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1 **Three or more BoNTA interventions are associated with altered medial gastrocnemius**
2 **volume and echo-intensity in children with spastic cerebral palsy**

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1 **ABBREVIATIONS**

2 SCP Spastic cerebral palsy

3 MG Medial gastrocnemius

4 MRI Magnetic resonance imaging

5 TD Typically developing

6 GMFCS Gross motor function classification system

7 BoNTA Botulinum neurotoxin-A

8 3DfUS Three-dimensional freehand ultrasonography

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1 **AIM** Children with spastic cerebral palsy (SCP) have smaller medial gastrocnemius (MG)
2 volumes and higher echo-intensity values with respect to typical developing (TD) peers. These
3 alterations associate with the Gross Motor Function Classification System (GMFCS). Botulinum
4 neurotoxin A (BoNTA) interventions are a common treatment with proven functional outcomes.
5 This cross-sectional investigation evaluates whether recurrent BoNTA interventions to the MG
6 have an additional influence on muscle morphology, beyond GMFCS level.

7 **METHOD** A TD-cohort (n=67, median age 9:11 (7:10-11:6)), a SCP-cohort naive to BoNTA
8 interventions (No-BoNTA n=19, median age 9:3 (8:5-10:10)) and a SCP-cohort with a minimum
9 of three recurrent BoNTA interventions to the MG (BoNTA n=19, 9:8 (7:3-10:7)) were recruited.
10 Three-dimensional freehand ultrasound was used to estimate MG volume normalised to body
11 mass, and echo-intensity.

12 **RESULTS** Normalised MG volume and echo-intensity significantly differed between the two SCP
13 cohorts ($p \leq 0.050$), with the BoNTA cohort having larger alterations. Associations between
14 normalised MG volume and echo-intensity were highest in the No-BoNTA cohort, followed by
15 the BoNTA cohort. Multiple regression analyses revealed that both GMFCS level and BoNTA
16 intervention history significantly associated with smaller normalised MG volume and higher
17 echo-intensity.

18 **INTERPRETATION** Recurrent BoNTA interventions may induce alterations to MG volume and
19 echo-intensity, beyond the natural history of the SCP pathology.

20 **SHORTENED TITLE** Altered muscle morphology due to BoNTA

21 **WHAT THIS PAPER ADDS**

- 22 • SCP MG volumes are smaller with higher echo-intensities compared with TD
- 23 • The alterations in the BoNTA cohort are larger than in the No-BoNTA cohort
- 24 • GMFCS level and BoNTA history significantly associate with smaller MG volume and
25 higher echo-intensity

1 Muscles are the motor components of the body, with their rate of growth in the early years of life
2 crucial for achieving developmental milestones, such as crawling, standing and walking. The
3 force-generating capabilities of a muscle are largely dependent on its gross-morphological
4 properties, such as volume. In children with spastic cerebral palsy (SCP), fewer satellite cells,
5 overlengthened sarcomeres and abnormal motor unit recruitment and activation characterise the
6 underlying pathophysiology of muscle growth and joint contractures.^{1,2} Consequently, deficits in
7 medial gastrocnemius (MG) growth have been reported,³⁻⁵ a muscle with an important power
8 generation function during walking.⁶ To confound matters, magnetic resonance imaging (MRI)
9 revealed adipose tissue replacing contractile tissue in the lower limb muscles of children and
10 adolescents with bilateral SCP.^{7,8} This was corroborated using B-mode ultrasound, where the MG
11 appeared hypo-echoic with respect to typically developing (TD) peers.^{9,10} Two of these
12 investigations also identified that the extent of changes within the muscle associated with gross
13 motor function classification system (GMFCS) level,^{8,9} as did muscle volume,⁴ inferring that muscle
14 activation and level of functional ability play a meaningful role in these alterations. It is therefore
15 likely that there will be a high association between the volume and echo-intensity of the MG in
16 SCP individuals.

17 The use of botulinum neurotoxin A (BoNTA) is a common treatment modality in children with
18 SCP, targeting the hyperactive stretch reflex.¹¹ The short-term chemo-denervation at the pre-
19 synaptic membrane, combined with serial-casting and physiotherapy, has been shown to
20 improve gait function.¹² Its use is not without concern, as discussed in a review on muscle
21 deformity in children with SCP.¹³ Attempts to address the veracity of these concerns have been
22 undertaken in several longitudinal investigations, where MG volume was evaluated following
23 BoNTA interventions. A reduction was reported five weeks post-infiltration,¹⁴ but a six month
24 follow-up with a targeted strength training protocol revealed eventual gains in both volume and
25 strength.¹⁵ A long-term investigation one year post intervention found absolute MG volume to
26 increase in two cohorts of BoNTA-naive SCP children.¹⁶ However, the use of absolute volume,
27 instead of a normalised parameter, might have biased the results (not taking the interim one

