mixed low-/high-velocity-dependent patterns had significant reduced muscle volumes and reduced muscle belly lengths compared to muscles with pure velocity-dependent muscle activation patterns.	
<u>Study 4</u>: Joint and muscle assessments of the separate effects of Botulinum Neurotoxin-A and lower-leg casting in children with cerebral palsy **Chapter 2.4.** ⇨ **PhD objective 3**	BoNT-A injections reduces the hyperactive stretch reflex	Confirmed	• Two weeks after the BoNT-A treatment, the hyperactive stretch reflex of the medial gastrocnemius was reduced. • Joint resistance, measured during high-velocity ankle rotations was significantly reduced two weeks after BoNT-A injections. • The muscle length estimated at the resting ankle angle was increased two weeks after BoNT-A injections.	This study provided more insight into the isolated effects of serial casting and BoNT-A at the level of the muscle, tendon and joint. These findings might eventually contribute to the development of individualized treatment protocols in children with SCP.
	Casting results in lengthening of the muscle belly and tendon and thereby reduces the resistance around the joint	Partly confirmed	• Joint angles shifted toward greater dorsiflexion and the medial gastrocnemius tendon length was significantly increased after two weeks of lower leg casting. • The joint resistance, measured during low-velocity ankle rotations, was not significantly reduced two weeks after casting. • There were association between the changes in muscle or tendon lengths and the changes in the stretch reflexes.	

Study 5: The short-term impact of integrated BoNT-A treatment on spasticity, muscle morphology and gait in children with spastic cerebral palsy Chapter 2.5. ⇨ PhD objective 3	Integrated BoNT-A treatment of the medial gastrocnemius and medial hamstrings reduce the hyperactive stretch reflex	Confirmed	• The hyperactive stretch reflex was significantly reduced after BoNT-A injections.	This study provided objective data of the effects of BoNT-A treatment on muscle morphology and gait, which may support clinical decision-making. Due to the heterogeneous response to treatment in SCP, the data provided by the objective measures may provide additional insight and ultimately contribute to the development of more individually tailored treatment plans.
	Integrated BoNT-A treatment causes reductions in muscle volume and negatively influences muscle quality.	Partly confirmed	• BoNT-A injections, administered according an integrated approach, resulted in a reduced muscle volume of the medial gastrocnemius and distal compartment of the semitendinosus after 8-10 weeks, while the echogenicity intensity remained unchanged.	
	Integrated BoNT-A treatment of the medial gastrocnemius and medial hamstrings improves that ankle and knee kinematics in the sagittal plane.	Confirmed	• Ankle gait kinematics significantly improved 8-10 weeks post BoNT-A treatment, combined with limited effects on knee kinematics.	

BoNT-A = Botulinum Neurotoxin-A, SCP = Spastic cerebral palsy

3.2. Methodological considerations

3.2.1. Participants

The overall aim of this doctoral thesis was to gain more insight into the heterogeneous macroscopic alterations of the muscle-tendon unit of different lower limb muscles in children with SCP. These insights may contribute to improved understanding of the heterogeneous nature of the disorder and the large variation in treatment response.

The overall heterogeneous clinical picture was one of the challenges in this doctoral project. We aimed to include study samples that were good representations of the population of children with SCP. To limit the heterogeneity, we included only children with pure SCP and GMFCS levels I, II and III. Additionally, we included only pre-pubertal children in **chapter 2.5**. However, despite the fact that we included reasonably large sample sizes in most studies (sample sizes ranged between 13 and 74 children with SCP), the sample sizes were often too small to perform sub-analyses, such as for subgroups defined by GMFCS-level, by bilateral- or unilateral involvement or by treatment history (for example for BoNT-A naive children or children with BoNT-A history).

Some other limitations related to the study samples have to be acknowledged. This doctoral thesis focused on ambulatory children with SCP. As muscle development and morphology are highly influenced by mechanical loading (17), the findings of this thesis may not apply to non-ambulatory children. All included children were diagnosed with pure SCP, since the presence of any dystonic features in the lower limb was an exclusion criterion. While SCP is the most common subtype, mixed types are also possible (18). Since dystonic features can be complex to detect in children with mixed, but predominantly spastic type of cerebral palsy, there is no full guarantee that the enrolled children did not have any dystonic features. The presence of length-dependency features in muscle activation patterns during passive stretch, as identified in the study sample of **chapter 2.3**, has been suggested to be potentially related to clinically undetected dystonic features (19).

The majority (51%) of included children included in the different PhD studies were classified as gross motor function classification level I. This reflects the distribution of gross motor

function classification levels in the total population of cerebral palsy (20). However, there was an under-representation (16%) of GMFCS III in all studies. Children with GMFCS III were often excluded due to previous surgery or comorbidities, such as cognitive problems that would have impeded the performance during assessments. GMFCS level III is a very heterogeneous population on itself and therefore, future studies should focus on further investigating and promoting muscle development and gross motor function in children with GMFCS III.
Additionally, it should be noted that the distinction between different GMFCS levels is not always easy to make. Therefore, future studies should consider using more detailed functional scales, like the gross motor function measure (GMFM) to define the participants' functional level.

The included cohorts of the different studies included both unilateral as bilateral involved children. The current project did not investigate to what extend this might have influenced our results. Barber et al. indicated that unilaterally involved children might be more severely affected, which could be explained by the fact that these children are able to compensate with their non-involved side, resulting in less biomechanical loading of the affected side (21). On the other hand, the children with the higher GMFCS levels, especially GMFCS level III, were mainly bilaterally involved children. As it has been previously shown that the degree of morphological alterations is related to the GFMCS level, this might explain the more affected muscle morphology in the SCP cohort with bilateral involvement (22). More research is required to comprehensively delineate and understand the differences in muscle morphology between children with unilateral and bilateral SCP.

The minimum age for participation in the presented studies was three years. This age was primarily chosen for practical reasons, as most assessments, like the instrumented spasticity assessment and three-dimensional gait analysis, require a certain level of cooperation. However, within our research group, lower limb muscle ultrasound protocols have been established for younger children (i.e., between 6 months and 3 years of age)(23). Additionally, previous studies suggested that the level of spasticity stabilizes from the age of five to six years (24). Since some of the children will receive their first treatment before the age of five years, these young ages were considered relevant to investigate.

3.2.2. Sample size

All sample sizes were based on power calculations. It should be noted that these power calculations were performed at an early stage of the PhD project. At that time, only limited data were available to be used for power calculations. Therefore, most sample size estimations had to be updated during the project years, partly with novel data provided by our own research group. While there were no clear indications that the different studies of this doctoral thesis were underpowered to test the study hypotheses, we recommend to replicate our findings in larger cohorts. Moreover, larger cohorts will enable sub-analysis that can improve our knowledge on the heterogeneous nature of SCP and the response to treatment.

The results of the fifth study, described in **chapter 2.5**, can be used to make first steps in the prediction of the BoNT-A treatment outcome. Indeed, data of baseline measures, including instrumented spasticity assessments and three-dimensional freehand ultrasound, may have a predictive value for the response to BoNT-A. For example, baseline muscle volume or echogenicity intensity, or baseline spasticity levels or spasticity patterns may partly predict the changes in spasticity and/or gait following BoNT-A treatment. The development of such prediction algorithms requires large sample sizes. Currently, our database does not yet include a sufficient number of children who received instrumented spasticity assessments, three-dimensional freehand ultrasound as well as gait analysis before and 8-10 weeks after BoNT-A injections. Therefore, the current dataset needs to be expended to perform prediction studies in the near future.

3.2.3. Methodological considerations of muscle imaging

Muscle imaging was the primary focus of this doctoral thesis. In the next section some methodological considerations related to data collection and data analysis of muscle imaging using three-dimensional freehand ultrasound are discussed.

<u>The selected muscles</u>

This doctoral thesis was limited to the investigation of the muscle morphology of four lower limb muscles. These muscles were selected due to their functional importance, the extent to which they are affected in children with SCP, the fact that they are often targeted by treatment

and their superficial location. However, other muscles might also be relevant to investigate. First, the largest plantar flexor muscle, the soleus, has an important role in gait (25,26). The soleus is often weak and reduced in size in children with SCP and has therefore been associated with excessive knee flexion and crouch gait (27–29). Hence, the soleus might be an important muscle to monitor. Second, the gluteus medius is known to be often weak and is characterized by low selective neuromotor control in children with SCP. Thirdly, spasticity and shortening of the hip adductor muscles may result in scissoring gait and/or may contribute to hip subluxation, especially in more severely impaired children. The hip adductors and abductors would thus also be clinically relevant to investigate in future studies. Fourthly, because the psoas muscle is often targeted by treatment modalities to increase the hip range of motion, the psoas morphology might also be of interest in future studies. Yet, since this muscle is not superficial and therefore not accessible using ultrasonography. Finally, the proximal compartment of the semitendinosus was found to be challenging to visualize using three-dimensional freehand ultrasound, due to its deep origin (30,31). Therefore, other imaging techniques, such as magnetic resonance imaging (MRI), might be required to investigate these deeper located muscles.

