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Quantitative assessment of spasticity-related and treatment-induced changes in muscle properties of hemiparetic patients

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Summary

Stroke is a leading cause of long-term disability worldwide. Many stroke survivors develop spasticity syndrome, a complex condition resulting from the interaction between neural abnormalities and changes in the muscles and soft tissues. While spastic muscle overactivity is well recognized and routinely targeted in clinical practice, the non-neural component of spasticity syndrome remains insufficiently understood. Structural and mechanical changes of muscles may contribute substantially to movement limitations, reduced mobility, and impaired independence and quality of life after stroke.

The overall objective of this thesis was to improve the understanding of post-stroke muscle changes and to investigate the effects of Botulinum toxin type A (BoNT-A), one of the most commonly used treatments for spastic muscle overactivity, on muscle properties. Particular attention was given to the triceps surae muscle group, which plays a key role in gait and is frequently affected after stroke.

To address these objectives, a series of complementary studies was conducted. First, a systematic review and meta-analysis synthesized the available evidence regarding structural and mechanical muscle changes following BoNT-A injections in neurological populations. Second, a quantitative MRI study investigated the effects of repeated BoNT-A injections on calf muscle structure in individuals with chronic stroke. Third, an ultrasound elastography study aimed to optimize the assessment of muscle stiffness by evaluating the influence of ankle position and muscle activation on measurement reliability. Finally, a longitudinal study was designed to characterize the evolution of muscle structure and stiffness from the acute phase after stroke using MRI and shear wave elastography.

By combining advanced imaging techniques, including magnetic resonance imaging and ultrasound, this work provides new insights into the mechanisms underlying post-stroke muscle remodeling and their evolution over time. Ultimately, a better understanding and quantification of these changes may contribute to more individualized rehabilitation strategies and improved management of spasticity syndrome after stroke.

Quantitative assessment of spasticity-related and treatment-induced changes in muscle properties of hemiparetic patients.

Part I. General introduction

Chapter 1. Pathologies of the central nervous system and motor impairments

Pathologies of the central nervous system such as stroke, cerebral palsy, multiple sclerosis, traumatic brain injury and other, impact functioning of an individual. Their global prevalence has increased in the past years. Stroke currently remains the leading contributor to mortality and disability in neurologic disorders among adults (1).

Stroke, or a cerebrovascular accident, is a leading cause of long-term disability worldwide. It is estimated that 12 million people in the world (2) and 1.8million people in Europe (3) become stroke victims every year, with a lifetime risk of stroke around 25%. In Belgium, number of all stroke admissions in 2023 was approximately 29000 individuals (4). In 2021, there were approximately 94 million people living with the effects of stroke worldwide (2). Stroke constitutes an important part of health care cost. The estimated global cost of stroke is over US\$890 billion per year, and is projected to almost double by 2050 (5). In the EU, the direct healthcare cost of stroke was estimated at €60 billion in 2017 (6).

Stroke is defined as a neurological deficit persisting for more than 24 hours and resulting from a focal vascular injury within the central nervous system (7).

This event arises from a disruption of cerebral blood flow, which deprives neurons of oxygen and essential nutrients, leading to cellular injury and death (8). Two major subtypes are recognized: ischemic stroke, accounting for approximately 70-80% of cases, results from the occlusion of a cerebral vessel by a clot, whereas hemorrhagic stroke (around 20-30% of cases) is caused by the rupture of a blood vessel, leading to intracerebral or subarachnoid bleeding (2,9) (Figure 1). The incidence of ischemic stroke tends to be higher in high-income countries (2).

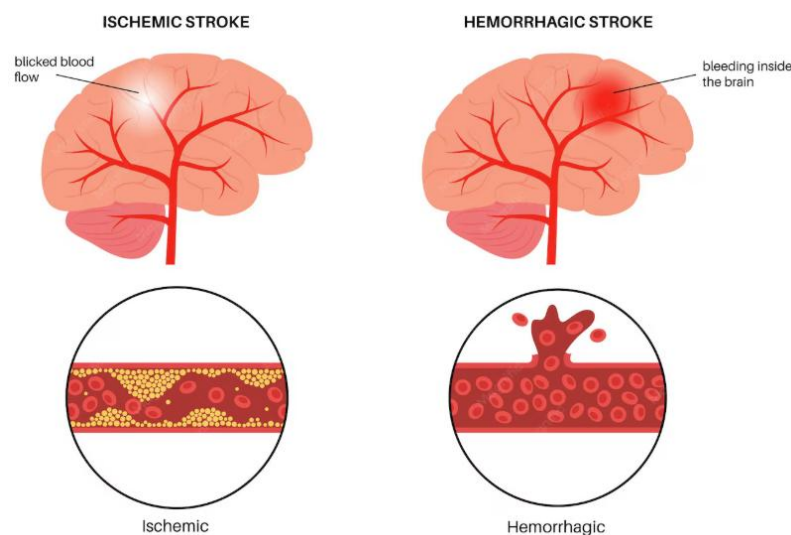


Figure 1. Ischemic and hemorrhagic stroke. Reproduced from magnifik.com.

The International Classification of Functioning, Disability and Health (ICF), developed by the World Health Organization and adopted in 2001, provides a standardized and comprehensive framework for describing health and health-related states (10) (Figure 2).

According to this model, the consequences of a health condition can affect three components: body function (e.g. muscle tone; ICF code b735) and structure (e.g. muscles of lower leg; ICF code s75012), activities (e.g. walking; ICF code d450), and participation (e.g. recreation and leisure; ICF code d920). Impairments (which refer to a problem in body function or structure, e.g. spasticity), activity limitations, and participation restrictions are dynamically interconnected. Furthermore, these components are influenced (positively or negatively) by both environmental factors (such as the availability of the family and/or friends; ICF code e3) and personal factors (such as the patient's age). The ICF has been increasingly integrated into clinical practice and research for stroke rehabilitation. It provides a common language between healthcare professionals, facilitating interdisciplinary communication and ensuring that interventions target not only impairments but also functional consequences and contextual barriers.

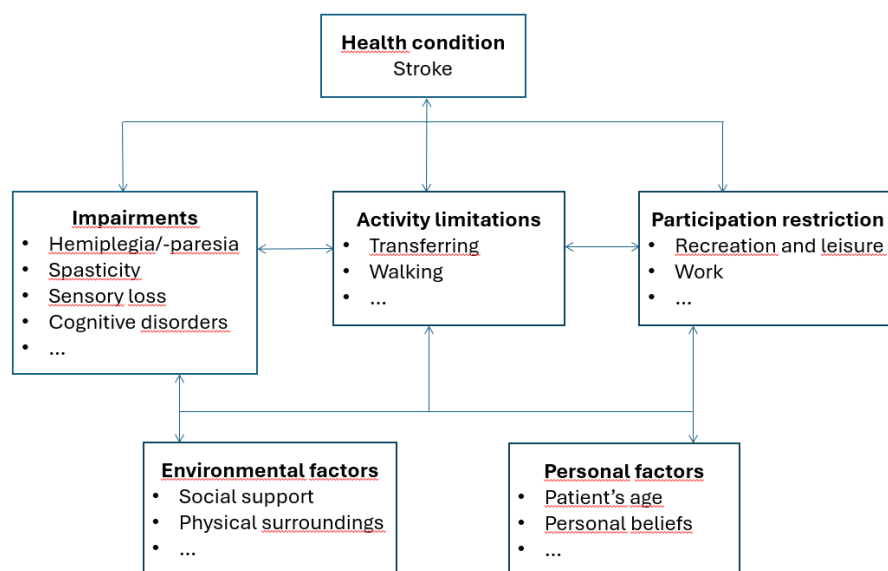


Figure 2. International Classification of Functioning, Disability and Health (ICF) framework, adapted for stroke survivors.

The clinical consequences of a stroke largely depend on the location, extent and severity of the cerebral lesion. The neuronal loss can induce a wide range of motor, sensory, and cognitive impairments, which frequently lead to limitations in daily activities and restrictions in participation with decreased quality of life (11). Neurological deficits are typically contralateral to the lesion site and are commonly associated with the upper motor neuron syndrome. This syndrome encompasses both negative and positive signs. Negative signs include muscle weakness or paresis and fatigability. Positive signs are characterized by hyperreflexia, spasticity, and various forms of abnormal muscle overactivity, such as clonus, spastic dystonia, co-contraction, associated reactions, and spasms (12,13).

In clinical practice and research, the type and severity of impairments, activity limitations, and participation restrictions are assessed using standardized rating scales. Numerous tools have been developed to capture the wide range of symptoms and impairments observed after a stroke, yet the large number of available outcome measures leads to considerable variability in clinical use. Standardization efforts have therefore led to the development of stroke-specific evaluation core sets, which also specify recommended time points for conducting assessments. Regarding clinical motor rehabilitation after stroke, the study by Pohl *et al.* identified a core set of nine outcome measures to be used across seven measurement time points that reflect the stages of post-stroke recovery (14).

Chapter 2. Spasticity syndrome

Poststroke spasticity constitutes a significant therapeutic challenge. It represents a major contributor to motor impairment, disability, reduced health-related quality of life, and elevated healthcare costs (15). It commonly develops within days to weeks following stroke. A recent meta-analysis found a prevalence of spasticity after a first-ever stroke to be around 27% (16). However, the reported prevalence of post-stroke spasticity varies markedly across studies, ranging from 4% to 64% (13,17). This heterogeneity likely reflects differences in sample size, temporal phase of assessment (prevalence appears to increase with time post-stroke (13)), functional level at admission (incidence appears to be higher in stroke patients with severe paresis and higher NIHSS score (16,18,19)), evaluated lower and/or upper limb (only a few studies focused specially on the lower limb), case definitions, diagnostic thresholds,...This underscores the need for a standardized definition and reliable measurement framework to enhance comparability across studies and strengthen the evidence base for targeted therapeutic interventions.

2.1 Definition(s) of spasticity and spasticity syndrome

The concept of spasticity was first introduced by Lance in 1980, who defined it as “a motor disorder characterized by a velocity-dependent increase in tonic stretch reflexes (muscle tone) with exaggerated tendon jerks, resulting from hyperexcitability of the stretch reflex, as one component of the upper motor neuron (UMN) syndrome” (20). This seminal definition rapidly gained wide acceptance among clinicians and researchers, underscoring the need to delineate the phenomenon more precisely.

Since then, numerous alternative definitions of spasticity have been proposed. Over the subsequent decades, these definitions have evolved in parallel with advances in the understanding of the underlying neurophysiological mechanisms (21–23). Despite these developments, no universal consensus was yet achieved until now regarding a single, comprehensive definition of spasticity. The main divergence among authors concerned whether spasticity should be defined based on its physiological mechanisms or its observable clinical manifestations.

Since 2024, an international spasticity redefinition workgroup (including many authors of the previous definition) has been examining the issue and has reached a consensus definition, which was presented at the congress of the International Society of Physical and Rehabilitation Medicine Congress in November 2025 and is accepted for publication. Spasticity can now be defined as “a disorder of sensorimotor control resulting from upper motor neuron disease. It is characterized by velocity- and length-dependent involuntary muscle overactivity, which is intermittent or sustained, during passive stretch.”(24). However, in both clinical and research contexts, the term “spasticity” is not only used to describe a spasticity “stricto sensu” (as described above) but also frequently as an umbrella term to encompass all abnormal muscle overactivity associated with upper motor neuron lesions. In a review of 250 studies, 35% simply equate spasticity with increased muscle tone, and nearly the same number either fail to define it or use their own definitions of spasticity (25,26).

An European Consensus published in 2017 emphasized the need to move beyond a purely neuro-centric view of “spasticity”. Indeed, van den Noort *et al.* (27) highlighted that hyper-resistance (describing the phenomenon of impaired neuromuscular response during passive stretch) results from both

neural and non-neural components, underlining that changes in peripheral tissues contribute significantly to the patient's clinical presentation. The non-neural (tissue related) component consists of muscular adaptations such as muscle stiffness (loss of extensibility) but also soft tissue and articular changes. Muscle stiffness can be further divided into elasticity (the ability to deform under an external force and return to baseline), viscosity (the internal resistance of muscle tissue to motion or deformation) and muscle shortening. Neural components (central nervous system related) include velocity dependent stretch increase in muscle tone (spasticity stricto sensu) and non-velocity dependent spastic dystonia and co-contractions. The authors recognize that the two components interact and that distinguishing between them can be challenging. However, hyper-resistance refers to passive stretch and not active assessment (i.e. spastic co-contraction). Moreover, the concept of spastic muscle paresis is not included within this framework.

Building on this dual-component framework, Baude *et al.* (22) examined in 2019 the interactions between UMNS and the muscles and soft tissues in which it manifests. They proposed the term “deforming spastic paresis” to describe the clinical syndrome resulting from lesions of the corticospinal pathways. This syndrome encompasses two intrinsically linked and mutually influencing components (22). The muscular disorder, referred to as spastic myopathy, arises from hypo-mobilization of the muscle in a shortened position during the early phase of paresis. This leads to progressive muscle shortening and loss of extensibility (stiffness) by increased muscle viscosity and elasticity, particularly obvious when applying high tension. The neurologic disorder is caused by the CNS lesion itself and further promoted by sensorimotor restriction in the context of paresis and by the muscle disorder itself.

It is characterized by two principal components that coexist around each joint and act synergistically to impede voluntary motor performance: muscle overactivity in antagonist muscles and stretch-sensitive paresis affecting the agonist muscles. Spastic muscle overactivity comprises 3 major forms of increased involuntary recruitment of motor units:

- 1) spastic dystonia defined as an unwanted, involuntary muscle activity at rest, in the absence of any phasic stretch or voluntary activity, but sensitive to tonic stretch;
- 2) spastic co-contraction, defined as unwanted, involuntary muscle activity in the antagonist muscle, during voluntary effort of the agonist, aggravated by antagonist muscle stretch;
- 3) spasticity, an enhancement of the velocity-dependent responses to phasic stretch, detected and measured at rest. As for stretch-sensitive paresis, it corresponds to a decreased central command to the agonist muscle, aggravated by antagonist muscle stretch.

However, all these definitions and descriptions are inadequate in describing the totality of the patient's problems and reported symptoms related to UMNS (i.e. tightness, stretch-induced pain,...). A broader, more comprehensive description was then proposed instead of a specific definition. To integrate these observations into a comprehensive framework, the ToxNet group agreed on introducing a new term to describe the clinical presentation of "spasticity syndrome" (28). It represents a constellation of neurological signs and symptoms secondary to upper motor neuron pathology, accompanied by non-neural peripheral tissue changes.

The non-neural component of spasticity syndrome refers to the structural and mechanical alterations in muscles and soft tissues that contribute to hyper-resistance during passive movement, independently of neural reflex activity. Following upper motor neuron lesions, chronic muscle overactivity, immobility, and altered loading conditions promote a cascade of peripheral adaptations. Passive musculoskeletal stiffness refers to the resistance offered by muscles, tendons, ligaments, and other soft tissues when a joint is moved passively, that is, without any voluntary muscle activation. This passive musculoskeletal stiffness arises from the intrinsic properties of the tissues (such as elasticity and viscosity) as well as from pathological adaptations including muscle shortening, increased collagen deposition, connective-tissue thickening, fibrosis, and the development of contractures, which represent a fixed and often irreversible loss of joint range of motion due to chronic tissue remodelling. These alterations reduce the ability of muscle and fascial tissues to lengthen, thereby increasing the passive resistance encountered when a joint is moved without voluntary activation. Importantly, both neural and non-neural components interact: sustained neural overactivity can accelerate structural changes, while increased passive stiffness can further amplify reflex responses. Recognizing the non-neural contribution and direct quantification of the different components is therefore essential for accurate assessment and the selection of targeted therapeutic interventions within the broader framework of spasticity syndrome.

In this manuscript, the term “muscle overactivity” will be used to describe the broader pathophysiological phenomenon, while “hyper-resistance” designates the assessment outcome characterized by excessive resistance during passive joint movement, resulting from both neural and non-neural

components. The term “spasticity” refers to the new definition also referred as “spasticity stricto sensu”. The constellation of clinical manifestations following upper motor neuron lesions, including symptom, spastic muscle overactivity, spastic muscle paresis and soft tissue changes, is herein designated as the “spasticity syndrome”. Furthermore, throughout this thesis, the term “muscle structure” refers to morphological and compositional characteristics of skeletal muscle, whereas “muscle stiffness” is discussed as a mechanical property resulting from these structural features. The term “muscle properties” is used in this thesis as an umbrella term encompassing both structural characteristics and mechanical properties (stiffness) of skeletal muscle.

It should be noted that in clinical context, the terms stiffness, elasticity, and viscosity are often used in a functional rather than strictly mechanical sense, which may differ from their formal definitions in classical mechanics. To clarify interpretation, these properties can be conceptualized using a spring–damper model. Stiffness refers to the resistance of a material or tissue to deformation, typically represented as the slope of the force (F) – displacement (Δx) relationship of a spring ($K = F/\Delta x$, unit: N/m). The inverse term, elasticity (compliance), describes the ability of a structure to deform under load ($C = 1/K$, unit: m/N). Viscosity (unit: N·s/m), describing the damping effect, can be defined as the ratio between an external force expressed in N and the speed of deformation expressed in m/s. However, the terms used throughout this manuscript are used in a clinical and functional sense, rather than according to strict biomechanical definitions.

2.2 Assessment of neural and non-neural components

Functional assessment is a cornerstone of post-stroke management, providing objective measures of impairment and recovery potential. It supports prognostic evaluation, guides the design of individualized rehabilitation programs, and enables clinicians to monitor treatment efficacy and adjust therapeutic strategies (29). Moreover, functional assessment informs decisions regarding discharge planning, long-term care needs, and social reintegration. By offering standardized, quantifiable data, it also underpins evidence-based practice and contributes to the continuous improvement of stroke care quality (14).

Clinical scales have no instrumental requirement; they can only classify subjective information. The Modified Ashworth Scale (MAS) is one of the most widely used clinical tools for assessing “spasticity”. It grades the perceived increase in muscle tone by passively moving a limb at a constant, moderate speed (that of the movement of a body segment if let to fall under gravity, approximately 1 second) through its range of motion and scoring the examiner’s impression of resistance (30) (Table 1). The MAS is simple and quick to administer, which makes it widely accepted and familiar, facilitating comparisons across studies and clinical settings. However, several studies have questioned the validity of the MAS for measuring spasticity, as it often does not correlate strongly with objective neurophysiological (31) or biomechanical (32) measures. Moreover, it primarily measures resistance to passive movement, which may reflect not only spastic muscle overactivity (neural component) but also non-neural factors such as muscle stiffness.

Furthermore, its reliability has shown to be insufficient.

A meta-analysis published in 2018 evaluated the inter- and intra-rater reliability of the MAS across 33 studies involving 1065 neurological participants with spasticity (33). Mean inter-rater ICC for lower limbs was 0.686 (95% CI 0.563–0.780) and for upper limbs 0.781 (95% CI 0.679–0.853), while intra-rater ICCs were 0.644 (lower limbs) and 0.748 (upper limbs), indicating only moderate reliability. Kappa coefficients were generally low to moderate (e.g., $\kappa \approx 0.360$ lower limbs, $\kappa \approx 0.625$ upper limbs), highlighting variability in consistency between raters. These findings suggest that MAS reliability is variable and often limited, especially for lower limb assessments.

Table 1. Modified Ashworth Scale.

Grade	Description
0	No increase in muscle tone
1	Slight increase in tone, characterized by a catch and release or by minimal resistance at the end of the range of motion (ROM)
1+	Slight increase in tone, manifested as a catch, followed by minimal resistance throughout the remainder (less than half) of the ROM
2	More marked increase in muscle tone through most of the ROM, but affected part(s) easily moved
3	Considerable increase in muscle tone, passive movement difficult
4	Affected part(s) rigid in flexion or extension

The Modified Tardieu Scale (MTS) was developed to overcome some of the limitations of the MAS by considering the velocity-dependent nature of spasticity. It assesses the muscle's response to passive stretch at 3 different velocities and records both the angle of muscle reaction and the quality of the muscle response (34) (Table 2). The limb is moved passively as slow as possible (V1) (to assess passive range of motion and non-neural stiffness), at the speed of limb segment falling under gravity (V2) and as fast as possible (V3) (to elicit the stretch reflex). The angle (R1) at which the first "catch" or clonus is felt during a fast stretch (V3), reflects neural contribution to hyper-resistance. The full passive range of motion (R2) during slow stretch (V1), reflects mechanical properties. R2–R1 difference indicates spasticity (larger differences suggest greater spasticity).

Studies on reliability have shown that the MTS demonstrates slightly better results than the MAS. Shu *et al.* systematically reviewed the reliability of the MTS in upper limbs among adults with neurological conditions (35). Six articles reported data on reliability. ICC values ranged from 0.56-0.851 for inter-rater reliability, and from 0.70-0.998 for intra-rater reliability. Although the selected papers showed positive results regarding the reliability of MTS in upper limbs, further analysis revealed methodological weaknesses and low study quality affected the credibility of the results. To date, no systematic review or meta-analysis has specifically synthesized the reliability evidence of the MTS for lower limb muscles. The lower limb evidence remains scattered across individual reliability studies (36,37).

Table 2. Modified Tardieu Scale.

Velocity	Description
V1	As slow as possible
V2	Speed of the limb segment falling under gravity
V3	As fast as possible (> natural drop)

Grade	Description
0	No resistance throughout passive movement
1	Slight resistance throughout, no clear catch at a precise angle
2	Clear catch at a precise angle, followed by release
3	Fatigable clonus (< 10 seconds) at a precise angle
4	Infatigable clonus (> 10 seconds) at a precise angle

Angle	Description
R1	Angle of catch following a fast-velocity stretch (V3)
R2	Passive range of movement achieved following a slow-velocity stretch (V1)
R2-R1	The difference between these angles indicates the dynamic component of spasticity. A large difference suggests significant dynamic spasticity, while a small difference suggests structural contracture

These two scales are widely used in research and are considered the gold standard for evaluating the construct validity of other assessment tools. Therefore, they should not be excluded from clinical practice; rather, their results should be integrated into a comprehensive evaluation that includes additional assessment methods. However, their limitations must be acknowledged: the velocity of stretch and the level of muscle activity are not controlled, and the outcome of these tests ultimately relies on a numeric value assigned by the examiner, based both on subjective perception and on joint angle measurement with goniometry. Finally, as mentioned above, both the MAS and the MTS are unable to distinguish between the neural and non-neural components of muscle hyper-resistance. The resistance perceived during passive stretch reflects the combined contribution of reflex-mediated muscle overactivity and alterations in the passive mechanical properties of muscles and surrounding soft tissues. Consequently, a given score may result from markedly different underlying mechanisms. For example, a patient presenting with a MAS score of 2 may exhibit hyper-resistance predominantly related to stretch reflex hyperexcitability, structural muscle changes such as fibrosis, muscle shortening, and increased passive stiffness, or a combination of both. Since neither the MAS nor the MTS allows these mechanisms to be quantified separately, interpretation of the results remains challenging. This limitation has important clinical implications, as treatment strategies differ according to the predominant underlying mechanism. For example, interventions targeting neural overactivity, such as BoNT-A injections, may be less effective when muscle hyper-resistance is mainly attributable to fixed structural changes. Therefore, relying solely on those two clinical scales may lead to an incomplete characterization of the patient's condition and potentially suboptimal therapeutic decision-making.

A goniometer is a widely used and practical clinical instrument allowing more precise measurement of joint range of motion. However, it presents several limitations. While providing an objective and quantitative method to evaluate joint mobility, it does not explain the underlying cause responsible for movement limitation, such as muscle contracture, structural alterations in tendons and joints or spasticity (38). Furthermore, although goniometric measurement is relatively simple and inexpensive, its accuracy depends on proper positioning, consistent technique, and the examiner's experience, making standardized procedures essential for reliable results (38).

The mechanisms through which an initially neurological disorder evolves into a pathological interplay between muscular and nervous system dysfunction—each amplifying the other—need to be better understood to move beyond the repetitive assessment of ‘increased tone’ in paretic patients. To address the shortcomings of existing clinical scores, objective measures have been investigated in recent decades. Researchers and engineers have applied technical advances to obtain objective data for the quantitative measurement and study of spasticity syndrome. One published review discusses several advanced quantitative post-stroke spasticity measurement methods for both research and clinical purposes (39). A first step involves quantifying the neural and non-neural components of post-stroke spasticity syndrome to understand the individual patient's present condition and evaluate different intervention strategies. The degree of involvement of neural and non-neural components is specific to each patient and changes over time. Distinguishing between the two components is highly relevant to provide tailored treatment.

2.2.1 Quantification of non-neural contribution

Although the primary injury occurs in the brain, many interventions—such as strengthening and stretching—are applied at the level of the muscles and tendons. However, these treatments are often insufficient for achieving long-term symptom control. Therefore, gaining a clearer understanding of how muscle properties are altered in spastic muscles after stroke is essential for guiding more effective treatment decisions. In the following section, selected tools for the assessment of muscle properties are presented; nevertheless, this overview is not exhaustive.

A) Elastography

Elastography is a medical imaging technique based on ultrasonography principles that measures variations in elastic stiffness. Clinicians using elastography sometimes use the term “elasticity” to refer to elastic stiffness, employing this terminology to differentiate between “stiff” and “elastic” tissues. In an ideal sense, a perfectly “elastic” structure would exhibit minimal “stiffness,” and vice versa. However, elastography devices specifically quantify elastic stiffness. Today, elastography is used as a diagnostic and assessment tool in various medical contexts, especially for evaluating breast, liver, and muscle tissues. The two major techniques used in musculoskeletal elastography are strain sonoelastography and shear wave elastography (SWE).

Strain elastography measures the deformation of a tissue when a manual compressive force is applied with the handheld US transducer. The underlying principle of this technique is that soft tissues deform, or strain, more than stiff tissues when exposed to the same applied stress.

The resulting differences in tissue strain are displayed on the elastogram, usually through color or grayscale contrasts. In these images, variations in color or shading reflect different levels of tissue stiffness. In SWE, an acoustic compressive radiation force is applied to tissues instead of external compression. The generated shear waves in the tissue then propagate perpendicular to the ultrasound beam, allowing for the measurement of tissue elastic stiffness (Figure 3). In the medical literature, the shear wave velocity (SWV) (displayed in meter per second), the shear modulus (expressed in kPa) and the Young's modulus have been used in reporting outcomes. SWV is influenced by material properties, with shear wave traveling faster through stiffer tissues. The distinction between SWE and strain elastography lies in how they assess tissue stiffness. Strain elastography primarily measures elastic stiffness longitudinally by applying manual compression with the probe, whereas SWE evaluates elastic stiffness perpendicular to the ultrasound beam without requiring manual compression. This ability to measure stiffness perpendicularly gives SWE a major advantage, allowing for objective and quantifiable assessments of tissue elastic stiffness.

In a systematic review and meta-analysis of Miller *et al.* (40) the reliability of US elastography was shown to be moderate in evaluating in vivo muscle elastic stiffness in neurological populations. It also showed good convergent validity with relevant clinical assessments, and good divergent validity in discriminating tissue changes within and between groups. However, there are several limitations associated with the use of SWE. Measurements are highly dependent on muscle state, as SWV varies with muscle length, passive tension, and even low-level involuntary activation, which are difficult to fully control in patients with spasticity syndrome.

In addition, the presence of increased subcutaneous fat or oedema may further compromise signal quality and measurement reliability. Therefore, SWE is more suitable for superficial muscles, as the assessment of deeper muscles is technically challenging due to signal attenuation, reduced spatial resolution, and interference from overlying tissues. Finally, no standardized measurement protocol exists regarding which method (type of USE, control of the exerted pressure, number of regions of interest (ROI)/measurements, probe position, joint angle, etc.) may be optimal for assessing muscle elastic stiffness among patients with neurological conditions.

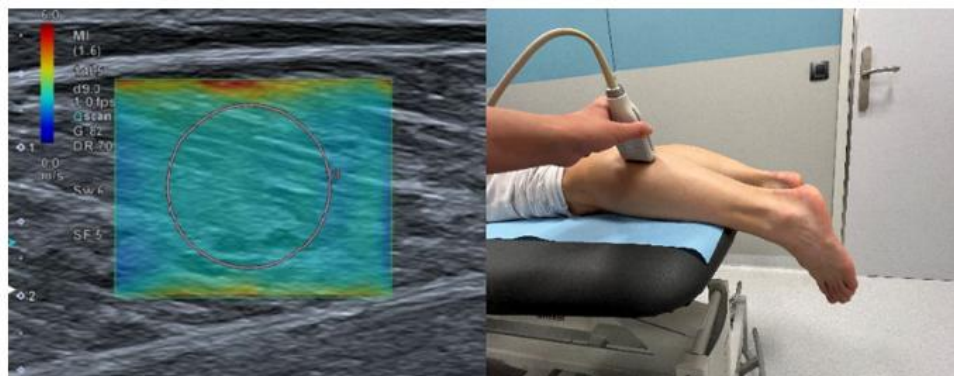


Figure 3. Shear wave elastography. Author's illustration.

B) Myotonometry

The MyotonPRO (Myoton AS, Tallinn, Estonia) is a portable, non-invasive myotonometer device designed to measure the viscoelastic properties of superficial skeletal muscles, tendons and other soft tissues (Figure 4). It operates by delivering a brief (0.015 seconds), low-force mechanical impulse (typically 0.4 Newton) under a constant compression force of 0.18 Newton to the skin surface over the muscle, inducing damped oscillations in the underlying muscle tissue.

These oscillations are then recorded by an integrated accelerometer, and the resulting signal is analyzed to calculate several biomechanical parameters, such as elastic stiffness (Newton/meter).

A recent systematic review and meta-analysis evaluated the validity and reliability of myotonometry in patients with stroke (41). Nine studies were included in the qualitative synthesis and data from 5 of these were pooled in a meta-analysis. They concluded that myotonometry seems to be a valid and reliable complementary tool to assess muscle elastic stiffness in stroke survivors. However, this conclusion should be interpreted with caution. A closer look at the included studies reveals substantial heterogeneity: different myotonometry parameters were used across studies, results for the lower limb muscles were generally weaker than for the upper limb, and the so-called “gold standard” methods used to establish construct validity are themselves questionable. These methodological inconsistencies, along with several other small but important limitations, make it difficult to confidently state that myotonometry is unequivocally valid and reliable in stroke populations. Therefore, more high-quality and standardized studies are needed before drawing firm conclusions.



Figure 4. MyotonPro ®. Adapted from images provided by Myoton AS.

C) Magnetic resonance imaging

One approach to better understand how the underlying mechanisms of spasticity affect muscle properties is to investigate their morphological characteristics at a macroscopic level. The development of new sequences and softwares in magnetic resonance imaging (MRI) and ultrasound (US) opens up the field of non-invasive muscle structure assessment. MRI is currently regarded as the most accurate, reliable, non-invasive, and quantitative technique for evaluating regional skeletal muscle (42).

Beyond providing high-resolution visualization of muscle architecture, it can quantify structural alterations such as muscle volume loss, fatty infiltration, connective tissue accumulation, and changes in fascicle orientation (43); features that are particularly relevant in the assessment of spastic muscle. Recent advances in MRI sequences and software have expanded its capabilities to include the evaluation of additional muscle characteristics. Among the most promising applications are the study of BoNT-A diffusion while specialized imaging approaches can monitor the distribution of BoNT-A within muscles (44). In addition to visual assessment, quantitative biomarkers such as T2 relaxation times, apparent diffusion coefficients (ADC), and fractional anisotropy (FA) can be extracted from tailored MRI sequences and followed over time to evaluate treatment-induced changes (45,46). An overview of the principal MRI biomarkers used for muscle assessment throughout this thesis, including their definitions and physiological significance, is provided in Table 3." Although MRI offers detailed morphological information, it remains expensive and is not well suited for joint repositioning or dynamic imaging. Therefore, the number of studies in this field remains limited.

Future work would benefit from comparing different MRI biomarkers with one another, as well as correlating them with comprehensive clinical assessments of the patient.

Table 3. Overview of the principal MRI biomarkers used for muscle assessment throughout this thesis

MRI biomarker	Definition/ physiological significance
Muscle volume	Three-dimensional MRI-derived measure of total muscle size, providing an indicator of muscle trophicity and the amount of contractile tissue.
Fat fraction	Indicator of extent and severity of muscle fibers replacement and destruction. The higher the fat fraction, the greater the muscle destruction. Assessed on 3D axial IDEAL IQ images.
T2 relaxation time	Quantitative MRI biomarker sensitive to changes in muscle composition, particularly increased water content related to edema, inflammation, or other pathological processes.
Apparent diffusion coefficient	Magnitude of water molecule diffusion within tissue; influenced by microstructural factors (extracellular space, barriers to water movement). Quantitative parameter derived from DWI and DTI.

D) Ultrasound

US, on the other hand, is a cost-effective, accessible, and non-invasive tool for examining skeletal muscles properties. Several US parameters provide valuable information on muscle structure and composition, which are key factors that determine its functional capacity.

Muscle thickness and cross-sectional area reflect muscle size, which is an important determinant of strength. Beyond muscle size, strength is influenced by muscle composition, reflecting the intrinsic quality of the tissue. In general, a greater proportion of contractile tissue relative to non-contractile components is associated with higher muscle quality and an increased capacity for force generation. Furthermore, fascicle length and pennation angle offer additional functional information, with fascicle length indicating the potential range and speed of contraction, and pennation angle reflecting the muscle's force-generating capacity (47,48). Beyond the contractile fibers, the overall muscle belly length and tendon length influence the muscle's excursion and the transmission of force to the skeleton (49).

In addition, echo-intensity analysis allows the assessment of muscle quality, including fatty infiltration and fibrotic changes (50). This parameter provides complementary information to structural measurements, helping to better characterize the extent of post-stroke muscle alterations. The most commonly used visual scale to assess muscle echo-intensity is the Modified Heckmatt scale (Figure 5) which demonstrated good reliability and validity to assess the pathologic muscle changes that occur in patients with spasticity (51).