1 year of growth into consideration). Based on their reported data, normalising mean MG volume
2 to mean body mass revealed a mean decrease from 1.70ml/kg to 1.62ml/kg in one cohort, and a
3 mean increase from 1.54ml/kg to 1.56ml/kg in the other cohort.¹⁶ This strengthens the notion
4 that BoNTA interventions may lead to reduced MG volume in the long-term. These insightful
5 findings regarding the use of BoNTA are of interest to the wider clinical community, but there
6 are some limitations to consider. Firstly, in two of the investigations,^{14,15} the cohorts had
7 received an unspecified number of previous BoNTA interventions, providing a challenge to
8 differentiate the influence of previous BoNTA interventions from the natural history of the SCP
9 pathology. Secondly, muscle volume does not provide insight into alterations to the
10 intramuscular composition, where a high recurrence of BoNTA infiltrations, or BoNTA
11 infiltrations delivered with a short interval, associated with neurogenic atrophy.¹⁷ In addition,
12 the number of BoNTA infiltrations to the MG significantly associated with type-1 fibre loss and
13 type-2 fibre predominance, which may impair long-term muscle function.¹⁷ Therefore, future
14 investigations evaluating the influence of BoNTA interventions should also consider alterations
15 to both contractile (myo-fibers) and non-contractile (adipose/connective tissue) tissue within
16 the injected muscle.

17 Three-dimensional freehand ultrasonography (3DfUS) is a static-condition measurement
18 modality that enables anatomical regions to be reconstructed in three-dimensions. It consists of a
19 B-mode ultrasound and motion-tracking system, where the spatial coordinates of reflective
20 markers rigidly affixed to the ultrasound probe are recorded for each acquired image.¹⁸ It requires
21 minimal acquisition time, which is favourable in measurements with younger children. Using
22 3DfUS, alterations to MG volume and echo-intensity in children with SCP were reported.^{10,19,20}
23 When visualising healthy muscles, non-contractile properties such as tendons and adipose tissue
24 appear lighter (hyper-echoic), whilst fluid and bones appear darker (hypo-echoic). A biopsy
25 analysis revealed a high association between echo-intensity and proportion of fibrous tissue in
26 the extracted sample,²¹ whilst MRI revealed high associations between echo-intensity of the MG

1 with the quantity of intramuscular adipose tissue.²² These findings suggest that echo-intensity can
2 be used as an indirect quantification of alterations to the properties within a muscle.

3 This investigation will explore the influence of BoNTA interventions on the MG volume and echo-
4 intensity in individuals SCP. A cross-sectional design will be employed, where two SCP cohorts are
5 identified, 1) a minimum of three BoNTA interventions (BoNTA cohort) and, 2) naive to BoNTA
6 interventions (No-BoNTA cohort). A matched TD control cohort will also be identified. The first
7 aim is to compare normalised MG volumes and echo-intensities between the two SCP cohorts, and
8 with respect to the TD cohort. The hypothesis states that both SCP cohorts have significantly
9 smaller MG volumes and higher echo-intensities with respect to the TD cohort, whilst the
10 alterations in the BoNTA cohort will be significantly greater with respect to the No-BoNTA cohort.
11 The second aim is to explore the association between MG volume and echo-intensity in all three
12 cohorts. The hypothesis states that there will be no meaningful association in the TD cohort, but
13 strong negative associations in the SCP cohorts. The third aim is to explore if BoNTA intervention
14 history significantly associates with smaller MG volume and higher echo-intensity, in addition to
15 GMFCS level. The hypothesis states that both GMFCS level and BoNTA intervention history will
16 significantly associate towards smaller MG volume and higher echo-intensity.

17 **METHOD**

18 **Participants**

19 Data collection was performed as part of an ongoing research project (muscle morphology and
20 function in spastic cerebral palsy), where TD children and adolescents, as well as those diagnosed
21 with SCP, were recruited through the clinical motion analysis laboratory at the University Hospital
22 of Leuven between January 2015 to September 2017. The initial diagnosis of SCP was confirmed
23 by a neuro-paediatrician, based on MRI imaging of the brain and clinical examination. To minimise
24 bias and ensure appropriate matching of two SCP cohorts, the following inclusion criteria were
25 applied to all previously acquired data: a) between 5-16 years of age, b) GMFCS levels I-II, c) no
26 previous orthopaedic or neurological surgery, d) naive to BoNTA interventions or at least six