The challenges related to echogenicity intensity

As the intrinsic muscle quality may be reduced in children with SCP due to excessive connective tissue and potential increased fat infiltration, and because muscles with reduced intrinsic quality may respond differently to BoNT-A treatment (32–34), it is considered useful to estimate muscle quality in a clinical setting. In the studies of the doctoral thesis, intrinsic muscle quality was estimated based on the mean echogenicity intensity value, i.e. the average grey value of the manual segmented ultrasound images. This parameter has often been used as an indirect estimation of the intrinsic muscle quality or integrity (35–37). We believe in its potential to provide a first estimate of muscle quality, without invasive or time-consuming methods. However, this parameter has been criticized. It is not fully understood to what extend muscle composition influences echogenicity intensity. Some studies have suggested that the echogenicity intensity of the ultrasound image is influenced by fibrous tissue (38–40), whereas other studies indicated that it is largely influenced by intramuscular adipose tissue (41,42). Since both muscle composition alterations have been described in SCP, the echogenicity intensity values in this population are expected to represent both fibrous as well

as adipose tissue (36). For a comprehensive understanding of muscle composition using echogenicity intensity, there is need for validation studies in SCP.

Additionally, the method of assessment, including applied pressure and probe tilting, can influence the echogenicity intensity outcome, as mentioned in **chapter 2.1**. The location within the muscle where the echogenicity intensity values are defined (such as the echogenicity intensity of the cross-sectional area at the mid-muscle belly, or the echogenicity intensity of the entire three-dimensional muscle reconstruction) and the used method to define the echogenicity intensity outcome can cause large differences in findings (36,43,44). Therefore, it is important that the methods of both data acquisition and data processing are standardized when analyzing echogenicity intensity, especially in a longitudinal follow up and repeated measures design. Lastly, the settings of the ultrasound device can influence the gray value of the images and thereby the estimation of echogenicity intensity. It is therefore important to keep the device settings constant during the study and to report the device settings in all research reports.

Assessments of muscle extensibility

Muscle morphology is often assessed in muscles at a resting length. However, it is unknown how the findings in this condition translate to the functional level. Furthermore, it does not provide any insight into the extensibility of the muscle. In **chapter 2.4** and **chapter 2.5**, we showed that the muscle belly length at rest increased after injection with BoNT-A. However, the results of **chapter 2.4** showed that this increased resting length was not accompanied with an increased muscle belly length in the maximum dorsiflexion position. Besides that, the resting joint angles during the ultrasonography assessments changed towards increased dorsiflexion in **chapter 2.5** and a tendency towards increased dorsiflexion in **chapter 2.4** post-BoNT-A treatment. These results suggested that the increase in resting muscle belly length may be a direct result of the BoNT-A-induced tone reduction instead of actual muscle remodeling (i.e. potential addition in sarcomeres in series), as spastic muscles usually have a higher resting muscle tone (45). Indeed, such muscle remodeling is expected to result in an increased muscle belly length at maximum dorsiflexion. Therefore, future studies should consider assessing the muscle in different conditions, i.e. at different lengths, in order to gain more insight into muscle belly lengthening behavior. In **chapter 2.1**, we have established a measurement setup which allows assessments of muscle lengths at different limb and joint

positions (46). The results of this study showed that this method can be a valuable tool to assess the muscle belly at different lengths and levels of stretch. In **chapter 2.1,** we compared three different conditions based on joint angles, i.e., a resting joint angle, a common joint angle and a maximum joint angle. However, it might be insightful to add an additional assessment at a common torque in studies that are primarily investigating muscle extensibility, like stretching interventions. Previous studies have used for example four Newton meters as a common torque, in order to compare muscle characteristics under the same amount of stretch (46,47). This condition might be easier to standardize and more relevant for the translation to muscle function. Indeed, at a common angle, the muscles of some children might not even be stretched, whereas for other children, these muscles are nearly maximally stretched. For the same reason, it might also be useful to apply a minimal torque, for example 0.5 Newton meters, in the resting condition, to apply slight stretch to the muscle belly and reduce the tendon slack, and to further standardize this condition. Furthermore, it should be noted that a maximum joint angle is highly dependent on the participant's stretch tolerance and might not represent the actual maximum joint angle.

In order to gain more insight into muscle morphology and the relation with function, ultrasound assessments during activity, like walking, have been introduced and are considered insightful (48,49).

3.2.4. Methodological considerations of the instrumented spasticity assessment

The use of the instrumented spasticity assessment has contributed to our insight into the nature of the hyperactive stretch reflex in SCP. Nonetheless, there are some methodological considerations that should be discussed.

<u>EMG electrode placement</u>

If repeated measurements are performed using surface electromyography, for example during the instrumented spasticity assessment or three-dimensional gait analysis before and after treatment, electrode placement has to be identical between repeated assessments. These identical electrode locations, as well as proper preparation of the skin, are needed because it may influence the registration of muscle activity. Therefore, it is important to provide good training on electrode placement for the different assessors. Furthermore,

regular refresh courses and small reliability and repeatability studies are recommended to standardize the electrode placement. During the instrumented spasticity assessments within this doctoral thesis, electrodes were always placed by a trained and experienced assessor. In case of repeated assessments, the assessments were always performed by the same assessor.

EMG signal normalization to the maximum voluntary contraction

Because larger muscles can produce higher levels of muscle activation than smaller muscles, and because the EMG amplitude is influenced by many factors (as mentioned above), it may be relevant to normalize EMG. Therefore, muscle activation is often expressed as a percentage of the maximum voluntary contraction. However, performing a maximum voluntary contraction can be challenging for children with SCP, because of their reduced selective neuromotor control (50). The spasticity patterns, as described in **chapter 2.3,** were based on the electromyography data expressed as a percentage of the maximum voluntary contraction. A change of 3% of the maximum voluntary contraction was used as a threshold to define length-dependency. If children with SCP are less able to produce a maximum voluntary contraction, their maximal EMG amplitude might have been underestimated and the level of length-dependency might have been overestimated. Besides the potential underestimation of the maximum voluntary contraction, this maximum EMG response can be influenced by muscle tone reducing therapies like BoNT-A injections. Therefore, the use of absolute instead of normalized electromyography values are recommended in short-term intervention studies concerning tone reduction (such as in **chapter 2.4** and **chapter 2.5**).

Challenges of joint torque data

The studies of this doctoral dissertation primarily focused on the neural factors of spasticity. The torque data of the instrumented spasticity measures were not analyzed in **chapter 2.3** and **chapter 2.5**. During this PhD project, we faced some technical challenges to extract the torque data relative to the joint angles. The technical issues have recently been solved, so future studies will be able to assess both the combined response on EMG as well as torque data. Moreover, assessments of joint torque during passive stretches at different velocities allows to decompose the neural and non-neural contributions to joint hyper-resistance and may thus add more insight specifically into the non-neural components of joint hyper-resistance, like muscle stiffness (51).

3.3. Spasticity and the alterations at the muscle level

In **chapter 2.3**, we have tried to gain more insight into the complex association between spasticity and muscle morphology. Only a very limited number of studies had previously investigated these associations (52–56). The study described in **chapter 2.3** showed several significant but mainly weak associations between muscle morphology and spasticity levels. The negative correlation between the muscle volume of the medial gastrocnemius and the change in EMG response (change in EMG signal between a low- and high-velocity stretch) was rated as moderate, suggesting that a smaller muscle volume is associated with higher spasticity levels (i.e. more muscle excitation during passive stretch). These findings were in contrast with a study in SCP by Pierce et al. who showed that higher levels of spasticity and peak knee extensor passive torque were positively associated with larger quadriceps volumes (53). This research group hypothesized that the larger muscle volume was a compensation for the muscle atrophy resulting from muscle disuse. Apart from these conflicting associations between muscle morphology and spasticity levels, the data described in **chapter 2.3** showed a weak positive correlation between echogenicity intensity and spasticity parameters, suggesting that reduced intrinsic muscle quality is slightly associated to the hyperactive stretch reflex. We further showed that muscles with spasticity patterns including pathological muscle activation during both low- and high-velocity passive stretches had a smaller muscle volume and a shorter muscle belly length in comparison to muscles with pure velocity-dependent spasticity patterns. Besides the shorter length of the semitendinosus, the range of motion of the knee flexors was also reduced, which pointed towards contractures and less extensible muscles. However, surprisingly, the echogenicity intensity of muscles with mixed patterns was not different than the echogenicity intensity values of the muscles with pure velocity-dependent spasticity patterns. These heterogeneous findings highlight the complexity of the association between spasticity and muscle morphology or composition and the underlying mechanisms of these findings remain unclear.

Multiple mechanisms have been proposed in literature for the interaction between neural factors and alterations at the muscle level. Most hypothesis are based on multiple assumptions as many interactions remain unknown. Based on the current evidence and the

findings in our own research we created a hypothetical framework on the potential underlying mechanisms of spasticity and altered muscle morphology, which is presented in Figure 2.