It grades muscle echogenicity on a 4-point ordinal scale by visual assessment of the US image, with 1 being closest to normal muscle architecture and 4 showing the greatest increase in EI that is typically described as hyperechoic like bone echo.

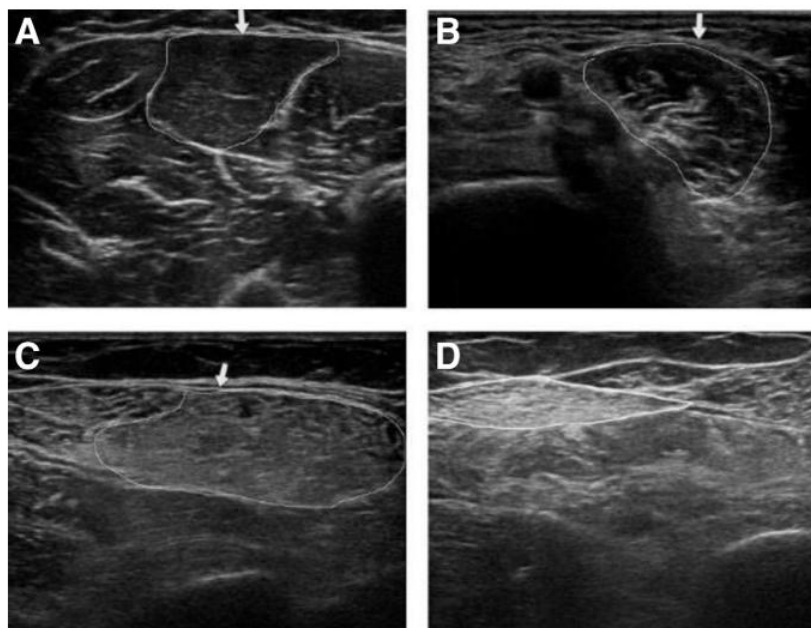


Figure 5. Traced muscles demonstrating the Modified Heckmatt grades. (A) Grade 1: normal echogenicity in more than 90% of the muscle that is distinct from bone echo. (B) Grade 2: increased muscle echogenicity in 10% to 50% of tissue, but with distinct bone echo and some areas of normal muscle echo. (C) Grade 3: marked increase in muscle echogenicity between 50% and 90% of tissue with reduced distinction of bone echo from muscle. (D) Grade 4: very strong muscle echogenicity, with near complete loss of distinct bone echo from muscle in more than 90% of tissue.

By combining all these parameters, US can give a comprehensive picture of both structural and composition changes in spastic muscles after stroke. In recent years, numerous studies have assessed post-stroke changes in muscle structure using ultrasound.

A recent systematic review demonstrated moderate-to-excellent reliability for measuring muscle architecture parameters, including muscle thickness, pennation angle, cross-sectional area and muscle length across various muscles in post-stroke patients (52). Furthermore, in 2021, Schillebeeckx *et al.* published a systematic review examining the muscle and tendon properties of the spastic lower leg after stroke using US (53). After including only 9 studies, a trend in reduced muscle thickness and reduced fascicle length of the hemiplegic spastic lower limb was observed; however, no uniform conclusion could be drawn. While US is a valuable tool for assessing spastic muscles after stroke, it has several limitations. Measurements are highly operator-dependent, which can lead to variability between examiners. Moreover, quantitative evaluation of muscle quality, such as fatty infiltration or fibrosis, may be less precise than with MRI. Furthermore, joint position can affect the accuracy of architectural measurements. Finally, deep or large muscles may be difficult to visualize fully, and muscle length or muscle volume cannot be directly measured.

To overcome this gap, recent research involves a 3D free-hand ultrasonography (3DfUS). It is an imaging technique that extends conventional 2D US into a system capable of reconstructing 3D anatomical datasets of muscles and tendons. This is achieved by combining standard B-mode US with a 3D motion-tracking system that records the position and orientation of the transducer during freehand sweeps (Figure 6). By integrating spatial information with sequential 2-D images, 3DfUS enables direct quantification of muscle structural parameters such as volume, length, thickness and echo intensity.

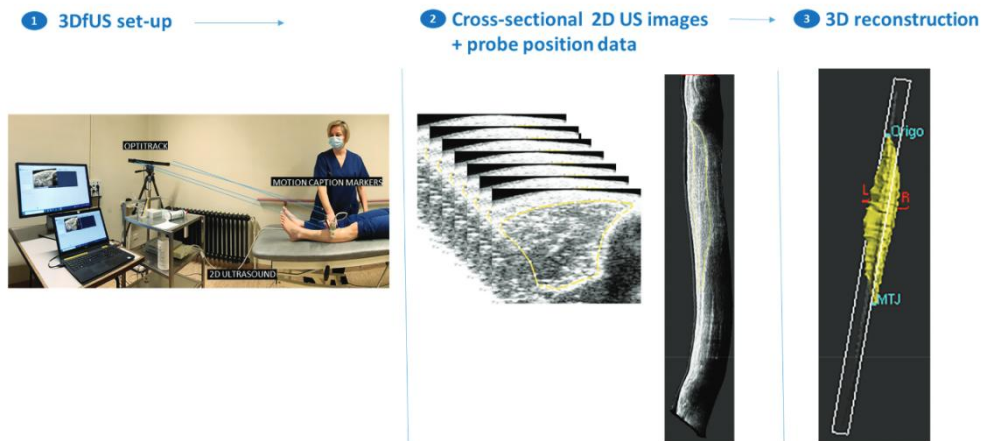


Figure 6. Overview of the 3DfUS setup and acquisition, 2D and 3D reconstruction of tibialis anterior muscle belly volume. Reproduced from Schillebeeckx *et al.* (54).

Compared with static 2D US, this approach provides a more comprehensive assessment of muscle structure, while remaining portable and cost-effective when compared with MRI. Validation studies of 3DfUS conducted in vivo investigations of the medial gastrocnemius muscle in healthy adults, demonstrated good agreement between 3DfUS-derived measurements and those obtained using MRI (55). Furthermore, it has been increasingly applied in neuromuscular research to assess muscle structural changes after neurological injury. Schillebeeckx *et al.* demonstrated that 3DfUS is a reliable method for quantifying tibial anterior muscle structure (muscle volume, muscle length) and composition (echo-intensity) in chronic stroke survivors (54). Moreover, 3DfUS seems to be reliable for assessing medial gastrocnemius muscle and tendon properties (muscle thickness, muscle length, tendon length) in patients with stroke (56).

2.2.2 Integrated Neural and Non-Neural Quantification

Only an assessment that simultaneously collects electrophysiological and biomechanical signals during passive muscle stretch has the potential to differentiate the influence of neural and non-neural components in a valid and reliable way.

Other authors have focused on measuring body segment kinematics and torque during passive stretch, together with EMG activity, in order to quantify both neural and non-neural components. Nevertheless, torque is a resultant measure that reflects the combined effects of active and passive structures crossing the joint. As torque depends not only on muscle force but also on biomechanical factors such as moment arm length and joint angle, caution is required when attributing the measured response to a specific muscle or mechanism, even combined with EMG.

For example, Bar-on *et al.* developed in children with cerebral palsy a manually controlled instrumented hyper-resistance analysis, which combine biomechanical (position and torque) and surface EMG signals during passive muscle stretch at different velocities (57) (Figure 7). Hyper-resistance parameters are extracted from the change in surface EMG and in torque between low velocity and high velocity. This approach aims to differentiate between neural and non-neural contributions to the resistance felt, to assess hyper-resistance severity in the lower limb muscles, and to evaluate the response to spasticity treatments in children with cerebral palsy. Recently, Schillebeeckx *et al.* demonstrated for this tool discriminative validity for both affected and non-affected leg in chronic stroke patients, as well as for the leg of stroke patients compared to healthy controls (58).

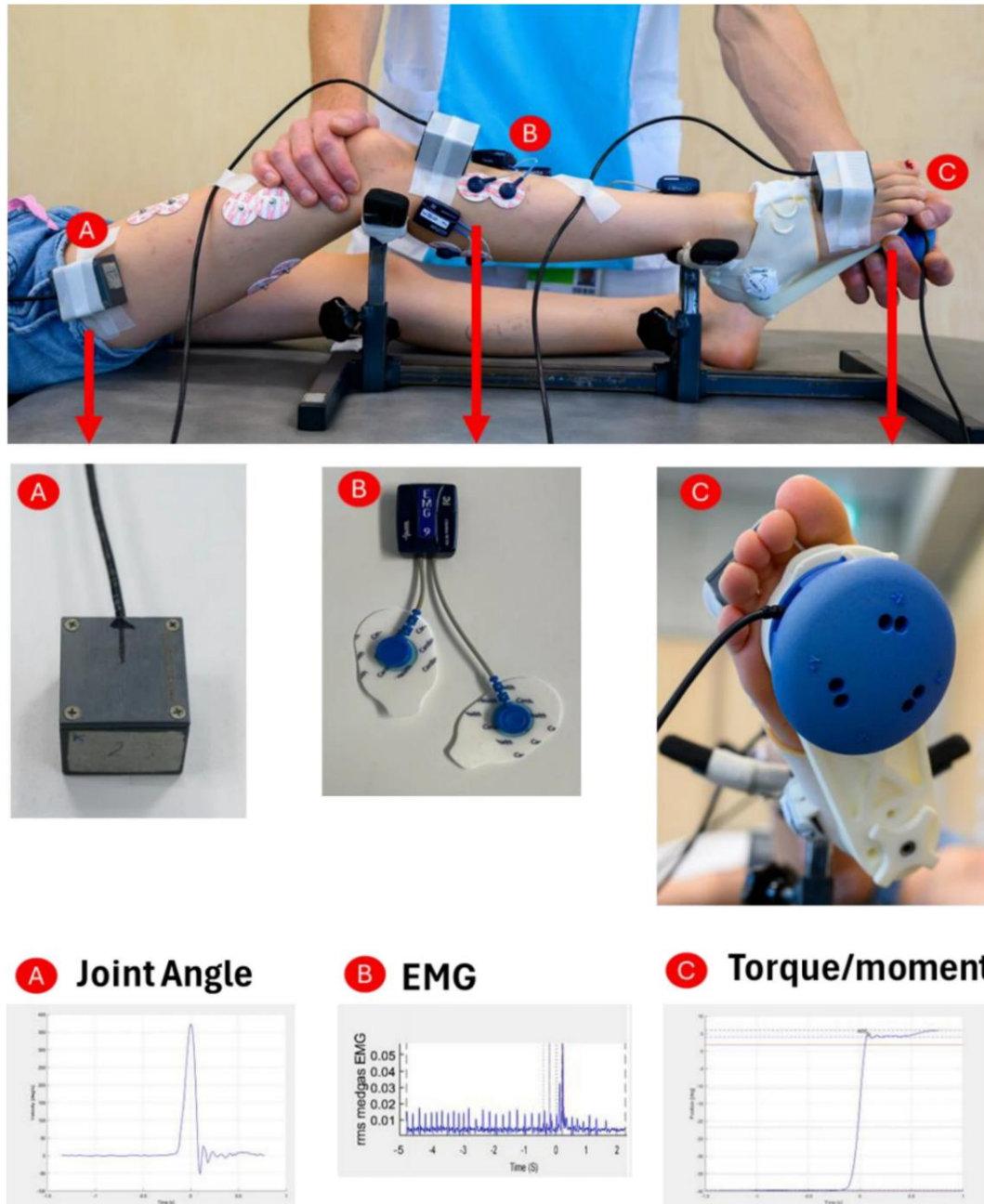


Figure 7. Overview of the instrumented assessment setup. Reproduced from Schillebeeckx *et al.* (58).

2.3 Management of spasticity through BoNT-A injections

Poststroke spasticity syndrome constitutes a significant therapeutic challenge. According to international guidelines, spasticity syndrome management should be part of a well-coordinated and comprehensive multidisciplinary rehabilitation program that is patient-centered and goal-specific (59). There are a variety of therapeutic options available, either alone or in combination (multimodal and/or adjunctive treatment) delivered as both generalized and focal interventions with a transient or permanent effect.

Importantly, it should be remembered that spasticity syndrome treatment is a dynamic process requiring constant re-evaluation and updating (59). To maintain clarity and brevity and to remain aligned with the objectives of this thesis, the focus will be limited to treatments involving BoNT-A injections, in accordance with current recommendations.

Botulinum toxin, first identified in the late 19th century as the agent responsible for botulism, has evolved from a feared neurotoxin into a widely used therapeutic tool. Since the 1980s, it has become a cornerstone in the management of spasticity due to its ability to temporarily block acetylcholine release at the neuromuscular junction, thereby exercising a paralyzing effect on muscle. This approach results in localized chemical denervation. It has largely been established that BoNT-A injections reduce spasticity (60–62). Clinically detectable reduction in muscle tone is evident after 24–72h, and a maximal effect occurs within 10 days to 4 weeks and can last for up to 3–6 months. These timelines must be recognized and included in the management plan (59). Nevertheless, many patients complain with the relatively short-lasting effects of the treatment and the return of symptoms between injection cycles.

As a result, there have been increasing suggestions to administer injections earlier, use higher doses, and/or combine BoNT-A injections with additional therapeutic interventions. By modifying the target muscles, the BoNT-A dose (per session, per muscle, and/or per injection site), the interval between treatments, and the number of target sites, focal BoNT-A treatment can be tailored to individual patient's symptoms. However, muscle selection and dosing are based on the clinical experience of the treating physician (59).

2.3.1 Doses

The three most commonly used BoNT-A are onaBoNT-A, incoBoNT-A and aboBoNT-A. The agents are not interchangeable and there are no validated conversion ratios. Since incoBoNT-A has a similar clinical efficacy and adverse event profile as onaBoNT-A, it is suggested that the dose ratio should be 1:1 or 1:2; whereas a variable dose ratio from 1:3 to 1:5 has been proposed for the conversion between onaBoNT-A and aboBoNT-A (63).

Indications, maximally recommended doses and reimbursement vary for each molecule and country. In Belgium, maximal doses are 400U for onaBoNT-A, 500U for incoBoNT-A, and 1500U for aboBoNT-A. These doses can represent a limit to optimal management of multifocal spastic muscle overactivity.

Therefore, in the past few years, there was a great interest in the use of higher doses, especially in case of severe spastic muscle overactivity. Higher doses do not necessarily mean the increase of the dose per muscle, but also the increase of the number of the injected muscles using conventional doses, thus increasing the total amount of injected BoNT-A.

The aim of this approach is to reduce the impact of spastic muscle overactivity, to prevent secondary complications and to obtain specific functional goals. Several studies have now been published about the injection of higher doses of BoNT-A in subjects with post-stroke spastic muscle overactivity, and some reviews have focused on this topic (61,63,64). Globally, many reports have suggested that higher doses of BoNT-A can be used efficaciously in reducing severe focal spastic muscle overactivity in adult post-stroke (65). However, reviews did not find evidence that higher doses improve either limb functionality, overall functional outcomes, or quality of life. (64). Moreover, the risks of diffusion, muscle weakness, and occurrence of severe side effects represent important concerns regarding the injection of high doses, even though the available studies indicate that such adverse effects remain rare (66,67). Overall, higher doses may be beneficial in selected patients but the evidence remains insufficient to recommend the use of higher doses for every patient. Furthermore, increasing BoNT-A doses also implies higher treatment costs, raising important economic considerations regarding the long-term sustainability of such approaches. Further studies are therefore required to confirm these findings and to evaluate the cost-effectiveness and cost-utility of high-dose BoNT-A in post-stroke spastic muscle overactivity management.

2.3.2 Adverse effects of BoNT-A

Although BoNT-A is generally considered a safe treatment for spastic muscle overactivity, both local and systemic adverse effects have been reported.

Local adverse effects are the most common, are primarily related to the injection procedure and typically resolve within a few days to several weeks.

These effects mainly include injection-site pain, bruising, swelling and local muscle weakness with an incidence of typically 5–20% (68,69). Systemic adverse effects are less common but can be significantly more severe. They are thought to result from diffusion or distant spread of the toxin. Reported symptoms include botulism-like syndromes, generalized muscle weakness, dysphagia, dysarthria, severe allergic reactions, and respiratory complications (<1%). In rare instances, these complications can be life-threatening or even fatal (70). Higher doses injections have often been suggested as potential risk factors, yet current evidence does not support this assumption (66).

Overall, most adverse effects are mild, self-limiting, and resolve spontaneously within a few weeks. Severe adverse events are uncommon but have been reported. The risk of adverse effects following BoNT-A injections can be minimized through careful treatment planning and adherence to good injection practices. Appropriate patient selection, individualized dose adjustment, and consideration of muscle size and severity of spasticity muscle overactivity are essential to avoid excessive chemodenervation. The use of guidance techniques such as US or EMG can improve injection accuracy and reduce unintended toxin spread to adjacent structures (71). In addition, respecting recommended maximum doses and allowing sufficient time between injection sessions may help limit cumulative muscular alterations and systemic exposure. Particular caution is warranted in vulnerable patients, such as those with pre-existing swallowing or respiratory difficulties. Patient education regarding potential adverse effects further contribute to the safe use of BoNT-A in clinical practice.

Interestingly, recent evidence suggests that the safety profile may not substantially differ between BoNT-A formulations. In the DIRECTION study, a large phase IV randomized double-blind crossover trial involving 464 adults with upper limb spasticity, Esquenazi et al. directly compared aboBoNT-A and onaBoNT-A (72). Treatment-emergent adverse events occurred in 20.3% of patients following aboBoNT-A and 23.0% following onaBoNT-A, demonstrating non-inferiority of aboBoNT-A with respect to safety. Importantly, no major differences in the nature or frequency of adverse events were observed between the two formulations. While aboBoNT-A was associated with a slightly longer duration of clinical response, both toxins exhibited a comparable safety profile. These findings suggest that formulation choice is unlikely to be a major determinant of adverse event risk when BoNT-A is administered according to recommended dosing and injection procedures.

2.3.3 BoNT-A and non-neural components

Besides its effect on spastic muscle overactivity, some authors have shown that injections of BoNT-A are responsible for changes in muscle structure (muscle morphology and composition). This possible effect is critical, as it could negatively impact the effectiveness of BoNT-A interventions, and the cumulative effect of injections could even be worse. However, the effect of BoNT-A on muscle structure remains controversial. In studies conducted to investigate the histopathologic changes in animals and humans, authors observed a reduction of the muscle cross-sectional area, a long-term muscle neurogenic atrophy (up to 1 year post-injection), a decreased fiber size, a decreased contractile material, an increase of the collagen proportion and of the intramuscular lipid accumulation after BoNT-A treatment (73–76).

A first systematic review, performed by Mathevon *et al.* (77) synthesized the literature covering the muscle structure assessment after BoNT-A injection, by means of imaging and histological evaluations. Their review, published in 2015, included 21 articles and observed that “BoNT-A injections lead to structural changes in the muscle: lingering atrophy, with a remodeling of the muscles contractile proteins, which is probably not completely reversible”. These potential effects may support the consideration of discontinuing or lengthening injection intervals when clinically appropriate.

Furthermore, such structural alterations could theoretically contribute to muscle shortening and the development of contractures, potentially necessitating more invasive interventions such as surgical procedures. Moreover, a degradation in overall muscle quality may also occur, potentially characterized by reductions in strength and endurance capacities.

This further underscore the importance of implementing adjunct therapeutic approaches, particularly rehabilitation interventions aimed at muscle strengthening, stretching, and functional training, to mitigate potential deleterious changes and optimize functional outcomes. Ultimately, a precise quantification of the potential adverse structural effects of BoNT-A on the muscle’s non-neural component is required to refine treatment decision-making and to more accurately assess the benefit–risk ratio of its use.

2.4 Spasticity syndrome and triceps surae

The triceps surae is a major muscle group of the posterior compartment of the lower leg, primarily responsible for plantarflexion of the ankle. It is composed of two muscles: the gastrocnemius and the soleus. The gastrocnemius is a bi-articular muscle with two heads (medial and lateral) that cross both the knee and ankle joints, contributing to knee flexion as well as powerful plantarflexion. In contrast, the soleus is a mono-articular muscle that lies deep to the gastrocnemius and acts exclusively on the ankle joint. Together, these muscles converge into the Achilles tendon, which inserts on the posterior aspect of the calcaneus, enabling effective force transmission during stance, push-off, and propulsion during gait. Architecturally, the gastrocnemius has a relatively larger pennation angle and shorter fascicle length compared to the soleus, which has longer fascicles and a predominantly slow-twitch fiber composition, making it well-suited for postural control and endurance activities (78). This combination of structural and fiber-type characteristics allows the triceps surae to generate both rapid, high-force contractions and sustained tonic activity, which are critical for locomotion and balance.

Therefore, focusing on the triceps surae is particularly relevant when investigating post-stroke spastic muscle overactivity and its structural consequences, as this muscle group plays a central role in gait, posture, and functional mobility.

The gastrocnemius and soleus are among the muscles most commonly affected by spastic muscle overactivity after stroke, contributing significantly to equinus/equinovarus foot deformity (79) (Figure 8).

Clinically, those deformities may present as reduced ankle dorsiflexion and abnormal foot–ground interaction with impaired foot clearance, leading to an impaired gait, augmented fall risk, poor functional performances, and reduced quality of life (80,81). Spastic equinus/equinovarus is due to an interaction of several main contributing factors: spasticity of calf muscles, triceps surae/Achilles tendon shortening, ankle dorsiflexor weakness, and tibialis anterior/peroneus muscle imbalance (82). The contribution of the main causes of those deformities differs between patients, and therapeutic decisions are therefore challenging.

Triceps surae accessible structure allows detailed assessment using imaging and biomechanical measurements. This makes the muscle group an excellent model for understanding the effects of spastic muscle overactivity and its treatment on muscle properties after stroke, as well as a key target for post-stroke rehabilitation. Given these considerations, this thesis is dedicated to investigating the triceps surae, aiming to better understand its structure, mechanical properties, and response to spasticity and its treatment after stroke.



Figure 8. Spastic equinovarus foot. Source: ResearchGate.

Chapter 3. Objectives of this thesis

The overall objective of this doctoral thesis was to enhance our understanding of the non-neural components involved in the spasticity syndrome and to clarify how BoNT-A injections influence this component. In parallel, this work aimed to better characterize post-stroke muscle alterations and to investigate their evolution over time. More specifically, this thesis sought to isolate the contributions of stroke-related changes, spastic muscle overactivity progression, and BoNT-A treatment, and to assess their temporal dynamics in the evolution of post-stroke muscle alterations.

As indicated in the above sections, several gaps remain in the literature regarding the effects of BoNT-A injections on spastic muscle properties. It has largely been established that BoNT-A injections reduce spastic muscle overactivity (neural component of HR), but some authors have also shown that injections of BoNT-A are responsible for changes in non-neural components (muscle stiffness, muscle structure and composition) of HR (77). This possible effect is critical, as it could negatively impact functional capacity as well as the effectiveness of further BoNT-A interventions. Despite these concerns, the impact of BoNT-A injections on spastic muscle properties remains controversial, highlighting the need for a rigorous evaluation of its influence on non-neural components of HR. Therefore, the first objective of this thesis was to evaluate structural and mechanical muscle adaptations following BoNT-A injections in neurological populations. In this regard, we conducted a systematic review with meta-analysis focusing on noninvasive analysis of spastic muscle structure and elastic stiffness after BoNT-A injection.

As BoNT-A injections are routinely repeated every 3–6 months in clinical practice, and because several histological studies in animals have shown that repeated injections may exacerbate deleterious effects on muscle structure, we carried out a second study related to this first objective. This clinical study consisted of a quantitative MRI evaluation of post-stroke spastic calf muscle structure following repeated BoNT-A injections.

The third study was developed in response to the lack of standardized ultrasound elastography protocols identified in Study 1 (chapter 4). Although standardization is essential in order to compare results across studies, certain parameters—such as the number or size of regions of interest—have little impact on elastography outcomes and were therefore not prioritized. Instead, this third clinical study focused on parameters that can considerably influence shear-wave measurements, particularly joint position and the muscle’s activation state. The objective was therefore to determine which ankle position provides the most valid and reliable assessment of gastrocnemius elastic stiffness in individuals with spasticity and examining the effects of muscle activation.

While working on this thesis, we identified a substantial gap in the literature in early-pathophysiology understanding. It is well established that post-stroke patients exhibit alterations in muscle structure (83,84); however, very few longitudinal studies have been conducted, and patients are rarely assessed during the acute phase. Understanding when these structural changes first appear and the temporality of these changes is crucial, as it could help us to guide strategies to prevent them and/or allow for earlier and potentially more effective interventions.

Therefore, the aim of our fourth study is to evaluate muscle structure (through MRI) and stiffness (using SWE) from the acute phase after stroke and to perform a longitudinal follow-up to monitor its evolution over time. This will allow us to determine when specific modifications in muscle structure occur, to investigate which types of structural alterations may contribute to spasticity, whether spasticity further modifies muscle structure, and to compare changes between the affected and unaffected limbs throughout the recovery process.

In the final section of this PhD thesis, we synthesize and analyze the principal findings of the conducted studies and consider how they integrate with and extend current literature. This concluding section also examines how our results may influence clinical practice and outlines key directions for future research.

Part II. Investigations

Chapter 4. Noninvasive analysis of overactive muscle structure and elasticity after Botulinum toxin type A injection: a systematic review and meta-analysis

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4.1 Study rationale, objectives and hypotheses

As indicated in the above sections, several gaps remain in the literature regarding the effects of BoNT-A injections on spastic muscle properties. It has largely been established that BoNT-A injections reduce spasticity (neural component of HR), but some authors have also shown that injections of BoNT-A are responsible for changes in non-neural components (muscle stiffness and structure) of HR (77). This possible effect is critical, as it may negatively influence functional capacity as well as the effectiveness of further BoNT-A interventions. Despite these concerns, the impact of BoNT-A injections on spastic muscle properties remains controversial, highlighting the need for a rigorous evaluation of its influence on non-neural components of HR.

Therefore, the first objective of this thesis was to evaluate structural and mechanical muscle adaptations following BoNT-A injections in neurological populations. In this regard, we conducted a systematic review with meta-analysis focusing on noninvasive analysis of spastic muscle structure and elastic stiffness after BoNT-A injection.

Based on the existing literature, and particularly on the findings of the systematic review by Mathevon et al. (77), we hypothesized that BoNT-A injections would induce measurable changes in the mechanical and structural properties of spastic muscles, characterized by reductions in muscle elastic stiffness and modifications in muscle architecture detectable through non-invasive imaging techniques. However, we further hypothesized that histological analyses are more sensitive than non-invasive imaging techniques for detecting these alterations, particularly during the early stages following injection.

Consequently, changes identified through MRI or US were expected to be less sensitive to early alterations and therefore detected later and less consistently than those reported in biopsy-based studies.

It should be noted that the term “elasticity” was used in this published manuscript in a clinical and functional sense and should be interpreted as elastic stiffness (i.e., the mechanical resistance to deformation of the muscle–tendon unit).

To complement the findings of this thesis, which included studies published up to April 2023, I conducted an updated search of the recent literature to identify newly available evidence on the effects of BoNT-A on muscle stiffness and muscle structure in neurological individuals with hyper-resistance. This search identified one additional study on elastic stiffness and one additional study on muscle structure.

Because the newly identified stiffness study did not allow for a meaningful update of the quantitative synthesis, no new meta-analysis was performed for elastic stiffness, and its findings are therefore discussed qualitatively in the general discussion. In contrast, the newly identified study on muscle structure provided sufficient data to conduct a series of new meta-analyses. The results of these analyses are presented in the section that follows the published article, as the article has already been published and could therefore not be modified to incorporate these additional data. The interpretation and discussion of these new findings are reserved for the general discussion of the thesis (section 8.2).

4.2 Abstract

Background. Injections of botulinum toxin type A (BoNT-A) are the first-line treatment for spastic muscle overactivity (MO). Some authors observed that BoNT-A injections lead to changes in muscle structure and muscle elasticity that are probably not completely reversible. This possible effect is critical, as it could lead to negative impacts on the effectiveness of BoNT-A interventions.

Objectives. Our study aimed to evaluate the current literature regarding changes in muscle elasticity and structure after BoNT-A injection, by diagnostic imaging, in neurological populations with MO. Our second objective was to pool all articles published on this topic in order to provide a quantitative synthesis of the data.

Methods. A systematic search was conducted between October 2021 and April 2023 using different databases in accordance with PRISMA guidelines. Two independent reviewers screened articles for inclusion, extracted data, and evaluated methodological quality of the studies. A meta-analysis was performed to compare muscle elasticity and structure before and after BoNT-A injections.

Results. A sample of 34 studies was selected for qualitative review and 19 studies for quantitative review. Meta-analysis of pre-post studies demonstrated significant improvement with a medium effect size (standardized mean difference = 0.74; 95% CI 0.46-1.02; $P < 0.001$) of muscle elasticity assessed by ultrasound elastography (USE) 4 weeks after BoNT-A injection. No statistically significant difference was found for muscle thickness, pennation angle, and muscle echo-intensity assessed by magnetic resonance imaging and/or ultrasonography at short-term.

On the other hand, normalized muscle volume decreased with a small effect size (standardized mean difference = -0.17; 95% CI -0.25 - -0.09; $P < 0.001$) 6 months after BoNT-A injection.

Conclusion. Muscle elasticity measured by USE improves with a temporary effect at short-term following BoNT-A injections. Synthesis of studies that assesses muscle structure is hindered by methodological differences between studies. However, based on a small amount of data, normalized muscle volume seems to decrease at long-term after BoNT-A injections in children with CP suggesting that the timing of re-injection should be considered with caution in this population. Further work should focus on the long-term effect of repeated injections on muscle structure and elasticity in neurological populations.

Prospero registration: CRD42022310902

Keywords: muscle overactivity, muscle spasticity, hyper-resistance, botulinum toxins, muscular atrophy, elasticity

4.3 Manuscript

1. Introduction

Muscle overactivity (MO) is one of the consequences of upper motor neuron syndromes that can affect children and adults. MO was at first englobed by the term “spasticity”, which 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 hyperexcitability of the stretch reflex, as one component of the upper motor neuron syndrome” (85). However, MO can present different clinical forms that are not covered by this definition. Consequently, in order to refine the taxonomy, Baude *et al.* (86) proposed a model where MO is a part of a larger neurological disorder and includes 3 components: spastic dystonia, spastic cocontraction and spasticity. Furthermore, a European consensus has developed a model to represent the hyper-resistance to passive stretch in patients with MO (27). This model distinguishes 2 main components of hyper-resistance: a non-neural (tissue-related) component and a neural (central nervous system related) component.

Injections of botulinum toxin type A (BoNT-A) are the first-line therapeutic method to treat focal MO (87,88). BoNT-A inhibits the release of acetylcholine at neuromuscular junctions resulting in blocking neuromuscular transmission (89–91). Therefore, BoNT-A injections act directly on the neural component of hyper-resistance (27). Besides its effect on MO, BoNT-A injections also act on other body functions and structures according to the International Classification of Functioning, Disability and Health (ICF) (92).

Indeed, the effects of a single injection of BoNT-A on overactive muscles have been largely studied and it has been well established that they lead to a reduction of hyper-resistance, as assessed by clinical scales (93). However, these measurements have poor intra-/inter-rater reliability (36,94). So far, no gold standard assessment of hyper-resistance, whether clinical or instrumental, is available. Therefore, objective and reliable measures of the two components of muscle hyper-resistance should be implemented to determine the efficacy of BoNT-A injections over time. Muscle elasticity is a reflect of the non-neural component of hyper-resistance. Some authors have used ultrasound elastography (USE) to assess this elasticity in qualitative and quantitative ways. In recent years, USE has been used in the assessment of muscle elasticity, which is closely associated with clinical assessment of MO (95). The two major techniques used in musculoskeletal elastography are strain sonoelastography and shear wave elastography (SWE). Strain elastography measures the deformation of a tissue when a manual compressive force is applied with the handheld US transducer. In SWE, an acoustic compressive radiation force is applied to tissues instead of external compression.

Furthermore, besides its effect on muscle elasticity, some authors have shown that repeated injections of BoNT-A are responsible for changes in muscle structure (96). This possible effect is critical, as it could negatively impact the effectiveness of BoNT-A interventions. However, the effect of BoNT-A on muscle structure remains controversial. In animal models, authors observed a reduction of the muscle's cross-sectional area, a long-term muscle neurogenic atrophy (up to 1 year post-injection), a decreased fiber size, a decreased contractile material, an increase of the collagen proportion and of the intramuscular lipid accumulation after BoNT-A treatment (75,76).

These findings are mainly based on anatomopathology sections studied with optic and electronic microscopy. In studies conducted to investigate the histopathologic changes in humans, there was a reduction of the muscle's cross-sectional area, a widespread neurogenic atrophy and a reduced size of type I and II fibers (73,74). The development of new sequences and softwares in Magnetic Resonance Imaging (MRI) and ultrasonography (US) opens up the field of non-invasive muscle structure assessment.

A first systematic review, performed by Mathevon *et al.* systematically reviewed the literature covering the muscle structure and stiffness assessment after BoNT-A injection, by means of imaging and histological evaluations (97). Their review, published in 2015, included 21 articles and observed that “BoNT-A injections lead to structural changes in the muscle: lingering atrophy, with a remodeling of the muscles contractile proteins, which is probably not completely reversible; and changes in muscle elasticity”. Thanks to improvements in the aforementioned new technologies and the increasing interest on the topic, many studies have been published since.

The primary objective of the present systematic review was to synthesize changes in muscle structure and elasticity after BoNT-A injection, as assessed by noninvasive imaging methods, in neurological populations presenting with MO. The secondary objective was to pool all selected articles in order to provide a quantitative synthesis of the effects of BoNT-A injections on muscle structure and muscle elasticity, as it has never been done before.

2. Methods

The systematic review and meta-analysis protocol is registered with PROSPERO (CRD42022310902) and conducted in accordance with PRISMA guidelines.

2.1 Data sources and search strategy

A systematic electronic search was conducted by two independent reviewers (MD and CR) between October 2021 and April 2023 using PubMed, Scopus, Embase, and the Cochrane Library databases.