1 months after a third or more recurrent BoNTA intervention. Following this retrospective analysis,
2 42 children and adolescents with SCP were identified that fulfilled the criteria. A BoNTA and No-
3 BoNTA cohort were then defined, matched in order of GMFCS level, topographical paresis, body
4 mass and age. This resulted in two groups of 19 participants, with the remaining four participants
5 excluded. The earliest clinical examination for each participant was also reviewed to ensure
6 comparable baseline clinical characteristics for joint range of motion, spasticity and strength at
7 the ankle (Table S1, online supporting information). The BoNTA cohort had a median five previous
8 interventions (IQR 3-6), with a median frequency of 12 months between interventions (IQR 10-
9 16). All 38 participants were under the same multi-disciplinary management, whilst the BoNTA
10 cohort all followed the same post-BoNTA treatment protocol, consisting of serial casting and
11 physiotherapy.²³ An overview of the quantity and timing of the BoNTA interventions can be found
12 in Figure S1 (online supporting information). A TD cohort was also defined, matched with respect
13 to mass and age, resulting in 67 participants (Table I). The experimental protocol was approved
14 by the local Ethical-Committee (s57234), whilst written informed consent was acquired from all
15 parents/guardians and children over the age of 12.

16 **Instrumentation**

17 A 3DfUS method was utilised for this investigation.¹⁸ This consisted of a Telemed-Echoblaster 128
18 Ext-1Z B-mode ultrasound system with a 5.9cm 10MHz linear US probe (HL9.0/60/128Z), and an
19 OptiTrack V120:Trio motion-tracking system with three fixed optical cameras (NaturalPoint,
20 USA). As echo-intensity is influenced by the ultrasound parameters, care was taken to ensure they
21 remained the same for all of the acquisitions (frequency, 10MHz; depth, 5cm; focus, 1.8-2.8cm;
22 gain, 46%; dynamic range, 44dB and unaltered time-gain compensation), whilst images were
23 recorded at 30Hz. The motion-tracking system has a 1mm spatial resolution, and images were
24 recorded at 120Hz. To acquire synchronized data, a signal trigger was utilised.¹⁸

25 **Acquisition**

1 For each SCP participant, the most affected leg was measured according to their most recent
2 clinical examination, whilst a coin-flip determined the measured leg in the TD cohort. Age and
3 body mass was recorded for each participant. In prone lying, a triangular cushion was placed
4 under the shank to provide approximately 25 degrees of knee flexion, and a cushioned belt was
5 strapped over the thigh to prevent movement of the leg (Figure 1). Two physiotherapists
6 experienced with 3DfUS and visual identification of the MG acquired all of the data, using sufficient
7 gel to avoid excess pressure on the leg. Care was taken to hold the ultrasound probe perpendicular
8 to the deep aponeurosis of the MG throughout the acquisition, enhancing the visibility of the
9 muscle border and minimising erroneous echoes. The ultrasound probe was moved in a
10 transverse orientation over the condyles of the knee, distally along the MG until the end of the
11 calcaneus. In cases of visually detected muscle contraction or movement of the leg, the acquisition
12 was repeated. Two acquisitions were acquired for each participant.

13 **Processing**

14 Three-dimensional reconstructions were created using a custom-made software package,¹⁸ and
15 processed using HOROS (www.horosproject.org). The same physiotherapist performed all of the
16 processing using the first of the two acquisitions. The MG was defined from the inferior margin of
17 the medial condyle of the tibia, until the most distal visible image of the MG.²⁴ Volume and echo-
18 intensity were estimated by drawing equally spaced transverse plane segmentations along the
19 inside of the muscle border, summing 10% of all the available ultrasound images of the MG. An
20 interpolation was applied to create segmentations in the remaining 90% of images. This reduced
21 the time required to extract MG volume and echo-intensity, whilst also maintaining sufficient
22 reliability (standard error of measurement values for estimating MG volume and echo-intensity
23 are 2.6ml and 3.3 values, respectively).²⁰ MG volume was reported in ml, and echo-intensity on an
24 8-bit greyscale (0-255 values). For the latter, the value 0 represented black and 255 white, with
25 in-between values 254 shades of grey. To enable inter-participant comparisons, MG volume was

1 normalised to body mass, reported as ml/kg.³⁻⁵ An illustration of the three-dimensional
2 acquisition and processing can be found in Figure 1.