Hyper-resistance measured at the level of the joint includes a neural and non-neural component, which are expected to interact (57). The alterations in the muscle's sensitivity to stretch can originate at three levels: at the biomechanical level, disturbances in the reflex loop and due to alterations in the muscle spindle itself. However, some proposed mechanisms seem to interact at different levels. A schematic representation of the reflex loop and muscle spindle is presented in Figure 1.

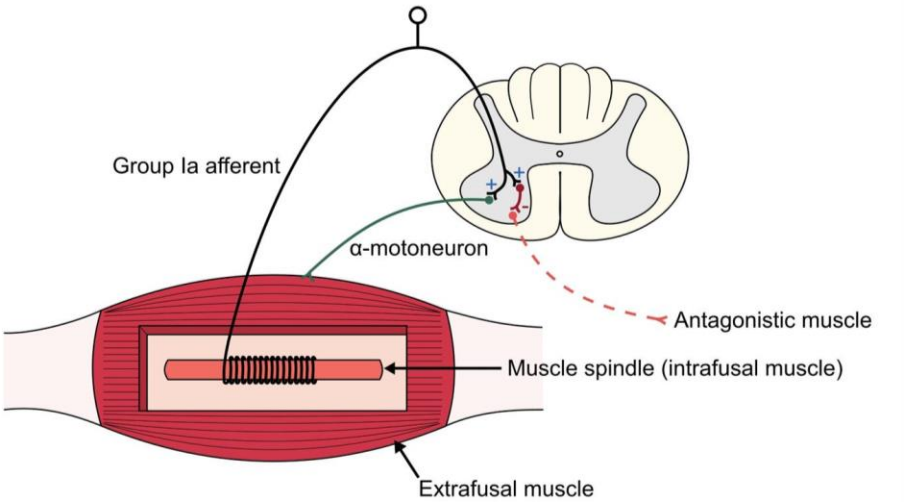


Figure 1. Schematic representation of a muscle spindle and the reflex loop (*image by Moises Dominguez, open access*)

At the first level, the biomechanical alterations can be due to disturbances in the muscle or tendon tissue. Due to enlarged tendon compliance and increased muscle stiffness, muscle spindles might detect less of the imposed movement. On the other hand, Gracies hypothesized that muscle activation in response to passive stretch may be enhanced in contractured muscles (58), suggesting that shorter and less extensible muscles would respond earlier. Gracies assumed that the stretching force would be more efficiently transmitted to the muscle spindle, resulting in lowered muscle spindle activation threshold, as indicated in an earlier animal study (59). Additionally, Rosant et al. showed muscle spindle sensitivity was increased in the soleus of rats after hind limb unloading, which was largely proportional to the increase in muscle-tendon stiffness (60). The influence of altered muscle properties, including the reduced extracellular matrix quality and increased intrinsic muscle stiffness resulting in

less compliant muscles, is a likely cause of the pathological muscle activation during low-velocity stretches as described in **chapter 2.3**, (61). While the complete pathophysiology of dynamic and fixed contractures remains unknown, reduced fascicle length, hypertrophic extracellular matrix and/or increased collagen content have been suggested as potential contributors to increased muscle stiffness (40,62–64).

Alterations at the second and third level include disturbances in the reflex loop and muscle spindle itself, which are difficult to evaluate separately. As stated above, previous studies highlighted the role of muscle spindle activation in the underlying pathophysiology of the hyperactive stretch reflex (58,65). In a healthy condition, the stretch reflex is responsible for maintaining the muscle at a constant length. If the muscle is stretched, force is transmitted through the muscle fibers and detected by the muscle spindles. These muscle spindles are wrapped around the muscle fibers (Figure 1) and respond to alterations in length and stretching velocity. Indeed, elongation of the muscle fiber causes stretch, whereby the spindle loses its spiral shape. The afferent sensory neuron transfers this stretch signal to the spinal cord, triggering the muscle to contract until the spindle is back to its original, spiral, state. In case of a pathological hyperactive stretch reflex, which was observed in the pure high-velocity muscle activation patterns described in **chapter 2.3**, it is assumed that the high-velocity elongation of the muscle belly is detected by the muscle spindles and due to imbalanced signals from the central nervous system, the contraction of the muscle is more intense and prolonged. The mixed muscle activation patterns showed additional muscle activation during a low-velocity passive stretch. Previous studies have hypothesized that this might be related to the persistent inward currents (66), which are the result of spinal neuron plateau potentials (prolonged depolarized states generated by neurons within the central nervous system) or to changes in motor neuron membrane potential or conductance (58). For example, plateau potentials may result in maintenance of motor unit activity despite the disappearance of the stimulus (67–69).

Furthermore, the muscle spindle discharge rate is dependent on both absolute as well as relative muscle fiber length and fiber movement velocity (70,71). Therefore, as stated above, less compliant, stiffer, muscles may cause spindle hypersensitivity, potentially due to increased activation of the gamma motor neurons (72,73).

Additionally, the stiffness of attached cross-bridges, which was initially described as a non-neural phenomenon, might interact with spindle firing, a neural component, as it determines the response of the muscle to stretch. Stecco et al. suggested that the viscosity of the loose connective tissue might be increased, resulting in reduced gliding between the different layers of collagen fibers, giving the patient a sense of muscle stiffness (74). A longitudinal immobilization study in rats showed reduced hyaluronic acid concentrations and reduced sarcomere length in the soleus muscle after one week (75). It is hypothesized that these changes can lead to an increased number of attached cross bridges during contraction (61,76). Additionally, the number of attached cross-bridges and thus the cross-bridge stiffness upon stretch is movement history dependent, with stiffness being higher after the muscle has been isometric in comparison to pre-movement (77). When involuntary background activation is increased, it might influence the measured joint resistance, but also the history dependent transient increase in stiffness upon stretch of a muscle that has been isometric, which in turn might elicit increased spindle firing (77,78). Blum et al. confirmed the complexity of muscle spindle afferent firing properties, despite the fact that there is still a lot unknown (79). Furthermore, animal studies by the same research group showed that sensory signals from the muscle spindles are temporarily increased in a passive muscle that is being stretched after being held at a constant length (80,81).

Additionally, Bar-On et al. described a tendency towards higher baseline tone in muscles with mixed low-/high-velocity-dependent spasticity patterns compared to pure velocity-dependent patterns (15). Continuous involuntary muscle activity in the absence of voluntary muscle activation or muscle stretch is also referred to as spastic dystonia (19). Yet, the estimation and decomposition of spastic dystonia should be addressed in future research.

We can conclude that there are still many gaps in the understanding of the association between intrinsic muscle properties and spasticity. We summarized the current hypothesized associations between peripheral alterations at the muscle level and the neural factors in Figure 2. The potential contribution of other alterations that are described for SCP muscles, including loss of sarcomeric titin, overstretched sarcomeres and fatty infiltration, should also be further explored.