The search terms were developed for PubMed and adapted for the other databases (additional material, T1). In addition, the reference list of the publications selected was also manually screened to detect references not found during the initial electronic search. There were no restrictions on the publication date of the included papers. Published works until April 2023 were considered. No filter was used in terms of language. All the databases were limited to humans.

2.2 Eligibility criteria

The PICO method was used to assess eligibility criteria. Full-text articles were included if i) Patient: studies were conducted in individuals with MO related to an upper motor neuron syndrome in upper and/or lower limb, no matter the age, the severity or neurological condition; ii) Intervention: botulinum toxin injection; iii) Comparison: individuals suffering from MO which didn't received BoNT-A injection or comparison before and after BoNT-A injection iv) Outcomes: muscle structure or muscle elasticity evaluation by noninvasive imaging methods, such as MRI, US, and elastography.

Articles were excluded if there were other neurological diseases or health condition that could influence MO or the paretic limb function. Systematic reviews, meta-analyses, theses, letters, dissertations, guidelines, scientific congress abstracts were also excluded.

2.3 Study selection process

After removing duplicates, two independent researchers (MD, CR) screened titles and abstracts according to the selection criteria of this review to identify potentially eligible studies. Full texts were independently evaluated for inclusion criteria by the same two researchers. In case of disagreement, discussion was initiated until consensus was reached.

2.4 Data extraction and analysis

For each study, relevant data was extracted by two independent researchers (MD, CR) as follows: first author, publication year, study design, sample size, participant characteristics (neurological condition, gender, age), comparisons, measurement tool, main outcomes involving structural and muscle elasticity adaptations, injected/evaluated muscles, BoNT-A injection characteristics (molecule, doses, dilution), previous BoNT-A injections, and time of evaluation.

2.5 Methodological quality assessment

The methodological quality was evaluated by two independent researchers (MD, CR) using the modified Downs and Black assessment tool (98). Higher scores indicate higher methodological quality. If there was any disagreement, the researchers discussed it to reach a consensus.

2.6 Statistical analysis

Meta-analyses were conducted across studies that made similar comparisons and used similar outcome measurements expressed in the same or different units. Case reports/studies were excluded because of their low proof of evidence. In addition, grouping required that there were at least 2 studies without excessive clinical heterogeneity based on intervention, participant characteristics, and study design. All analyses were conducted using Review Manager (version 5.4) (99) following the guidelines in the Cochrane Handbook for Systematic Reviews of Interventions (100). Meta-analyses were displayed as forest plots. I^2 statistics was used to determine statistical heterogeneity among the studies (101). Heterogeneity was considered to be acceptable for a $I^2 < 60\%$ (101). For all pre and post studies included in the meta-analyses, baseline and post-intervention means and standard deviations (SD) were extracted to perform the analyses. In case of mean and SD missing data, an estimation was calculated using the following formula proposed by Wan *et al* (102) and the Cochrane handbook (100): $\text{Mean} = (q1 + \text{median} + q3)/3$ and $\text{SD} = (q3 - q1)/1.35$ where $q1$ is first quartile and $q3$ third quartile. Where needed, standard error (SE) of mean differences was calculated using the following formula proposed by the Cochrane handbook: $\text{SE} = (\text{upper limit} - \text{mean difference})/1.96$ or $\text{SE} = \text{SD}/\sqrt{n}$ where n is the sample size. A random effect model was used because of heterogeneity. Because different units (meter/second, kPa,...) were pooled together to determine the effect of BoNT-A injection before and after intervention on muscle elasticity, the effect size was reported as standardized mean difference with 95% CI. With regards to the muscle structure, mean difference or standardized mean difference were used to express the effect size depending on the units of the different outcome measures.

No funnel plots were included in the review to visually explore publication bias due to an insufficient number of studies (<10) included in each meta-analysis (103). Sensitivity analysis was conducted to verify results robustness in case of statistical heterogeneity or presence of outliers.

3. Results

3.1 Selection of the studies

A total of 1280 studies published until April 2023 were found in the databases (PubMed = 344, Embase = 773, Scopus = 163, Cochrane Library = 0). After removing duplicates, the titles and abstracts of 803 articles were screened and from these, 727 were excluded for not fulfilling the eligibility criteria. Thereafter, the full text of 76 articles was read. A sample of 34 studies was selected for qualitative review. Of those 34 studies, 7 were published before 2015 and were already included in Mathevon's systematic review (97) and therefore not detailed in our systematic review. Finally, of those 34 studies included in our systematic review, 19 studies were selected for our meta-analyses in order to quantitatively synthesize the effects of BoNT-A injections on muscle structure and muscle elasticity (Figure 1).

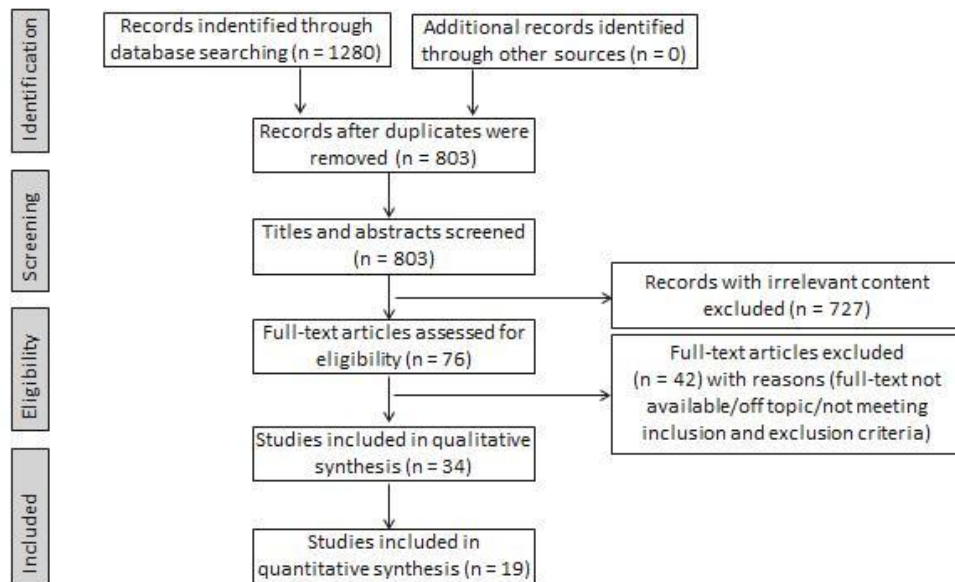


Figure 1. Flow chart illustrating article screening and selection in accordance with PRISMA guidelines.

3.2 Studies and patients characteristics (Table 1 (45,95,104–135))

Included articles were mostly observational prospective longitudinal cohort studies ($n=19$), case reports/series ($n=6$), and randomized controlled trials (RCT) ($n=3$) (108,125,128). The quality of the reviewed articles based on the modified Downs and Black was variable but most part of the studies were of low (total score $\leq 14/28$) ($n=13$) to fair (total score between 15-19/28) ($n=14$) quality (Table 2 (45,95,104–135)). The main items not reported were those related to external validity, internal validity (confounding), and power.

The sample size ranged from 1 to 105 subjects. A total of 577 injected patients (666 limbs, 957 injected and evaluated muscles), 90 non-injected individuals with neurological condition presenting MO (98 limbs) and 111 healthy control subjects were included in the studies.

The majority of the treated subjects were male (64%), and the mean age ranged from 2 to 66 years. Twenty-two studies assessed children with CP, 11 studies evaluated adults with chronic stroke, and 1 study included a child with spastic paresis of unknown origin.

Regarding comparisons, 9 studies included 1 control group or more and 9 studies compared the injected paretic muscles to the non-paretic contralateral muscles. Moreover, 16 studies did not include a control group.

The most used methods of analysis were US (n=17), followed by USE (n=15), MRI (n=6) and dual-energy X-ray absorptiometry (n=1).

The different evaluated outcomes were muscle elasticity (n=15), muscle thickness (n=8), muscle echo-intensity (n=9), absolute and/or normalized muscle volume (n=7), pennation angle (n=5), muscle length and tendon length (n=5), cross-sectional area (n=4), fascicle length (n=4), and muscle mass (n=1).

Most of the studies evaluated their outcomes at short-term (2 weeks – 6 weeks) (n=23) and mid-term (7 weeks – 12 weeks) (n=14) after BoNT-A injection. Only 5 studies performed long-term evaluations (> 6 months). Furthermore, 3 studies (107,112,130) did not specify the time after BoNT-A injection.

The injected and evaluated muscles varied among studies. However, the most evaluated muscles were the gastrocnemius medialis in lower limb and the biceps brachialis in upper limb.

3.3 Muscle elasticity

Initially, the results of 14 pre-post studies that examined the effect of BoNT-A injection on muscle elasticity using SWE and/or strain USE were meant to be pooled in a meta-analysis. However, due to excessive statistical heterogeneity explained by the various measurement protocols and the poor methodological quality of some studies, 3 of them (109,113,124) were excluded from the meta-analysis after sensitivity analysis was performed (additional material). Moreover, 1 study was excluded because of missing data (121) and 1 additional study was excluded because of its design (case report) (116). Finally, 9 pre-post studies evaluating 11 different muscles were included. Five studies used strain USE and 5 studies made use of SWE. The method used did not seem to influence the results. However, due to different units of measurement, a standardized mean difference was used to pool the results of both methods instead of mean difference.

Analyses were made for short-term (4 weeks) and mid-term (12 weeks) effect. At short-term, 5 studies evaluated lower limb muscles while 2 studies evaluated upper limb muscles. Results were comparable with an improvement of elasticity of the same magnitude. Heterogeneity was considered to be acceptable with $I^2 = 37\%$. Moreover, 2 studies evaluated both upper and lower limb muscles at short-term and did not make any distinction between both in their results. The most evaluated muscles were the gastrocnemius in lower limb (7 of the 9 studies) and the biceps brachialis in upper limb (3 of the 9 studies). Related to the neurological condition and age, 4 studies evaluated children with CP and 5 studies evaluated adults suffering from chronic stroke. In summary, the analysis at short-term showed a statistically significant improvement of muscle elasticity with a medium effect size (standardized mean difference = 0.74; 95% CI 0.46-1.02; $P < 0.000001$) (Figure 2

(95,105,108,110,111,117,118,128,129)). The analysis at mid-term included only gastrocnemius muscles of children with CP and showed no statistically significant within-group difference (Figure 2 (95,105,108,110,111,117,118,128,129)). Furthermore, out of the 5 studies excluded from the meta-analysis, 4 (109,113,121,124) found a significant decrease (-23% to -56%) of USE values 4 to 12 weeks after treatment. In Furukawa's report of 2 cases (116) both patients showed improvement in muscle elasticity at 2, 4, and 8 weeks after BoNT-A injection. However, these two measures followed different patterns through week 12 of evaluation.

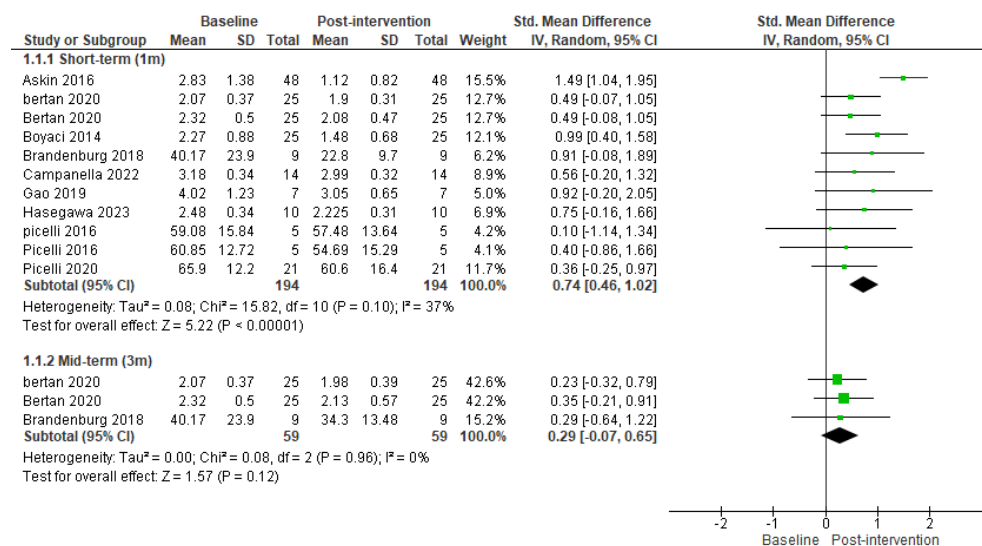


Figure 2. Effect of BoNT-A injections on muscle elasticity.

3.4 Muscle volume

Six studies mentioned measurement of muscle volume assessed by US (n=3) or MRI (n=3). The results of 2 pre-post studies (104,134) that examined the effect of BoNT-A injection at short-term on muscle volume normalized to tibia length in children with CP were pooled in a meta-analysis. However, given the statistical heterogeneity ($I^2=70\%$), this quantitative synthesis has not been kept in this paper, but is available as additional material. Both studies observed a significant decrease of normalized muscle volume at short- and/or mid-term.

The results of 2 pre-post studies that examined the long-term effects (24 weeks) of BoNT-A on muscle volume normalized to tibia length (104) or to the product of body mass and body length (114) in children with CP were pooled in a meta-analysis. A statistically significant decrease of normalized muscle volume, with a small effect size (standardized mean difference = -0.17; 95% CI -0.25 - -0.09; $P < 0.0001$) was observed (Figure 3 (104,114)). In the study of De Beukelaar *et al.* (114) the amount of changes in normalized muscle volume assessed by US tended to be lower in BoNT-A patients who received a first BoNT-A injection (i.e. BoNTA-naive treated patients) compared to untreated spastic patients. However, no significant between-group difference was found between BoNT-A naive treated cohort and BoNT-A non-naive treated cohort.

One study could not be included in the meta-analysis because of missing data. The normalized muscle volume showed a significant reduction up to 16.2% at 8 weeks post BoNT-A injection (126).

Furthermore, 2 studies were not included in the meta-analysis because of clinical heterogeneity. Schless *et al.* compared normalized muscle volume in untreated spastic patients and in spastic patients treated with BoNT-A (at least 3 injections). They showed that, more than 6 months after the intervention, the treated cohort had a smaller muscle volume. Furthermore, a higher number of previous injections was significantly associated with a lower normalized muscle volume (130). On the contrary, Weide *et al.* (133) did not report any significant change in normalized muscle volume at mid- nor at long-term after BoNT-A injection in their case report.

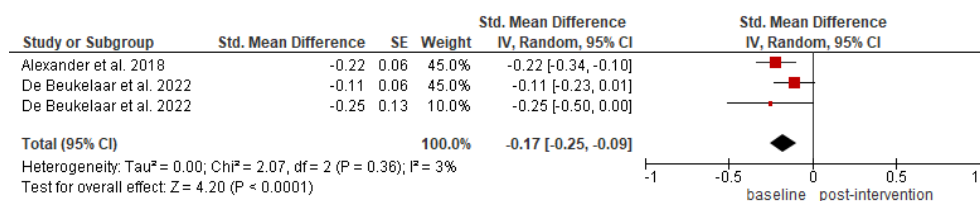


Figure 3. Effect of BoNT-A injections on normalized muscle volume at long-term.

3.5 Cross sectional-area

Results were very conflicting in the 4 studies that assessed cross sectional-area. No meta-analysis was made because of clinical heterogeneity. Cosenza *et al.* (112) reported no significant difference in cross-sectional area of BoNT-A treated rectus femoris compared to untreated spastic muscles. On the contrary, De Beukelaar *et al.* (114) observed a significant decrease (up to 17%) of normalized cross-sectional area of gastrocnemius muscle at long-term, while Weide *et al.* (133) showed that gastrocnemius cross sectional-area normalized for body mass slightly increased at mid- and long-term. Furthermore, two studies (107,114) didn't show any significant correlation between the number of previous BoNT-A injections in treated patients and cross-sectional area alteration over time.

3.6 Muscle mass

Only 1 study (122) evaluated the effect of BoNT-A injection on muscle mass. Lean body mass of the affected lower limb, assessed by dual-energy X-ray absorptiometry, decreased (-1.8%) without statistically significant difference at 4 weeks ($p = 0.316$). However, 12 weeks after injection, lean body mass was significantly increased compared to preinjection (6.7%, $p = 0.003$) and 4 weeks after injection (8.7%, $p = 0.0008$).

3.7 Muscle thickness

Muscle thickness is defined as the longest distance between the superficial and the deep aponeuroses. Muscle thickness of the lower leg was investigated in 7 studies. The results of 5 pre-post studies (95,119,129,131,135) that examined the effect of BoNT-A injection on gastrocnemius muscle thickness in children with CP and adults suffering from chronic stroke were pooled in a meta-analysis. All 5 articles expressed muscle thickness in millimeter. Thus, results are comparable and the mean difference could be used to perform our meta-analysis. No statistically significant within-group difference was found ($p > 0.05$) at short-term nor at mid-term (Figure 4 (95,119,129,131,135)). Furthermore, Cosenza *et al.* (112) reported no significant difference in muscle thickness of BoNT-A treated muscles compared to untreated spastic muscles. Battaglia *et al.* (107) observed a significant smaller muscle thickness in BoNT-A treated limbs compared to unaffected limbs but without any significant correlation between BoNT-A injection and muscle thickness alteration over time.

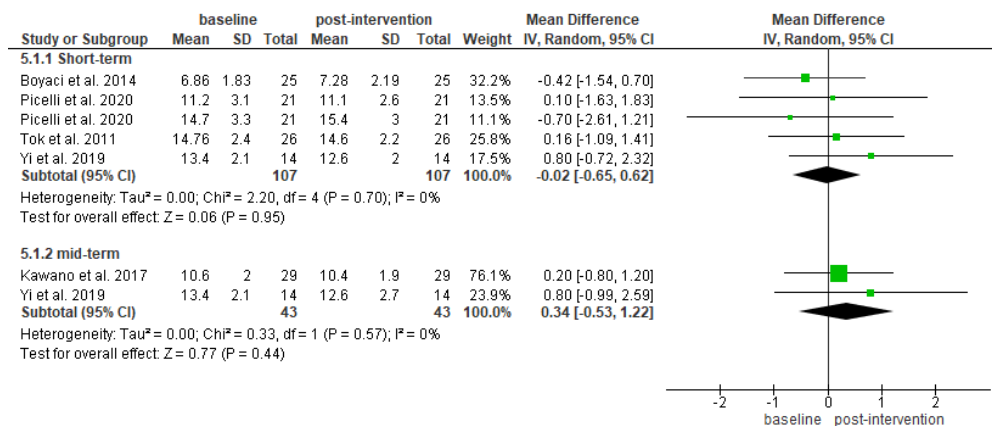


Figure 4. Effect of BoNT-A injection on muscle thickness.

3.8 Pennation angle

Pennation angle was investigated by 5 studies in the lower leg. The results of 3 pre-post studies that examined the effect of BoNT-A injection on pennation angle of 4 different gastrocnemius muscles in resting position at short-term were pooled in a meta-analysis (Figure 5 (129,131,135)). No statistically significant within-group difference was found ($p > 0.05$) at 4 weeks. Due to statistical heterogeneity ($I^2 = 73\%$), meta-analysis at mid-term was not retained for the present paper (additional material). The 3 studies (119,131,135) found a smaller pennation angle at mid-term depending on the ankle position. Furthermore, 1 non-included study (107) reported significantly smaller pennation angle in muscles of hemiparetic BoNT-A treated limb compared to the unaffected limb at rest, but without any significant correlation between the number of previous BoNT-A injections and pennation angle alteration over time.

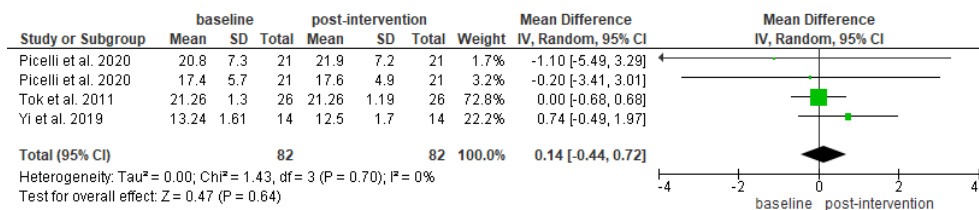


Figure 5. Effect of BoNT-A injection on pennation angle at short-term.

3.9 Fascicle length

Four studies assessed fascicle length (119,131,133,135). No meta-analysis was made because of clinical heterogeneity. Results were contradictory in all studies with an increased or decreased fascicle length at short-, mid-, and/or long-term after BoNT-A injection in the gastrocnemius medialis, depending on the ankle position.

3.10 Muscle and tendon length

Four studies took muscle and tendon length in consideration. No meta-analysis was made because of clinical heterogeneity. Three studies showed an increase in muscle and/or tendon length in the gastrocnemius muscle at short-term (106,136) and mid-term (133). Furthermore, Weide *et al.* (133) observed a slightly decreased normalized muscle length at long-term while normalized tendon length had slightly increased. On the other hand, De Beukelaer *et al.* (114) did not observe any significant change in normalized muscle length and normalized muscle-tendon complex in the gastrocnemius muscle at long-term following BoNT-A injection.

3.11 Muscle echo-intensity

Nine of the included studies mentioned measurement of muscle echo-intensity after BoNT-A injection. The results of 4 pre-post studies (111,127–129) evaluating muscle echo-intensity with the Heckmatt scale of 6 different muscles in children with CP and adults with chronic stroke were pooled in a meta-analysis. Five studies (107,112,114,126,130) were excluded due to excessive clinical heterogeneity. The analysis at short-term (4 weeks) showed no statistically significant within-group difference (Figure 6) (106,122–124). In the same way, Peeters *et al.* (126) observed no statistically significant differences in muscle echo-intensity at mid-term (8 weeks). Furthermore, Cosenza *et al.* (112) showed no statistically significant differences in muscle echo-intensity between treated and untreated spastic cohorts. On the contrary, two studies (114,130) found a significantly higher muscle echo-intensity in BoNT-A treated patients compared to untreated spastic patients. Furthermore, Schless *et al.* (130) observed a positive significant fair association between muscle echo-intensity and the number of previous BoNT-A interventions, while two studies (107,114) didn't find any significant correlation with number of previous treatment cycles.

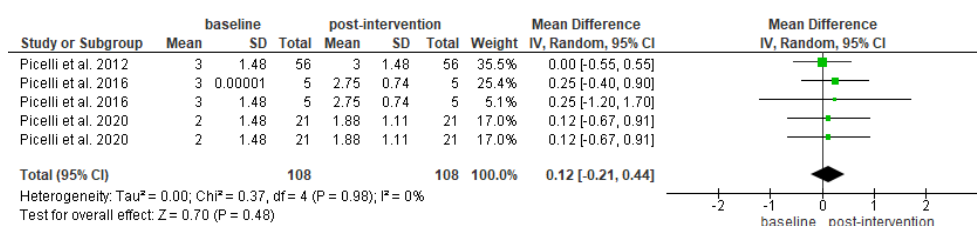


Figure 6. Effect of BoNT-A injections on muscle echo-intensity at short-term.

3.12 Other MRI-related outcomes

Three studies (45,115,123) reported hyperintensity on T₂w scans and increased T₂ from 6 weeks up to 34 months. Weidensteiner *et al.* (45) also observed a trend to an increase of fat fraction in the hyperintense region of interest at 12 weeks but not at 6 weeks post-BoNT-A injection. Furthermore, Weidensteiner *et al.* (45) observed an increased fractional anisotropy and an unchanged apparent diffusion coefficient in hyperintense regions of interest.

4. Discussion

The present study reviewed muscle elasticity and muscle structure changes as reported in the literature after BoNT-A injection in overactive muscles measured by non-invasive imaging methods in humans with neurological conditions. For the first time, a quantitative analysis was performed on this topic. Overall, this systematic review and meta-analysis found, at short-term, a significant improvement of muscle elasticity assessed by USE 4 weeks after BoNT-A injection.

Furthermore, no statistically significant difference was found for muscle structure (muscle thickness, pennation angle, and echo-intensity) at short-term. At long term, however, normalized muscle volume decreased.

4.1 muscle elasticity

According to Mathevon, muscle stiffness, as any viscoelastic tissue, depends on its intrinsic structure and contractile status at rest (97). In our systematic review, 14 studies have addressed muscle elasticity measured by USE.

In a systematic review and meta-analysis of Miller *et al.* (40), the reliability of USE was shown to be moderate in evaluating in vivo muscle elasticity in neurological populations.

It also showed good convergent validity with relevant clinical assessments, and good divergent validity in discriminating tissue changes within and between groups. The same authors (40) showed a linear relationship between USE values and clinical assessment of “spasticity”. Our results are in the same line as 6 authors reported faster shearwave velocities when MAS/TS score is greater. However, no standardized measurement protocol exists regarding which method (type of USE, control of the exerted pressure, number of regions of interest/measurements, probe position,...) may be optimal for assessing muscle elasticity among patients with neurological conditions.

Based on our quantitative data analyses, we can assume that muscle elasticity measured by USE improves with a temporary effect at short term following BoNT-A injections. These results are in line with Mathevon’s systematic review (97), which included 4 studies that analyzed viscoelastic properties of spastic muscles with USE. These studies already showed an improvement in muscle elasticity at short-term after a single BoNT-A injection. These results raise questions, as the reasons explaining this improvement remain unclear. Indeed, the impaired resistance to passive stretch in central neurological disorders, named hyper-resistance, can have neural and non-neural components (27). By decreasing MO, BoNT-A is supposed to act on the neural component of hyper-resistance. However, BoNT-A seems to also act on the non-neural component of hyper-resistance as this reduction of elasticity is a priori linked to tissue-related properties.

Bollens *et al.* also showed a decrease of the neural- and non-neural component as measured by a mechanical oscillatory device 2 and 6 months following BoNT-A injection (137). In the same line, Bar-on *et al.* (106) observed a decrease of both the neural (-96%) and non-neural (-92%) components of ankle joint hyper-resistance, as measured by an instrumented spasticity assessment 2 weeks post BoNT-A injection.

4.2 Muscle structure

Seventeen studies reported outcome parameters related to muscle structure: muscle volume, cross-sectional area, muscle mass, muscle thickness, muscle and tendon length, fascicle length, pennation angle, and echo-intensity.

4.2.1 Muscle atrophy

Muscle atrophy is defined as the loss of muscle tissue, and can include decreased muscle volume, muscle thickness and cross-sectional area. Muscle atrophy is critical, as it could affect the muscle's functional ability and decrease the effectiveness of future BoNT-A injection.

Regarding muscle volume, the quantitative data analysis provides some arguments for a decreased normalized muscle volume at long-term after BoNT-A injection, even if these results should be taken with caution given the small sample size. These results are in line with the numerous studies reporting muscle atrophy in humans with MO undergoing BoNT-A injections. Among others, a decrease of muscle volume had already been reported in the systematic review of Mathevon *et al.* (97), as 3 studies (132,134,138) included in their review observed moderate muscle atrophy (from 4 to 30%), assessed by MRI, and lasting from 5 weeks to 1 year after a single BoNT-A injection.

Data from animal studies in Mathevon's systematic review are in the same line and report post-BoNT-A injection muscle atrophy (from 30% to 60%), starting from 2 weeks post-injection (139). Higher doses of BoNT-A injections increased and prolonged muscle atrophy (75,140,141). The recovery was complete by 12 weeks post-injection in 1 study (141), while 2 other studies reported an incomplete recovery of 89% (140) and 96% (139) up to 1 year later. Furthermore, Fortuna *et al.* (76) observed that repeated injections major this atrophy and increase its duration in animals. However, our qualitative data analysis does not allow us to draw any conclusion in humans about this cumulative effect, as results were contradictory in 2 studies (114,130).

Regarding muscle thickness, no significant change can be identified in the 5 studies included in the meta-analysis at short- and mid-term (95,119,129,131,135). No conclusions can be drawn about the effects of BoNT-A on muscle thickness at long-term. Results at short-term were conflicting in the review by Mathevon *et al* (97).

Regarding cross-sectional area, results were also very conflicting in the 4 studies.

Thus, while muscle atrophy has been evidenced, its magnitude, duration, and reversible nature remain unclear.

This lack of consensus may result from multiple factors related to the heterogeneity between studies, including measurement techniques, normalization, assessed muscles, neurological condition, ambulation status, BoNT-A dosage, and previous BoNT-A exposure. These factors were not studied or not described in our included studies, making interpretation difficult.

4.2.2 Pennation angle

Pennation angle indicates the position of muscle fascicles in relation to the aponeurosis and is an important parameter related to musculoskeletal function. Based on measurements of pennation angle, estimations of the orientation of fascicles can be made (142). In pennate muscles, such as the gastrocnemius, muscle fascicles are obliquely arranged (119).

A decrease in pennation angle indicates an increase in fascicle length, which indicates that the muscle fiber has elongated, leading to an improvement in muscle extensibility (119).

Pennation angle was investigated in 5 studies on the lower leg. Based on our quantitative data analysis, there are some arguments for an unchanged pennation angle in resting position at short-term after BoNT-A injection. However, those results should be taken with caution due to the small sample size. Furthermore, based on our qualitative synthesis, we can assume that pennation angle decreases in resting angle position at mid-term following BoNT-A injections.

Statistical heterogeneity of 73% in the mid-term meta-analysis could be explained by the different timing of assessment in the 3 included studies. Indeed, 2 articles reported a greater reduction in pennation angle 12 weeks after BoNT-A injection (119,135) then at 8 weeks (131). No studies evaluated the effects of BoNT-A injections on pennation angle at long-term.

4.2.3 Fascicle length

Fascicle length is the primary determinant of muscle excursion because it represents the number of sarcomeres working in series (40). This outcome was investigated by 3 studies on the lower leg. Based on our qualitative data analysis, we were unable to draw conclusions about the effects of BoNT-A

injection on fascicle length. Indeed, the fascicle length is dependent of the ankle position and the 3 studies measured fascicle length at a different ankle positions (119,133,135). Moreover, there was a lack of normalization to account for natural muscle/skeletal growth, as only one study (133) investigated normalized fascicle length. Since skeletal growth is found to be altered in spastic CP children compared to typically developing children, normalization of muscle outcomes to skeletal growth should be done (114).

4.2.4 Muscle echo-intensity

Increased echo-intensity on US is supposed to reflect increased muscle fibrosis and intramuscular fat content (127). Picelli et al. (127) reported that patients with higher spastic muscle echo-intensity have a reduced response to BoNT-A and thus hypothesized that the toxin dosage needs to be increased in order to obtain a significant reduction of spasticity. On the other hand, it has been shown that repeated BoNT-A injections decrease the percentage of contractile material, and that muscle fibers are primarily replaced by fibroadipose tissue (76). This leads to a vicious circle which has a possible critical effect, as it could lead to negative impacts on the effectiveness of BoNT-A interventions. In the present review, 9 of the included studies mentioned measurements of muscle echo-intensity after BoNT-A injection. Some articles used a computer-aided gray scale analysis, others used the Heckmatt Scale as an outcome measure for muscle echo-intensity. Six studies, including 3 papers written by the same author, showed no statistically significant difference at short-term (105,121–123) nor between treated and untreated hemiparetic limbs (107,112).

Thus, based on our literature search and data analysis, there are some arguments for an unchanged muscle echo-intensity after a single BoNT-A injection but no conclusion can be drawn about the effects of previous BoNT-A exposure. Based on 1 study (127), Mathevon et al. (97) reached the same conclusion in their systematic review.

5. Perspectives

The results of the present study underline the effects of BoNT-A on muscle elasticity and structure. To date, MRI is considered the most precise and reliable, non-invasive and quantitative method, for measuring regional skeletal muscle mass (42). Besides morphological changes, new MRI sequences and softwares are now able to assess other muscle characteristics. The most promising ones are muscle composition and BoNT-A diffusion. First, fatty infiltration could allow monitoring the extent and severity of changes in muscle composition after BoNT-A injection. Second, the use of MRI is also a promising approach to study the movement of BoNT-A within human muscles (123). Based on animal studies, BoNT-A diffusion can occur up to a distance of 4.5-5cm from the injection site and even into adjacent muscles (45,76,132,143). The precise displacement of BoNT-A could be influenced by different factors, like modality of injection or dilution. Indeed, some studies (144,145) demonstrated that higher doses and/or dilutions result in wider BoNT-A muscle distribution.

Determining BoNT-A diffusion would improve the understanding of its action and could help to optimize injection technique. In the study of Weidensteiner *et al.* (45) and O'dell *et al.* (123) T₂w images and T₂ maps showed hyperintensity but only in parts of the muscles indicating changes at or near the site of BoNT-A injection.

Beyond visual analysis, quantitative biomarkers, in particular T2 relaxation times, average apparent diffusion coefficients, and fractional anisotropy can be derived from tailored MRI sequences and followed up over time to monitor changes induced by the treatment. In the study of Weidensteiner *et al.* (45) fractional anisotropy increased while apparent diffusion coefficient did not change in hyperintense regions of interest, indicating a BoNT-A induced increase in extracellular space and a simultaneous decrease of muscle fiber diameter. In summary, MRI is a promising tool to evaluate the effect of BoNT-A injections on muscle properties. However, further studies are needed on this topic to draw firmer conclusions. There is still place for carefully designed long-term studies using multiparametric MRI studies in neurological patients with MO undergoing BoNT-A injections.

Finally, the long-term effect of BoNT-A on muscle structure remains a matter of debate. Clinically, BoNT-A has a plateau of its peak effect on MO reduction by approximately 1 month post-injection and its pharmacological effect washes out by approximately 3 months. Therefore, injections are repeated every 3-6 months in clinical routine.

The lack of recovery of muscle properties following a BoNT-A injection might reduce the effects of the following injections, and the cumulative effect of injections could even be worse. Thus, it is important to understand the long-term impact of BoNT-A beyond this period and to understand the effects of repeated injections on muscle structure and elasticity as the denervation effects of BoNT-A may be still present in the injected muscle.