3 **Statistical analysis**

4 Data were analysed in SPSS version 22 (SPSS, Inc., Chicago, Illinois). Normal distribution of the
5 primary outcome parameters was accepted by plotting the data and visually inspecting the
6 symmetry of the histogram. In addition, the data was also reported as median and interquartile
7 range, revealing the extent of skewness in each parameter. Equality of variance was assessed
8 using Levene's test ($p \geq 0.050$), and treated accordingly. The threshold for statistical significance
9 was two-tailed. To determine if the SCP cohorts differed at the time of their earliest clinical
10 examination, age, ankle dorsiflexion (with the knee extended), modified Ashworth score, modified
11 Tardieu scale and manual muscle testing of the plantar flexors were compared using an
12 independent samples Mann-Whitney U test ($p \leq 0.050$). To answer the first hypothesis, a one-way
13 analysis of variance and Tukey HSD post-hoc test compared age, body mass, absolute and
14 normalised MG volume, and echo-intensity between the three cohorts. To answer the second
15 hypothesis, a Pearson's correlation was used to determine the strength of the associations
16 between echo-intensity and normalised MG volume in the two SCP and TD cohort (0-0.2, poor;
17 0.21-0.4, fair; 0.41-0.6, moderate; 0.61-0.8, good and 0.81-1.0, very good).²⁵ To answer the third
18 hypothesis, multiple regression analyses were performed on the combined SCP cohorts. Both
19 GMFCS level and BoNTA intervention history (No-BoNTA cohort = group 0, BoNTA cohort = group
20 1) were entered as independent variables to determine if they both significantly associate with
21 normalised MG volume and echo-intensity. The adjusted r^2 and p-value of the model, and p-value
22 of each independent variable was calculated.

23 **RESULTS**

24 The Mann-Whitney U test revealed no significant differences in age, body mass, or outcome
25 parameters between the two SCP cohorts at their earliest clinical examination, except for
26 maximum ankle dorsiflexion ($p=0.030$) (Table SI). The Tukey HSD post-hoc test revealed no

1 significant differences between the three cohorts for age ($F_{2,102}=0.023$; $p=0.977$) or body mass
2 ($F_{2,102}=1.237$; $p=0.295$). Statistically significant differences were found for all the primary outcome
3 parameters, except absolute MG volume between the SCP cohorts (Table II).

4 In the BoNTA cohort, echo-intensity had a positive significant fair association with the number of
5 previous BoNTA interventions ($r=0.58$, $p=0.009$), and a negative non-significant fair association with
6 the frequency between recurrent BoNTA interventions ($r=-0.41$, $p=0.078$). Echo-intensity had a
7 negative significant fair, moderate and good association with normalised MG volume in the TD,
8 BoNTA and No-BoNTA cohorts, respectively (Table II, Figure 2.)

9 The multiple regression analyses revealed that both GMFCS level and BoNTA intervention history
10 significantly associate towards explaining both echo-intensity (adjusted $r^2=0.64$, model $p\leq 0.001$,
11 GMFCS level $p\leq 0.001$, BoNTA history $p\leq 0.001$), and normalised MG volume (adjusted $r^2=0.52$,
12 $p\leq 0.001$, GMFCS level $p\leq 0.001$, BoNTA history $p=0.003$).

13 **DISCUSSION**

14 The first aim of this investigation was to compare normalised MG volume and echo-intensity
15 between a SCP cohort with a minimum of three recurrent BoNTA interventions, a SCP cohort naive
16 to BoNTA interventions, and a control TD cohort. The first finding was that the normalised MG
17 volume of the TD cohort was comparable to that of previous reported values in literature,^{4,5,24} and
18 significantly larger than the normalised MG volumes of both SCP cohorts. The SCP BoNTA cohort
19 had the largest deficits in normalised MG volume, which were also significantly smaller with
20 respect to the No-BoNTA cohort. Therefore, it may be that the repeated sessions of BoNTA
21 interventions further inhibit muscle growth, beyond the natural history of the pathology.
22 Similarly, both SCP cohorts had significantly higher MG echo-intensity values with respect to the
23 TD cohort, with the BoNTA cohort also presenting with significantly higher echo-intensity values
24 with respect to the No-BoNTA cohort. Therefore, the first hypothesis, stating that both SCP cohorts
25 have smaller normalised volumes and higher echo-intensities with respect to the TD cohort, was
26 confirmed. The employed 3DfUS technique has previously been used to quantify higher MG echo-

1 intensity values in children with SCP,^{10,20} strengthening the concept that not all of the measured
2 muscle volume is functional contractile or connective tissue. This may be due to a higher
3 percentage of intramuscular fat,^{7,8} and changes to the collagen content, like the extracellular
4 matrix.²⁶ It remains to be seen at what age these alterations occur, but the importance of
5 stimulating cross-sectional muscle growth as early as possible is imperative for maximising long-
6 term muscle function.²