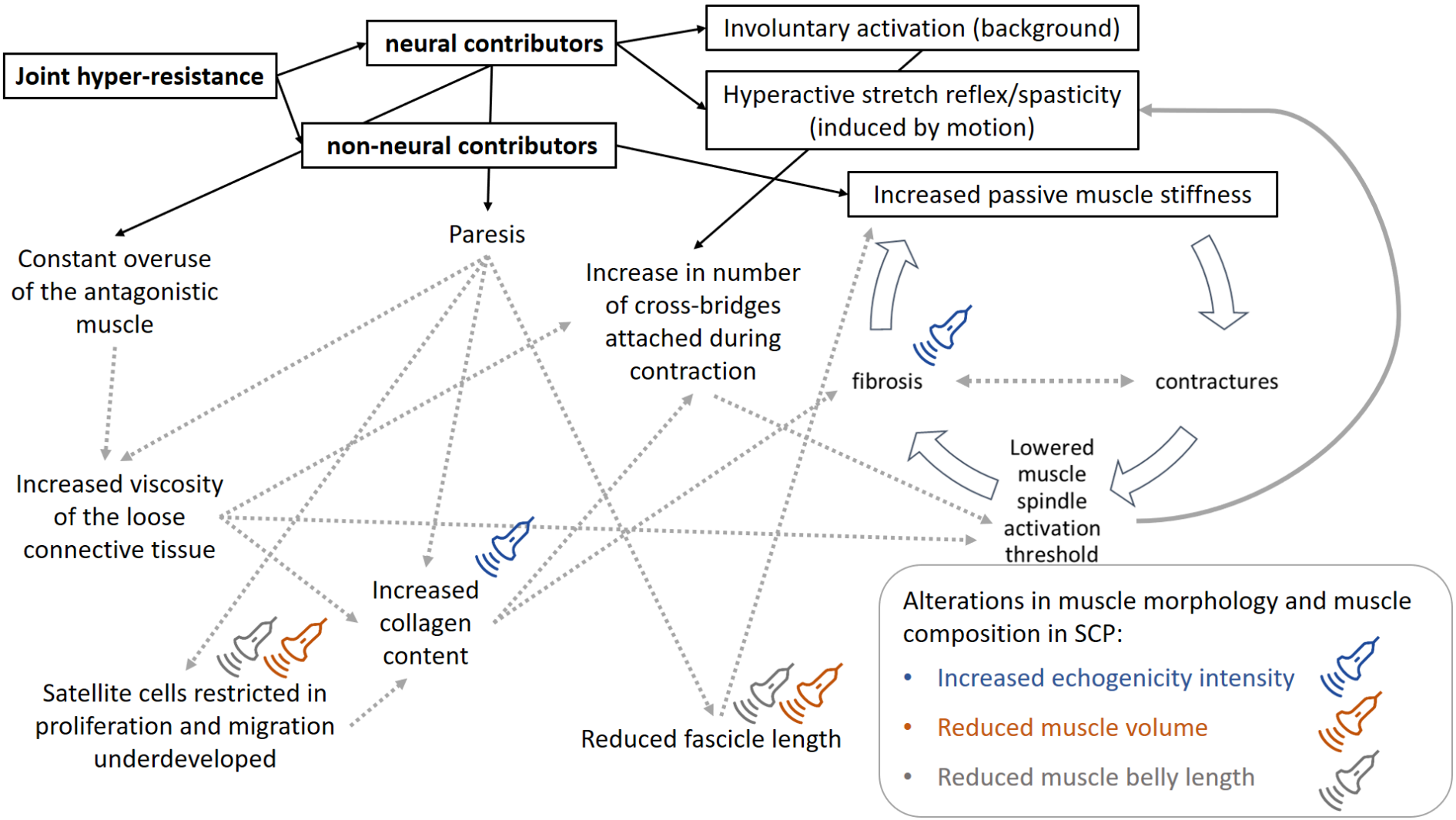


Figure 2. A hypothetical framework based on the current evidence on the association between spasticity, and other neural contributors to joint hyper-resistance, and alterations at the muscle level.

The colored ultrasound probes indicate which parameters are expected to be able to detect the proposed differences in the muscle.

Today, no conclusion can be drawn on the causal relation between muscle morphological alterations and spasticity. Willerslev-Olsen showed that there are indications that muscle morphology and muscle growth are hampered before the presence of a hyperactive stretch reflex or increased stiffness around the ankle joint (82). Herskind, et al. (83) confirmed that alterations in muscle morphology are present from an early age on, which was recently confirmed by our own research group (23). Finally, recent studies have shown that muscle weakness might have a more significant impact on impaired daily functioning than spasticity (84–87). Therefore, it could be argued that alterations in muscle morphology and intrinsic muscle properties might potentially be more influenced by the negative signs (reduced neuromotor control and impaired voluntary movement – muscle weakness) of the upper motor neuron lesion than by the positive signs (spasticity).

3.4. The toxic benefits of Botulinum Neurotoxin-A in young children with spastic cerebral palsy

Recently, concerns have raised on the use of BoNT-A in young children with SCP (14). As muscle development is already hampered in SCP and the regenerative capacity of the muscles might be reduced, there are concerns that muscles might not completely recover after BoNT-A treatment (14,88–92). BoNT-A is often applied to improve motor function, as confirmed by multiple studies of our research group (13,15,93–95). However, a recent study questioned the positive effects of BoNT-A on gait (96). Additionally, this same research group suggested that the use of subjective outcomes can result in overestimation of the positive effects. They stated: "Current literature shows that it is becoming as difficult to find a black cat in a dark room as to demonstrate objective, clinically verifiable, positive outcomes from botulinum toxin therapy in children with cerebral palsy" (97). Today, high quality controlled trials, including objective assessments considering both the effects at the muscle and functional level are scarce. Therefore, we have performed two studies investigating the effect of BoNT-A by objective assessments exclusively. In **chapter 2.4**, we have investigated the isolated effects of BoNT-A injections to the medial gastrocnemius, without lower leg casting, on the muscle and tendon lengths, spasticity and the joint hyper-resistance. In **chapter 2.5**, we quantified the effects of integrated BoNT-treatment on spasticity, and muscle volume and echogenicity intensity, in order to monitor the possible paralysis-induced alterations at the muscle level, and the effects on gait to evaluate the functional gains. Treatment-induced alterations in muscle morphology and composition were compared to an untreated control group. These two studies provided objective data on the potential benefits and harms of BoNT-A injections and might contribute to well-considered treatment selection.

3.4.1. The impact of Botulinum Neurotoxin-A on muscle morphology

In **chapter 2.5**, we have confirmed that BoNT-A injections result in reduced volume of the medial gastrocnemius and semitendinosus muscles. However, the echogenicity intensity remained unchanged. Previous studies showed higher echogenicity intensity values in children with a history of BoNT-A injections compared to children who were naive to BoNT-A treatment

(3,89). As previously mentioned, the validity of the echogenicity intensity should be further investigated, and these findings should thus be interpreted with caution.

Furthermore, we found an increased muscle belly length, which was in line with our findings in **chapter 2.4**, investigating the isolated effects of BoNT-A and casting (1). We believe that the increase in muscle belly length was potentially due to the more relaxed muscle belly following tone reduction treatment, as the muscle belly length measured at maximum dorsiflexion remained unchanged after BoNT-A. If the muscle belly would have lengthened by adding sarcomeres in series, it was expected to also result in an increased muscle belly length at maximum dorsiflexion. Furthermore, the findings of **chapter 2.5** confirmed that, on short-term, BoNT-A negatively reduces volume, potentially as a direct result of the BoNT-A-induced paralysis. Previous studies showed that the reductions in muscle volume remain after the clinical effects of BoNT-A and that muscle recovery is still ongoing at six months post-BoNT-A treatment (89,98,99). The highest degree of muscle atrophy and quality reduction were reported in earlier animal studies and trials in healthy adults, showing only partial recovery of muscle morphology and function at 12 months post-BoNT-A treatment (88,100,101).

The degree of muscle volume reduction of the medial gastrocnemius (-5.2%), as reported in **chapter 2.5**, was in line with previous studies (98,102), but lower compared to volume reductions after six months (-16%), as reported by our own research group (89). It should be noted that this latter previous study evaluated the BoNT-A-effects in children who received their first BoNT-A treatment, while the current study included children who were BoNT-A naive as well as children with a history of BoNT-A-treatment. It should be noted that muscle volume reductions at short-term post-BoNT-A were expected because of the temporal chemo-denervation. Yet, muscle recovery should be monitored closely, especially because of the partial muscle recovery observed in these BoNT-A naive children after six months (89). The need for specific guidelines to facilitate muscle recovery following the first BoNT-A versus repeated BoNT-A treatment should be investigated in future studies.

The volume reduction of the semitendinosus (-16.2%) was larger in comparison to the medial gastrocnemius (-5.2%). However, this effect of BoNT-A on the semitendinosus cannot yet be compared to literature. A previous study by Van Campenhout et al. reported 20.5% volume reduction in the psoas two months after BoNT-A injections (103). These findings strongly suggest that muscle atrophy after BoNT-A is muscle-specific and that proximal lower limb muscles might potentially be more affected than distal muscles. Additionally, the results

described in **chapter 2.5** revealed that muscle volume reductions are patient-specific. Muscle volume (normalized to height*body mass) reductions exceeding the previously reported standard error of measurement values (medial gastrocnemius = 0.07 mL/(kg*m), semitendinosus = 0.04 mL/(kg*m)) (9) were present in 54% of the injected medial gastrocnemius muscles and in 67% of the injected semitendinosus muscles. The changes in normalized muscle volume per subject are presented in Figure 3 for the two muscles for the intervention as well as the control group. These individual responses clearly highlight the variability amongst patients. The muscle- and patient-specific response to BoNT-A should be further addressed in future studies, and point towards the need for individualized follow-up of muscle morphology.