6. Limitations

6.1 Limitations of the studies reviewed

The quality of the reviewed articles was variable but most studies had low (n=13) to fair (n=14) quality and only 7 studies had good quality. In half of the studies, the cohorts had received an unspecified number of previous BoNT-A interventions, precluding the study of the effect of previous injections or even the natural evolution of the spastic pathology. Moreover, only one study evaluated the effects of repeated BoNT-A injections, despite those injections being repeated every 3-6 months in clinical practice. Furthermore, all studies but one included patients with CP or stroke. Thus, we cannot draw any conclusion regarding other neurological conditions (TBI, SCI,...). Another limitation is the use of complementary treatments.

Although adjunct treatments are now considered as standard of care, it is difficult to attribute the measured effects solely to the BoNT-A injections. For example, extracorporeal shock wave therapy could have both a neurological effect and a non-neural rheological effect while casting could have an additional non-neural rheological effect. Moreover, rehabilitation (strengthening) could counteract the muscle atrophy effect of BoNT-A injections.

6.2 Limitations of our systematic review and meta-analysis

There are some limitations to consider. First, we included randomized and nonrandomized clinical trials (including pre and post studies) because there were few RCTs on this topic. Indeed, BoNT-A for MO management is now best practice care; therefore RCTs are not ethically appropriate. The number of RCTs was very small (n=3), therefore a pre-post design was the only option.

Furthermore, except for the meta-analysis on muscle elasticity, forest-plots included a small number of subjects and/or studies. Therefore, in their current forms, our statistical analyses might lack some power to detect a significant difference.

In addition, protocols were highly variable between the different studies, which can explain the small meta-analyses and the heterogeneous and sometimes contradictory results. Furthermore, due to the small meta-analyses, no subgroup analysis was made at short-term for children and adults nor for upper and lower limb muscles. However, the studies included in our systematic review lead us to believe that BoNT-A injections induce the same effects on muscle elasticity and muscle structure in children and in adults or in upper and lower limb muscles at short-term. Indeed, Furukawa *et al.* (116) showed equivalent improvements in muscle elasticity in both the biceps brachii and medial gastrocnemius at four weeks after BoNT-A injection. Furthermore, Picelli *et al.* (128) did not find any significant difference in muscle echo-intensity in both upper and lower limb muscles at short-term. Moreover, all studies included in this systematic review showed statistically significant improvements in muscle elasticity at short-term both in children and in adults. In addition, all studies included in this systematic review showed no statistically significant difference in muscle echo-intensity and pennation angle at short-term both in children and in adults.

7. Conclusions

Our study synthesized the current literature regarding changes in muscle structure and elasticity after BoNT-A injection, by diagnostic imaging, in individuals with neurological conditions presenting MO. This review demonstrates that muscle elasticity measured by USE improves temporarily at short-term following BoNT-A injections. Synthesis of existing clinical studies that include post-treatment assessment of muscle structure is hindered by methodological differences between studies.

However, muscle thickness, pennation angle and muscle echo-intensity do not seem to be modified at short-term. On the other hand it appears that, based on a small amount of data, normalized muscle volume decreases at long-term following BoNT-A injections in children with CP suggesting that the timing of re-injection should be considered with caution in this population. No conclusions on other outcome parameters related to muscle structure can be formulated due to a cruel lack of studies investigating the effects of BoNT-A injections on muscle structure at long-term. US and MRI seem to be promising tools to evaluate the effects of BoNT-A injections on muscle structure and elasticity. Further work should focus on the long-term effects of repeated injections on muscle structure and elasticity in neurological populations.

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Analysis and interpretation of Data: Marine Devis, Gaëtan Stoquart

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Tables

Table 1 Summary table of the results.

Author, year	Neurological condition	Age (years) (mean +/- SD)	Population	Control group	Measurement tool	Study criteria	Injected and evaluated muscles	BoNT-A, dose, dilution	BoNT-A naive?	Time of assessment
Alexander <i>et al.</i> 2018 (104)	CP	8.8 +/- 3.1	11	No	MRI	MV	GC, medial hamstrings	Botox, 1.6-12U/kg, 60-340U, 100U/1-2ml	Yes	T-1w, T+4w, T+13w, T+25w
Aşkın <i>et al.</i> 2016 (105)	Stroke	58.5 +/- 8.8	48	Unaffected limb	USE	ME	BB	Botox, 150U, ?	?	T, T+4w
Bar-on <i>et al.</i> 2017 (106)	CP	11	1	No	US	ML	GCM	?, 6U/kg, ?	?	T, T+2w, T+4w
Battaglia <i>et al.</i> 2021 (107)	Stroke	63.78 +/- 11.21	53	Unaffected limb	US	CSA, MT, PA, EI	GCM, soleus	?	No	?
Bertan <i>et al.</i> 2020 (108)	CP	4.6 +/- 1.2	17	Yes	USE	ME	GCM, GCL	Botox, 50U/head, 100U/2ml	Yes	T-4w, T+4w, T+12w
Bilgili <i>et al.</i> 2017 (109)	CP	8.58 +/- 2.48	12	No	USE	ME	GCM	Botox, 200-300U, ?	?	T, T+4w
Boyaci <i>et al.</i> 2014 (95)	CP	4 +/- 1.4	16	Yes	USE, US	ME, MT	GCM	Botox, 1.5U/kg, ?	Yes	T, T+4w
Brandenburg <i>et al.</i> 2018 (110)	CP	5.2 +/- 1.7	9	No	USE	ME	GCL	?, 3.3U/kg, ?	Both	T-4w, T+4w, T+12w

Campanella <i>et al.</i> 2022 (111)	Stroke	61	14	Unaffected limb	USE, US	ME, EI	GC, soleus, BB, brachialis, flexor digitorum superficialis	Xeomin, 20-100U, 400U, 50U/ml	?	T, T+4w
Cosenza <i>et al.</i> 2020 (112)	Stroke	64 +/- 10.87	37	Unaffected limb	US	MT, CSA, EI	Rectus femoris	?	Both	?
Dağ <i>et al.</i> 2020 (113)	CP	6 +/- 2.8	24	No	USE	ME	GCM	Botox, 3-6U/kg, ?	Both	T, T+4w
De Beukelaar <i>et al.</i> 2022 (114)	CP	3.5 +/- 0.6	26	Yes	US	MV, ML, TL, CSA, EI	GCM	Botox, 2.7U/kg, ?	Both	T, T+24w
de Coulon <i>et al.</i> 2021(115)	CP	4.5	2	Unaffected limb	MRI	Muscle intensity	GC, soleus	?, 4-6U/kg, 100-170U, ?	?	T, T+1y
Furukuwa <i>et al.</i> 2021 (116)	Stroke	64	2	No	USE	ME	GCM, BB	Botox, 30-50U/head, ?	?	T, T+2w, T+4w, T+8w, T+12w
Gao <i>et al.</i> 2019 (117)	Stroke	45	7	Unaffected limb	USE	ME	BB	Botox, 50-100U, 100U/2-4ml	?	T, T+2-4w
Hasegawa <i>et al.</i> 2022 (118)	Stroke	62.7 +/- 8.7	10	No	USE	ME	GCM	Botox, 20-70U, ?	No	T, T+4w
Kawano <i>et al.</i> 2017 (119)	CP	6.2	18	Yes	US	PA, MT, FL, muscle elongation rate	GCM	Botox, 4U/kg, ?	Yes	T, T+12w
Kwon <i>et al.</i> 2012 (120)	CP	2.3	1	No	USE, US	ME, MT	GCM	Botox, 20U, ?	?	T, T+4w

Kwon <i>et al.</i> 2021 (121)	CP	6.4 +/- 2.5	15	Yes	USE	ME	GCM	Meditoxin, 4- 6U/kg, ?	No	T, T+4w, T+12w
Lee <i>et al.</i> 2021 (122)	CP	5.3 +/- 2.5	15	Unaffected limb	DXA	Muscle mass	GCM, GCL, soleus, TP, peroneus longus	Botox, 2-8U/kg, ?	Both	T, T+4w, T+12w
O'Dell <i>et al.</i> 2017 (123)	Stroke	61.8 +/- 12.5	5	No	MRI	MV, T2RT	GCL, soleus	Botox, 200U, ?	Yes	T, T+8w, T+12w
Park <i>et al.</i> 2012 (124)	CP	4.7 +/- 1.8	17	No	USE	ME	GCM, GCL	Botox, ?, ?	?	T, T+4w
Peeters <i>et al.</i> 2020 (125)	CP	7.7 +/- 3.7	17	Yes	US	ML, TL	GCM	Botox, 1.93-3.66U/kg, ?	?	T, T+2w
Peeters <i>et al.</i> 2022 (126)	CP	7.25 +/- 2.5	25	Yes	US	MV, EI, ML, TL	GCM, semitendinosus	Botox, 1.7-2.7U/kg, 50U/site, 100U/5ml	Both	T, T+8w
Picelli <i>et al.</i> 2012 (127)	Stroke	59.2 +/- 14.1	56	No	US	EI	GCM, GCL	?, 250U/head, ?	No	T, T+4w
Picelli <i>et al.</i> 2016 (128)	CP	9.7	10	Yes	USE, US	ME, EI	GC, medial hamstrings, elbow flexors, wrist flexors	Botox, ?, 100U/1ml	?	T, T+4w
Picelli <i>et al.</i> 2020 (129)	Stroke	65.6 +/- 14.5	21	No	USE, US	ME, EI, MT, PA	GCM, GCL	Dysport, 250U/head, 500U/2ml	Both	T, T+4w
Schless <i>et al.</i> 2019 (130)	CP	8.8 +/- 2.2	19	Yes	US	MV, EI	GCM	?	No	?
Tok <i>et al.</i> 2011(131)	Stroke	55 +/- 14	26	Unaffected limb	US	PA, FL, MT	GCM, GCL	?, 100-150U, ?	?	T, T+10d, T+8w

Van Campenhout <i>et al.</i> 2012 (132)	CP	12	7	No	MRI	MV	Psoas	Botox, 2U/kg, 20U/1ml	No	T, T+8w, T+24w
Weide <i>et al.</i> 2019 (133)	SP of unknown origin	6	1	No	US	ML, TL, CSA, MV, FL	GCM	Botox, 360U, ?	No	T-1w, T+9w, T+26w
Weidensteiner <i>et al.</i> 2021 (45)	CP	11.6	6	No	MRI	T2RT, FF, ADC, FA	GCM, GCL, soleus	Botox, 2.8-8.8U/kg, 50-100U/head, 50U/1ml	Both	T, T+6w, T+12w
Williams <i>et al.</i> 2013 (134)	CP	8.4 +/- 1.8	15	No	MRI	MV	GC, medial hamstrings, soleus, adductors, rectus femoris, TP	Botox, 1-6U/kg, ?	No	T-2w, T+5w
Yi <i>et al.</i> 2019 (135)	CP	4.6 +/- 2.3	14	Unaffected limb	US	MT, FL, PA	GCM	Botox, 2.6-4.4U/kg, 100U/2ml	No	T,T+4w, T+12w
ADC: apparent diffusion coefficient; BB: biceps brachialis; CP: cerebral palsy; CSA: cross-sectional area; DXA: dual-energy X-ray absorptiometry; EI: echo-intensity; FA: fractional anisotropy; FF: fat fraction; FL: fascicle length; GCM: gastrocnemius medialis; GCL: gastrocnemius lateralis; ME: muscle elasticity; ML: muscle length; MRI: magnetic resonance imaging; MT: muscle thickness; MV: muscle volume; PA: pennation angle; SD: standard deviation; SP: spastic paresis; T2RT: T2 relaxation time; TL: tendon length; TP: tibialis posterior; US: ultrasonography; USE: ultrasound elastography; ?: unknown										

Table 2 Modified Downs and Black.

	Reporting	External validity	Biais (internal validity)	Confounding (internal validity)	Power	Total (/28)
Alexander <i>et al.</i> 2018 (104)	9	2	5	3	1	20
Aşkın <i>et al.</i> 2016 (105)	7	0	6	0	0	13
Bar-on <i>et al.</i> 2017 (106)	5	0	4	1	0	10
Battaglia <i>et al.</i> 2021 (107)	7	1	4	2	1	15
Bertan <i>et al.</i> 2020 (108)	9	1	6	5	0	21
Bilgici <i>et al.</i> 2017 (109)	8	0	6	0	0	14
Boyacı <i>et al.</i> 2014 (95)	9	1	5	4	0	19
Brandenburg <i>et al.</i> 2018 (110)	8	1	5	2	0	16
Campanella <i>et al.</i> 2022 (111)	9	0	6	1	0	16
Cosenza <i>et al.</i> 2020 (112)	7	0	4	1	0	12
Dağ <i>et al.</i> 2020 (113)	6	0	6	1	0	13
De Beukelaar <i>et al.</i> 2022 (114)	10	2	6	3	0	21
de Coulon <i>et al.</i> 2021 (115)	6	0	5	1	0	12
Furukuwa <i>et al.</i> 2021 (116)	6	0	4	1	0	11
Gao <i>et al.</i> 2019 (117)	7	1	5	3	0	16
Hasegawa <i>et al.</i> 2023 (118)	9	1	6	3	0	19
Kawano <i>et al.</i> 2017 (119)	7	1	5	2	0	15
Kwon <i>et al.</i> 2012 (120)	6	1	4	1	0	12
Kwon <i>et al.</i> 2021 (121)	8	0	5	1	0	14
Lee <i>et al.</i> 2021 (122)	7	0	5	0	0	12
O'Dell <i>et al.</i> 2017 (123)	6	0	4	0	0	10
Park <i>et al.</i> 2012 (124)	8	1	5	3	0	17
Peeters <i>et al.</i> 2020 (125)	9	1	6	4	0	20

Peeters <i>et al.</i> 2022 (126)	10	1	5	4	0	20
Picelli <i>et al.</i> 2012 (127)	9	1	6	4	1	21
Picelli <i>et al.</i> 2016 (128)	10	1	6	6	0	23
Picelli <i>et al.</i> 2020 (129)	7	1	5	2	0	15
Schless <i>et al.</i> 2019 (130)	8	1	4	4	0	17
Tok <i>et al.</i> 2011 (131)	8	1	5	3	0	17
Van Campenhout <i>et al.</i> 2012 (132)	8	1	6	2	0	17
Weide <i>et al.</i> 2019 (133)	6	0	4	1	0	11
Weidensteiner <i>et al.</i> 2021 (45)	6	0	4	1	0	11
Williams <i>et al.</i> 2013 (134)	9	1	5	3	0	18
Yi <i>et al.</i> 2019 (135)	7	0	5	0	0	12

4.4 Update of the systematic review and meta-analysis

To complement the findings of this thesis, which included studies published up to April 2023, I conducted an updated search of the recent literature. This search identified a particularly informative study investigating morphological changes in skeletal muscle after BoNT-A injections in children with CP, with longitudinal follow-up at 3, 6, and 12 months (146). The results of this study were subsequently incorporated into our meta-analyses on different muscle outcomes, allowing for a more comprehensive assessment of the effects of BoNT-A on skeletal muscle structure. Results are presented here below while interpretation and discussion of these new findings are reserved for the general discussion of the thesis (section 8.2).

4.5.1 Muscle volume

In the initial meta-analysis assessing the short-term effects of BoNT-A on muscle volume, statistical heterogeneity was considered too high to allow for a reliable pooled estimate, and the meta-analysis was therefore not retained. Following the inclusion of data from our prospective study (chapter 5), the analysis was updated, allowing for a more robust and comprehensive synthesis of the available evidence. Three studies were included, comprising a total of 57 participants. The pooled analysis using standardized mean differences showed a moderate but significant reduction in muscle volume already at 1 month post-injection (SMD = -0.45, 95% CI -0.74 to -0.17, $p < 0.002$) (Figure 9).

Regarding the mid-term effects of BoNT-A on muscle volume, no quantitative synthesis could be performed in the initial systematic review due to the limited number of available studies reporting comparable outcomes.

However, the inclusion of this new study (146) allowed us to identify additional relevant data, making it possible to conduct a meta-analysis on the effects of BoNT-A injections on normalized muscle volume at 3 months post-injection in children with CP. The results of this analysis indicate that normalized gastrocnemius muscle volume remained reduced at 3 months following BoNT-A injections, with a moderate effect size of -0.43 (95% CI: -0.80 to -0.06 ; $p = 0.02$) (Figure 10).

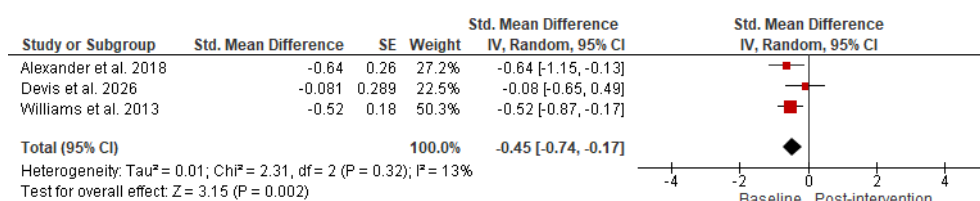


Figure 9. Effect of BoNT-A injections on muscle volume 1 month after intervention.

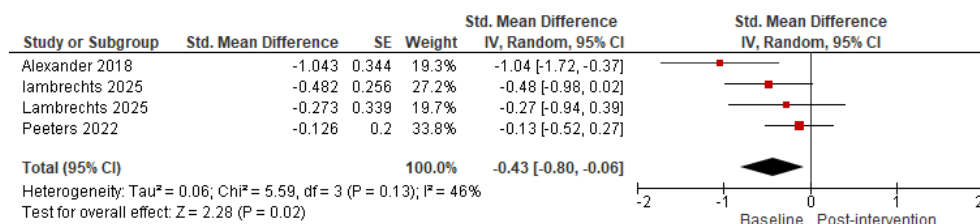


Figure 10. Effect of BoNT-A injections on normalized muscle volume in children with CP 3 months after intervention.

Furthermore, this new study (146) was subsequently incorporated into our meta-analysis on the long-term effects (6 months) of BoNT-A on medial gastrocnemius normalized muscle volume in children with CP. It allowed us to highlight two important observations. First, it increased the overall effect size for muscle volume reduction, which was previously minimal (-0.17 ; 95% CI -0.25 - -0.09 ; $p < 0.0001$).

With the addition of this study, the overall effect size is now -0.45 (95% CI: -0.81 to -0.10, $p = 0.01$), which can be interpreted as a moderate effect (Figure 11). Second, examination of the forest plot reveals a clear distinction between injection-naïve patients (3 studies) and non-naïve patients (2 studies). For naïve patients, the effect size is -0.70 (95% CI: -1.04 to -0.37, $p < 0.0001$), which can be considered a large effect, whereas for non-naïve patients it is -0.07 (95% CI: -0.47 to 0.33, $p = 0.74$).

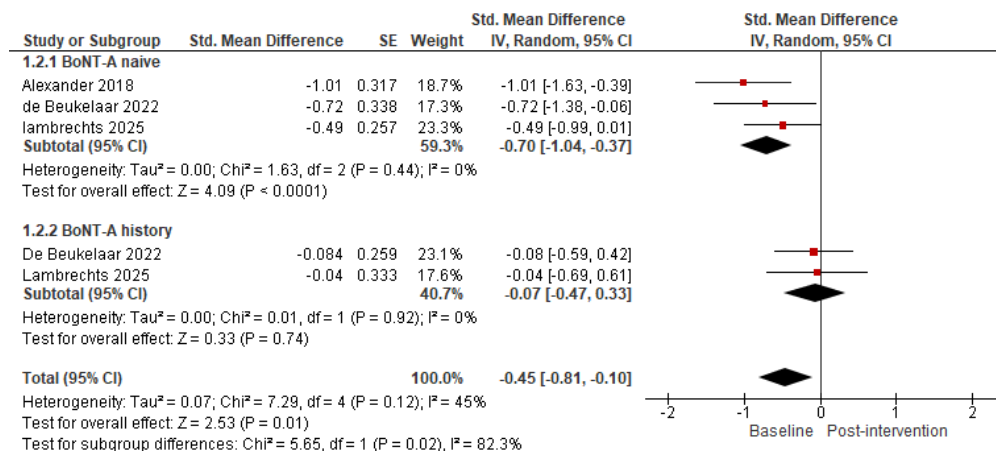


Figure 11. Effect of BoNT-A injections on normalized muscle volume in children with CP 6 months after intervention.

Finally, although a meta-analysis could not be performed for longer follow-up periods due to the limited number of available studies, the newly included study also provides valuable long-term data up to 12 months after BoNT-A injection. In this study (146), normalized muscle volume remained significantly reduced one year after treatment, with a decrease of 8% compared with baseline ($p = 0.0086$) in BoNT-A naïve children.

4.5.2 Cross-sectional area

In the initial systematic review, a quantitative synthesis of the mid- and long-term changes in CSA following BoNT-A treatment could not be performed due to the limited number of studies reporting comparable outcomes. The inclusion of this new study (146) provided additional relevant data, enabling the conduction of a new meta-analysis evaluating CSA at long-term post-injection in children with CP.

The meta-analysis of 4 independent cohorts of children with CP showed a significant reduction in muscle CSA normalized to body weight (mm^2/kg) 6 months following treatment. The pooled mean difference was $-1.95 \text{ mm}^2/\text{kg}$ (95% CI: -2.48 to -1.42 ; $p < 0.001$), with no heterogeneity observed ($I^2 = 0\%$), indicating a consistent effect across studies (Figure 12).

A meta-analysis at 1-year follow-up could not be performed due to limited data. However, the study by Lambrechts *et al.* (146) reported that the CSA of the muscle was no longer significantly different from baseline, suggesting a trend toward recovery at one year. More specifically, the mean difference between baseline and 12 months was -0.35 ($p = 0.10$), compared with -0.69 at 6 months ($p = 0.0009$).

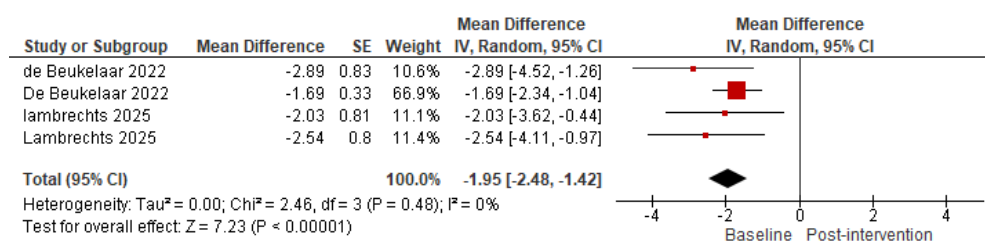


Figure 12. Effect of BoNT-A injections on normalized cross-sectional area in children with CP 6 months after intervention.

4.5.3 Muscle length

Regarding muscle length, with the inclusion of the recent study of Lambrechts *et al.* (146), a meta-analysis could be performed to evaluate changes in normalized muscle length at mid-term and long-term follow-up. Three months following BoNT-A injection, the pooled analysis showed no significant change in muscle length, with a mean difference of 0.07% (95% CI: -0.34 to 0.49) (Figure 13).

Similarly, 6 months post BoNT-A treatment, no significant effect was observed, with a mean difference of 0.00% (95% CI: -0.34 to 0.35), indicating stable muscle length over time and consistent findings across studies (Figure 13).

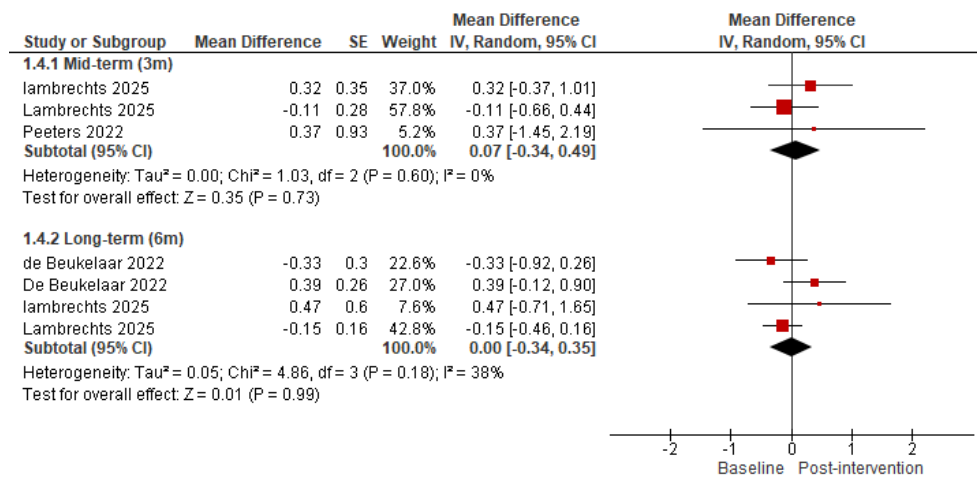


Figure 13. Effect of BoNT-A injections on normalized muscle length in children with CP 3 and 6 months after intervention.

4.5.4 Muscle quality

As a reminder, in our previously published meta-analysis, no statistically significant difference in echo-intensity was found at short-term following BoNT-A injection. Furthermore, in the initial systematic review, a quantitative synthesis of the mid- and long-term effects of BoNT-A on muscle echo-intensity could not be performed due to the limited number of available studies reporting comparable outcomes. However, the inclusion of this new study (146) allowed us to identify additional relevant data, making it possible to conduct a meta-analysis on the effects of BoNT-A injections on muscle echo-intensity at 3 and 6 months post-injection in children with CP. At mid-term, the pooled analysis showed a mean increase in muscle echo intensity of 8.9 units (95% CI 4.6–13.2, $p < 0.0001$), corresponding to a moderate effect size (Figure 14). At long-term follow-up (6 months), this effect was no longer statistically significant, with a pooled mean difference of 4.18 units (95% CI -1.48 to 9.84; $p = 0.15$) (Figure 14). In addition, heterogeneity was moderate ($I^2 = 58\%$), indicating variability in the effect across studies.

4.5.5 Summary of the results

A synthesis of the updated evidence together with the results from the original systematic review that were not modified by the update, thereby providing a comprehensive overview of the available literature, can be found in Table 4.

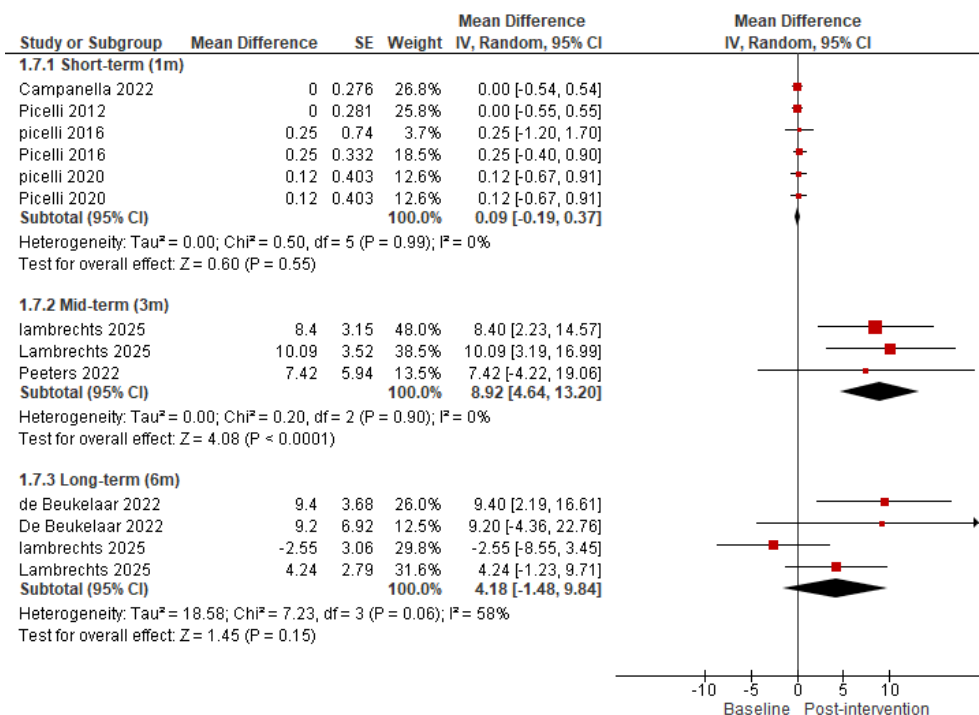


Figure 14. Effect of BoNT-A injections on muscle echo-intensity in children with CP 1, 3 and 6 months after intervention.

Table 4. Summary of the results.

Outcome	Short-term (1month)	Mid-term (3months)	Long-term (6months)
Muscle volume	Decreased	Decreased	Decreased
Cross-sectional area	?	?	Decreased
Muscle length	?	Unchanged	Unchanged
Muscle echo-intensity	Unchanged	Increased	Unchanged
Muscle thickness	Unchanged	Unchanged	?
Pennation angle	Unchanged	?	?

Chapter 5. Evaluation of post stroke spastic calf muscle structure using quantitative MRI following repeated injections of Botulinum toxin type A

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Manuscript under review.

5.1 Study rationale, objectives and hypotheses

As BoNT-A injections are routinely repeated every 3–6 months in clinical practice, and because several histological studies in animals have shown that repeated injections may exacerbate deleterious effects on muscle structure, we carried out a second study related to this first objective. This clinical study consisted of a quantitative MRI evaluation of post-stroke spastic calf muscle structure following repeated BoNT-A injections.

Considering evidence from animal studies (76,96,140) suggesting cumulative muscle damage following repeated BoNT-A administrations, we hypothesized that repeated injections would be associated with greater structural muscle alterations in individuals with chronic post-stroke spasticity, including increased muscle atrophy and fat infiltration.

It should be noted that the protocol presented here below was included in a broader research project recently published by Selves *et al.* examining the effects of 3 cycles of progressively higher doses of BoNT-A on functional parameters (147) and sagittal kinematic gait parameters (148) in individuals with multifocal spasticity following stroke. Consequently, the study we conducted on the effects of repeated BoNT-A injections on muscle structure assessed by MRI constituted a secondary outcome of this larger project. For completeness, the main results of the studies by Selves *et al.* are briefly presented before introducing the MRI-based analyses.

Twenty-five participants were included. Activities remained stable after the 1st injection but improved after the 2nd and 3rd cycles on the 6MWT (+0.07 and +0.12 m/s, respectively, $p < 0.05$) and TST (-13.7 s and -14.8 s, respectively, $p < 0.05$). Spasticity significantly reduced after each injection cycle, with greater reductions after the 2nd and 3rd cycles.

Sagittal kinematic improvements were not observed after the first injection cycle but became apparent after the third. Significant improvements were observed in two postural gait patterns: pes equinus during stance, with a mean increase in ankle dorsiflexion of 3° ($SD = 6^{\circ}$, $p = 0.008$), and stiff knee gait, with a mean increase in knee flexion during swing of 7° ($SD = 7^{\circ}$, $p < 0.001$). Those studies showed that progressively higher doses of BoNT-A led to improvements in walking activities, spasticity, and sagittal knee and ankle kinematics in individuals with multifocal post-stroke spasticity, with the greatest effects emerging after successive treatment cycles; suggesting either cumulative benefits, dose-dependent effects, or a combination of both.

5.2 Abstract

Background. Botulinum toxin type A (BoNT-A) injections reduce lower-limb muscle overactivity (MO), but their long-term impact on muscle structure remains debated.

Aim. This prospective study used MRI to quantify the following changes induced in muscles by repeated BoNT-A Injections: muscle volume, fat fraction, T_2 relaxation time (T_2 -RT) and apparent diffusion coefficient (ADC) in the BoNT-A injected muscles and in their homologue muscles of the less-affected limb.

Methods. Twelve individuals with plantar flexors spasticity post-stroke received 3 cycles of BoNT-A in the calf muscles at 3 months intervals. Participants were evaluated immediately before and one month after first and third BoNT-A injections. A paired-sample t-test compared affected and less-affected muscles, while repeated-measures ANOVA assessed changes over time. Correlations between MRI parameters and impairments were evaluated.

Results. The injected muscles had a significantly smaller muscle volume, higher fat fraction, and increased T_2 -RT compared to the less-affected side. Muscle volume of the affected gastrocnemius muscles decreased from V1 to V3 in almost all patients. However, ANOVA was not significant. Fat fraction significantly increased between V1 and V3 in the affected GM muscles. T_2 -RT significantly increased between the different timepoints and V3 in the affected gastrocnemius muscles. No correlations were found between MRI parameters and other impairments.

Conclusion. Repeated BoNT-A injections in chronic stroke patients may cause some muscle atrophy, increased muscle fat content and water content, suggesting progressive muscle alterations. This study highlights the need for tailored rehabilitation to mitigate potential muscle deterioration following BoNT-A treatment.

ClinicalTrials registration: NCT04544280

Keywords: stroke, muscle spasticity, botulinum toxins, muscular atrophy, magnetic resonance imaging, lower limb

5.3 Manuscript

Introduction

Muscle overactivity (MO) is one of the consequences of upper motor neuron syndromes that can affect children and adults. This often leads to gait impairment after stroke, impacting functional independence and quality of life (149).

Botulinum toxin type A (BoNT-A) injections are the first line recommended treatments to manage focal MO after stroke (87). Clinically, BoNT-A has a plateau of its peak effect on MO reduction by approximately 1 month post-injection and its pharmacological effect washes out by approximately 3 months. Therefore, injections are repeated every 3-6 months in clinical routine. Evidence supports the effectiveness of a single BoNT-A injection in reducing lower-limb MO, but long-term impact on muscle structure is controversial (150). Indeed, some authors have shown that a single BoNT-A injection could be responsible for changes in muscle structure (96). This possible effect is critical, as it could negatively impact the effectiveness of BoNT-A interventions, and the cumulative effect of injections could even be worse.

A previous meta-analysis (151) reviewed the literature dealing with the muscle structure changes after BoNT-A injection, by means of noninvasive imaging. Synthesis of studies was hindered by methodological differences between studies. However, muscle thickness, pennation angle and muscle echo-intensity did not seem to be modified at short-term (1 month) following a single BoNT-A injection. On the other hand, it appeared that, based on a small amount of data, muscle volume decreases at 6 months following a single BoNT-A injection in children with cerebral palsy.

No conclusions on other outcome parameters related to muscle structure could be formulated due to a cruel lack of studies investigating the effects of BoNT-A injections on muscle structure at long-term. Moreover, studies on multiple BoNT-A injection cycles are limited.