7 The second aim of this investigation was to explore the association between echo-intensity with
8 normalised MG volume. In all three cohorts, significantly negative associations were found, with
9 the strongest in the No-BoNTA cohort ($r=-.72$), followed by the BoNTA ($r=-.54$) and TD cohorts
10 ($r=-.27$). Therefore, the second hypothesis, stating that there will be no meaningful association in
11 the TD cohort, but strong negative associations in the SCP cohorts, was confirmed. The negative
12 association in the TD cohort may be explained by attenuation of the ultrasound beam due to the
13 much larger MG muscles, leading to a reduction in returning echoes. The high association in the
14 No-BoNTA cohort is very informative, as it suggests that alterations to normalised MG volume and
15 echo-intensity may be explained by the natural history of the pathology, likely a lack of
16 myofibrillar growth.¹³ However, the weaker association in the BoNTA cohort infers that
17 something beyond the natural history of the pathology is inducing additional alterations to
18 normalised MG volume and echo-intensity. This may be explained by BoNTA interventions
19 targeting an increase in passive ankle joint resistance due to spasticity, when reduced muscle
20 growth and altered tissue properties may have been more culpable. The resulting chemo-
21 denervation induced atrophy,¹⁴ combined with ankle joint immobilisation (serial casting), could
22 have triggered the additional alterations identified in the BoNTA cohort. Quantifying ankle joint
23 resistance and muscle activation in an objective and quantitative manner, and evaluating the
24 individual influence of both BoNTA and serial casting, may help to provide more insight into what
25 is happening to the MG and the ankle joint.^{2,27,28} Smaller MG volumes will also likely result in a
26 reduced capacity to generate sufficient active force, but the consequence of higher echo-intensity
27 values is less clear. The understanding of the implications may be supported by earlier research.

1 An increase in passive tissue stiffness of the SCP MG has been identified, quantified according to
2 the shear wave velocity of ultrasound elastography.²⁶ A subsequent investigation found that
3 differences in shear wave velocity between the non-paretic and the paretic muscles of individuals
4 with stroke were strongly associated with differences in echo intensity.²⁶ Based on this, higher MG
5 echo-intensity may be indicative of an increase in passive stiffness of the muscle properties. If so,
6 it would be of great interest to determine whether the MG is able to increase in length following
7 BoNTA and serial casting, or whether it is inhibited by the altered tissue composition.
8 Additionally, the ability to recruit and activate motor units is also a known impairment in children
9 and adolescents with SCP.¹ Exploring if higher echo-intensity values with measures are related to
10 reduced isometric force- and rate of torque-generation, as seen in sarcopenia populations,²⁹
11 would also provide another clinical meaning to higher echo-intensity values in individuals with
12 SCP.

13 The third aim of this investigation was to explore whether in addition to GMFCS level and age,
14 BoNTA intervention history significantly associates with alterations in normalised MG volume
15 and echo-intensity. GMFCS level was included, as it has previously been shown to associate with
16 MG volume and echo-intensity, representing the natural history of the SCP pathology in the No-
17 BoNTA cohort.^{4,8,9,24} The multiple regression analyses revealed that both BoNTA history and
18 GMFCS level significantly associate with both echo-intensity and normalised MG volume in the
19 combined SCP cohort, thereby confirming the third hypothesis. To the best of our knowledge, this
20 is one of the strongest reported findings of alterations to MG morphology due to recurrent BoNTA
21 interventions. This is also supported from a clinical perspective, where it was suggested that the
22 efficacy of BoNTA interventions to improve overall functional ability in children with SCP may
23 diminish after three recurrent treatments.²³ However, as BoNTA interventions have been
24 reported to yield functional benefits, at least in the short-term, it is worthwhile to determine if
25 treatment goals can still be attained with a smaller BoNTA dosage, perhaps calculated with respect
26 to the normalised MG volume or echo-intensity, instead of body mass.³⁰

1 Limitations

2 Determining the effects of an intervention is preferable with a longitudinal investigation design.
3 However, evaluating the long-term influence of BoNTA interventions on muscle morphology
4 would span the course of many years, with various feasibility issues. Therefore, this investigation
5 utilised a cross-sectional design, where careful attention was placed on matching two SCP cohorts
6 with differing treatment histories (BoNTA/No-BoNTA). Based on the ankle impairments from
7 their earliest clinical examinations, we did not identify any statistically significant differences,
8 aside from slightly less dorsiflexion in the BoNTA cohort, which may have been due to insufficient
9 power. Nonetheless, it remains unclear why the treatment histories differed, but it is likely related
10 to a more comprehensive evaluation of impairments. This would also include the knee and hip
11 joint levels, degree of pathological gait, and also considering both patient and family preferences
12 with respect to treatment goals.¹¹ Furthermore, as a consequence of the research design, this
13 investigation is unable to delineate between the individual influence of BoNTA infiltrations, and
14 the subsequent protocol of serial casting and physiotherapy. As mentioned earlier, there was a
15 mild attenuation of the US beam when visualising the largest MG muscles in the TD cohort. This
16 effect seemed confined to the TD cohort, as the largest MG volume in the SCP cohort was around
17 the mean of the TD cohort. Future investigations may aim to negate this problem by altering the
18 ultrasound time-gain-compensation settings, however that may introduce new challenges for
19 inter-participant comparisons of echo-intensity. Another possibility is to apply a correction to the
20 ultrasound images that takes into consideration the thickness of the subcutaneous tissue and
21 depth of the muscle.²²