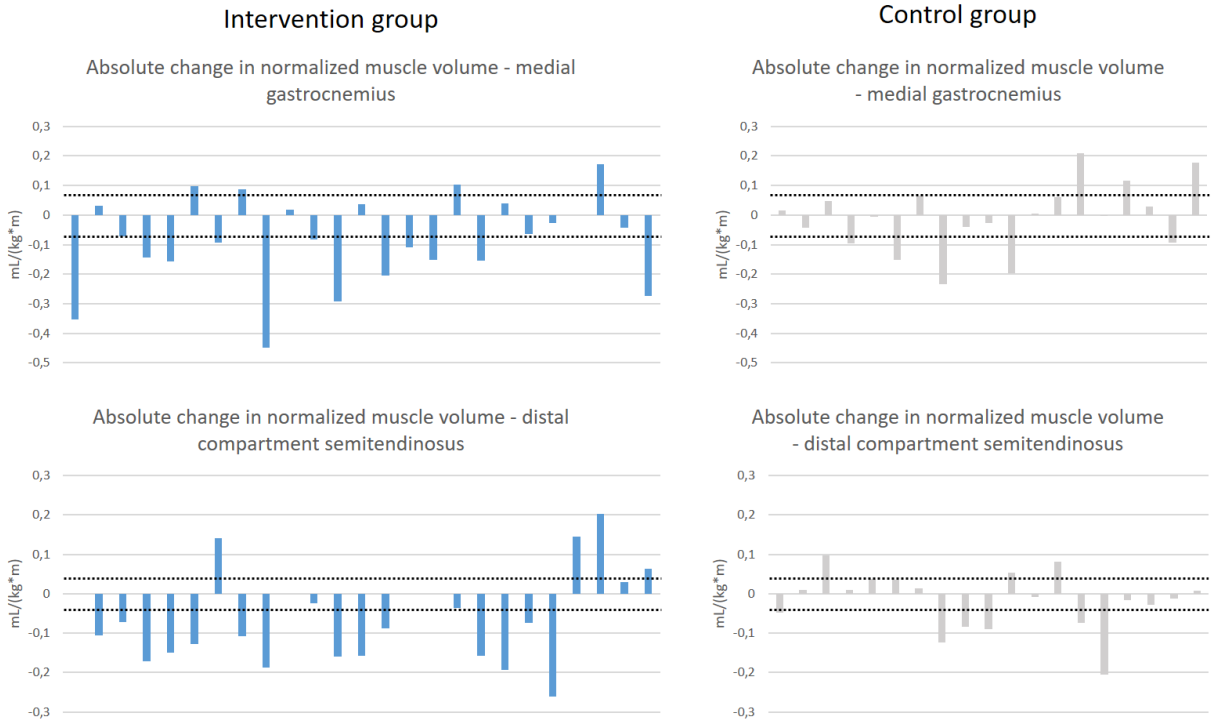


Figure 3. The absolute change in normalized muscle volume of the medial gastrocnemius and distal compartment of the semitendinosus per subject for the BoNT-A intervention and untreated control group.

*The horizontal lines indicate the standard error of measurement values (medial gastrocnemius = 0.07 mL/(kg*m), semitendinosus = 0.04 mL/(kg*m)) for normalized muscle volumes (9).*

kg = kilograms, m = meters, mL = milliliters, SEM = standard error of measurement

The intervention and untreated control group were matched on severity (GMFCS level) and age, in order to know whether the changes in muscle morphology have arisen as result of the

BoNT-A injections. However, the fact that the participants of the untreated control group had no indication for treatment, might have introduced bias. Additionally, the children in the intervention group received intensive physical therapy and casting if necessary, whereas the children in the untreated control group received usual care, including regular physical therapy and orthotic wear. In order to improve matching at baseline, a crossover design can be relevant, including only children with an indication for treatment. However, due to the long-lasting effects of BoNT-A on the muscle, a study with such a cross-over design can take up to 12 months, which is not feasible. Furthermore, postponing treatment in young children might face ethical dilemmas.

3.4.2. The impact of Botulinum Neurotoxin-A on gait

In **chapter 2.5**, we found significant improvements in ankle kinematics during gait. Interestingly, these functional benefits were not accompanied with improved maximum ankle dorsiflexion during the clinical examination. This could be explained by a potential ceiling effect, as the included sample was not characterized by severe ankle contractures at baseline. Besides the fact that some studies questioned the positive effects of BoNT-A on gait (14,96,104,105), some earlier studies showed that walking velocity, stride length and sagittal plane kinematics deteriorated in untreated children between five and 12 years of age (106–108). Furthermore, the positive effects of integrated BoNT-A treatment on gait have been shown in multiple studies by our research group (13,15,93–95).

In **chapter 2.5**, we described improved ankle dorsiflexion during gait as a positive effect of the BoNT-A treatment. In contrast, Hastings-Ison et al., expressed concerns about the increase in ankle dorsiflexion movement in children with bilateral SCP (96). They showed that the increased dorsiflexion was often combined wat increased knee flexion during stance in children that were bilaterally affected. As grouch gait is the most common gait abnormality in children with bilateral SCP, they were concerned that the BoNT-A-induced tone reduction may result in a crouch pattern that might remain even after the active period of BoNT-A. Our results did not show an increased incidence of crouch following BoNT-A treatment (13,93,109). Interestingly, in an earlier study, the same research group (Hastings-Ison et al.) showed that the prevalence of crouch gait dramatically reduced after the introduction of BoNT-A (110). Before the introduction of plantar flexor BoNT-A treatment, gastro-soleus lengthening procedures were often applied in young children with SCP, resulting in a rising prevalence of

progressive crouch patterns. The risk of crouch following BoNT-A treatment thus need to be further explored. Additionally, the increased dorsiflexion angle, which was present over the full gait cycle, might not be beneficial during push-off, if the total ankle range of motion remains unchanged. As there are concerns regarding power generation during push-off as a result of the induced muscle weakness, future studies should investigate kinetic data to monitor the effects of BoNT-A on the push-off. However, preliminary analyses by our research group, in a larger population, showed no negative effects on push-off power after BoNT-A treatment (Papageorgiou et al., unpublished data).

We believe that BoNT-A can be effective in reducing pathological gait when combined with goal-directed gait rehabilitation, which will be discussed later on in more detail.

3.4.3. Dose-dependent effects of Botulinum Neurotoxin-A

One of the questions that remained unanswered is whether the positive and negative effects of BoNT-A are dose-dependent. Previous animal studies showed that higher BoNT-A dosages were related to larger functional effects, together with larger reductions in muscle size, muscle mass and muscle strength (111,112).

A previous study by Molenaers et al. concluded that the total injected BoNT-A dose was not related to the goal attainment in children with SCP (94). It should be noted that, because the latter study was retrospective, all children that were enrolled in the study received a similar treatment approach, whereby the dosage variation was rather limited. On the other hand, Baker et al. showed that the increase in ankle dorsiflexion was most pronounced in patients who were randomly allocated to 20 units of BoNT-A (Dysport®) per kilogram in comparison to bilateral injections of placebo, 10 or 30 BoNT-A units per kilogram in the medial gastrocnemius (113). They reported only few potential toxin-related adverse events which were all classified as mild events, and hypothesized that these adverse events could potentially be minimized by selecting an appropriate dose of BoNT-A.

A recent trial on upper-limb BoNT-A (Dysport®) treatment of the wrist or elbow flexors showed that a dose of two units per kilogram body weight was less effective in reducing spasticity levels, assessed by the modified Ashworth score, compared to eight or 16 units per kilogram (114). Despite the fact of using a subjective outcome like the modified Ashworth score, this study confirmed that a sufficient dose of BoNT-A is required in order to achieve the

desired effect on the hyperactive stretch reflex. The challenge remains to find the balance between a BoNT-A dosage that is sufficiently low to limit the negative effects on muscle size, and sufficiently high to achieve functional improvements or reduce the patients' discomfort.

In conclusion, there is still insufficient evidence that the unwanted effects of BoNT-A are dose-dependent, and could potentially be avoided by lowering the injected dose. The used dosages in our studies were similar to two different studies by Williams et al. and Alexander et al. (98,102), who reported similar degrees of muscle volume reduction over the same interval as we reported in **chapter 2.5**.

The effect of the used dosage and the wanted and unwanted effects should be further investigated in future studies. This may help to further fine-tune the dosages at a patient-specific level. Additionally, it has been suggested to define BoNT-A dosage based on muscle volume or muscle thickness instead of body weight (3,34,115). We do realize that trials testing different dosages of BoNT-A in young children may face ethical problems. Therefore, ex vivo studies, using harvest muscle tissue, or in vitro experiments, using cultured muscle cells, might provide improved insight. Our research group has established a reliable and minimally invasive muscle micro-biopsy technique to investigate altered muscle properties in children with SCP (116), which is currently applied pre and post BoNT-A injections.

3.4.4. The timing of Botulinum Neurotoxin-A treatment

In our center, mainly young children are treated with BoNT-A. In **chapter 2.5**, we have only included children below the age of 12 years, aiming to limit the risk of severe secondary muscular and bony deformities. Some studies suggested that the effectiveness of BoNT-A declines over age (117). The optimal timing of BoNT-A treatment is believed to be between two and five years of age (90,118). This age-range is characterized by dynamic neuromotor development, creating an opportunity to positively influence its course. Love et al. showed that most clinically significant improvements were observed in spastic hemiplegia patients with equinus under the age of 4 years (119). Previous studies further showed that intensive treatment of spasticity during an early age could result in greater gross motor outcome (120–122).

With increasing age, the response to BoNT-A becomes less, whereas surgical lengthening of the gastro-soleus becomes more effective for treatment of equinus (14,123–125). The fact that BoNT-A is less frequently applied at older ages can also be influenced by the development of spasticity over age, which tend to decline after the age of five years (24).
Furthermore, the interval between repeated BoNT-A treatment sessions should be considered. A literature review by Kahraman showed that the interval between repeated BoNT-A treatment sessions varied from three to 12 months, between the different centers (90). As previous animal studies and one study including two healthy adults showed only partial recovery of muscle morphology and function 12 months after BoNT-A treatment (88,100,101), treatment intervals of at least 12 months are currently advised (14,94).