The development of new imaging sequences and image post-processing softwares in Magnetic Resonance Imaging (MRI) opens up the field of non-invasive muscle structure assessment (152). To the best of our knowledge, no previous study has investigated the changes induced in muscle structure measured by MRI following repeated BoNT-A injections. Thus, the aim of this exploratory study was to quantify the changes in muscle structure following repeated BoNT-A injections using the following MRI-derived parameters: muscle volume, fat fraction, T_2 relaxation time (T_2 -RT) and apparent diffusion coefficient (ADC) in the BoNT-A injected muscles and in their homologue muscles of the less-affected limb. The findings of this study could contribute to better understand the physiological and therapeutic effects of BoNT-A injections on muscles in patients with MO.

Materials and methods

This prospective, non-randomized, dose-titration, multicentre cohort study followed the STROBE checklist for cohort studies (153). The protocol was registered on clinicaltrials.gov (NCT04544280) as part of a larger study recently published by Selves *et al.* (147) and approved by the local ethics committee. All participants provided written informed consent before participating.

Participants

Patient recruitment was performed as part of the study of Selves *et al.* (147). Participants were recruited from the Physical Medicine and Rehabilitation Departments of the Cliniques universitaires Saint-Luc and Centre Hospitalier Universitaire de Namur site Godinne (Belgium), from the in and outpatient clinic. Inclusion criteria were as follows i) age ≥ 18 years old, ii) hemiparesia/-plegia following stroke, iii) at least 3 months after injury, iv) lower limb spasticity, including the plantar flexors (Modified Ashworth Scale (MAS) ≥ 1) resulting in gait impairment objectified by the clinical assessment, v) indication for BoNT-A injections of up to 800 U of Inco BoNT-A, as assessed by the investigators (experienced PM&R physicians). Patients were excluded if they had i) any other neurological or orthopaedic condition that might interfere with the affected limb function, ii) aphasia or major cognitive impairment limiting functional assessment, iii) previous BoNT-A injection in the last 3 months, iv) underlying neuromuscular disease.

Study design

The study design is shown in Figure 1. Participants received 3 cycles of progressively higher doses of incoBoNT-A at 3 months intervals (50 U/ml in normal saline). At screening, investigators (physicians in PM&R with 8-20 years of experience in spasticity management) selected the target muscles and doses for the first injection cycle. Muscle selection was based on participants' needs. The primary impairment targeted was pes equinus or equinovarus foot, thus treatment focused on the ankle plantar flexors (gastrocnemius lateralis (GL), gastrocnemius medialis (GM), and soleus). The total dosage for the lower limb was systematically increased with each cycle, although the dose per muscle was not necessarily increased.

Muscle and dose selection are not reported here as they fall outside the scope of the paper, but can be found in the article of Selves *et al* (147). Participants were evaluated immediately before (V1) and one month after first (V2) and third (V3) BoNT-A injections, resulting in 3 MRIs and clinical evaluations.

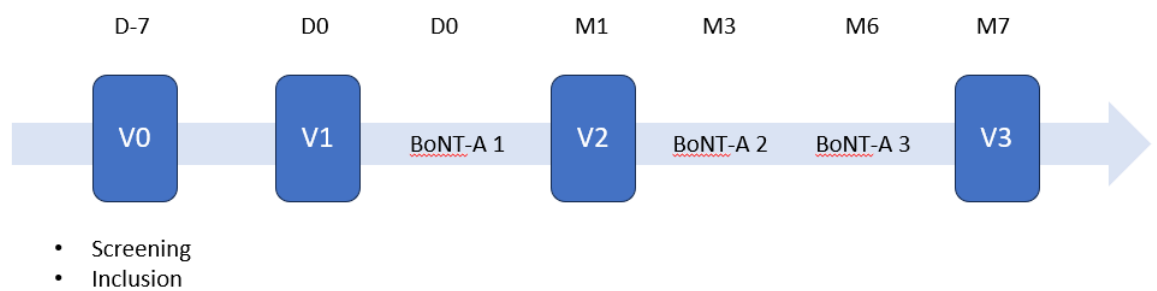


Figure 1. Study design. BoNT-A: Botulinum toxin type A injection; D: day; M: month; V: visit.

Outcome

MRI

MRI studies were performed with a whole-body research-dedicated 3T Signa Premier system (GE HealthCare). The patients were positioned in supine position, feet first, on the MRI table. Anatomical images consisted in a 2D T₂-weighted fast spin echo sequence using a Dixon water-fat separation technique and the following parameters: 66 axial slices with 5 mm thickness and 1 mm gap; 40x20x50 cm³ FOV; 1x1x5 mm³ acquisition voxel; TE/TR = 60/4567 ms; echo-train length = 17; acceleration factor = 2 (ARC); bandwidth = 250 kHz; NEX = 2 and acquisition time = 2min36s. A 3D IDEAL IQ (GE HealthCare) protocol was used for fat quantification.

It features a multi-echo fast spoiled gradient echo sequence along with an automated post-processing tool to calculate water-only, fat-only images and fat fraction maps. The sequence was implemented with the following parameters: 6 echoes; axial acquisition, $1.6 \times 1.6 \times 10 \text{ mm}^3$ acquisition voxel; $40 \times 40 \times 32 \text{ cm}^3$ FOV; TE/TR = 1.2, 2.2, 3.2, 4.1, 5.1, 6.1/8 ms; flip angle = 4° ; acceleration factor = 2 (ARC); bandwidth = 111.11 kHz and acquisition time = 1min21s. A 2D spin-echo with echo-planar imaging readout diffusion tensor imaging sequence was used to compute ADC maps with the following parameters: 50 axial slices with 7 mm thickness and 1 mm gap; $40 \times 20 \times 40 \text{ cm}^3$; $3.7 \times 1.6 \times 7 \text{ mm}^3$ acquisition voxel; TE/TR = 43.1/4000 ms; 24 monopolar diffusion gradient directions; b-values = 0 and 400 s/mm^2 , fat saturation, acceleration factor = 2 (ASSET); 65% partial Fourier acquisition; two shim volumes placed manually on each calf and acquisition time = 1min48s. Fat fraction and ADC maps were calculated inline by the MRI software. For T_2 -mapping, an axial T_2 -weighted fast spin echo with 8 echoes was implemented to cover both calfs entirely. Fifty 7 mm thick slices were used with 1mm gap, TR/TE=2800/7.8-62.3 ms, FoV= $40 \times 20 \text{ cm}^2$, voxel size= $1.2 \times 1.6 \text{ mm}^2$, Bandwidth=62.5KHz, NEX=1, Acceleration factor=1 (Asset), Acquisition time=12min08. Image analysis was performed in the affected as well as in the less-affected lower limb. The following muscles were delineated: gastrocnemius lateralis, gastrocnemius medialis, and soleus. Muscle volume was assessed by segmenting the involved muscles on out-of-phase Dixon images (axial FLEX V1). A semi-automatic segmentation was performed by a physician (MD) on a limited number of slices, and a semi-automatic method using a combination of diffeomorphic registrations deployed as an FSL-based plugin was used to propagate these segmentations to the remaining slices (154,155). Manual corrections were performed after each propagation.

The measurement of fat fraction in muscles monitors the extent and severity of muscle fibers replacement and destruction. The higher the fat fraction, the greater the muscle destruction. Fat fraction was assessed on 3D axial IDEAL IQ images. The masks obtained previously for muscle volume analysis were resampled in order to fit on the IDEAL IQ images. Finally, T_2 -RT and ADC were calculated on a PACS workstation (Carestream Vue, Philips, Best, The Netherlands) from parametric maps generated by the scanner after each acquisition. T_2 -RT was used as a quantitative marker of inflammation and muscle oedema (156). Indeed, an increase in water content leads to an increase in the T_2 -RT value that manifests as hyperintense areas on T_2 -weighted images. As for ADC, it is a quantitative parameter derived from diffusion weighted imaging and diffusion tensor imaging. It measures the magnitude of diffusion of water molecules within tissue and is influenced by microstructural factors such as cellularity, extracellular space, and barriers to water movement. To calculate T_2 -RT and ADC, 3 regions of interest (ROI) of 100mm² were delineated in each muscle on 3 consecutive slices (slice of appearance of the popliteus muscle $\pm$ 1 slice). The exact positioning of each ROI (on the two extremities and in the mid portion of the studied muscle) was verified in consensus by clinicians and radiologists. Mean ADC and mean T_2 -TR values derived from the 9 ROIs for each muscle were used for statistical analysis.

Clinical examination

MO of the calf muscles was evaluated using the MAS and Modified Tardieu Scale (MTS) in the supine position with both a flexed and extended knee. The ankle dorsiflexion range of motion was measured in the supine position while extending and flexing the knee using a hand goniometer.

Motor control of lower extremity was assessed with the Fugl-Meyer test (subscale of motor function /34 points). Ongoing medication and rehabilitation treatments were recorded at each visit with no changes allowed from 2 weeks prior to screening and throughout the study. Participants were actively questioned about adverse events.

Statistical analysis

Statistical analyses were performed using SPSS Statistics, v. 28 (IBM, New York). Continuous data are presented as mean and standard deviation or median and first-third quartile. Categorical data are presented as counts and percentages. Normal distribution of variables was evaluated with statistical (Shapiro-Wilk) and graphical approaches (Q-Q plot). A paired-sample T test was performed to assess differences between the affected and less-affected muscles. An equivalent Wilcoxon signed rank test was used instead if results were abnormally distributed. A one-way repeated measures analysis of variance (ANOVA) with Bonferroni adjustment for multiple comparison was performed to compare the different parameters between the different visits. In addition, if the one-way repeated measures ANOVA was non-significant, a paired-sample T test was performed between V1 and V3 to increase statistical power by removing a possible confounding visit given the small sample size. Moreover, equivalent ANOVA Friedman analyses with Bonferroni adjustment for multiple comparison were performed if results were abnormally distributed. Correlations between MRI-derived parameters and impairments were evaluated. Pearson/ Spearman correlation was considered good, moderate, or poor if the correlation coefficient (r/ρ) was >0.6 , $0.3 < r/\rho < 0.6$, <0.3 , respectively (157). Missing data were not replaced. For all tests, the statistical significance was set at $p < 0.05$.

Results

Twelve participants with chronic stroke were included in the study. Of those 12 participants, 58% were males, mean age was 54 ± 10 years, 67% suffered from an ischemic stroke, and 52% were due to a left-brain lesion. Mean time since stroke was 9 ± 6 months. Five (42%) participants had already received past BoNT-A injection in the gastrocnemius and/or soleus muscle with a mean duration since last BoNT-A injection of 6.4 ± 3 months.

Clinical examination

MO of the calf muscles, evaluated with MAS and MTS, knee-extended, significantly improved after each injection cycle compared to baseline (V2: MAS: -1.2 , $p < 0.001$, MTS-V2: -0.8 , $p = 0.001$, MTS-V3: -0.8 , $p = 0.01$; V3: MAS: -1.6 , $p < 0.001$, MTS-V2: -1.2 , $p = 0.001$, MTS-V3: -1.4 , $p = 0.001$). A more pronounced decrease was observed after the 3rd injection (V3) compared to the 1st injection (V2) (MAS: -0.8 ; $p = 0.02$, MTS-V3: -0.6 ; $p = 0.04$). Similarly, MO of the calf muscles, knee-flexed, significantly decreased (MAS and MTS, $p < 0.001$) after each injection cycle. Moreover, the reduction in spasticity was significantly greater after the 3rd injection (V3) compared to the 1st injection (V2) as evaluated with MTS-V3 (-0.6 ; $p = 0.01$). FMA-LE score remained stable throughout the study ($p > 0.05$).

Table 1. Mean (SD) or median [Q1-Q3] muscle volume, fat fraction, T2-RT and ADC of the affected and less-affected muscles at baseline.

Muscle	Side	Muscle volume (mm ³)	Fat fraction (%)	T2-RT (ms)	ADC (10 ⁻⁶ mm ² /s)
GL	A	67246 (16971)	10.5 (2.4)	43.7 (3.6)	1899 (243)
	LA	74563 (20652)	8.3 (2.1)	41.3 (2.2)	1976 (170)
GM	A	132005 (28908)	10.4 [8.1-14.6]	47.7 (3.3)	1729 [1602-1882]
	LA	160715 (48270)	8.8 [7.4-10.2]	43.5 (2.6)	1784 [1695-1827]
Soleus	A	377433 (72848)	11.2 (3.1)	46 (3.1)	1773 (212)
	LA	418183 (91276)	9 (1.9)	42.3 (1.8)	1847 (174)

A: affected; ADC: apparent diffusion coefficient; GL: gastrocnemius lateralis; GM: gastrocnemius medialis; LA: less-affected; T2-RT: T2 relaxation time.

Muscle volume

Results were normally distributed. The mean muscle volume of the affected and less-affected muscles at baseline can be found in Table 1 and Figure 2. At baseline, the GL, GM and soleus muscles of the affected side had a significantly smaller muscle volume of -9.8% (p=0.03), -17.9% (p=0.02), -9.8% (p=0.02) respectively, compared to the less-affected side. There was no significant change over time in muscle volume for the affected as well as the less-affected limb. However, it should be noted that the GL and GM muscle volume decreased from V1 to V3 in all patients except for 2 and 1 patient, respectively (Figure 4). Moreover, a significant paired-sample T test was observed between V1 and V3 (-9.5%, p=0.03) for the affected GM muscle.

In addition, the ratio between the affected and less-affected limbs was analyzed over time. Repeated-measures ANOVA revealed a significant effect of time for both the GL ($p=0.007$) and GM ($p=0.03$) muscle volume ratios. However, post hoc pairwise comparisons did not identify significant differences between individual visits. Nevertheless, paired-sample t-tests comparing V1 and V3 demonstrated a significant decrease in the affected-to-less-affected limb ratio for both the GL ($p=0.017$) and GM ($p=0.02$) muscles, whereas no significant change was observed for the soleus muscle. Finally, a significant positive correlation was observed between the baseline affected-to-unaffected volume ratio and the change in muscle volume between V1 and V3, but only for the GM muscle ($p=0.028$).

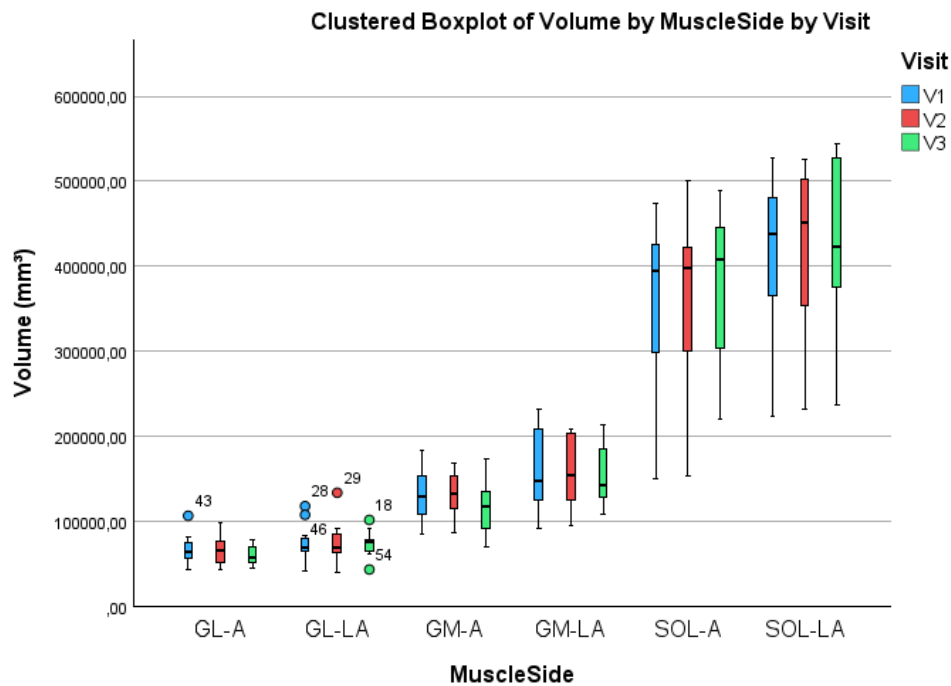


Figure 2. Muscle volume of the different muscles at different visits. A: affected; GL: gastrocnemius lateralis; GM: gastrocnemius medialis; LA: less-affected; SOL: soleus; V:visit

Fat fraction

Results were normally distributed for GL and soleus muscles only. Mean $\pm$ SD or median [Q1-Q3] fat fraction of the affected and less-affected muscles at baseline can be found in Table 1 and Figure 3. At baseline, the GL, GM and soleus muscles of the affected side had a significantly higher fat fraction of +32.2% ($p < 0.001$), +27.3% ($p = 0.02$), and +33.1% ($p < 0.001$) respectively, compared to the less-affected side. On the other hand, there was no significant difference between GL, GM, and soleus muscles of the affected side. Regarding the affected GM muscle, a significant increase was observed between V1 and V3 (+23.3%, $p = 0.01$). Furthermore, a significant increase was observed between V2 and V3 for GL (+16.1%, $p = 0.008$) and soleus (+12.8%, $p = 0.04$) muscles. On the other hand, the one-way repeated measures ANOVA for the affected GL and soleus muscles was not significant between V1 and V3. However, fat fraction increased between V1 and V3 in all patients (Figure 4) with a significant paired-sample T test for GL (+14.3%, $p = 0.001$) and soleus muscles (+13.9%, $p = 0.007$). In addition, the ratio between the affected and less-affected limbs was analyzed over time. A repeated-measures ANOVA revealed a significant effect of time for the GL fat fraction ratio ($p = 0.002$). Post hoc pairwise comparisons showed a significant difference between V2 and V3. A significant effect of time was also observed for the soleus fat fraction ratio ($p = 0.036$). However, post hoc pairwise comparisons did not identify significant differences between individual visits. As the GM fat fraction ratio was not normally distributed, a Friedman test was performed instead of a repeated-measures ANOVA. No significant effect of time was observed, and subsequent Wilcoxon signed-rank tests did not reveal any significant differences between visits.

Interestingly, a significant negative correlation ($p < 0.001$) was observed between the baseline affected-to-unaffected fat fraction ratio and the change in fat fraction between V1 and V3 for all 3 muscles (GL, GM, and soleus).

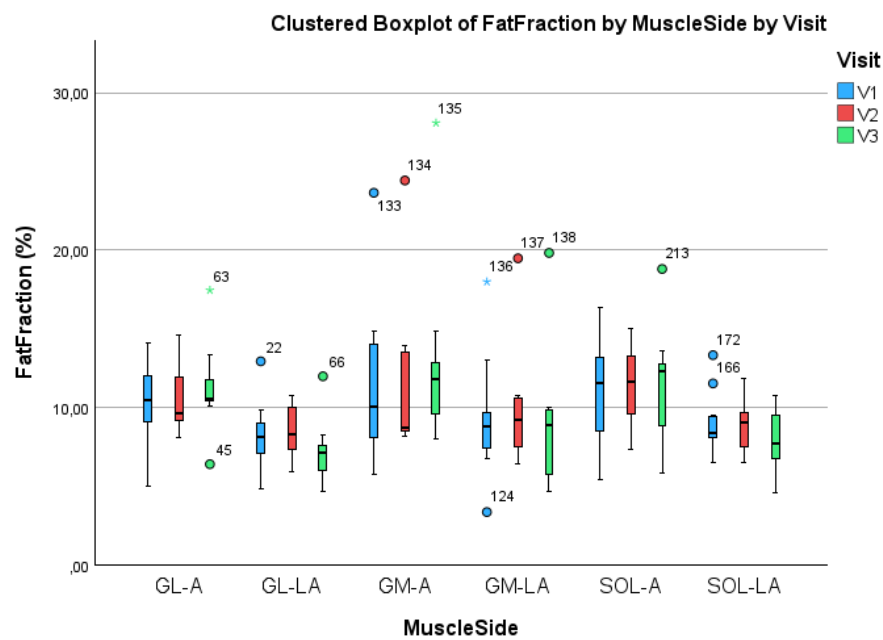


Figure 3. Fat fraction of the different muscles at the different visits. A: affected; GL: gastrocnemius lateralis; GM; gastrocnemius medialis; LA: less-affected; SOL: soleus; V: visit.

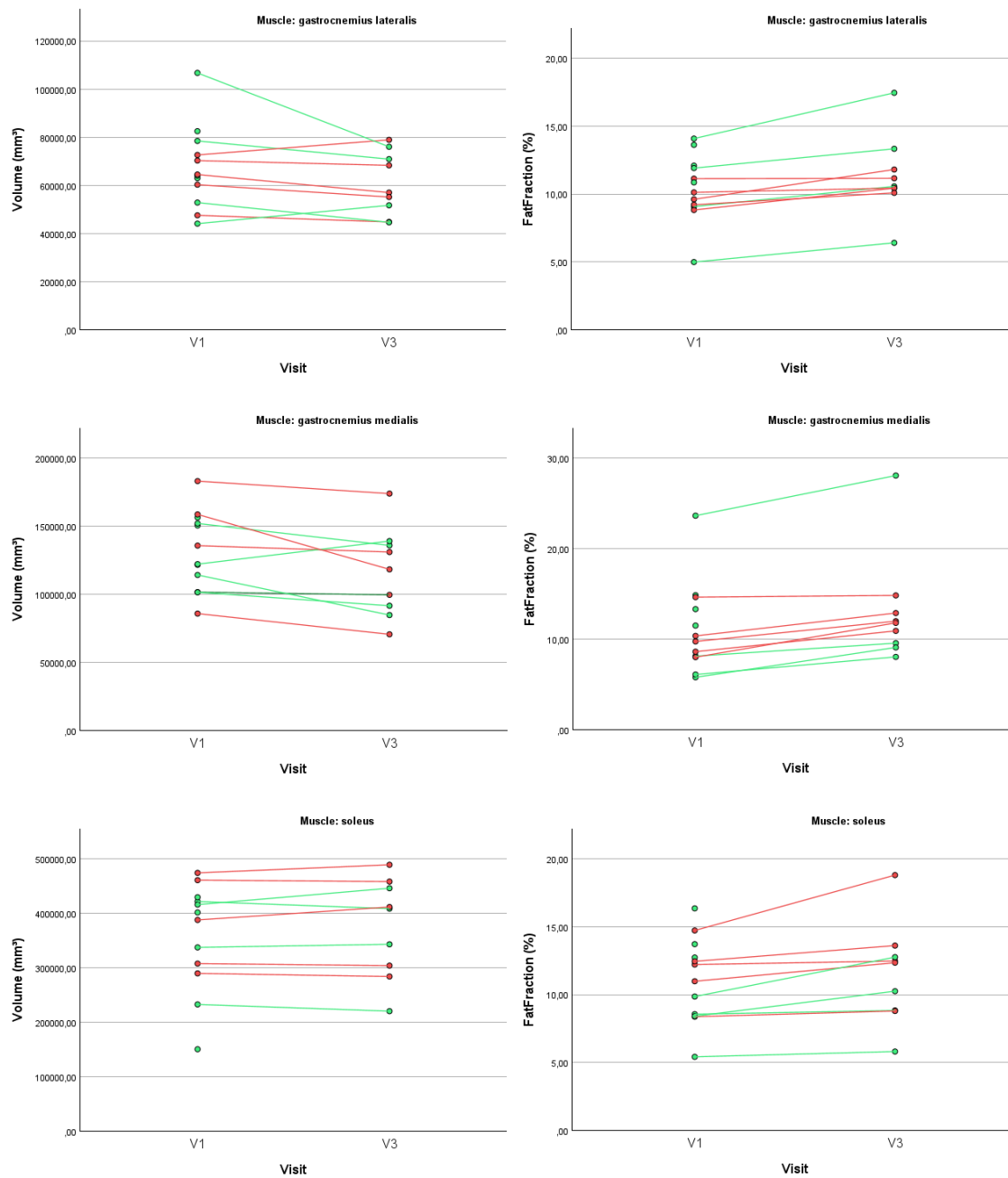


Figure 4. Individual values of muscle volume and fat fraction for the different muscles at V1 and V3. BoNT-A naïve participants are represented in green, non-naïve participants in red.

T₂ relaxation time

Results were normally distributed. The mean T₂-RT of the affected and less-affected muscles at baseline can be found in Table 1 and Figure 5. At baseline, the GL, GM and soleus muscles of the affected side had a significantly increased T₂-RT of +5.8% (p=0.03), +9.7% (p=0.003), +8.8% (p<0.001) respectively, compared to the less-affected side. Moreover, T₂-RT of the affected GM muscle was significantly higher than the GL and soleus muscle at the 3 timepoints. Furthermore, T₂-RT significantly increased between V1 and V3 in the affected GL (+15.4%, p=0.001) and GM (+14.4%, p=0.003) muscles as well as between V2 and V3 in the affected GL (+12.7%, p=0.02) and GM (+13.4%, p=0.02) muscles. For the soleus muscle, all patients except one increased their T₂-RT between V1 and V3 (+5.4%, p=0.01) with a significant paired-sample T test but no significant difference on the one-way repeated measures ANOVA. T₂-RT remained stable between all the different timepoints in the less-affected limb.

ADC

Results were normally distributed for GL and soleus muscles only. Mean $\pm$ SD or median [Q1-Q3] ADC of the affected and less-affected muscles at baseline can be found in Table 1. The ADC of the GL, GM and soleus muscles of the affected side were not statistically significantly different than the less-affected side. Moreover, there was no significant difference between GL, GM, and soleus muscles of the affected side. Finally, ADC remained stable between all the different timepoints in the affected as well as in the less-affected limb.

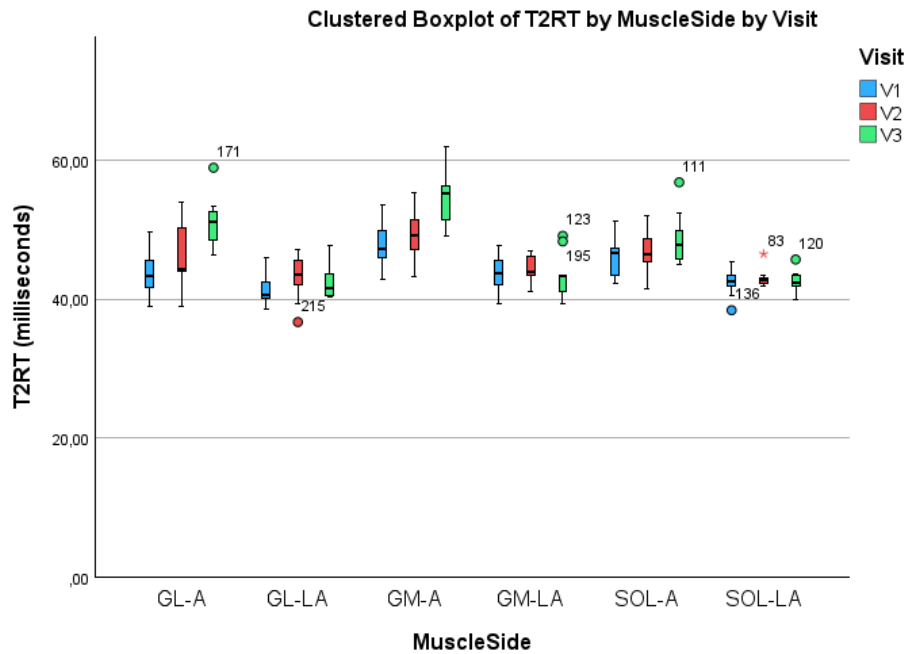


Figure 5. T2-RT of the different muscles at the different visits. A: affected; GL: gastrocnemius lateralis; GM; gastrocnemius medialis; LA: less-affected; SOL: soleus; T2-RT: T2 relaxation time; V: visit.

Correlations

No correlations were found between the different MRI outcomes (muscle volume, fat fraction, T₂-RT, ADC) of the affected limb and other impairments (MAS, MTS, pROM, and Fugl-Meyer). Moreover, no correlations were observed between the different MRI outcomes and the injected BoNT-A doses nor the status of prior BoNT-A exposure, including the number of previous injections and the time elapsed since the last injection. Finally, no correlations were found between the different MRI outcomes and demographic characteristics of the sample.

Discussion

The present exploratory study evaluated several potential muscle tissue changes following repeated BoNT-A Injections. The effect of those injections into calf muscles of chronic stroke participants with MO was quantitatively assessed using MRI over 7 months. Our study first showed that the injected muscles had a significantly smaller muscle volume, higher fat fraction, and increased T₂-RT compared to the less-affected side at baseline. This confirms the consequences of spastic paresis on muscle structure. Moreover, some degree of muscle volume reduction, increased fat fraction and increased T₂-RT was observed after the 3rd BoNT-A injection.

Muscle volume

Muscle atrophy is defined as the loss of muscle tissue and can include decreased muscle volume. Muscle atrophy is critical, as it could affect the muscle's functional ability and decrease the effectiveness of future BoNT-A injection. Given that muscle volume of the affected lower limb is reduced in chronic stroke patients compared to the less-affected limb or to healthy patients (158,159), research has focused on the potential for BoNT-A injection to further reduce muscle volume. Documenting a reduction in muscle volume could reflect either BoNT-A effect and/or disuse atrophy over time. Recent publications have raised concern regarding the effect of BoNT-A on muscle volume.

Indeed, data from animal (75,76) and healthy human (74) studies have both found muscle atrophy up to 1 year following BoNT-A injections. Moreover, a moderate muscle atrophy (from 4 to 11%), assessed by MRI, and lasting from 4 weeks to 25 weeks after a single BoNT-A injection was found in 3 studies conducted in children with cerebral palsy (154–156).

Fortuna *et al.* (76) observed that repeated injections major this atrophy and increase its duration in animals. To the best of our knowledge, this study is the first to investigate the effect of repeated BoNT-A injections on muscle volume in humans. Although our results are not statistically significant, there is a trend towards a greater reduction in muscle volume after the 3rd BoNT-A injection.

Furthermore, higher doses of BoNT-A injections increased and prolonged muscle atrophy in an animal study of Choi *et al.* (75). However, our study could not detect a correlation between the change in muscle volume and injected doses.

Regarding the effect of previous BoNT-A exposure, our results corroborate those of De Beukelaer *et al.* (163). Indeed, they found no significant between-group difference for the amount of morphological muscle changes 6 months after BoNT-A injection in the gastrocnemius muscle of BoNT-A naïve and not naïve children with cerebral palsy.

Finally, the ratio between the affected and less-affected limbs was analyzed over time. The use of this ratio minimizes inter-individual variability associated with factors such as body size, sex, and baseline muscle mass, thereby providing a more sensitive measure of changes in muscle asymmetry over time. The positive correlation between the baseline affected-to-unaffected volume ratio and longitudinal changes in GM muscle volume indicates that muscles with a lower degree of baseline asymmetry (reflecting a muscle volume on the affected side that is close to that of the non-affected side) exhibited a greater reduction in muscle volume over time. Although derived from a different context, this finding is consistent with previous reports from our meta-analysis showing a greater reduction in muscle volume after botulinum toxin injections in treatment-naïve patients compared to

previously treated individuals, supporting the concept that baseline muscle status may modulate longitudinal structural changes.

Thus, while muscle atrophy has been evidenced, its magnitude, duration, and reversible nature remain unclear. This lack of consensus may result from multiple factors related to the heterogeneity between studies, including measurement techniques, normalization, assessed muscles, neurological condition, ambulation status, BoNT-A dosage, and previous BoNT-A exposure (151).

Fat fraction

The measurement of fat fraction in muscles monitors the extent and severity of muscle fibers replacement and destruction. The higher the fat fraction, the greater the muscle destruction and replacement. In this study, a significantly increased fat fraction was observed after the 3rd BoNT-A injection. Thus, a greater percentage of muscle volume was attributed to fat after treatment. In a study of Fortuna *et al.* (76), twenty rabbits received monthly repeated injections of BoNT-A into the quadriceps femoris during 3 and 6 months. The percentage of contractile material was reduced for the 3 and 6 months animals to 80 and 64%, respectively, and was replaced primarily by fibroadipose tissue after 3 months and the effect became more evident after 6 months. Moreover, in an animal study from Minamoto *et al.* (96), a single BoNT-A injection showed about a 7-fold increased lipid accumulation, while double injected muscles showed over a 12-fold increase.

In humans, Weidensteiner *et al.* (45) observed a trend for an increase in fat fraction measured by MRI at 12 weeks and not at 6 weeks following a single BoNT-A injection in paretic calf muscles of children with cerebral palsy. Our results are in line with these findings.

To the best of our knowledge, this is the only existing study measuring the fat content of spastic BoNT-A injected muscles using MRI.

Interestingly, a significant negative correlation was observed between the baseline affected-to-unaffected fat fraction ratio and the change in fat fraction between V1 and V3 for all 3 muscles, indicating that greater baseline inter-limb asymmetry was associated with a larger increase in muscle fat infiltration over time. One possible explanation is that muscles already exhibiting substantial structural impairment may be more vulnerable to further fatty degeneration, suggesting a self-perpetuating process once a certain degree of muscle involvement has been reached.

T₂-relaxation time

Tissue water content is an important factor that may affect relaxation times (164). Therefore, T₂-RT may indicate inflammation and muscle oedema. An increase in water content leads to an increase in the T₂-RT value that manifests as hyperintense areas on T₂-weighted images.

The present study found that BoNT-A injected GL and GM muscles had increased signal intensity (increase in T₂-TR) on the MRI performed after the 3rd BoNT-A injection. This increase of approximately 15% is greater than the difference of T₂-RT between affected and less-affected side. This observation suggests an effect related to the BoNT-A injection and not a time effect. Furthermore, a trend towards a greater signal intensity was observed in soleus muscle after the 3rd BoNT-A injection. The high signal intensity that we observed is consistent with an increase in extracellular fluid seen in muscles 1-6 months after denervation (74,165).