22 CONCLUSION

23 This investigation identified that a history of three or more BoNTA interventions has an additional
24 negative influence on normalised MG volume and echo-intensity, beyond the natural history of
25 the SCP pathology. This is a novel finding and warrants further confirmation. It may be that BoNTA
26 interventions associate with differences seen at the muscle (smaller volume, higher echo-

1 intensity), as we cannot conclude whether they are the cause of the altered muscle properties.
2 However, as a lack of muscle growth and increased passive stiffness seem to be key factors in the
3 presence of joint contractures, it is important to be aware of the potential risks of recurrent
4 BoNTA interventions in individuals with spastic cerebral palsy.

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25

Table I. Overview of participant characteristics

Cohorts	TD (n=67)	SCP No-BoNTA (n=19)	SCP BoNTA(n=19)
	Median (IQR)		
Age (y:mo)	9:11 (7:10-11:6)	9:8 (7:3-10:7)	9:3 (8:5-10:10)
Mass (kg)	29.6 (24.3-39.8)	29 (20.5-42)	26.5 (22.9-29.7)
Sex	43M / 24F	10M / 9F	13M / 6F
Topographic Paresis	NA	9BL / 10UL	9BL / 10UL
GMFCS levels	NA	13 I / 6 II	13 I / 6 II

TD, typically developing; SCP, spastic cerebral palsy; SD, standard deviation; BL, bilateral; UL, unilateral; GMFCS, gross motor function classification system; NA, not applicable

Table II. Overview and comparison of the primary outcome parameters between all three cohorts, and the associations between medial gastrocnemius mean echo-intensity and normalised muscle volume.

Outcome Parameters	TD (n=67)	SCP No-BoNTA (n=19)	SCP BoNTA (n=19)	TD vs. SCP No-BoNTA	TD vs. SCP BoNTA	SCP No-BoNTA vs. SCP BoNTA
	Median (IQR)			Independent samples t-test (p≤0.050)		
Echo-intensity (0-255)	99.8 (93.6-106.5)	117.1 (110.7-121.2)	124.9 (120.6-132.9)	≤0.001	≤0.001	0.004
MG volume (ml)	62.0 (49.7-81.8)	44.4 (33.7-58.9)	31.5 (25.3-44.0)	=0.004	≤0.001	0.140
Normalised MG volume (ml/kg)	2.1 (1.8-2.3)	1.5 (1.3-1.7)	1.2 (1.0-1.6)	≤0.001	≤0.001	0.032
Pearson Correlation						
(echo-intensity vs. normalised MG volume)		TD (n=67)	SCP NoBoNTA (n=19)	SCP BoNTA (n=19)		
r		-0.27	-0.72	-0.54		
p value (significance at ≤0.050)		0.025	≤0.001	0.016		

TD, typically developing; SCP, spastic cerebral palsy; NoBoNTA, cohort naïve to previous BoNTA interventions; BoNTA, cohort with a minimum of three previous BoNTA interventions