Multiple studies reported that the first session of BoNT-A injections is the most effective, resulting in larger increases in ROM, significant improvements in gait and larger improvements on the gross motor function measure (GMFM-88) compared to repeated injections (105,126,127). However, both animal studies (128) and studies in SCP (98) suggest that the first injection is also the one with the most detrimental effects on the muscle. These conflicting findings of the desired clinical improvements in contrast to the unwanted effects on the muscle, suggest that BoNT-A has the most significant impact during the first exposure. This could explain the higher degrees of muscle volume reduction in our previous study including solely BoNT-A naive children compared to the mixed group in **chapter 2.5** (89). In **chapter 2.5**, 10 out of 25 (40% of the total intervention group) participants were naive for BoNT-A treatment at the time of inclusion. A descriptive sub-analysis on children who received their first BoNT-A and children who had a history of BoNT-A treatment, did not reveal consistent differences between the naive and non-naive participants in alterations at the muscle nor at the functional level.

3.4.5. Botulinum Neurotoxin-A and the added value of physical therapy

Multiple studies by our research group showed that multilevel BoNT-A injections as part of an integrated approach are successful in improving gross motor function and postponing the need for more invasive procedures (13,95,129). Franki et al. systematically summarized scientific and clinical evidence on the additional value of tone reduction in combination with (intensive) physical therapy (129). They showed that reduction of the hyperactive stretch

reflex by BoNT-A can provide a window of opportunity for functional training and targeted strengthening exercises (130–133). If the BoNT-A treatment is focused on the lower limb, goal directed gait training and functional training are recommended in order to improve gross motor function (105,119,132,134–136). Furthermore, strength training is a promising intervention to reduce the paralysis-induced negative effects in the injected muscles, including muscle weakness and reduced muscle volume (130,133,137–139). Additionally, the non-injected muscles, like the tibialis anterior and the glutei muscles, are easier to target after injecting for example the gastrocnemius and hamstrings or psoas, respectively.

In contrast, Schasfoort et al. recently concluded, from their partly randomized study, that lower limb BoNT-A treatment had no beneficial effects over 12-weeks comprehensive rehabilitation in children with SCP aged between four and 12 years (140). Directly after the rehabilitation program (12 weeks) and during a follow-measurement (24 weeks), children who received BoNT-A combined with rehabilitation showed similar levels of pain, muscle length, spasticity, muscle strength, walking speed, overall function and goal attainment compared to children who received comprehensive rehabilitation only. The used BoNT-A treatment protocol in combination with casting if necessary and orthotic management, followed by individualized physical therapy is similar to the protocol in our center. This study showed that other factors apart from BoNT-A may equally explain the observed improvements. However, the interpretation is challenging due to the partly randomized design, resulting in more children that already had repeated BoNT-A treatments, higher spasticity levels and limited range of motion in the BoNT-A group compared to the comprehensive rehabilitation group.
Due to our integrated approach, it is challenging to assess the isolated effects of BoNT-A. Even though the children in **chapter 2.4** did not receive casting, they still received regular physical therapy and orthotic wear.
As stated above, intensive physical therapy after BoNT-A might be required, as it is known that BoNT-A induces temporary paralyses. Muscle weakness in SCP is an insidious and silent problem and potentially underestimated or overlooked (86). As muscle weakness is shown to be more disabling than spasticity and BoNT-A may cause temporal additional muscle weakness, physical therapy intervention focusing on muscle strengthening is of paramount importance (86). Williams et al. already showed that immediate muscle volume reductions could be limited by additional strength training (138). Therefore, complementary

interventions should focus on accelerating muscle recovery after BoNT-A injections and promote muscle growth.

3.4.6. The balance between benefits and harms

As a result of the potential long-lasting effects on muscle morphology, for which we currently have no evidence, and the concerns on irreversible muscle atrophy, several experts have asserted the need to revise treatment protocols. Furthermore, animal studies suggested that the detrimental effects to the muscle might become more severe after multiple injections (141). Therefore, it is important to monitor the recovery profile of the individual muscle and to assess the state of the muscle at the macroscopic level (by muscle imaging) as well as at the microscopic level (using muscle biopsies).

The combined current evidence indicates that the selection of BoNT-A as tone reduction should be based on a well-considered review of potential befits and harms. As previous studies showed that muscles are not fully recovered after six months, an interval of at least 12 months between repeated BoNT-A interventions has been advised (14,94).

The challenge when using BoNT-A in young children with SCP is finding the right balance between the positive effects and some unwanted effects. We should improve the muscle regenerative capacity and anticipate to the unwanted effects, for example by providing progressive strength training to overcome the BoNT-A-induced muscle weakness and reduction in muscle volume (138). We hope that our studies and future investigations will contribute to improved insight and provide objective information on the use of BoNT-A for the patients, their parents and caregivers, such that they can make a well-considered decision for BoNT-A treatment.

3.5. Clinical relevance and the clinical implementation of objective assessments of muscle morphology and spasticity

A major challenge in the management of SCP is the heterogeneous nature of the disorder and the heterogeneous response to treatment, emphasizing the need for patient-tailored treatment. The objective assessments of muscle morphology and the instrumented spasticity assessments as applied in the current studies of this doctoral thesis can be of great value to assess the response to treatment. Moreover, the explored associations between observed symptoms, such as the hyperactive stretch reflexes, and altered muscle morphology, may help to uncover subtypes in the heterogeneous group of children with SCP, which can support personalized treatment.

The majority of the used assessments in this doctoral thesis are instrumented assessments, which are objective, easier to be standardized, and thus more reliable in comparison to manual subjective assessments. These objective assessments are particularly relevant, since previous studies showed that the subjective assessments can overestimate treatment effects of BoNT-A (97).

3.5.1. Clinical implementation of muscle ultrasound and future steps

Regularly muscle imaging will provide more insight into the condition of the muscle before and after treatment. We hypothesize that treatment outcome might be patient- and muscle-specific. Hence, close monitoring of each muscle for each individual child with SCP is of paramount importance. For example, the possible volume reduction after BoNT-A injections should be monitored before considering repeated injection.

Based on the findings in this doctoral thesis, some specific recommendations for future muscle morphology assessments can be made, to be applied in both research and clinical settings.

<u>Standardization of measurement setup</u>

In **chapter 2.1**, different measurement setups were introduced, allowing to change limb position and adjust joint angles if relevant for the study design (46). The assessments that allowed changes in the involved joint angles were well-tolerated and were shown to be useful for muscle belly length assessments in different conditions. Assessments of a muscle's resting

length may have limited relevance to be linked to functional assessments (as discussed before in the section methodological considerations). Muscle imaging at different muscle lengths might provide more insight into its extensibility and response of the muscle-tendon complex to stretch. We believe that these measurement setups can be of an added value for future studies where length assessments are of primary importance, such as for example in studies that investigate the effect of stretching on the muscle and tendon structure.

The evaluation of muscle volume and echogenicity intensity was more challenging in side position as the probe position, probe tilting and applied pressure were more challenging to control. Additionally, the multiple sweep technique, which is often required in older children (due to their larger muscle sizes), was shown to be more challenging when participants were positioned on their side, often resulting in three-dimensional reconstructions with insufficient overlap between the two parallel sweeps. Therefore, we recommend to perform assessments of muscle volume and echogenicity intensity in prone or supine position. A recent reliability study by Hanssen et al., performed on the same dataset as included in **chapter 2.1**, showed comparable good reliability of three-dimensional freehand ultrasound parameters for a typically developing cohort and a cohort of children with SCP (9). Within the cohort of children with SCP, the estimations of muscle morphology outcomes were found to be sufficiently reliable to detect differences between SCP children with different levels of gross motor function, which pointed towards a good validity. The differences in muscle volume and echogenicity intensity that were reported in this reliability study were potentially due to differences in performance of the assessment (applied pressure, probe tilting) between sessions and between assessors. Therefore, it is recommended to have repeated assessments, especially in a longitudinal design, performed by the same assessor. If different assessors are involved, a high level of training and agreement on acquisition guidelines is required and frequent comparisons of performance should be made.

An early study by our research group showed that the data processing of the ultrasound images to define muscle volume of the semitendinosus, rectus femoris and tibialis anterior muscles were reliable in both typically developing children and children with SCP (30). These findings were in line with the processing reliability of the medial gastrocnemius which was investigated in another previous study of our group (2). Hanssen et al. provided a clear

description of data processing guidelines and defined a training protocol, which could be used for training of future assessors (30).

All these reliability and validity studies contribute to the clinical implementation of the muscle morphology assessments.