Our findings corroborate that of several studies. Schroeder *et al.* (74) observed increased signal intensity up to 1 year in the gastrocnemius muscles of two healthy adults following a single BoNT-A injection. The treated muscles' biopsies showed also increased connective tissue and enlargement in extracellular space. Furthermore, Weidensteiner *et al.* and de Coulon *et al.* observed a considerably increased MRI signal intensity in the BoNT-A-injected gastrocnemius and soleus muscles of children with cerebral palsy at respectively, 6 and 12 weeks (45) and 22 and 34 months (166) post-injection. Finally, in the study of O'Dell *et al.* (165), an increase in T₂-RT was observed 2 and 3 months after BoNT-A injection in various lower limb muscles of stroke patients.

Apparent diffusion coefficient

Regarding the diffusion results, we didn't find the increase in ADC we might have expected with an increase in extracellular space. Our results are in the line with those of Weidensteiner *et al.* (45). Indeed, they could not detect a change in ADC 12 weeks after a single BoNT-A injection in gastrocnemius and soleus muscles of children with cerebral palsy. One possible explanation put forward by Weidensteiner *et al.* (45) is that an increase in extracellular space combined with a simultaneous decrease in muscle fiber size (96), may offset the effect on ADC, resulting in a stable ADC. We were unable to find any other studies evaluating the effect of BoNT-A injection on ADC.

Muscle structural changes and the link with clinical efficacy

Although some structural changes have been observed, the significance and impact of these changes remain unclear. Muscle atrophy, for example, could affect the muscle's functional ability and decrease the effectiveness of future BoNT-A injection.

However, this study demonstrated improvements in clinical parameters with reduced MO after each injection cycle. Moreover, greater reductions were observed after the 3rd injection cycle, suggesting cumulative effects. Furthermore, lower limb motor function, evaluated by the FMA-LE, did not deteriorate after repeated BoNT-A injections. On the other hand, Picelli *et al.* (127), reported that patients with higher spastic muscle echo-intensity (which likely reflects increased muscle fibrosis and intramuscular fat content) have a reduced response to BoNT-A and thus hypothesized that the toxin dosage needs to be increased in order to obtain a significant reduction of MO.

This study demonstrated MO reduction after each injection cycle but also showed enhanced effects with higher doses of BoNT-A. At the same time, an increased fat fraction was observed after the 3rd BoNT-A injection which could lead to a vicious circle of increased doses and fat fraction.

Finally, clinical efficacy of BoNT-A injections may be at its peak by approximately 1 month post-injection, structural muscle changes appear to be detectable after a larger period of time. Those results corroborate the findings of a previous published meta-analysis assessing the effect of a single BoNT-A injection on muscle structure (151). No statistically significant difference was found for muscle structure (muscle thickness, pennation angle, and echo-intensity) assessed by ultrasound 1 month after BoNT-A injection (151).

Limitations

Our study has some limitations. First, statistical tests lack some power due to small sample size which make a clear interpretation difficult. However, it should be noted that this study is a preliminary and exploratory study that now can be used to calculate a sample size for future studies.

Secondly, quantitative imaging (fat fraction, T_2 -RT, ADC) is generally longer and more sensitive to motion than clinical MRI, which therefore constitutes a particular challenge with spastic patients. Some patients moved their lower limbs during the examination making MRI sequences therefore uninterpretable and further reducing the number of useful images. Finally, T_2 -RT is rarely resulting from a single pure component, as MRI acquisition voxels are likely to enclose different components like water, fat, muscle tissues, etc. Fat infiltration could actually cause the voxel T_2 -RT to increase, which could explain a part of the reported increases in T_2 -RT values. More advanced sequences (using 16 echoes instead of 8) and/or post-processing techniques (using multi-exponential models) could allow T_2 -RT quantification of different sub-components and thus, may be considered for future studies focusing on evaluating the relationship between T_2 -RT and the physiopathology.

Conclusion

To conclude, our study shows that MRI is a useful, non-invasive tool for monitoring the effects of BoNT-A injections on muscle structure in a population of chronic stroke survivors suffering from muscle overactivity. Repeated BoNT-A injections in chronic stroke patients may cause some degree of muscle atrophy, increased muscle fat content and water content, suggesting progressive muscle alterations. However, it did not negatively alter function. This highlights the need for tailored rehabilitation to mitigate potential muscle deterioration following BoNT-A treatment.

To our knowledge, our study is the first to employ a non-invasive imaging method to monitor the effects of repeated BoNT-A injections on muscle structure of spastic muscles. Further studies with larger samples should assess the long-term impact of repeated BoNT-A injections on muscle structure to draw firmer conclusions.

Statements and Declarations

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Declaration of Competing Interest: the authors report no conflicts of interest.

Ethical approval and informed consent: this study was approved by the Comité Hospitalo-Facultaire UCLouvain-Saint Luc (403) (approval no. 2018-002499-42) on August 29th, 2018. All participants provided written informed consent before participating.

Data availability : the authors confirm that the data supporting the findings of this study are available within the article or are available on request from the corresponding author.

Chapter 6. Shear wave elastography in the assessment of gastrocnemius spastic muscle elasticity: influences of ankle position and muscle contraction.

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6.1 Study rationale, objectives and hypotheses

The third study was developed in response to the lack of standardized ultrasound elastography protocols identified in Study 1 (chapter 4). Although standardization is essential in order to compare results across studies, certain parameters—such as the number or size of regions of interest—have little impact on elastography outcomes and were therefore not prioritized. Instead, this third clinical study focused on parameters that can considerably influence shear-wave measurements, particularly joint position and the muscle's activation state.

The objective was therefore to determine which ankle position provides the most valid and reliable assessment of gastrocnemius elastic stiffness in individuals with spasticity and examining the effects of muscle activation.

We hypothesized that SWE measurements would be strongly influenced by joint position and muscle activation state. Specifically, we expected that measurements acquired with the ankle in a resting position and under fully passive conditions would yield the most reliable and clinically relevant estimates of gastrocnemius muscle elastic stiffness in individuals with spasticity, as this position minimizes passive tension and reduces variability related to muscle length.

Once again, it should be noted that the term “elasticity” was used in this published manuscript in a clinical and functional sense and should be interpreted as elastic stiffness (i.e., the mechanical resistance to deformation of the muscle–tendon unit).

6.2 Abstract

Background. Following upper motor neuron syndromes (UMNS), intrinsic viscoelastic muscle properties such as elastic stiffness may be altered, which leads to muscle hyper-resistance to passive mobilization. So far, no gold standard assessment of hyper-resistance, whether clinical or instrumental, is available. Shear wave elastography (SWE) has been increasingly used for non-invasive evaluation of elastic stiffness of skeletal muscles in people with hyper-resistance.

Aim. Our study aimed to evaluate the validity of SWE at ankle neutral resting position (ANRP). Additional objectives included assessing the influence of ankle position, muscle contraction, and laterality on elastic stiffness, and evaluating the reproducibility and responsiveness of SWE.

Design. Observational cohort study.

Setting. Outpatients of the physical and rehabilitation medicine department of a university hospital in Brussels.

Population. 30 hemiparetic patients following UMNS with hyper-resistance in gastrocnemii muscles.

Methods. Elastic stiffness was quantified by shear wave velocity (SWV) measurements of gastrocnemii muscles. A higher SWV corresponds to a higher elastic stiffness. Measurements were performed on the affected and less-affected limbs in ANRP, in passive dorsiflexion and during isometric contraction. Assessments were performed 3 times. Criterion validity, reproducibility, and responsiveness were evaluated. A linear mixed model was used to study position and laterality effect.

Results. In ANRP, reproducibility was excellent and SWV was significantly higher in the affected limb than in the less-affected limb. This laterality effect disappeared in passive dorsiflexion and was even reversed during isometric contraction. SWV was significantly higher on both sides in passive dorsiflexion and during contraction than in ANRP.

Conclusion. Our results suggest that SWE measurements in ANRP are reliable and may provide a more valid measurement of gastrocnemii elastic stiffness following UMNS.

Clinical Rehabilitation Impact. SWE may be a useful clinical tool as an extension of the physical exam for longitudinal monitoring of passive muscle elastic stiffness, to assist with treatment decisions and to better quantify the therapeutic effect of procedures to reduce muscle overactivity. However, a standardized protocol should be used. ANRP seems to be the most valid position for assessing gastrocnemius elastic stiffness in neurological populations. This should be kept in mind for the choice of positioning in further studies.

ClinicalTrials registration: NCT05510726

Keywords: muscle spasticity, hyper-resistance, elasticity, Elasticity Imaging Techniques

6.3 Manuscript

Introduction

Muscle overactivity is one of the consequences of upper motor neuron syndromes (UMNS) that affects children and adults. This often leads to functional loss, limitations in the ability to perform activities of daily living, and decreased quality of life (167). Hyper-resistance is one of the clinical features of muscle overactivity. It is still too often referred to as “spasticity”, despite a European consensus recommending the use of the term hyper-resistance to describe the phenomenon of impaired neuromuscular response during passive stretch (27). This model distinguishes 2 main components of hyper-resistance: a non-neural (tissue-related) component and a neural (central nervous system related) component. The non-neural component consists of muscular adaptations such as muscle stiffness (loss of extensibility). Muscle stiffness can be further divided into elasticity (the deformability of a muscle tissue as a result of an external impulse and the capability of returning to its original state) and viscosity (the internal resistance of muscle tissue to motion or deformation). Neural components include velocity dependent stretch hyperreflexia (spasticity stricto sensu) and non-velocity dependent spastic dystonia and co-contractions. Evaluation of muscle hyper-resistance is mostly based on clinical assessments, such as the Modified Ashworth scale (MAS) and Modified Tardieu scale (MTS). They are easy to use and require no equipment, but these measurements have poor intra-/inter-rater reliability (36,94). Moreover, these measurements cannot distinguish neural from non-neural components (168). So far, no gold standard assessment of hyper-resistance, whether clinical or instrumental, is available.

Ultrasound elastography has been increasingly used for non-invasive evaluation of elastic stiffness of skeletal muscles in people with hyper-resistance. SWE generates a transient shear wave propagating in the tissue. In the medical literature, the shear wave velocity (SWV) (displayed in meter per second), the shear modulus (expressed in kPa) and the Young modulus have been used in reporting outcomes (169).

Some studies have shown that muscle elasticity is also joint angle specific. Saeki *et al.* (170) and Lee *et al.* (171) showed that elastic stiffness increases respectively with ankle dorsiflexion and during active voluntary contraction in healthy volunteers. However, to our knowledge, no study has evaluated the influence of the ankle position and muscle contraction on the elasticity of gastrocnemius muscle in the affected and less-affected leg simultaneously in neurological adults suffering from hyper-resistance.

The first objective of our study was to evaluate the validity of SWE at ankle neutral resting position (ANRP) in hemiparetic subjects with hyper-resistance in gastrocnemii muscles. Our second objective was to assess the influence of ankle position and muscle contraction on the elasticity of gastrocnemius muscle in the affected and less-affected leg simultaneously. Our third objective was to evaluate the reproducibility and responsiveness of SWE.

Materials and Methods

This study was registered in ClinicalTrials under NCT05510726.

Subjects

In this observational single-center cohort study, patients were prospectively enrolled at the Department of Physical Rehabilitation Medicine of the “Cliniques universitaires Saint-Luc” (Brussels, Belgium) between September 2022 and May 2023.

Inclusion criteria were as follows i) age ≥ 18 years old, ii) hemiparesia/-plegia following upper motor neuron syndrome, iii) at least 3 months after injury, iv) muscle overactivity in affected lower limb. We excluded patients with i) any other neurological or orthopedic condition that might interfere with the affected limb function, ii) aphasia or major cognitive impairment limiting functional assessment, iii) previous BoNT-A injection in the last 3 months, iv) previous treatment of muscle overactivity with surgical procedures in the affected limb. This study was approved by the relevant ethics committee and all patients signed a written informed consent after comprehensive explanation of the procedures, according to the Declaration of Helsinki.

Evaluations

All evaluations were performed by the same physician. Assessments on T1 and T2 were performed consecutively (short-term reliability) and assessment on T3 was performed after a 10-day interval, at the same time of the day (long-term reliability). Measurements were taken at T1, T2, and T3 in the gastrocnemii medialis and lateralis, bilaterally. Participants were asked to lie prone with their feet sticking out from the examination table to prevent contractions. Measurements were performed in the neutral position, in passive dorsiflexion as well as during muscle contraction. During passive dorsiflexion, a constant external force of 80 kPa was applied bilaterally by a second investigator using a MicroFet dynamometer¹. During muscle contraction, the patient was asked to apply a force of 50 kPa on the Microfet dynamometer bilaterally when possible.

¹ MicroFET hand-held dynamometer: force transducer converting applied force into kPa using a known and fixed contact surface area.

If the patient could not achieve a force of 50 kPa, he was asked to perform the maximal force possible on the affected leg. The same force was then applied with the less-affected leg.

Shear-wave elastography

Ultrasound elastography examinations of the patients were made with SWE (DIAGNOSTIC ULTRASOUND SYSTEM Canon Aplio i800) using a linear probe (5-14 MHz) PLT 1005 BT. Those measurements were conducted by a physician (M.D.) with 3 months training in SWE and US. Transmission gel was applied. The transducer was placed parallel to the longer axis of the muscles with as little compression as possible (as any additional pressure may lead to overestimation of muscle stiffness). A region of interest that was approximately 10 mm in diameter was placed in the middle depth of the muscle units such that muscle fascia borders, tendon, and blood vessels were excluded. SWV (displayed as meters per second) were measured in the region of interest after a shear wave quality map automatically generated by the software revealed homogeneous color. Three measurements were made for each position, and the mean values were used for further analysis. SWV is influenced by material properties, with shear wave traveling faster through stiffer tissues.

MyotonPro

The Myoton (Myoton AS, Tallinn, Estonia) is a noninvasive hand-held myotonometer device aimed at recording, among others, the elastic stiffness of myofascial tissues (172). It produces a series of damped oscillations induced by a single external mechanical impulse. The probe of the MyotonPro was placed perpendicularly where SWV had previously been obtained.

With a pre-force of 0.18 N, a quick mechanical impulse of 0.4 N was released for 15 ms. The total force applied to the structure was 0.58 N. Following the single external mechanical impulse, free oscillations were recorded for 400 ms. Then, the device needed 10 ms for processing raw signals and five more to compute all the parameters. The sequence was looped five times for a total duration of 2.15 s. The mean value of the five sequences corresponds to one assessment allowing the calculation of stiffness (displayed as Newton per meter). One measurement was made for each position.

Clinical assessment

The following clinical assessments were performed for the affected leg at T1 and T3: MAS, MTS, passive range of motion, and Fugl-Meyer Assessment(173). Hyper-resistance of the calf muscles was evaluated using the MAS and MTS in the supine position with both a flexed and extended knee. The passive range of motion of ankle dorsiflexion was measured in the supine position while extending and flexing the knee using a manual goniometer. The muscles and soft tissues were stretched at slow speed to avoid a stretch reflex. Regarding the Fugl-Meyer Assessment, only the subscale of motor function (/34 points) of lower extremity was performed.

Data availability

The data associated with the paper are not publicly available but are available from the corresponding author on reasonable request.

Statistical analysis

Statistical analyses were performed using SPSS Statistics, v. 28 (IBM, Armonk, New York). Continuous data are presented as mean and standard deviation or median and first-third quartile. Categorical data are presented as counts and percentages. Normal distribution of variables was evaluated with statistical (Shapiro-Wilk) and graphical approaches (Q-Q plot). In order to validate SWE, we followed the standards proposed by the Consensus-Based Standards for the Selection of Health Measurement Instruments (COSMIN) checklist (174). We assessed the criterion validity, reliability, internal consistency, and responsiveness.

Criterion validity was assessed by studying the correlation between muscle elasticity measured with SWE and (i) myotonometer, (ii) clinical assessments of hyper-resistance and function. Spearman correlation was considered good, moderate, or poor if the correlation coefficient (r) was >0.6 , $0.3 < r < 0.6$, <0.3 , respectively (157).

A linear mixed model with random subject effect was used to compare SWV and search for interactions. SWV was considered as dependent variable while side, muscle, position and time were considered as factors. Fixed effects with non-nested build terms were used. Residuals were saved to check for normality based on graphical approaches (Q-Q plot and histogram). Test-retest reliability (reproducibility) was assessed using intraclass correlation coefficients (ICCs), which were calculated with average measures, two-way random-effects model for absolute agreement with 95% confidence intervals (CI). ICCs < 0.50 were considered poor, 0.50 – 0.75 moderate, 0.75 – 0.90 good, and >0.90 excellent (175).

ANOVA Friedman analyses were performed to see whether more than two related groups differed.

Measurement error was evaluated by use of the standard error of measurement (SEM) (176). Responsiveness was estimated using the minimum detectable change (MDC). The MDC, which represents the magnitude of change necessary to exceed the measurement error of 2 repeated measures at a specified CI was calculated for the 95% CI (MDC95) (176). MDC95 was also expressed as a percentage (MDC95 %).

The power calculation based on the results of a previous SWE study (177) , estimated that a sample size of 28 patients was needed to obtain a power of 80% with a first type error of 0.05. Missing data were not replaced. For all tests, the statistical significance was set at $p < 0.05$.

Results

We included 30 participants in our study. Of those 30 patients, 24 were males (80%), 20 (69%) suffered from stroke, and median age was 56 [47-64.75] years. Ten (33.3%) patients had already received past BoNT-A injection in the gastrocnemius muscle with a median duration since last BoNT-A injection of 17.5 [9.5-26.25] months. The detailed sample characteristics are shown in Table 1. All variables were abnormally distributed except for Fugl-Meyer Assessment. SWV of every measurement can be found in Table 2.

Regarding the results of the mixed model (Fig. 1), no time effect was observed ($p > 0.05$). Interaction muscle-side was not statistically significant ($p > 0.05$) and thus removed from the mixed model. On the contrary we found a statistically significant effect of muscle, side and ankle position on SWV.

Moreover, the interactions muscle-position and side-position were statistically significant (Table 3). Residuals were normally distributed according to the graphical approaches (supplementary material).

Ankle neutral resting position

A side effect was observed with a mean SWV of the affected limb significantly higher than the less-affected limb in the GL muscle (+10%, $p<0.05$) as well as in the GM muscle (+22.5%, $p<0.004$). Furthermore, a muscle effect was found in the affected limb as mean SWV was significantly higher in the GM (+0.429 m/s, $p<0.017$) than in the GL muscle. No muscle effect was observed in the less-affected limb.

Influences of ankle position and muscle contraction

No side effect was observed in passive dorsiflexion as no significant difference was found between the affected and the less-affected limb, both for the GM and the GL muscle. During muscle contraction, on the other hand, mean SWV was significantly higher in the less-affected limb than in the affected limb, both for the GM and the GL muscle. Furthermore, a position effect was observed as mean SWV was significantly higher during muscle contraction and in passive dorsiflexion than in neutral position, both in the GM and GL muscle of the affected and less-affected limb ($p<0.001$). No muscle interaction was analyzed in those two positions and only mean SWV of GM muscle are represented in Fig 1.

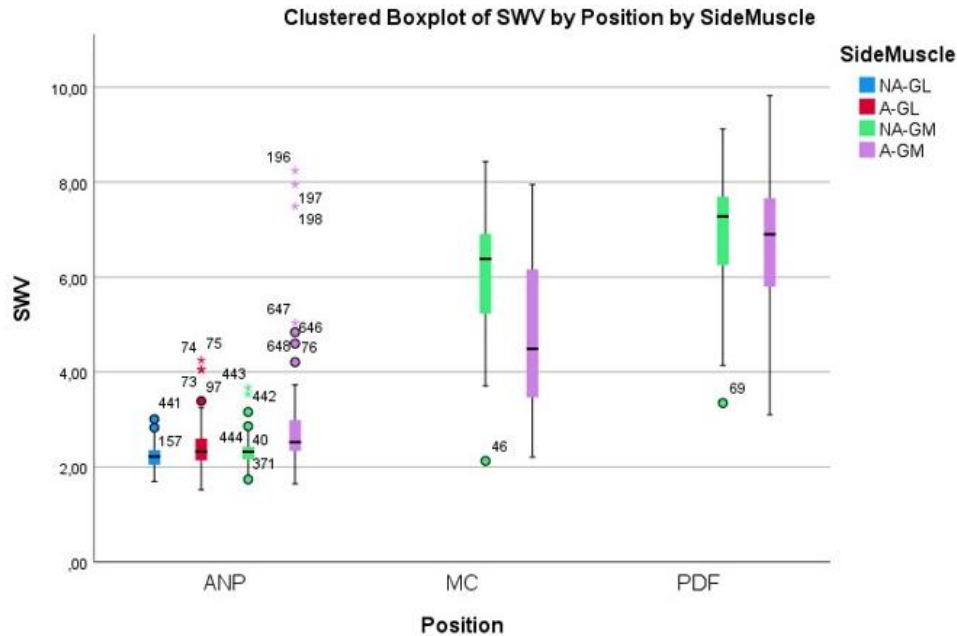


Figure 1. Effect of position, side, and muscle on shear wave velocity. A: affected; ANRP: ankle neutral resting position; NA: non-affected; PDF: passive dorsiflexion; SWV: shear wave velocity.

Criterion validity

Criterion validity was evaluated in ANRP as only ANRP could correctly distinguish the affected- and less-affected limb. A moderate positive correlation was found at T3 between SWV of GL muscle and MAS ($r=0.42$, $p=0.02$) and between SWV of GL muscle and number of previous BoNT-A injections ($r=0.42$, $p=0.02$). No other correlations were found in neutral position between SWV and clinical assessments (MAS, MTS, Fugl-Meyer, passive range of motion) nor between SWV and elasticity measured with MyotonPro, nor between SWV and demographical and clinical characteristics (disease duration, previous Botulinum-toxin injections).

Reliability

ICCs were calculated for SWE measures of the affected limb in neutral position. Reproducibility was excellent ($ICC > 90$) at short and long term for both muscles. ICCs are shown in Table 4 and illustrated in Fig 2. Furthermore, according to ANOVA Friedman's test, no time effect was observed as the mean SWV was not significantly different ($p > 0.05$) between T1, T2, and T3 in the affected limb in neutral position.

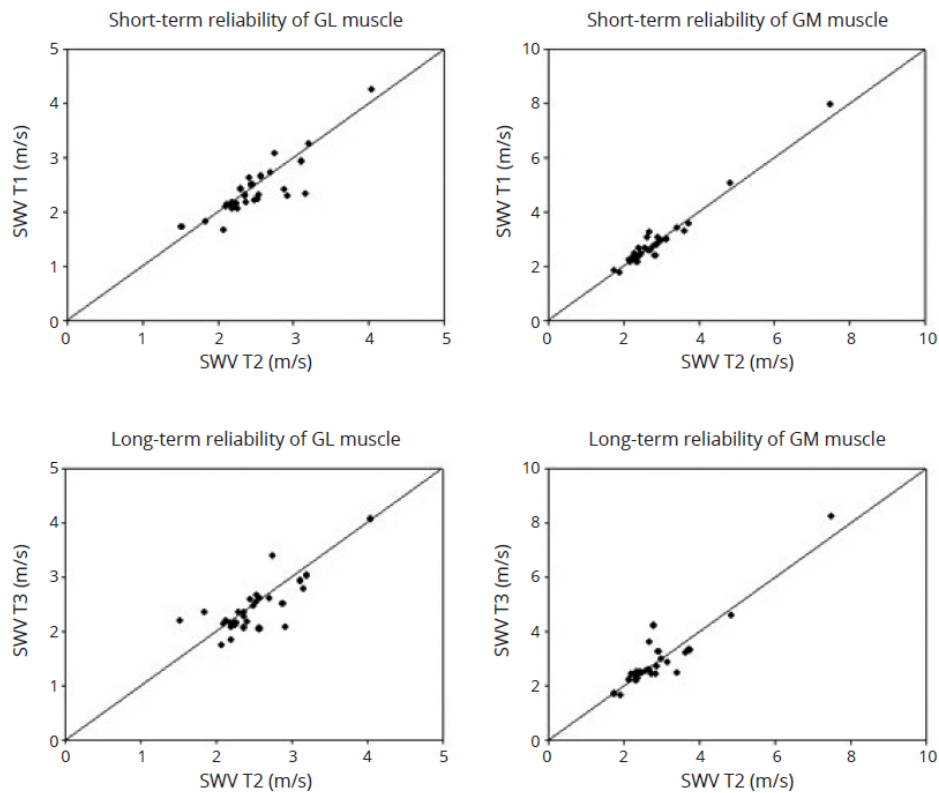


Figure 2. Agreement plots of short-term and long-term reliability of SWE in the affected limb in ankle neutral resting position. GL: gastrocnemius lateralis; GM: gastrocnemius medialis; SWV: shear wave velocity.

Responsiveness

In neutral position at short term SEM was 0.14 m/s and 0.11 m/s and MDC was 0.38 m/s (16%) and 0.30 m/s (11%) for GL and GM, respectively. At long term, SEM was 0.16 m/s and 0.22 m/s with an MDC at 0.45 m/s (18%) and 0.62 m/s (22%) for GL and GM, respectively (Table 4).

Discussion

The present study evaluated the validity of SWE in ANRP in hemiparetic subjects with hyper-resistance in gastrocnemii muscles. It also assessed the influence of ankle position and muscle contraction on the elastic stiffness of gastrocnemius muscle in the affected and less-affected leg simultaneously. Finally, we aimed to study the reliability and responsiveness of SWE.

Influence of ankle position and muscle contraction

There is currently no consensus regarding which method may be the most optimal for assessing gastrocnemius muscle elasticity among neurological populations. In the present study, the choice was made not to strictly control joint angles in neutral position.

We opted for allowing the patient to adopt a natural resting position, which can be more readily replicated during a routine examination, as opposed to fixed-angle “neutral” postures that might necessitate an external device for replication and furthermore would stretch the muscle, thus increasing stiffness. In neutral position, a side effect was observed with a mean SWV of the affected limb significantly higher than the less-affected limb in the GM muscle as well as in the GL muscle, indicating greater elastic stiffness.

In the same way, Hong *et al.* (178) and Kesikburun *et al.* (179) observed a significantly higher elasticity index of the spastic GM and GL muscles compared with the unaffected side in stroke survivors. Our results are also in line with studies conducted in upper limb muscles (174–176).

These results suggests that the spastic paretic muscles have altered muscle properties which may result in changes in the elastic characteristics of the muscle. The mechanisms behind muscle stiffness changes in spastic muscle are unclear and current measurements of SWV cannot distinguish between the factors that may contribute to this increased elastic stiffness. However, several potential mechanisms can be highlighted such as muscle composition changes and muscle architectural alteration. Those changes include changes in connective tissue, increased collagen content (183,184), increased extracellular matrix (185), stiffer fibres (184,186), shorter resting sarcomeres (178), changes in intracellular proteins such as titin, accumulation of extracellular fat in the muscle (183), quantifiable fibrosis (187), and pennation angle and fascicle length changes (178,188,189).

Furthermore, a muscle effect was found in the affected limb as mean SWV was significantly higher in the GM than in the GL muscle. This has never been highlighted before. No muscle effect was observed in the less-affected limb, supporting the concept that GM muscle was probably more affected in our patients than GL muscle.

While measurements taken in neutral position offer a physiological baseline, the elastic stiffness observed in contracted or stretched muscles was more variable between pathological and healthy muscles.

Our results showed that during isometric muscle contraction, mean SWV was significantly higher in the less-affected limb than in the affected limb, both for the GM and the GL muscle. Those results raise questions as during muscle contraction, the patient was asked to apply a force of 50 kPa on the Microfet dynamometer bilaterally. Furthermore, a position effect was observed as mean SWV was significantly higher during muscle contraction than in neutral position, both in the GM and GL muscle of the affected and less-affected limb. Thus, SWV is sensitive to muscle activity. Those results are in line with several studies carried out on healthy volunteers where SWV of gastrocnemius muscle increased with active muscle contraction (164,183–185). Moreover, our findings align with studies conducted in upper limb muscles (181,193).

Regarding stretching in our study, no side effect was observed in passive dorsiflexion as no significant difference was found between the affected and the less-affected limb, both for the GM and the GL muscle. On the other hand, a position effect was observed as mean SWV was significantly higher in passive dorsiflexion than in neutral position, both in the GM and GL muscle of the affected and less-affected limb. Thus, SWV increases with muscle length. These observations are related to length-dependent structural and mechanical properties of skeletal muscles. Our results are in line with the study of Chernak *et al.* (191) and Dubois *et al.* (194) where average GM SWV increased significantly with passive dorsiflexion in healthy volunteers. Moreover, Jakubowski *et al.* (195) and Lee *et al.* (196) found no significant difference in GM SWV at maximum dorsiflexion between the affected and the less-affected side in chronic stroke survivors and in children with cerebral palsy, respectively.

To summarize, muscle contraction and stretching of gastrocnemius increases SWV but seems to alter the differences between the affected and the less-affected limb. Ankle neutral resting position seems therefore to be the most valid position for assessing gastrocnemius muscle elastic stiffness in neurological populations. This should be kept in mind for the choice of positioning in further studies.

Criterion validity

Criterion validity was assessed by studying the correlation between muscle elastic stiffness measured with SWE and (i) myotonometer, (ii) clinical assessments.

No correlations were found between muscle elastic stiffness measured with SWE and with MyotonPro in neutral position, both for GL and GM muscle. These results are conflicting with the results of several studies (171,190,197) who observed a moderate but significant correlation in medial gastrocnemius muscle elastic stiffness between measurements of SWE and MyotonPro at rest in healthy volunteers. Several possible reasons could explain this difference. First, those studies showing significant correlations between elastic stiffness measured with SWE and MyotonPro were undertaken in healthy subjects and not in neurological populations. Frölich-Zwahlen *et al.* (198) and Garcia-Bernal *et al.* (199) showed that elastic stiffness measured with the MyotonPro did not differ between the affected and the less-affected gastrocnemius muscles in chronic stroke survivors.

The potential validity of MyotonPro for the assessment of gastrocnemius elastic stiffness in neurological populations may thus require further investigation. Secondly, the measurement of muscle elastic stiffness using MyotonPro did not include an assessment of subcutaneous fat.

Previous evidence indicates that MyotonPRO is less suitable for measuring elastic stiffness when the muscles are covered by more than 20 mm of subcutaneous fat (171).

The MAS and MTS scores of spastic gastrocnemius muscles didn't show any significant correlation with SWE measurements. MAS and MTS are easy to use clinical assessment of hyper-resistance and require no equipment, but these measurements have poor intra-/inter-rater reliability (36,94). Moreover, these measurements cannot distinguish neural from non-neural components (168). If one of the components is predominant, it will be overlooked by those clinical scales (200). Thus, MAS and MTS might not be as sensitive as SWE in detecting muscle elastic stiffness which is part of the non-neural component. The degree of involvement of neural and non-neural components is specific to each patient. It may be that our study population had predominant non-neural components. Our results are in the same line as several other studies. Brandenburg *et al.* (169), Kesikburun *et al.* (179), and Hong *et al.* (178) didn't find any significant correlation between SWE measurements and MAS of the GL and/or GM muscle in children with cerebral palsy and stroke survivors, respectively. Our results are also in line with studies conducted in upper limb muscles such as the biceps brachialis (180,189,201) and the flexor carpi radialis (182,202) of stroke survivors.

On the other hand, contrary findings were reported by several studies (203,204). Moreover, in the systematic review of Roots *et al.* (205) the MAS correlated with ultrasound elastography stiffness in 10 studies and did not correlate in 4 studies. Further studies should be conducted to develop quantitative measurement of hyper-resistance.

Reliability

In healthy volunteers, the intra-rater and inter-rater reliability of SWE has been shown to be good to excellent in several muscles including the gastrocnemius muscle (171,194,206). Prior studies have also highlighted differences in probe orientation. They demonstrated that the longitudinal plane provided the best reproducibility for normal skeletal muscles (207,208). In a systematic review and meta-analysis of Miller *et al.* published in 2020, reliability varies across neurological populations and assessment conditions (probe orientation, ankle joint, active/passive muscle condition), with most studies indicating moderate to good reliability (40). Since our results show that ANRP seems to be the most valid position to evaluate gastrocnemius muscle elastic stiffness through SWE, reliability was only analyzed in ANRP.

Based on our results, short-term and long-term intra-rater reproducibility of SWE in longitudinal plane is excellent in spastic gastrocnemius muscle in ANRP ($ICC > 90$). In comparison, Mathevon *et al.* (209) found a good intra-rater reproducibility ($ICC=0.87$) for shear elastic modulus in longitudinal plane of paretic gastrocnemius muscle in neutral position. In the same way, intra-observer agreement on the use of SWE to measure SWV in post-stroke spastic biceps brachialis was good ($ICC = 0.82$) in the study of Gao *et al.* (201).

Regarding responsiveness of SWE, MDC% results at long term in neutral position were 18% and 22% for gastrocnemius lateralis and medialis respectively. This is an important information to consider in order to evaluate the effectiveness of a treatment for example Botulinum toxin type A injections.

A difference of +/- 20% has to be found before and after the injection to conclude that there is a real improvement in muscle elastic stiffness. Our results are better compared to the results of Mathevon *et al.* (2009) with a MDC% of approximatively 30% for gastrocnemius medialis at long-term.

Limitations

Firstly, the sample size is relatively small. Even if the study power is adequate, the generalizability of our results to the neurological population is not warranted.

Secondly, the freehand technique used in SWE leads to technical challenges in reproducing images due to the difficulty of controlling the applied pressure. To decrease the effect of this problem we took the mean of several successive acquisitions.

Finally, most SWE techniques assume that the underlying tissue is isotropic, homogenous and with negligible viscosity. However, muscle is neither isotropic nor homogenous and has known viscous properties, thus caution is advised when interpreting SWE measurements.

Conclusion

SWE is a promising tool for assessing muscle elastic stiffness in spastic gastrocnemius muscles. However, a standardized protocol should be used. Using SWE, we demonstrated that in ANRP, shear waves travel faster in the affected limb than less-affected limb of hemiparetic subjects with hyper-resistance in gastrocnemii muscles. SWV was also demonstrated to increase with increasing ankle angle and with active muscle contraction. However, muscle contraction and stretching of gastrocnemius muscle seems to alter the differences between the affected and the less-affected limb.