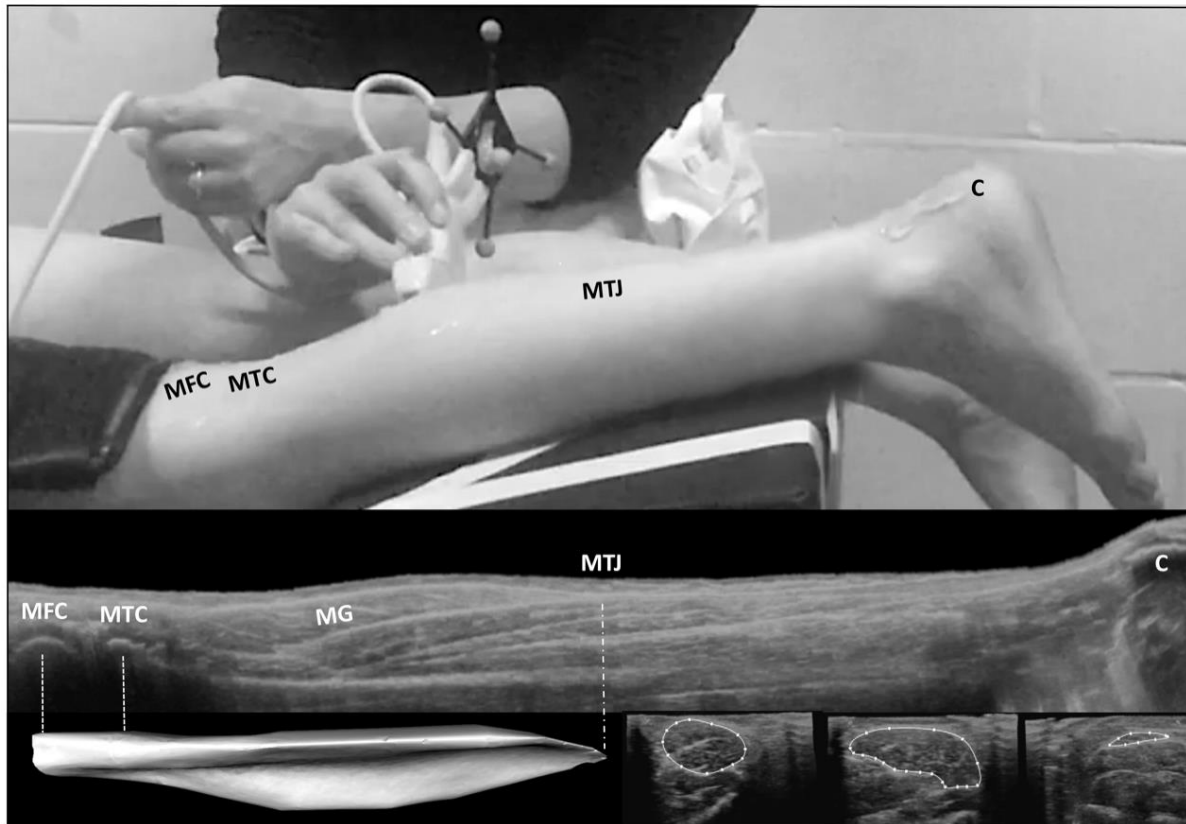


Figure 1: Upper image: An example of the 3DfUS measurement acquisition with the instrumented US probe. Centre image: A sagittal plane image extracted from the 3D reconstruction. Lower image: Examples of muscle border segmentations in the transverse plane images, and the resulting interpolated 3D reconstruction of the medial gastrocnemius. MFC, medial femoral condyle; MTC, medial tibial condyle; MTJ, muscle-tendon junction; C, calcaneus; MG, medial gastrocnemius.