Normative muscle morphology database

In **chapter 2.2**, we have created a large normative database and described muscle development with increasing age. This study confirmed that muscle development is muscle- and gender-specific and more heterogeneous from the pubertal ages onwards. Therefore, we recommend to match typically developing children and pathological pediatric cohorts primarily based on age or body dimensions. Gender-specific matching should be considered if pubertal or post-pubertal children are included. This dataset can serve as a normative database for pediatric populations and may provide a cross-sectional reference frame to better understand both physiological and pathophysiological muscle development. However, in order to gain more insight into the muscle development in pubertal ages, the dataset should be extended and adequate measurements of pubertal stage, like Tanner stages or hormone levels, should be included (142). Alternatively, the peak height velocity might serve as an objective marker for the timing of puberty (143). More complex statistical methods that can handle the changing variability in the dataset with increasing age (such as the Lambda-Mu-Sigma (LMS) method (144)) are required to describe muscle development over the full age range. An important limitation of this study was the cross-sectional design. In order to investigate the variability in muscle development between children, repeated assessments over time according a longitudinal design will be required.

The assumptions of simple linear regression equations, to describe muscle development over age, have recently been explored in a longitudinal dataset on muscle morphology of the medial gastrocnemius in children with SCP (23). This recent study showed piecewise profiles with increasing age with different break points and non-linear trajectories of muscle growth. Therefore, growth rate estimations over a large age range, assuming a linear relation, should be interpreted with caution.

Scaling techniques to normalize muscle morphology data

In order to compare cohorts with different body dimensions, due to pathology or diversity in age, muscle morphology outcomes are often normalized. While most studies have used ratio scaling, multiple methods have been used and clear guidelines are lacking (8). Besides ratio scaling, allometric scaling has been used for normalizing strength assessments to body size (145). Yet, allometric scaling was only rarely used to normalized muscle morphology, and never for growing children (12).

In **chapter 2.1**, we compared the ratio and allometric scaling to normalize muscle morphology outcomes of children with a typical development. Ratio scaling, also called isometric scaling, assumes a simple linear relationship between muscle morphology and measure of body size (for example body mass) (11). The morphological outcomes can be normalized by dividing the outcome by a measure of body size. Allometric scaling on the other hand, assumes that all humans have the same shape and architecture, however are different in size (11).
As not all body parts grow at the same rate, since the limbs grow often faster compared to the trunk, scaling to leg length might be more valid compared to scaling to total body height.
Even though the scaling methods might not yet be optimal, normalizing the muscle morphology data will ease the comparison between different cohorts and reduce the impact of growth in repeated assessments following a longitudinal design.

Valorization

During the umbrella TAMTA project, important steps towards clinical implementation, and introducing the three-dimensional freehand ultrasound technique in other medical centers, were taken. Parallel to the investigations in this doctoral project, different reliability studies have been performed to estimate the expected measurement errors and clinical detectable differences for each morphological parameter. Manuals on the data collection and data processing were developed. The consortium organized several training sessions, with hands-on sessions and video tutorials, after which improvements were made in the training protocols. Furthermore, a simplified processing method, which allows muscle volume estimations without 3D motion tracking, was developed (7). As the data processing is quite time-consuming, semi-automatic processing techniques will be further explored. Besides that,

a data management platform was designed within the University Hospitals Leuven, which can be transferred to other medical or research centers, if relevant.

The large clinical and normative muscle morphology databases that were created, will contribute to monitoring of patient-specific muscle development in different pathologies. During the TAMTA project, different templates for clinical reports were designed, to improve the interpretation and clinical translation of the muscle morphology data.

The TAMTA consortium will continue organizing training sessions including data acquisition, data processing and data interpretation. In the future, steps will be taken to establish reimbursement for muscle morphology evaluations by three-dimensional freehand ultrasound in different patient populations.

3.5.2. The clinical implementation of instrumented spasticity assessments and future steps

This doctoral thesis primarily focused on the neural components of joint hyper-resistance. However, in order to improve treatment efficacy, it is essential to distinguish between the neural and non-neural components contributing to the increased resistance at the joint and to get a better understanding of the interaction between both components (146). Instrumented, objective, assessments of joint hyper-resistance are therefore considered essential.

Furthermore, the different muscle activation patterns have been related to treatment outcome after BoNT-A injections in the medial hamstrings (15). While these findings suggested that muscle activation patterns can predict treatment outcome, this should be verified for other muscles and for other treatment modalities.

<u>Assessments of muscle stiffness</u>

The assessment of muscle stiffness was beyond the scope of the doctoral thesis. However, objective assessments of muscle stiffness can provide additional insight into the pathophysiology of dynamic and fixed contractures and the impact of different treatment modalities. Estimations of muscle stiffness can be based on muscle morphology assessments at different degrees of muscle stretch or by the use of dynamometry during passive muscle stretches, like in our instrumented spasticity assessments. However, dynamometry applied at the joint cannot distinguish between stiffness of the muscle belly and other soft tissue structures like tendons or the articular capsule (147). More recently, studies investigating

muscle stiffness have used shear wave elastography methods, which estimates the passive muscle tension (148). Shear wave elastography has thus been proposed as valuable tool to quantify muscle stiffness in children with SCP (147,149) and has been suggested to be more sensitive to intra-muscular changes in comparison to torque measures (150). Previous research showed that shear-wave elastography was well tolerated by children with SCP (147,151,152). However the previously used protocols for shear wave elastography should be further standardized and reliability should be evaluated, before clinical implementation could be considered (151–155).

3.5.3. Translation to other pathologies

The assessments of muscle morphology and the instrumented assessments, used in this thesis are valuable tools for other disorders with impaired muscle development or upper motor neuron syndrome. In our research group, these methods are currently being used to investigate populations with Duchenne muscular dystrophy, low-back pain, hereditary spastic paraplegia and stroke survivors.

As the upper motor neuron damage in children with SCP occurs in an immature central nervous system, the findings of this doctoral thesis cannot be generalized to central nervous lesions that occur at an adult age. Due to the plasticity of the central nervous system in young children and the muscle plasticity that is influenced by age and biomechanical loading and neural activity, muscles of children with SCP might response differently to altered stimuli from the central nervous system compared to adults with neurological disorders, such as stroke (32,156,157).

As stated before, lower limb muscle morphology is highly influenced by biomechanical loading. Therefore, findings of this doctoral thesis, focusing on the lower limb, might not be completely translatable to the muscles of non-ambulatory children or to upper limb muscles.

3.5.4 Individualized treatment: the way to go

The ultimate goal of the research line on altered muscle morphology in children with SCP within our research group is to develop patient-specific and muscle-specific treatment algorithms (158). Patient- and muscle-specific baseline characteristics may have a predictive value for treatment outcome. In this way, a more personalized treatment paradigm that

considers the current state of the muscle itself, rather than indirect clinical measures, like manual spasticity assessments or passive range of motion, can be developed. The latter clinical measures are commonly used for treatment selection. However, due to their subjective nature and the lack of sensitivity, the treatment algorithms based on these clinical scores might potentially be incomplete or wrong. The intervention studies (**chapter 2.4** and **chapter 2.5**) that were performed in this doctoral thesis are preparatory studies for the future prediction studies and for subsequent consensus meetings on treatment algorithms. In the final phase of the TAMTA project, in which this PhD project was situated in, a Delphi consensus survey will be organized, whereby a panel of experts in the use of muscle imaging in clinical context, will seek agreement on the clinical value of muscle morphology assessments in children with SCP. The goal of this consensus project is to develop guidelines on the use of muscle morphology parameters to define patient-specific treatment.

<u>The use of personalized goals</u>

In order to improve patient satisfaction, strengthen the patient's sense of autonomy and to enhance treatment outcome, personalized goals are considered beneficial. Franki et al. recommended that older children should be more actively involved in the process of goal setting (159).

Studies by Van den Broeck and Franki et al showed tendencies towards larger improvements in functional goals like walking, after personalized physical therapy programs over a general program (159–161).

We believe that the rather limited functional improvements that were described in **chapter 2.5** after BoNT-A injections can be improved when further individualizing the intensive physical therapy period. Multidisciplinary discussion and personalized reports are of paramount importance in the personalized care for children with SCP. Furthermore, future studies should consider to evaluate treatment success by a goal attainment score (158). This score is based on pre-defined goals. The patient and his/her caregivers can be actively involved in the identification of problems and goal setting (158,162).

3.6. Critical reflection and future research in a wider perspective

While this doctoral thesis has contributed to improved understanding of the heterogeneous nature of SCP, it could only add a small piece in a very complex puzzle. For example, muscle alterations in relation to the different types and onset of brain abnormalities, gait patterns, physical activity and comorbidities should be further investigated.

3.6.1. Quality of life and social participation

Quality of life and social participation are important factors to consider when monitoring a child with SCP or selecting treatment. One of the most important things is the attainment of life skills that can allow children with SCP to take part in society. Another import factor is promoting physical activity. Apart from the physical advantages, physical activity can improve quality of life and emotional well-being (163). Therefore, sports participation should be promoted in children with SCP.

In our intervention studies (**chapter 2.4** and **chapter 2.5**), we have only investigated the effect of interventions on the muscular and functional level and not on quality of life or social participation (164,165). A recent study highlighted that BoNT-A treatment may significantly improve quality of life, as it reduces pain (164). Regular evaluations of quality of life, physical activity and social participation are essential in clinical follow-up and future intervention studies. Factors that can influence quality of life in a positive or negative way should be further investigated and considered in future treatment planning.