Ankle neutral resting position seems therefore to be the most valid position for assessing gastrocnemius muscle elastic stiffness in neurological adult populations. In the future, SWE may be a useful clinical tool as an extension of the physical exam for longitudinal monitoring of passive muscle stiffness, to assist with treatment decisions and to better quantify the therapeutic effect of procedures to reduce muscle overactivity.

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Conception and design: Marine Devis, Gaëtan Stoquart

Acquisition of data: Marine Devis

Analysis and interpretation of Data: Marine Devis, Gaëtan Stoquart

Drafting the article: Marine Devis

Revising it for intellectual content: Gaëtan Stoquart, Thierry Lejeune, Frédéric Lecouvet

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Declaration of Competing Interest: the authors report no conflicts of interest.

Tables

Table 1. Demographic and clinical characteristics of the sample.

Gender	
Males, N (%)	24 (80)
Females, N (%)	6 (20)
Age, median [Q1-Q3] (years)	56 [47-64.75]
Disease	
Stroke, N (%)	21 (70)
Brain tumor, N (%)	4 (13.5)
Multiple sclerosis, N (%)	3 (10)
Cerebral palsy, N (%)	2 (6.5)
Disease duration, median [Q1-Q3] (months)	25 [13.25-48.75]
BoNT-A naive patients, N (%)	20 (66.7)
Number of past BoNT-A injections/patient in the gastrocnemius muscle, median [Q1-Q3]	2.5 [1.25-3.75]
Duration since last BoNT-A injection, median [Q1-Q3] (months)	17.5 [9.5-26.25]
Motor function score Fugl-Meyer Assessment (/34), mean $\pm$ SD	20.9 $\pm$ 5.5
Modified Ashworth scale, N (1/1+/2/3)	4/8/17/1
Modified Tardieu scale, N (1/2/3/4)	3/16/9/2

BoNT-A: Botulinum neurotoxin type A; N: number; Q: quartile; SD: standard deviation.

Table 2. Shear wave velocities.

Position	Muscle	Side	SWV (m/s), median [Q1-Q3]
ANRP	GL	A	2.33 [2.14-2.61]
		NA	2.22 [2.05-2.36]
	GM	A	2.53 [2.34-2.99]
		NA	2.33 [2.16-2.43]
MC	GL	A	5.08 [3.47-6.25]
		NA	6.12 [5.18-6.67]
	GM	A	4.49 [3.41-6.21]
		NA	6.39 [5.21-6.93]
PDF	GL	A	5.10 [4.18-6.37]
		NA	5.67 [4.93-6.76]
	GM	A	6.90 [5.76-7.69]
		NA	7.15 [5.97-7.65]

A: affected; ANRP: ankle neutral resting position; GL: gastrocnemius lateralis; GM: gastrocnemius medialis; MC: muscle contraction; NA: non-affected; PDF: passive dorsiflexion; SWV: shear wave velocity.

Table 3. Type III tests of fixed effects.

Source	F	Sig.
Side	23.62	<.001
Muscle	50.81	<.001
Position	939.77	<.001
Time	1.56	0.212
Side*Position	26.10	<.001
Muscle*Position	24.08	<.001

Table 4. Intraclass correlation coefficients and responsiveness SWE of the affected limb in ankle neutral resting position.

	ICC (T1-T2)	ICC (T2-T3)	SEM Short term (m/s)	SEM long term (m/s)	MDC short term (m/s) (%)	MDC long term (m/s) (%)
GL	0.92	0.88	0.14	0.16	0.38 (16)	0.45 (18)
GM	0.99	0.96	0.11	0.22	0.30 (11)	0.62 (22)

ICC: intraclass correlation coefficient; MDC : minimal detectable change ; SEM : standard error of measurement.

Chapter 7. Shear wave elastography in the assessment of gastrocnemius spastic muscle elasticity: influences of ankle position and muscle contraction.

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Ongoing recruitment; preliminary findings.

7.1 Study rationale, objectives and hypotheses

While working on this thesis, we identified a substantial gap in the literature in early-pathophysiology understanding. It is well established that post-stroke patients exhibit alterations in muscle structure (83,84); however, very few longitudinal studies have been conducted, and patients are rarely assessed during the acute phase. Understanding when these structural changes first appear and the temporality of these changes is crucial, as it could help us to guide strategies to prevent them and/or allow for earlier and potentially more effective interventions. Therefore, the aim of our fourth study is to evaluate muscle structure (through MRI) and stiffness (using SWE) from the acute phase after stroke and to perform a longitudinal follow-up to monitor its evolution over time. This will allow us to determine when specific modifications in muscle structure occur, to investigate which types of structural alterations may contribute to spasticity, whether spasticity further modifies muscle structure, and to compare changes between the affected and unaffected limbs throughout the recovery process.

We hypothesized that muscle structural and mechanical adaptations following stroke would follow a temporal pattern. Based on findings from animal studies, we expected muscle atrophy-related changes to occur relatively early after stroke, whereas alterations in muscle composition, particularly fat infiltration, would emerge more gradually over time. However, because histological analyses are likely more sensitive than MRI for detecting early tissue changes, we anticipated that some alterations may only become detectable using non-invasive imaging at later stages.

Furthermore, we hypothesized that differences between the paretic and non-paretic limbs would progressively increase throughout recovery and become clearly distinguishable during the chronic phase after stroke. This hypothesis was supported by previous literature as well as by the findings of our second study, which demonstrated significant baseline differences in muscle structure between the paretic and non-paretic limbs prior to any BoNT-A injection in chronic stroke survivors.

7.2 Manuscript

Introduction

Despite growing interest in post-stroke muscle alterations, data from the early phase after stroke and longitudinal follow-up studies remain scarce. We therefore conducted a longitudinal study to investigate the natural evolution of muscle stiffness and structure from the early subacute phase to the chronic phase post-stroke. As data collection is still ongoing, the results presented in this work should be interpreted as preliminary.

Materials and Methods

Subjects

In this observational single-center cohort study, patients are prospectively enrolled from the neurology inpatient unit of the “Cliniques universitaires Saint-Luc” (Brussels, Belgium) since December 2024. Inclusion criteria are as follows i) $18 \leq \text{age} \leq 80$ years old, ii) hemiparesia/-plegia following a first stroke, iii) ≤ 2 weeks post-stroke. We excluded patients with i) any other neurological or orthopedic condition that might interfere with the affected limb function, ii) major cognitive impairment limiting functional assessment, iii) bilateral limb paresis, iv) contraindication to perform MRI. This study was approved by the relevant ethics committee and all patients signed a written informed consent after comprehensive explanation of the procedures, according to the Declaration of Helsinki.

Outcome

The patient's medical history is taken into account. We record information about the type of stroke, underlying risk factors for stroke, other medical condition that can affect muscle and nerves, functional status prior to stroke. Patients are evaluated at 2 weeks (V1), 1 month (V2), 3 months (V3) and 6

months (V4) after stroke. All the evaluations which follow are carried out at the four visits.

Muscle structure was investigated using MRI. The MRI protocol was strictly identical to that implemented in the TRITITOX study (chapter 5) and is therefore not described in detail in the present work. In brief, quantitative MRI was employed to characterize muscle structural properties by measuring muscle volume (MV), fat fraction (FF), T2 relaxation time (T2-RT), and apparent diffusion coefficient (ADC). These parameters were extracted for the medial gastrocnemius, lateral gastrocnemius, and soleus muscles of the paretic limb, as well as for the corresponding muscles of the contralateral non-paretic limb.

Muscle elastic stiffness was evaluated using SWE in the gastrocnemius muscles of both the paretic and non-paretic limbs. Participants were preferentially positioned in a prone lying position with their feet extending beyond the examination table in order to minimize passive muscle tension and involuntary activation. Measurements were performed with the ankle in a neutral position, as this joint configuration was shown in our previous study to provide the most valid and reliable assessment conditions. However, in the acute phase, prone position was sometimes difficult to achieve due to clinical constraints and patient tolerance. In such cases, participants were assessed in a supine position with the leg placed in slight rotation to allow access to the gastrocnemius muscle while minimizing muscle activation. To ensure consistency and reliability of longitudinal measurements, each participant was evaluated in the same body position across all visits.

Patient's functional assessment was performed according to the most recent guidelines (14):

- the impairment (motor control, muscle strength and hyper-resistance) was assessed with the Fugl-Meyer test (210) and the 10 meter walk test. Regarding the Fugl-Meyer Assessment, only the subscale of motor function (/34 points) of lower extremity were performed. Hyper-resistance of the calf muscles was evaluated using the MAS and MTS in the supine position with both a flexed and extended knee. The passive range of motion of ankle dorsiflexion was measured in the supine position while extending and flexing the knee using a manual goniometer. The muscles and soft tissues were stretched at slow speed to avoid a stretch reflex.

- the activities of daily living were evaluated by means of the Timed Up And Go (TUG) and the Berg Balance Scale (BBS). The Timed Up and Go test is a reliable and valid test for quantifying functional mobility (211). The Berg Balance Scale is a valid and reliable quantitative measure of balance and risk for falls (212). Functional ambulation category was evaluated. Moreover, the ABILOCO questionnaire assessed activities (213).

- the participation and the quality of life were assessed by the Stroke Impact Scale (SIS) (214). This questionnaire is a reliable and valid patient-based, self-report questionnaire. It was designed to assess multidimensional stroke outcomes, including strength, hand function Activities of Daily Living / Instrumental Activities of Daily Living, mobility, communication, emotion, memory and thinking, and participation.

Statistical analysis

Statistical analyses were performed using SPSS Statistics, v. 28 (IBM, Armonk, New York). Continuous data are presented as mean and standard deviation or median and first-third quartile. Categorical data are presented as counts and percentages. Normal distribution of variables was evaluated with statistical (Shapiro-Wilk) and graphical approaches (Q-Q plot). A paired-sample T test was performed to assess differences between the affected and less-affected muscles. An equivalent Wilcoxon signed rank test was used instead if results were abnormally distributed. A one-way repeated measures analysis of variance (ANOVA) with Bonferroni adjustment for multiple comparison was performed to compare the different variables between the different visits. In addition, if the one-way repeated measures ANOVA was non-significant, a paired-sample T test was performed between V1 and V4 to increase statistical power by removing a possible confounding visit given the small sample size. Moreover, equivalent ANOVA Friedman analyses with Bonferroni adjustment for multiple comparison were performed if results were abnormally distributed. Correlations between MRI-derived parameters, SWE and impairments were evaluated. Pearson/Spearman correlation was considered good, moderate, or poor if the correlation coefficient (r/ρ) was >0.6 , $0.3 < r/\rho < 0.6$, <0.3 , respectively (157). The power calculation based on the results of our previous SWE study (215), estimated that a sample size of 27 patients was needed to obtain a power of 80% with a first type error of 0.05. Missing data was not replaced. For all tests, the statistical significance is set at $p < 0.05$.

Results

Since the start of recruitment, 26 patients met the inclusion criteria for the study. Among these, 15 patients were enrolled, while the remaining individuals declined participation. Of the 15 included participants, 6 have currently completed the full study protocol, 6 withdrew before completion, and 3 are still undergoing follow-up assessments at the time of writing. The mean age of the participants was 65.7 ± 9.1 years, and 60% were female. Brain lesion was left-sided in 53% of cases, and 87% of strokes were ischemic. At inclusion, stroke severity as assessed by NIHSS, showed a mean total score of 10.1 ± 4.8 , with a mean lower limb subscore of 2.3 ± 1.2 .

Clinical examination

Regarding clinical “spasticity” assessment, important inter-individual variability was already observed at the first evaluation performed 2 weeks post-stroke (V1). According to the MAS, 6 patients did not present any clinically detectable spasticity. In contrast, 5 patients exhibited a MAS score of 1, 2 patients presented a score of 1+, and 2 patients had a MAS score of 2, indicating that signs of increased muscle tone were already present in a substantial proportion of the cohort during the early subacute phase after stroke. Furthermore, no significant changes in MAS/MTS scores were observed over time throughout the follow-up period. These findings suggest that the overall level of clinically assessed spasticity remained relatively stable between the early subacute and chronic phases in the present cohort. At V1, the majority of patients were non-ambulatory, with 11 participants presenting a Functional Ambulation Category (FAC) score of 0. In addition, 3 patients had a FAC score of 1, indicating that walking was only possible with continuous physical assistance.

Functional impairment was substantial, with a mean lower extremity Fugl-Meyer Assessment score of 11.3/34. Balance abilities were also markedly altered, as reflected by a mean Berg Balance Scale score of 11.75/56. In addition, patients reported reduced activity and participation levels, with a mean SIS score of 151/295 and a mean ABILOCO score of 3.9/13. Overall, these findings indicate a cohort presenting with severe motor and functional limitations during the early subacute phase after stroke.

At the final follow-up assessment (V4, 6 months post-stroke), data were available for only 6 participants, as several patients had discontinued the study during follow-up. Nevertheless, a global improvement in functional outcomes was observed over time. Regarding ambulation, only 1 patient remained non-ambulatory, while 2 patients achieved a FAC score of 3 (ambulator, dependent on supervision), 2 patients a score of 4 (ambulator, independent level surface only), and 1 patient a score of 5 (ambulator, independent), indicating recovery of independent ambulation in a substantial proportion of the cohort. Motor impairment also improved, with the mean lower extremity Fugl-Meyer Assessment score increasing from 11.3/34 at V1 to 22.5/34 at V4. Similarly, balance performance improved markedly, with the mean Berg Balance Scale score increasing from 11.75/56 to 40.4/56. Walking capacity also improved substantially, as reflected by the increase in mean gait speed during the 10-meter walk test from 0.11 m/s at baseline to 0.55 m/s at 6 months post-stroke. Finally, patient-reported outcomes improved over time, with the mean SIS increasing from 151/295 to 193.8/295 at V4. Importantly, repeated-measures ANOVA demonstrated significant longitudinal improvements for all these functional outcomes throughout the follow-up period.

Muscle volume

Results were normally distributed. The mean muscle volume of the affected and less-affected muscles at baseline can be found in Table 1. No differences were observed between the affected and less-affected muscles at any time point. Furthermore, there was no significant change over time in muscle volume for the affected as well as the less-affected limb. Finally, no significant correlations were identified between muscle volume, SWE measurements, and clinical impairment outcomes.

Fat fraction

Results were normally distributed. The mean fat fraction of the affected and less-affected muscles at V1 can be found in Table 1. Significant differences were identified between the affected and non-affected limbs at specific time points. At 3 months post-stroke (V3), fat fraction was significantly higher in the affected muscles compared to the contralateral side for both the GL (+19.6%, $p = 0.007$) and the GM (+20.2%, $p = 0.004$).

Similarly, at 6 months post-stroke (V4), fat fraction remained significantly higher in the affected limb (GL: +25.2%, $p = 0.048$; GM: +25%, $p = 0.033$). We further examined differences in fat fraction between the 3 muscles within the affected limb. Fat fraction was consistently higher in the GM compared to the GL at all time points, reaching statistical significance at each assessment ($p < 0.05$). The relative increase in fat fraction in GM compared with GL ranged from 29% to 39%, depending on the time point. In contrast, no significant differences were observed between muscles in the non-affected limb. Moreover, no significant changes were observed over time, as evidenced by both repeated-measures ANOVA and paired t-tests.

Finally, no significant correlations were identified between fat fraction, SWE measurements, and clinical impairment outcomes.

Elastic stiffness

Results were normally distributed. The mean SWV of the affected and less-affected muscles at V1 can be found in Table 1. No significant differences were observed between the affected and non-affected limbs at any of the 4 assessment time points. Similarly, no significant longitudinal changes in SWV were identified over time, either in the paretic or in the contralateral limb. Finally, no significant correlations were identified between SWE measurements, MRI derived muscle parameters and clinical impairment outcomes.

Table 1. Mean (SD) muscle volume, fat fraction and SWV of the affected and less-affected muscles at V1.

Muscle	Side	Muscle volume (mm³)	Fat fraction (%)	SWV (m/s)
GL	A	52791 (6285)	10.8 (1.5)	2.03 (0.1)
	LA	53318 (4977)	10.8 (1.3)	1.96 (0.07)
GM	A	107101 (9563)	13.9 (2.1)	2.03 (0.09)
	LA	112413 (11148)	13.0 (2.3)	1.98 (0.06)
Soleus	A	338850 (29668)	12.0 (1.3)	/
	LA	328182 (30522)	12.1 (1.4)	/

A: affected; GL: gastrocnemius lateralis; GM: gastrocnemius medialis; LA: less-affected ;
SWV: shear wave velocity.

Discussion

First, the present findings should be interpreted with caution, as they represent preliminary results from an ongoing study. Although the estimated sample size required to achieve 80% statistical power was 27 participants, only 15 patients had been recruited at the time of the present analyses. In addition, a substantial proportion of participants discontinued the study before completion, with nearly half of the enrolled patients dropping out during follow-up. Consequently, the number of participants available at the final follow-up assessment (V4, 6 months post-stroke) was limited, resulting in reduced statistical power, particularly for longitudinal analyses. Therefore, the absence of significant findings in some outcomes should not necessarily be interpreted as evidence of absence of muscle alterations, but rather considered in light of the limited sample size and reduced ability to detect subtle effects.

Beyond these methodological considerations, several interesting observations emerge from the present preliminary results. First, contrary to what has been suggested in previous studies focusing on muscle wasting after stroke, no significant reduction in muscle volume was observed over time, nor were significant differences identified between the affected and less-affected limbs. These findings may appear surprising given the described effects of disuse and reduced neural activation on skeletal muscle mass in the early post-stroke phase (216). However, they may partly be explained by the relatively small sample size and limited statistical power of the present analyses.

In addition, the inclusion of both ambulatory and non-ambulatory patients may have contributed to increased inter-individual variability, as muscle wasting is known to be strongly influenced by mobility status and mechanical

loading. Importantly, although no significant muscle atrophy was detected, this does not exclude the presence of more subtle compositional or microstructural changes occurring within the muscle tissue.

Interestingly, fat fraction appeared to be the parameter most sensitive to early post-stroke muscle alterations in our cohort. While no significant longitudinal progression was observed over time, significant inter-limb differences emerged from 3 months post-stroke onward, with higher fat fraction values in the affected gastrocnemius muscles. These findings are consistent with previous DEXA-based studies reporting increased fat accumulation in the paretic limb during the subacute and chronic phases after stroke (217). Our results further extend these observations by showing that MRI-derived fat infiltration may already be detectable within the first months following stroke, even in the absence of clinically established spasticity. This supports the hypothesis that altered muscle composition may represent one of the earliest manifestations of stroke-related muscle remodeling.

The observation that fat fraction was consistently higher in the medial gastrocnemius compared with the lateral gastrocnemius in the affected limb is also noteworthy. This muscle-specific pattern suggests that post-stroke remodeling may not affect all muscles uniformly. Given the predominant role of the medial gastrocnemius in weight-bearing and propulsion during gait, as well as its greater activation compared with the lateral gastrocnemius during walking (218), reduced activation and altered loading of the paretic limb may disproportionately impact this muscle, thereby favoring adipose infiltration. Such findings reinforce the importance of considering muscle-specific adaptations rather than treating the triceps surae as a homogeneous muscle group.

In contrast, no significant differences or longitudinal changes were observed for muscle stiffness assessed by SWE. These findings differ from the majority of elastography studies performed in chronic spastic stroke populations, where increased stiffness of paretic muscles has consistently been reported (205). However, this discrepancy may precisely support the conceptual framework developed throughout this thesis. Since the present cohort was evaluated early after stroke and before the development of pronounced spasticity, the absence of stiffness alterations may suggest that increased passive muscle stiffness is not an immediate consequence of the neurological lesion itself, but rather develops progressively under the combined influence of spasticity, chronic disuse, connective tissue remodeling, and altered muscle architecture. Another possible explanation is that SWE may be less sensitive to early compositional changes such as fat infiltration when these alterations remain moderate and are not yet associated with substantial fibrosis or contracture formation. Importantly, these negative findings should also be interpreted in light of the limited sample size. The sample size calculation for the present study was specifically based on SWE measurements, which constituted the primary outcome, using effect sizes derived from our previous study (chapter 6). However, only 15 of the 27 required participants had been recruited at the time of the present analyses, with additional participant drop-out during follow-up. Consequently, the study was likely underpowered to detect subtle differences or longitudinal changes in muscle stiffness. The absence of significant SWE findings should therefore not be interpreted as definitive evidence of unchanged muscle mechanical properties, but rather as preliminary observations requiring confirmation in the full cohort.

Conclusion

Overall, the present preliminary findings suggest that muscle remodeling after stroke may begin early, even before the development of clinically significant spasticity. While no clear alterations in muscle volume or passive elastic stiffness were identified during the first 6 months post-stroke, MRI analyses revealed early side-specific changes in muscle composition, characterized by increased fat infiltration in the paretic gastrocnemius muscles. These observations support the concept that structural muscle adaptations may precede detectable mechanical changes and reinforce the hypothesis that post-stroke muscle remodeling is, at least initially, driven by altered activation and mechanical unloading rather than by spasticity alone.

Importantly, the muscle-specific differences observed within the triceps surae further suggest that post-stroke adaptations are not homogeneous across muscles, emphasizing the need for detailed and localized assessments of muscle properties. The longitudinal and multimodal design of the present study, combining quantitative MRI and SWE from the early subacute phase onward, provides a unique opportunity to investigate the natural history of post-stroke muscle changes independently of BoNT-A exposure and established spasticity.

Nevertheless, these findings remain preliminary and should be confirmed in the full cohort. Future analyses with a larger sample size and complete longitudinal follow-up will be essential to better determine the temporal relationship between structural muscle changes, passive stiffness alterations, spasticity, and motor recovery after stroke.

Part III. General discussion

The overarching objective of this doctoral thesis was to enhance our understanding of skeletal muscle properties alterations following neurological injury, by disentangling the respective contributions of therapeutic interventions, spasticity, and the neurological lesion itself. The present doctoral thesis was therefore built upon a deliberately reverse line of reasoning. Rather than adopting the classical path that starts from the central nervous system lesion and follows the downstream consequences on muscle properties, we chose to progressively disentangle the different factors that may contribute to muscle alterations in neurological populations. First, we focused on the impact of BoNT-A injections on muscle properties by investigating the effects of this therapeutic intervention on muscle structural and mechanical properties. Second, we addressed the role of spasticity as a clinical syndrome and investigated how spasticity, beyond neural hyperexcitability, may be associated with structural and mechanical muscle adaptations. This step was essential to determine whether the muscle changes observed in spastic muscles can be attributed to spasticity per se, rather than to its treatment. Finally, we extended this reasoning further upstream by exploring muscle properties changes occurring after stroke in the absence of established spasticity. By assessing muscle structure and stiffness from the acute phase and performing a longitudinal follow-up, we sought to characterize the earliest muscle alterations induced by the neurological insult itself, before the development of spasticity or exposure to BoNT-A.

This reverse and deconstructive approach allowed us to examine muscle adaptations from three complementary perspectives—treatment-related effects, spasticity-related effects, and lesion-related effects—and to better understand their respective and potentially overlapping contributions to muscle properties changes.

Chapter 8. Effects of Botulinum toxin type A on muscle properties

BoNT-A injections are widely used in the management of spasticity and primarily target the neural component of HR. While their effects on neural transmission and reflex excitability are well established, increasing attention has been directed toward their potential influence on the non-neural component of HR, i.e. on the skeletal muscle tissue itself. Beyond their neuromuscular blocking action, BoNT-A injections may be associated with changes in muscle properties, including both structural and mechanical characteristics. Understanding how BoNT-A injections affect muscle properties is of particular clinical importance, as these injections are commonly repeated over time. Potential treatment-related alterations in muscle structure or mechanical behavior could not only influence muscle function, but also modify the muscle's response to subsequent injections, thereby affecting long-term treatment efficacy.

Despite increasing interest in BoNT-A–related muscle changes, the literature remains heterogeneous and inconclusive. In this first section of the discussion, we examine the effects of BoNT-A injections on muscle properties by integrating the findings of the present systematic review and meta-analysis (chapter 4) and our first longitudinal clinical study (chapter 5) with the existing literature.

8.1 BoNT-A as a modulator of muscle stiffness

One of the central questions addressed in this chapter concerns whether BoNT-A injections modify the passive mechanical properties of spastic muscles. Muscle stiffness plays a critical role in the non-neural component of joint hyper-resistance, and alterations in elastic properties may directly influence movement limitations and functional capacity.

The results of our systematic review and meta-analysis demonstrate that muscle elastic stiffness measured by USE improves temporarily at short-term (4 weeks) following BoNT-A injections. In order to complement the findings of this study, which included studies published up to April 2023, I conducted an updated exploration of the recent literature to identify newly published evidence regarding the effects of BoNT-A on muscle stiffness in neurological individuals with HR. This search revealed only one additional study, published in 2025, that evaluated gastrocnemius muscle stiffness 6 weeks and 3 months after BoNT-A injection in post-stroke patients (219). Consistent with the conclusions of our meta-analysis the authors reported a significant reduction in elastic stiffness at 6 weeks, but not at 3 months, confirming the typical clinical peak effect of BoNT-A around 4 weeks post-injection. However, the interpretation of this new study warrants caution. Stiffness measurements were performed in maximal passive dorsiflexion and 30° plantarflexion, despite our previous work (215) demonstrating that these joint positions lack discriminatory capacity between the affected and unaffected sides. Indeed, in the study of Simic *et al.*, the reported SWV were nearly identical between sides (e.g., 1.55 m/s in the affected limb versus 1.52 m/s in the contralateral limb), making it unlikely that a truly meaningful lateral difference could be detected in these non-optimal positions. This reinforces the need for methodological rigor and standardization of joint positioning and measurement protocols when assessing muscle stiffness, to ensure that results across studies are interpretable, comparable, and above all valid. In light of these findings, it is relevant to revisit the meta-analysis conducted at the beginning of this thesis, which reported a decrease in muscle elastic stiffness measured by SWE 1 month after BoNT-A injection (151). Given the strong

influence of measurement conditions on elastography outcomes, this result should now be interpreted with caution.

In particular, it becomes important to examine the joint position and muscle activation state used in the studies included in the meta-analysis, as variations in muscle length, passive tension, or activation may have substantially affected stiffness estimates. A more detailed analysis of these methodological aspects could help determine to what extent the reported decrease in stiffness reflects genuine tissue-level changes induced by BoNT-A, as opposed to measurement-related variability. Among the 9 included studies in our meta-analysis, only 3 (118,177,220) assessed the gastrocnemius muscles in a resting position consistent with the ankle neutral resting position identified as optimal in our previous study (chapter 6) (215). Two studies did not report the joint position used during elastography measurements (129,221), while 1 study performed measurements at 0° of dorsiflexion (222). Another study referred only to “shortened” and “stretched” positions without providing precise joint configuration (111). The remaining two studies investigated upper limb muscles: one reported measurements on the biceps brachii and showed results consistent with our findings regarding the influence of muscle state (223), whereas the other performed assessments during passive elbow flexion (105). Overall, this heterogeneity in measurement protocols calls for caution when interpreting elastography-based findings and comparing results across studies. To further explore this issue, we conducted an additional meta-analysis including only the studies that assessed the gastrocnemius muscles in an ankle neutral resting position.

The results did not differ markedly from those of the initial meta-analysis, with a medium effect size still observed for the decrease in elastic stiffness 1 month after BoNT-A injection (standardized mean difference = 0.66; 95% CI 0.35–0.97; $p < 0.0001$), compared with the original estimate (standardized mean difference = 0.74; 95% CI 0.46–1.02; $p < 0.000001$) (Figure 15). These findings suggest that, within this restricted subset of studies, the overall conclusion remains qualitatively similar, while still warranting cautious interpretation given the limited number of studies and remaining methodological variability.

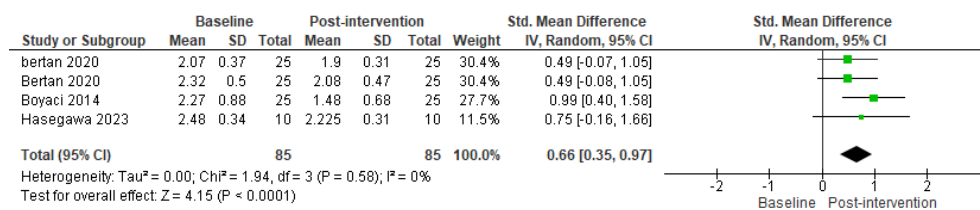


Figure 15. Effect of BoNT-A injections on elastic stiffness 1 month after intervention.

8.1.1 Mechanisms underlying changes in elastography-derived muscle stiffness following BoNT-A injections

Although BoNT-A primarily acts on the neural component of HR by inhibiting acetylcholine release at the neuromuscular junction, several mechanisms may explain the reduction in muscle elastic stiffness measured using SWE after injections.

A first explanation relates to the decrease in involuntary muscle activation following BoNT-A injection. Even in resting conditions, spastic muscles often exhibit persistent low-level motor unit activity due to reflex hyperexcitability (224).

Because SWE measurements are highly sensitive to muscle activation, the reduction in background neural drive following BoNT-A injection may lead to a decrease in measured SWV, thereby giving the appearance of improved passive muscle stiffness.

A second possible mechanism involves changes in cross-bridge formation within muscle fibers and the thixotropic properties of muscle tissue. Skeletal muscle exhibits thixotropic behavior, meaning that its mechanical properties depend on its recent contractile and movement history rather than being purely passive (225). In spastic muscles characterized by persistent activation and limited movement, the muscle maintains a number of weakly attached actin–myosin cross-bridges, contributing to the short-range stiffness (SRS). SRS corresponds to the high resistance observed during the initial phase of stretch and is known to arise from the population of attached cross-bridges present even in “resting” muscle (226). Consequently, SWE measurements obtained at rest are not purely reflective of passive tissue stiffness; they also capture the contribution of these cross-bridges and micro-activation. Experimental evidence suggests a relationship between short-range stiffness and elastography-derived measures of muscle stiffness. In an *in vivo* animal study, Bernabei *et al.* (227) compared direct mechanical measurements of SRS with SWV during muscle contraction. They reported that SWV is sensitive to changes in muscle stiffness, even when controlling for muscle tension, indicating that SWE may partly capture the intrinsic stiffness related to actin–myosin cross-bridge interactions. However, this relationship remains complex, as SWE reflects global tissue mechanical behavior rather than isolating specific microscopic mechanisms. This study remains, to my knowledge, the first and only study to directly compare mechanically measured SRS with SWV.

Since then, although both SRS and SWE have been further explored independently, direct experimental comparisons between these two constructs remain scarce. This highlights an important gap in the literature.

Following BoNT-A injection, neural drive to the muscle is reduced, leading to relaxation of motor units and detachment of residual cross-bridges. This results in a decrease in SRS and, correspondingly, a reduction in elastography-derived muscle stiffness, even without structural remodeling of muscle fibers or connective tissue. Recent biophysical models further support this interpretation by showing that SRS is highly dependent on the number of attached cross-bridges and behaves as a time-dependent viscoelastic element rather than a simple spring (228,229). These effects are transient: as neuromuscular transmission recovers over weeks to months, involuntary activation and residual cross-bridge attachment gradually return, and SWE stiffness values tend to revert toward baseline. This mechanism explains why BoNT-A can temporarily improve resting elastic stiffness through a combination of reduced neural activation, decreased short-range stiffness, and restoration of a less stiff thixotropic state, highlighting the complex interplay between neural and mechanical factors in spastic muscles. Another potential explanation proposed in the literature is that the reduction in spasticity following BoNT-A injections may indirectly facilitate rehabilitation. By decreasing involuntary muscle activity and improving joint mobility, patients may become more able to engage in physiotherapy, including stretching and active exercises. Such interventions could contribute to short-term improvements in muscle viscoelastic properties (116). However, the transient nature of the changes observed in elastography-derived stiffness raises questions regarding this hypothesis.

If structural or mechanical adaptations induced by rehabilitation were the primary driver, more sustained modifications would be expected. Instead, the return of stiffness values toward baseline as the pharmacological effect of BoNT-A diminishes suggests that the observed improvements are more likely related to temporary reductions in neural activation rather than lasting alterations of muscle tissue properties.

8.2 Effects of BoNT-A on muscle structure

Beyond mechanical stiffness, an increasing number of studies have investigated whether BoNT-A injections induce structural modifications in skeletal muscle. According to our updated meta-analysis, muscle volume consistently decreased over time. Cross-sectional area decreased at long-term follow-up, while muscle length remained unchanged from the mid term onwards. Muscle echo-intensity showed a transient increase at 3 months, whereas muscle thickness and pennation angle generally remained unchanged when data were available. Evidence was insufficient for several outcomes and follow-up periods.

To provide a clear and structured overview, the results will be discussed section by section according to specific muscle outcomes, including muscle volume, cross-sectional area, muscle length and muscle quality.

8.2.1 Muscle volume

Muscle volume consistently decreased across short-, mid-, and long-term follow-up periods in children with CP. These findings suggest that structural changes in skeletal muscle may occur relatively early after BoNT-A administration.

The early reduction in muscle volume observed as soon as 1 month post-injection raises important questions regarding the underlying mechanisms. Such rapid changes are unlikely to be solely explained by structural remodeling processes such as fibrosis or fat infiltration, which typically require a longer time course to develop. Instead, this short-term decrease in muscle volume is more likely to reflect acute changes induced by chemodenervation and inhibition of neuromuscular transmission including reduced muscle activation, decreased contractile protein synthesis, and early onset of disuse atrophy. These mechanisms are consistent with findings from experimental models, where muscle atrophy has been shown to occur rapidly following denervation (96). In this context, BoNT-A–induced chemodenervation may mimic a transient denervation-like state, leading to early and measurable reductions in muscle volume before more chronic structural adaptations take place.

Regarding the mid-term effects of BoNT-A on muscle volume, the observed reduction is unlikely to be explained solely by normal developmental changes, as muscle volume was normalized to account for body growth in this pediatric population. It may therefore reflect treatment-related alterations in muscle morphology. At this stage, the sustained decrease in muscle volume may reflect the progression from chemodenervation induced atrophy toward more established structural remodeling, potentially involving changes in muscle architecture and composition.