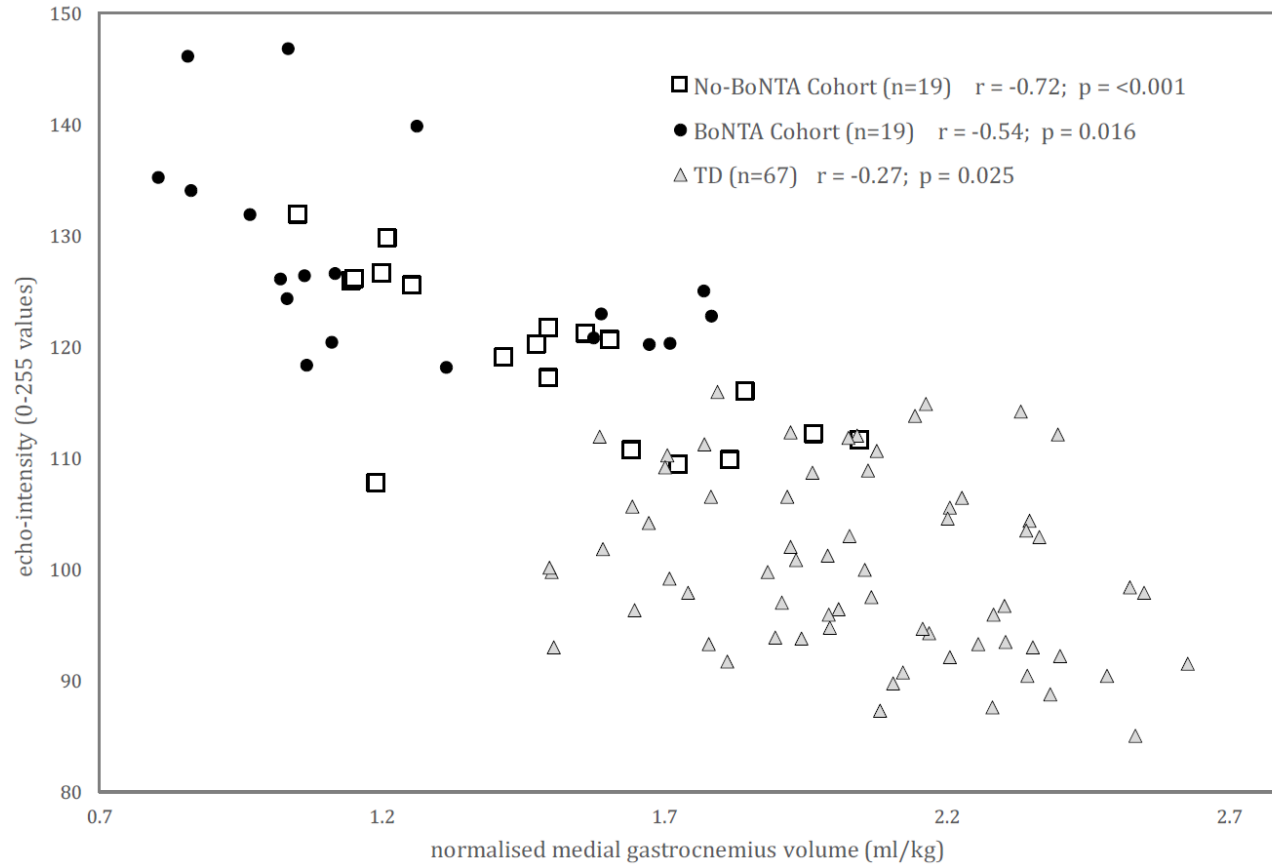


Figure 2: No-BoNTA, cohort naive to previous BoNTA interventions; BoNTA, cohort with a minimum of three previous BoNTA interventions; TD, typically developing control cohort.

SUPPLEMENTARY INFORMATION

Table SI – Comparisons between the two spastic cerebral palsy cohorts at the time of their earliest clinical examinations.

Cohort	TD	SCP No-BoNTA	SCP BoNTA	SCP No-BoNTA (n=19) vs.
	(n=67)	(n=19)	(n=19)	SCP BoNTA (n=19)
	Median (Interquartile Range)			p-value (significance at ≤ 0.050)
Age (y:mo)	-	6:2 (4:7 – 8:8)	5:3 (4:8-5:8)	0.093
Max DF (°)	-	10 (10 - 15)	5 (4 - 10)	0.030
MAS	-	2 (1.5 - 2)	2 (1.5 - 2)	0.523
MTS (°)	-	-10 (-15 - -5)	-10 (-16 - -5)	0.975
MMT - PF	-	3.6 (3.6 - 4)	3.6 (3.5 - 4)	0.684

TD, typically developing; SCP, spastic cerebral palsy; NoBoNTA, cohort naïve to previous BoNTA interventions; BoNTA, cohort with a minimum of three previous BoNTA interventions; Max DF, maximum dorsiflexion with the knee extended; MAS, modified Ashworth scale; MTS, modified Tarideu scale; MMT - PF, manual muscle testing of the plantar flexors.

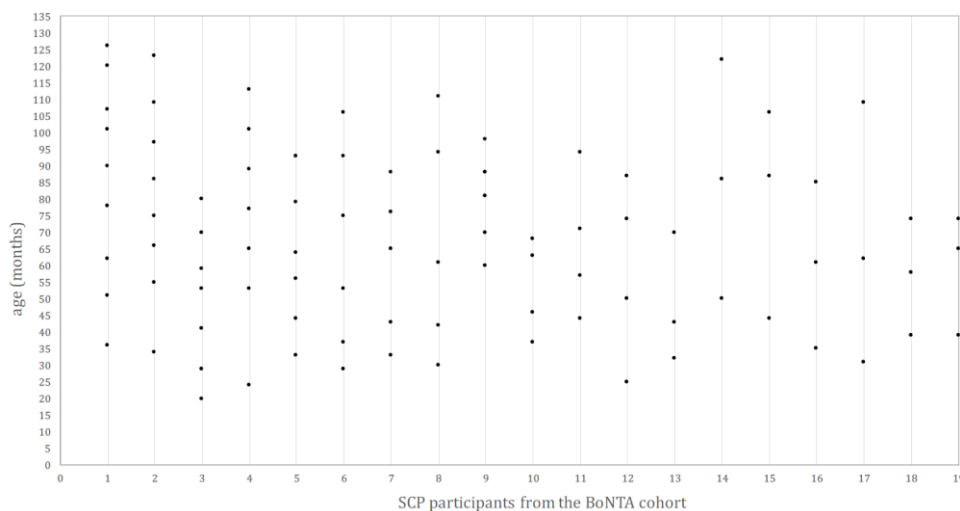


Figure S1: Each black dot represents a BoNTA intervention. The quantity of dots on each vertical line indicates the total number of previous BoNTA interventions to the medial gastrocnemius, whilst the gap in-between each dots indicates the time in months between recurrent interventions.