3.6.2. Epigenetic factors in spastic cerebral palsy

While SCP is not a genetic disorder in general, epigenetic factors could also alter the muscle (166,167). Epigenetic factors, usually behavioral or environmental aspects, can change the way the cells control gene activity, without alterations in the DNA sequence. A recent review of literature by Romero et al. summarized studies relating to epigenetic phenomena to SCP in all three major epigenetic systems (i.e., DNA methylation, histone modification, and noncoding RNA). They showed that there is a growing interest in contribution of epigenetic factors in the manifestation of SCP (168) or alterations in skeletal muscles (167,169). For

example, hampered muscle growth in SCP, caused by satellite cell dysfunction and reduced ribosome synthesis, is believed to be a result of hyper-methylation originated at the time of cerebral insult. Furthermore, muscle fibrosis (altered extracellular matrix and collagen deposition) (170) is often observed in SCP and expected to influence response to BoNT-A treatment (34,171). As the outcomes of histological studies in humans are less consistent in comparison to animal studies (91,171,172), it has been hypothesized that muscle fibrosis in SCP is caused by epigenetic components, potentially similar to conditions like systemic sclerosis or pulmonary fibrosis (173–175). Yet, more research is needed in this field to test these hypotheses. Additionally, more research into epigenetic factors contributing to the onset of SCP, alterations at the muscle level and treatment response, may provide more insight into the heterogeneous outcomes of therapy.

3.6.3. Treatment response

Future studies should include large and heterogeneous cohorts, such that patient and muscle characteristics that can predict treatment outcome can be identified. Additionally, those studies should include untreated control groups, investigate multiple muscles and combine assessments of the muscle, neuromuscular symptoms and motor function. Muscle evaluations should preferable include both macroscopic (muscle imaging) and microscopic (molecular and histological analyses) evaluations, in order to assess the muscle recovery profile (for example following BoNT-A treatment), regenerative abilities and intrinsic quality of the muscle (116).

In addition to the above mentioned suggestions, future studies with specific focus on BoNT-A interventions should specifically investigate the long-term effects, including both microscopic, molecular and functional assessments, in order to critically appraise the benefit-harm balance of BoNT-A treatment over a longer follow-up. These future intervention studies should focus on accelerating muscle recovering after BoNT-A and stimulating muscle growth.

Most studies, including the current studies (**chapter 2.4** and **chapter 2.5**), focused on the effect of BoNT-A in ambulatory children. Therefore, future studies should also include participants with less functional capacity in order to evaluate the benefits and potential harms for the total SCP population (131).

Overall Conclusion

Within this doctoral thesis, we aimed to provide more insight into the altered muscle properties and heterogeneous nature of SCP and the heterogeneous response to treatment.

In the first study, we showed that the heterogeneity in muscle morphology could be partly explained by methodological considerations. This methodological study highlighted the essence of standardization and detailed documentation of limb position and joint angles during muscle morphology assessments. In the second study, we showed that natural muscle growth can induce variation in muscle morphology. This study established a comprehensive dataset of normative muscle morphology data with recommendations for data normalization that may reduce the variability caused by natural growth. Successful application of muscle morphology data normalization will highlight the variation caused by pathology. In the third study, we showed that the heterogeneity in muscle morphology can be partially explained by the level of spasticity or by different spasticity patterns. However, mainly weak associations between the level of spasticity and alterations in muscle morphology were found, suggesting that other factors may co-influence this complex relation. In the fourth and fifth study, we confirmed that treatment history can influence muscle morphology and may thus also contribute to the heterogeneous muscle presentation in children with SCP. Furthermore, this thesis provided more insight into the isolated effects of BoNT-A injections and lower leg casting and the effects of BoNT-A injections as part of an integrated treatment approach. Potential harmful effects on the muscle were highlighted, emphasizing the need for close monitoring of muscle recovery after BoNT-A treatment. Treatment selection, specifically in young children with SCP, should be based on a patient-specific critical evaluation of the benefits and harms. These objective data on the effects of BoNT-A treatment may contribute to clinical decision making and individualized treatment.

We believe that the use of objective assessments, especially of muscle morphology and spasticity, will be a valuable contribution to clinical follow-up of children with SCP. We believe that the different studies in this doctoral thesis have provided a foundation for clinical implementation of the three-dimensional freehand ultrasound and instrumented spasticity assessments. These assessments will gain more insight into the natural course of the disorder and the impact of treatment and thereby eventually improve treatment efficacy.

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Appendices

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Personal contribution

At the start of my doctoral training, I was involved in the assessments of the PASTA (Patient-specific treatment) project, which resulted in my first publication, which is included in **chapter 2.4**. This project was led by Dr. Lynn Bar-On. I was involved in patient recruitment, study assessments, data processing, and statistical analyses. I wrote the manuscript with guidance and supervision of Dr. Lynn Bar-On.

The other studies in this doctoral thesis were situated in the TAMTA (Treatment algorithms based on muscle and tendon architecture) project.
The participant recruitment and data collection of the first study (**chapter 2.1**) was performed by Britta Hanssen and myself. The data included in the second study (**chapter 2.2**) were collected by Britta Hanssen, Nathalie De Beukelaer and myself. The typically developing children included in this study were mainly recruited by our graduate students or via acquaintances of our colleagues. The participant recruitment and assessments of the third study (**chapter 2.3**) was a joint effort by Britta Hanssen, Nathalie De Beukelaer, Tijl Dewit and myself. I was responsible for the recruitment and assessments of the intervention group of the fifth study (**chapter 2.5**). In case I was unavailable, our research co-workers Tijl Dewit or Ester Huyghe took over. The assessments of the control group were performed by Tijl Dewit, Ester Huyghe, Britta Hanssen and myself.

Regarding the data processing, the data of the first study (**chapter 2.1**) were processed by Tijl Dewit and myself. The data of the second study (**chapter 2.2**) were processed by Ester Huyghe and myself. During the processing of the data of **chapter 2.3**, I got assistance of Marjan Coremans, a graduate student of our research group, during processing and classification of the spasticity assessments. For the fifth study (**chapter 2.5**), I was responsible for the data processing of the intervention group, whereas Ester Huyghe and Tijl Dewit processed the data of the control group. Additionally, Lauraine Staut and Eirini Papageorgiou helped me during the data processing of the three-dimensional gait analyses.

Finally, I performed all data explorations and data analyses of the different studies, resulting in the different chapters of this doctoral thesis. I performed all statistical analyses, with exception of the statistical non-parametric mapping analyses of the fifth study (**chapter 2.5**), which was performed by Eirini Papageorgiou. I wrote the manuscripts of the five different chapters under supervision of Professor Dr. Kaat Desloovere and Professor Dr. Patrick Calders, and co-supervision of Professor Dr. Hilde Feys, Professor Dr. Anja Van Campenhout and Professor Dr. Christine Van den Broeck.

Conflict of interest statement

I have no conflict of interest to declare.

Appositions

1. The publication of negative findings should be stimulated in all research communities to enhance and drive scientific debate on negative results.

2. Teenagers should be educated about how to recognize and handle fake news in order to prevent them from being misinformed about the current situation in the world.

3. Bicycle helmets and reflective vests should be mandatory for all cyclists under the age of 18 years in order to reduce the number of bicycle accidents and accidents leading to severe brain damage.

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About the author



Nicky Peeters was born on the 6th of January 1993, in Oss, The Netherlands. She attended Maaslandcollege high school (pre-university level), from which she graduated in 2011. Nicky studied Biomedical sciences at the Radboud University Nijmegen (2011-2016). During her Masters she specialized in Human Movement Sciences with internships at the department of Clinical Neurophysiology (Radboud University Medical Center, Nijmegen, The Netherlands) and Children's exercise center (Wilhelmina Children's hospital, University Medical Center Utrecht, Utrecht, The Netherlands). Additionally she spent six months in New Orleans (Louisiana, United States of America) for a research stay at the Hayward genetics center at Tulane Medical School, Tulane University New Orleans, as part of her Minor subject in Human Pathobiology.

In December 2016, Nicky applied for a position at the Clinical Motion Laboratory of the University Hospitals Leuven. In February 2017 she moved to Leuven and joined the clinical team of the Clinical Motion Laboratory of the University Hospitals Leuven, campus Pellenberg. From September 2017 she started her PhD in the research group of Neurorehabilitation under the supervision of Professor Dr. Kaat Desloovere, Professor Dr. Patrick Calders, Professor Dr. Hilde Feys, Professor Dr. Anja Van Campenhout and Professor Dr. Christine Van den Broeck. This PhD project was a joint project with Ghent University. Her PhD research focused on alterations in muscle morphology in children with spastic cerebral palsy and the impact of treatment, with special focus on the impact of Botulinum Neurotoxin-A treatment.

When she is not working, Nicky enjoys traveling, running, cycling and scuba diving.