Interestingly, similar findings have recently been reported in another meta-analysis investigating the effects of BoNT-A injections on gastrocnemius muscle morphology in children with CP (230). In this study, which included 11 cohort studies, the authors also observed a significant decrease in gastrocnemius muscle volume or mass 2–3 months after injection, with a

standardized mean difference of -0.496 (95% CI: -0.810 to -0.181). These results are therefore remarkably consistent with those obtained in the present analysis, both indicating a moderate reduction in muscle volume at the mid-term following BoNT-A injections. Unfortunately, full access to this article was not available at the time of writing. Consequently, it was not possible to examine in detail the studies included in their quantitative synthesis, the methodological choices made for the analysis, or the corresponding forest plots. Further examination of this work would nevertheless be valuable to better understand the degree of overlap with the studies included in our analysis and to compare methodological approaches.

Furthermore, the inclusion of this newly identified study (144) reinforced the evidence for a long-term reduction in normalized muscle volume following BoNT-A injections in children with CP. Notably, the effect appeared to differ according to injection history, with more pronounced muscle volume loss observed in injection-naïve patients than in those who had previously received BoNT-A. This pattern suggests that the initial exposure to BoNT-A may trigger the greatest degree of muscle atrophy, while subsequent injections exert a more limited effect on muscle volume. This result not only strengthens the evidence for a measurable impact of BoNT-A on skeletal muscle morphology in the long-term but also highlights the importance of prior exposure status when interpreting structural changes and planning subsequent injections in pediatric patients.

In parallel with these meta-analytic findings, our prospective study (chapter 5) provides additional insights into the evolution of muscle volume 6 months following repeated BoNT-A injections. In this cohort, no significant change in muscle volume was observed over time for the injected GM, GL and soleus muscles as assessed by repeated-measures ANOVA. However, a more detailed examination of individual trajectories revealed a decrease in muscle volume of the GL and GM muscle from V1 to V3 in all patients except for 2 and 1 patient, respectively. This trend reached statistical significance as assessed by a paired-sample T test for the GM muscle, with a mean reduction of 9.5% between V1 and V3 ($p = 0.03$). It should be noted that this third time point corresponds to approximately 7 months after the first injection, but follows three successive BoNT-A injections. As such, the interpretation of these findings remains challenging, as it is difficult to disentangle the respective contributions of each injection. The observed reduction in muscle volume may reflect a cumulative effect of repeated injections, a delayed effect of the initial injection, or a combination of both. Moreover, the progressive increase in BoNT-A dose across treatment sessions may have further contributed to the observed changes, adding another layer of complexity to the interpretation. In addition, the absence of significant changes at the group level in our prospective study, which appears to contrast with the findings of the meta-analysis at 6 months post BoNT-A injection, may be partly explained by the limited statistical power of our study. The relatively small sample size increases the risk of type II error and may have prevented the detection of more subtle changes in muscle volume over time.

Furthermore, important differences in study populations should be considered when comparing these results with those of the meta-analysis. While the latter primarily included children with CP, our prospective study was conducted in

adult patients with stroke. Differences in muscle plasticity, growth, baseline muscle condition, and response to BoNT-A between these populations may contribute to the observed discrepancies.

Finally, although a meta-analysis could not be performed for longer follow-up periods due to the limited number of available studies, the newly included study also provides valuable long-term data up to 12 months after BoNT-A injection. In this study (146), normalized muscle volume remained significantly reduced one year after treatment, with a decrease of 8% compared with baseline ($p = 0.0086$) in BoNT-A naïve children. Interestingly, the magnitude of the reduction appeared to progressively decrease over time, with a 12% reduction at 3 months, 10% at 6 months, and 8% at 12 months post-injection. These findings suggest that the structural effects of BoNT-A on muscle volume may persist for several months, although a gradual recovery seems to occur over time. This temporal pattern may reflect the progressive reinnervation of the muscle and restoration of neuromuscular activity. Although reinnervation is known to begin within weeks following BoNT-A-induced chemodenervation, it is a gradual process that unfolds over several months, which may explain the delayed recovery of muscle volume observed over time. However, the persistence of a reduced normalized muscle volume at one year indicates that muscle recovery may either remain incomplete or simply require a longer time course than commonly assumed, particularly in growing children with CP. These findings are further supported by evidence from experimental human studies, which have demonstrated persistent muscle atrophy up to one year after a single BoNT-A injection in the lateral gastrocnemius muscle of healthy volunteers (74).

This suggests that the long-term structural impact of BoNT-A on skeletal muscle may primarily reflect a general biological response to chemodenervation, rather than being specific to pathological muscle. However, the extent and reversibility of these changes are likely modulated by the underlying muscle condition and developmental context.

Thus, while muscle atrophy has been evidenced, its magnitude, duration, and reversible nature remain unclear. This lack of consensus may result from multiple factors related to the heterogeneity between studies, including measurement techniques, normalization, assessed muscles, neurological condition, ambulation status, BoNT-A dosage, and previous BoNT-A exposure. Altogether, these results highlight the importance of considering treatment history, injection sequence, and population-specific factors when interpreting longitudinal changes in muscle morphology.

8.2.2 Cross-sectional area

Cross-sectional area showed a decrease at 6 months, whereas evidence at earlier time points was insufficient. A meta-analysis at 1-year follow-up could not be performed due to limited data. However, the study by Lambrechts *et al.* (146) reported that the CSA of the muscle was no longer significantly different from baseline, suggesting a trend toward recovery at one year. Importantly, the number of participants remained unchanged throughout the study, indicating that this loss of statistical significance at 12 months is unlikely to be explained by reduced statistical power, but rather reflects a true attenuation of the effect over time. Interestingly, although CSA appears to recover, muscle volume did not fully return to baseline values in the same study.

This apparent discrepancy can be explained by changes in muscle shape or geometry: CSA reflects a single-plane measure of muscle size, whereas volume integrates the entire 3-dimensional structure. Muscle remodeling after intervention may allow partial restoration of CSA in key sections without fully restoring total volume, possibly due to residual longitudinal shortening and altered fascicle length (suggesting the development of muscle contractures) or uneven recovery along the muscle length.

8.2.3 Muscle length

Muscle length remained unchanged at mid- and long-term follow-up. In children with CP, muscle growth is known to be impaired, particularly in terms of longitudinal development (231). In this context, the absence of change in normalized muscle length following BoNT-A injection suggests that the intervention does not further affect longitudinal muscle growth. This finding contrasts with the consistent reductions observed in muscle volume and cross-sectional area. One possible explanation for this dissociation lies in the different structural components underlying radial and longitudinal muscle adaptations. The reduction in muscle volume and cross-sectional area is likely driven by muscle fiber atrophy following chemodenervation, leading to a decrease in fiber diameter and overall muscle bulk.

In contrast, muscle length is primarily determined by the number of sarcomeres arranged in series, which may be less sensitive to mid- and long-term changes induced by BoNT-A. As a result, BoNT-A appears to predominantly affect muscle size (radial growth) rather than longitudinal architecture, at least within the time frame studied.

8.2.4 Muscle quality

Muscle echo-intensity was unchanged in the short term and increased at 3 months. Higher echo intensity is indicative of poorer muscle quality, potentially resulting from an increase in connective tissue, fibrosis, fat infiltration and a reduction in contractile components (126). However, since echo intensity is only an indirect indicator of muscle quality, it does not allow for distinguishing between the contributions of these different factors, underscoring the need for studies with high-quality measurement tools, such as MRI, to accurately assess muscle quality following BoNT-A injections. To my knowledge, only 1 study has directly assessed the effect of BoNT-A on fat fraction using MRI. Weidensteiner *et al.* (45) observed a trend for an increase in fat fraction at 12 weeks but not at 6 weeks following a single BoNT-A injection in paretic calf muscles of children with CP. This finding aligns with our meta-analyses showing no statistically significant difference at 1 month but a moderate increase in echo-intensity at 3 months following BoNT-A injection.

At long-term follow-up (6 months), this effect was no longer statistically significant. In addition, heterogeneity was moderate ($I^2 = 58\%$), indicating variability in the effect across studies. Interestingly, those results appear to contrast with the findings from our prospective study. In the latter, a significant increase in fat fraction was observed after the third BoNT-A injection, indicating that a larger proportion of muscle volume was attributable to fat following treatment. This measurement corresponds to approximately 7 months after the first injection. However, caution is required when interpreting these results, as it is difficult to disentangle the contributions of each individual injection.

Furthermore, the dose of BoNT-A was increased over the course of treatment, which may have further influenced the observed structural changes. These results are also in line with a study of Fortuna *et al.* (76) where twenty rabbits monthly received repeated injections of BoNT-A into the quadriceps femoris during 3 and 6 months. The percentage of contractile material was reduced for the 3 and 6 months animals to 80 and 64%, respectively, and was replaced primarily by fibroadipose tissue after 3 months and the effect became more evident after 6 months. Moreover, in an animal study from Minamoto *et al.* (96), a single BoNT-A injection showed about a 7-fold increased lipid accumulation 3 months following treatment, while double injected muscles showed over a 12-fold increase 6 months following first injection (96). These factors highlight the complexity of assessing long-term muscle remodeling following repeated BoNT-A treatments and suggest that cumulative effects may not be fully captured by studies with only single or short-term follow-ups.

In addition to changes in echo-intensity and fat fraction, our prospective MRI study also assessed T2 relaxation time as a complementary measure of muscle quality. T2-RT is an MRI-derived measure that reflects the microstructural and compositional properties of skeletal muscle, primarily by quantifying the amount of free water within the tissue. Increases in T2-RT can result from enhanced intramuscular water content, which may occur due to edema, local inflammatory responses, fibrosis, or the relative expansion of interstitial space following fiber atrophy. In our prospective study, T2-RT significantly increased between V1 and V3 in the injected gastrocnemius lateralis and medialis ($\pm 15\%$), as well as between V2 and V3 in the same muscles ($\pm 13\%$).

Interpretation of these results remains challenging in our cohort, as it is difficult to disentangle the respective contributions of cumulative effects from repeated injections and the increasing doses administered over time. Nevertheless, these findings indicate that BoNT-A–induced chemodenervation leads to early alterations in muscle composition, which are detectable as increases in T2-RT. Importantly, these changes may reflect a combination of early fiber atrophy, remodeling of the extracellular matrix, intramuscular water accumulation, and potentially a transient local inflammatory response rather than a single isolated process. T2-RT appears to provide a sensitive marker of structural muscle adaptations that can precede or accompany changes in muscle volume and cross-sectional area.

Overall, muscle quality, as assessed by echo-intensity, fat fraction and T2-RT, appears to be altered following BoNT-A injection, with measurable changes emerging from approximately 3 months post-injection. This delayed onset likely reflects the progressive nature of structural remodeling, including fiber atrophy, accumulation of connective tissue, and fat infiltration, which require time to become detectable by imaging techniques. Although long-term effects reported in the literature are heterogeneous and can trend in different directions, integrating evidence from histopathological studies, US, and MRI tends to suggest that BoNT-A has a persistent impact on muscle quality that can still be detected up to 6 months post-injection. These findings underscore the importance of considering both the temporal dynamics and the cumulative effects of repeated BoNT-A treatments when interpreting changes in muscle quality.

8.3 Limitations

A limitation inherent to both the present meta-analysis and the prospective clinical study concerns the difficulty in disentangling the specific effects of each BoNT-A injection on muscle properties from the pre-existing effects of spasticity and the underlying neurological lesion. In clinical practice, BoNT-A is administered to muscles that are already spastic, and therefore already exposed to chronic alterations in neural drive, passive tension, reduced voluntary activation, and changes in muscle use. Consequently, muscle structural and mechanical properties assessed after BoNT-A injections reflect the combined influence of the neurological injury, spasticity-related adaptations, and the therapeutic intervention itself, rather than the isolated effect of the toxin. Careful interpretation of post-injection muscle changes is therefore essential in neurological populations. This limitation is particularly relevant when interpreting post-injection changes in longitudinal designs, whereas it remains challenging to determine whether observed muscle alterations reflect cumulative effects of repeated injections or the long-term evolution of spasticity-related muscle changes. These issues contribute to the heterogeneity of findings reported in the literature and partly explain the persistent controversy regarding the impact of BoNT-A on non-neural components of HR. Several methodological approaches could help to better address this challenge in future studies. First, the inclusion of appropriate control muscles within-subject, such as non-injected spastic muscles, non-spastic muscles of the affected limb, and contralateral healthy muscles, thereby enabling a more accurate assessment of treatment-related effects. Second, longitudinal designs with assessments performed in acute phase, prior to the onset of spasticity and before the first BoNT-A injection would provide valuable insights into the temporal sequence of muscle structural

changes. Finally, experimental models and carefully designed dose–response or injection-spacing studies may further clarify the direct effects of BoNT-A on muscle tissue. Together, these approaches may contribute to a more precise understanding of how BoNT-A interacts with spastic muscle adaptations and help refine clinical decision-making.

8.4 Investigating injection parameters: a question-and-answer approach

The following section is structured around a series of questions that emerged progressively during the writing of this discussion. Rather than addressing BoNT-A as a single homogeneous intervention, each question specifically focuses on one parameter of the injection procedure. Indeed, despite the widespread use of BoNT-A in clinical practice, there is no fully standardized protocol regarding formulation, dose, dilution, injection volume, or treatment repetition.

In this context, a central issue is whether variations in these injection parameters may influence the muscular effects of BoNT-A, beyond its expected neuromodulatory action. More specifically, we sought to determine whether modifying one parameter of the injection—such as the type of toxin, the administered dose relative to muscle volume, the dilution protocol, or the frequency of injections—could alter the extent and nature of muscle structural remodeling and mechanical adaptations. Each question addressed in this chapter therefore examines whether BoNT-A–induced muscle changes are fixed and intrinsic to the molecule itself, or whether they are, at least partly, modifiable and dependent on how the treatment is delivered. These questions are subsequently discussed in light of our experimental findings and the existing literature.

Is there a dose–response relationship between BoNT-A and muscle properties adaptations?

The existence of a dose–response relationship between BoNT-A and muscle structural or mechanical adaptations remains a critical but insufficiently resolved question. From a mechanistic perspective, higher doses of BoNT-A induce a more pronounced chemodenervation, leading to greater inhibition of neuromuscular transmission. This would be expected to result in more substantial reductions in muscle activation and, consequently, more marked structural changes such as atrophy, reduced CSA, and alterations in muscle composition.

Experimental studies support this hypothesis, showing that the magnitude of muscle atrophy and structural alterations increases with the administered dose (75,96,140,141). However, translating these findings to clinical populations is challenging. In humans, doses are typically adjusted according to muscle size, severity of spasticity, and prior treatment history, making it difficult to isolate the independent effect of dose.

In contrast, clinical imaging studies investigating muscle morphology or composition in neurological populations have not specifically evaluated dose–response relationships. As a result, current evidence remains insufficient to determine whether increasing BoNT-A dose leads to greater structural muscle alterations in humans.

Moreover, dose is often correlated with other parameters, such as injection volume, dilution, and number of injection sites, further complicating interpretation. In our own work, dose was progressively increased across injections, which may have contributed to the observed changes in muscle composition.

However, as with repeated injections, it remains difficult to disentangle the specific contribution of dose from other factors, including cumulative exposure and temporal effects.

In summary, while a dose–response relationship is biologically plausible and supported by experimental data, current clinical evidence is insufficient to clearly establish how increasing BoNT-A dose influences muscle structural or mechanical adaptations. The effect of dose is likely context-dependent and intertwined with other injection parameters, highlighting the need for controlled studies specifically designed to isolate its impact.

Do repeated BoNT-A injections lead to cumulative muscle damage over time?

This question arises naturally from clinical practice, where BoNT-A injections are typically repeated every 3–6 months as the therapeutic effect diminishes over time. The potential for cumulative structural effects of repeated BoNT-A injections is therefore a key concern in both clinical practice and research. Several lines of evidence provide insight, although results remain somewhat heterogeneous.

Animal models consistently show that repeated BoNT-A injections induce structural muscle alterations, with effects that appear to depend on the number of prior injections, suggesting a cumulative component. (76,96,140).

In humans, evidence regarding the cumulative effects of repeated BoNT-A injections remains limited and somewhat inconsistent. To date, only a few studies have specifically investigated the relationship between the number of prior injections and structural muscle changes.

A prospective histopathologic study by Valentine *et al.* (73) on 10 ambulatory children with CP confirmed a positive association between number of BoNT-A injections and neurogenic atrophy between 4 months and 3 years after the

last treatment. Regarding the BoNT-A effect on the different muscle fiber types, they showed a slow type I fiber loss and a fast type II fiber predominance significantly related to the number of treatments of BoNTA. Furthermore, in children with CP, 2 studies (146,163) reported that the first BoNT-A injection leads to the most pronounced muscle atrophy, a finding that is further supported by our meta-analysis, which confirms a greater effect in injection-naïve patients. This suggests that the initial exposure to chemodenervation may induce the greatest structural impact on muscle tissue, while subsequent injections result in smaller or less detectable changes. However, findings are less consistent when considering muscle quality, as assessed by echo-intensity. While those 2 studies (146,163) did not observe significant differences between injection-naïve and non-naïve children, a third study (232) reported a positive association between echo-intensity and the number of previous BoNT-A injections, suggesting a potential cumulative effect on muscle composition.

Taken together, these results suggest that while muscle atrophy may be most pronounced after the first injection in children with CP, the effects of repeated injections on muscle quality remain uncertain and potentially progressive, highlighting the need for further longitudinal studies with standardized protocols.

In the stroke population, evidence is even more limited, with only one study specifically investigating the effects of repeated BoNT-A injections on muscle structure. This study did not find any significant correlation between the number of previous BoNT-A injections and structural muscle alterations over time (107).

Disentangling the effects of each injection is complex, because clinical studies often involve incremental dose adjustments, variable intervals between injections, and differences in baseline muscle condition. In our prospective study of stroke patients, structural muscle alterations were observed after the third injection, approximately 7 months after the first injection, but it is difficult to determine whether this reflects cumulative damage, delayed effects of the first injection, acute effects of the last injection, the increasing doses administered over time or a combination of the four.

In summary, the impact of repeated BoNT-A injections on muscle structure remains unclear. While some clinical studies suggest that the first injection may induce the most pronounced changes, experimental and histopathological findings indicate that repeated exposures can lead to progressive, cumulative structural alterations. In humans, available data are limited and heterogeneous, and do not allow a clear distinction between the effects of cumulative exposure, delayed responses to initial injections, or the influence of changing doses and injection protocols. Consequently, it remains difficult to determine whether repeated BoNT-A injections result in true cumulative muscle damage over time. Further longitudinal studies with standardized protocols are needed to better characterize the long-term structural consequences of repeated BoNT-A treatment.

Can BoNT-A–induced muscle remodeling affect the efficacy of subsequent injections?

This question is of particular clinical relevance, as BoNT-A injections are commonly repeated over time, often in the same muscles. If BoNT-A–induced structural remodeling alters muscle properties, it may in turn influence the responsiveness of the muscle to subsequent injections. As highlighted in the previous sections, BoNT-A injections are associated with muscle atrophy, increased connective tissue content, fat infiltration and/or fibrosis. These changes may modify the diffusion properties of the injected toxin within the muscle.

Increased fibrosis in particular could limit toxin spread, leading to a more localized and potentially less effective blockade of neuromuscular transmission. Therefore, muscle fibrosis is often considered a potential cause of treatment failure (233). Elevated echo-intensity on US, a parameter that reflects increased fibrotic and fatty changes within muscle, has been associated with reduced responsiveness to BoNT-A. In a cohort of 56 stroke patients with spastic equinus foot, those with higher gastrocnemius echo-intensity graded on the Heckmatt scale exhibited less clinical improvement in spasticity following the same dose of BoNT-A compared with patients with lower echo intensity (127). Similarly, in a larger retrospective study, post-stroke patients with greater muscle echo-intensity showed poorer motor function improvements after BoNT-A and multidisciplinary rehabilitation than those with lower echo intensity (234). These findings suggest that underlying structural muscle changes, including fibrosis, may modulate the effectiveness of BoNT-A treatment and emphasize the potential importance of assessing muscle quality prior to injection.

8.5 BoNT-A–induced muscle remodeling in other clinical contexts

Beyond the spasticity literature, valuable information regarding the structural effects of BoNT-A on skeletal muscle can also be derived from studies investigating the masseter muscle. BoNT-A injections are commonly administered into the masseter for the treatment of masseter hypertrophy, bruxism, temporomandibular disorders, and facial contouring. Because these individuals are generally free from neurological disease, such studies provide a useful model for examining the direct effects of BoNT-A on muscle tissue without the confounding influence of spasticity or paralysis.

Several recent studies have demonstrated that BoNT-A induces measurable structural changes in the masseter muscle. In an experimental study, Ramos *et al.* reported that a single BoNT-A injection into the masseter muscle of rats significantly reduced muscle fiber cross-sectional area and increased connective tissue content for up to 6 months after treatment (235). Similarly, human imaging studies have shown reductions in masseter muscle volume and thickness, together with increases in echo intensity following single and repeated BoNT-A injections, with some studies demonstrating persistent morphological changes for up to 12 months after treatment (236–238). These findings further support the notion that BoNT-A can induce structural muscle remodeling independently of the underlying neurological condition.

8.6 Comparison with Peripheral Denervation Disorders

The structural changes observed following BoNT-A injections raise the question of whether similar adaptations occur in other conditions characterized by reduced neuromuscular transmission. Since BoNT-A induces a temporary chemodenervation by blocking acetylcholine release at the neuromuscular junction, comparison with peripheral nervous system disorders associated with denervation may provide valuable insight into the mechanisms underlying muscle remodeling.

Lumbar radiculopathy provides a clinically relevant model of peripheral nervous system injury to contextualize BoNT-A-induced muscle changes. MRI studies have demonstrated selective atrophy of paraspinal muscles, particularly the multifidus, on the side and at the segmental level corresponding to the affected nerve root (239,240). This atrophy is accompanied by fatty infiltration and other compositional muscle changes (e.g. fiber atrophy, fiber type grouping) (241). Similar observations have been reported in other peripheral nerve disorders, supporting the concept that reduced neuromuscular input leads to predictable patterns of muscle remodeling. These findings closely mirror the structural adaptations observed after BoNT-A injections, suggesting that chemodenervation induced by BoNT-A may reproduce, at least in part, the biological consequences of focal denervation seen in radiculopathy.

The similarities between BoNT-A-treated muscles and chronically denervated muscles support the hypothesis that many of the observed structural changes represent a biological response to reduced neuromuscular activity rather than a phenomenon unique to the toxin itself.

8.7 Conclusions

Overall, the findings of this chapter indicate that BoNT-A injections exert measurable effects not only on the neural component of hyper-resistance but also on the structural and mechanical properties of skeletal muscle. While a transient reduction in elastography-derived muscle stiffness is consistently observed at short-term, this effect appears primarily driven by decreased neural activation and alterations in cross-bridge dynamics rather than by true modifications of passive tissue properties following BoNT-A injection. In contrast, structural adaptations of muscle tissue—including reductions in muscle volume and cross-sectional area, as well as remodeling in muscle quality reflected by increased echo-intensity, fat fraction, and T2 relaxation time—emerge progressively over time, typically from 3 months post-injection and may persist up to 6–12 months. However, the magnitude, persistence, and reversibility of these changes remain heterogeneous across studies and populations.

Despite these structural alterations, their functional implications remain unclear. Importantly, current clinical evidence does not demonstrate a deleterious impact of BoNT-A–induced muscle changes on patient function. On the contrary, BoNT-A is widely associated with improvements in spasticity, passive function, and in some cases active function, particularly when combined with rehabilitation (242,243). This suggests that the clinical benefits related to the reduction in neural hyperexcitability may outweigh potential deleterious effects on muscle structure, at least in the short to medium term. Nevertheless, it cannot be excluded that subtle or long-term structural changes may influence muscle performance or treatment responsiveness over time.

These considerations raise important questions regarding the optimal timing and repetition of injections. Emerging evidence (96) suggests that repeated injections administered before full neuromuscular recovery may exacerbate structural muscle alterations, particularly in experimental models. This highlights the potential importance of allowing sufficient recovery time between injections to limit cumulative muscle damage (73). However, such strategies must be balanced against the clinical need to control spasticity and maintain functional gains.

In this context, a more individualized approach to BoNT-A treatment may be warranted, taking into account not only clinical presentation but also muscle structural status and recovery dynamics. Future studies should aim to better define the relationship between structural muscle changes, functional outcomes, and injection protocols, in order to optimize the long-term safety and efficacy of BoNT-A treatment in neurological populations. It should also be noted that spastic muscles inherently exhibit structural abnormalities, even in the absence of botulinum toxin treatment. These pre-existing alterations may influence how muscle tissue responds to BoNT-A injections and should therefore be considered when interpreting treatment effects. This important aspect will be discussed in the following chapter.

Chapter 9. Spasticity as a driver of muscle adaptations

Spasticity “stricto sensu” can now be defined as “a disorder of sensorimotor control resulting from upper motor neuron disease. It is characterized by velocity- and length-dependent involuntary muscle overactivity, which is intermittent or sustained, during passive stretch.”. It is a common complication and an important cause of high rates of disability after stroke (15,16). A recent meta-analysis found an overall prevalence of spasticity after a first-ever stroke to be around 27%. A closer look at the results reveals that the incidence was the highest within 1 month, 31.6% (16). Evidence suggests that early detection and intervention for post-stroke spasticity may be more effective (244). Therefore, clarifying the risk factors can help to detect and treat post-stroke spasticity and the functional disorders caused by it at an earlier stage. The severity of the initial stroke is one of the strongest predictors of spasticity. Patients with higher scores on the NIHSS or those presenting with moderate to paresis are at greater risk (16). The same meta-analysis indicates that patients with hemorrhagic stroke are at higher risk of developing spasticity at 1-, 6-, and 12-months post-stroke. Additionally, patients with sensory impairments appear to be at increased risk between 3 and 6 months (16). Lesion site (e.g. basal ganglia) and lesion volume may also play a role, but there is currently insufficient evidence to draw definitive conclusions (18). Understanding these risk factors is critical, as the presence and severity of spasticity not only influence functional outcomes but also drive secondary changes in muscle structure and mechanical properties, which are the focus of the present chapter. The presence of spasticity exposes muscles to chronic muscle overactivity, altered patterns of muscle recruitment, reduced voluntary activation, and abnormal mechanical loading, among others.

Over time, these factors can induce a cascade of morphological, composition and mechanical changes within the affected muscles. Moreover, this process can become self-reinforcing. These peripheral changes further restrict joint mobility and contribute to persistent hyper-resistance, impaired force production, and functional limitations, thereby perpetuating the cycle of maladaptive muscle remodeling and reinforcing the clinical expression of the spasticity syndrome.

9.1 Structural and compositional muscle changes associated with spasticity

Several studies have documented structural alterations in spastic muscles across neurological conditions. However, the relation between spasticity and adaptive muscular properties changes still remains unclear.

Schillebeeckx *et al.* published a systematic review on this topic in 2021. Nine observational case control studies were included, reporting the outcome parameters of interest: muscle thickness, muscle-tendon length, fascicle length, pennation angle, and echo-intensity in spastic lower leg muscles of adults patients with first ever stroke (53). Unfortunately, no uniform conclusion could be formulated due to methodological differences and contradictory results. Nevertheless, there were some arguments for reduced muscle thickness and reduced fascicle length measured by US of the hemiplegic, spastic lower limb.

More recent studies published after this systematic review have provided additional insights into muscle structural alterations in spasticity. Two studies conducted in subacute and chronic stroke populations reported a reduction in muscle thickness on the spastic side compared with the contralateral, non-affected limb, supporting the presence of muscle atrophy (245,246).

In addition, one of these studies also observed higher echo-intensity in the spastic muscles, suggesting increased intramuscular fat and/or connective tissue content, although these changes did not appear to correlate directly with spasticity severity as assessed clinically (246). On the other hand, another recent study focusing on the gastrocnemius medialis in 26 chronic stroke survivors did not report clear differences in muscle thickness, pennation angle and echo-intensity between spastic muscles and contra-lateral unaffected muscles nor between spastic muscles and healthy control muscles (247). These discrepancies further highlight the heterogeneity of muscle adaptations in spasticity and the difficulty in establishing a direct and systematic relationship between structural muscle changes and clinical spasticity measures. Finally, another recent study investigating US characteristics of brachialis muscles in 50 stroke survivors reported a significant reduction in muscle thickness and increased echo-intensity on the affected side compared with the contralateral limb at baseline, 1 month, and 6 months post-stroke (49). Interestingly, when comparing the brachialis muscle within the affected limb at 1 month, the spastic muscle exhibited greater thickness and fascicle length with lower echo-intensity than the hemiparetic but non-spastic muscle. The authors hypothesized that this apparent preservation of muscle structure may be explained by the fact that spastic muscles are maintained in a state of tonic contraction, which could partially protect against disuse-related atrophy and architectural degradation.

Histological analyses further highlight modifications at the cellular level. Spastic muscles undergo histopathological alterations that contribute to their abnormal mechanical and functional properties. These changes include increased fatty infiltration (233) and a marked variability in muscle fiber size, with both atrophic and hypertrophic fibers (248), suggesting that spasticity can drive both quantitative and qualitative changes in muscle tissue. Concerning sarcomere length and fiber type conversion, results are more heterogeneous and hardly predictable, emphasizing the complex character of spastic muscle in human subjects (233,248). Beyond muscle fibers, alterations in intramuscular connective tissue and extracellular matrix have been reported. Biopsy studies indicate increased perimysial thickness and accumulation of collagen in spastic muscles (249,250). These structural changes likely contribute to increased passive stiffness and reduced extensibility observed clinically. Emerging evidence also implicates extracellular matrix components such as hyaluronan (251,252) and intracellular proteins such as titin (248,253) in modifying the viscoelastic properties of spastic muscle, further linking structural remodeling to altered mechanical behavior.

Even among spastic muscles, structural adaptations are not uniform. The extent and nature of remodeling depend on several muscle-specific factors. First, muscle architecture and functional role influence vulnerability. Even in healthy individuals, muscle architecture varies substantially across muscles and regions: fascicle length, pennation angle, and fiber orientation influence strain and mechanical behaviour independently of pathological conditions (254).

Building on this inherent heterogeneity, long fusiform muscles such as the triceps brachii may be more susceptible to fascicle shortening and sarcomere loss, while multi-pennate, postural muscles such as the gastrocnemius and soleus are frequently exposed to chronic loading in shortened positions, promoting connective tissue accumulation and fatty infiltration. Second, fiber type composition plays a role: muscles with a higher proportion of slow-twitch fibers (type I), such as the soleus, may resist atrophy but develop increased stiffness, whereas muscles rich in fast-twitch fibers (type II), such as the gastrocnemius or biceps brachii, may undergo more pronounced atrophy and fatty infiltration under chronic spastic activation. Third, the habitual pattern and intensity of spastic activation modulate the degree of remodeling: even if two muscles are spastic, the frequency and duration of involuntary contractions, together with the joint positions they maintain, can lead to very different structural outcomes. As a result, two spastic muscles—such as the gastrocnemius and triceps brachii—may both show adaptations, but the type, magnitude, and distribution of changes in architecture, composition, and mechanical properties can vary substantially. These muscle-specific differences are supported by both animal and human studies. In spastic rat models, the soleus and gastrocnemius exhibit distinct changes in passive stiffness and length-force properties under the same spastic conditions, indicating intrinsic differences in muscle response to chronic neural and mechanical stress (255). In humans, US analyses show that architecture differs within the triceps surae complex in spastic hemiplegia, with medial gastrocnemius, lateral gastrocnemius, and soleus showing varying fascicle lengths and pennation angles (256).

Moreover, associations between spasticity severity and muscle morphology are not uniform across muscles such as gastrocnemius and semitendinosus, highlighting that even similarly spastic muscles can undergo distinct structural adaptations (257). Recognizing these muscle-specific differences is essential for interpreting assessment results and for planning targeted interventions such as BoNT-A injections or rehabilitation programs.

While spasticity drives muscle adaptations in all neurological populations, the nature, severity, and timing of these adaptations vary according to the underlying pathology.

Cerebral palsy represents a unique condition in which spasticity is lifelong and develops during growth. In this population, muscles fail to grow longitudinally at the same rate as bones, leading to more profound structural alterations (258,259). These adaptations are more severe and diffuse than in adult-onset lesions, reflecting both chronic neural overactivity and developmental constraints.

In traumatic brain injury (TBI), spasticity often develops in a more heterogeneous and less predictable manner than after stroke, largely depending on lesion location, severity, and associated diffuse axonal injury. Patients with TBI frequently present with prolonged disorders of consciousness, extended periods of immobilization, and severe impairments in voluntary motor control, which expose muscles to a combination of neural overactivity, disuse, and abnormal mechanical loading (260). This unique clinical context may accelerate muscle atrophy, promote early connective tissue accumulation, and increase the risk of contracture formation.

Moreover, spasticity in TBI is often accompanied by dystonia, tremors, and myoclonus among others, leading to highly variable activation patterns across muscles (261). These complex motor manifestations may result in uneven and asymmetric muscle remodeling, with some muscles undergoing pronounced shortening and stiffness while others exhibit predominant atrophy and fatty infiltration. Compared with stroke, where spasticity often follows relatively stereotyped postural patterns, muscle adaptations after TBI may therefore be more diffuse and less predictable, reflecting the greater heterogeneity of central motor impairments in this population.

In multiple sclerosis (MS), spasticity is generally also less predictable in its onset and intensity, often fluctuating with disease activity and fatigue. As a result, muscle adaptations are more variable both between individuals and within the same patient over time. Fatty infiltration, connective tissue accumulation, and architectural alterations may be less pronounced than in chronic stroke but can accumulate over repeated relapses (262,263). While spasticity after TBI and MS is recognized clinically, there is a relative paucity of quantitative studies describing detailed muscle architecture and compositional changes in those populations compared with stroke or cerebral palsy. This gap in the literature highlights a need for focused research on muscle adaptations in TBI- and MS-related spasticity.

Collectively, these observations highlight that the temporal pattern of spasticity onset, duration of exposure, and the interaction with growth or aging significantly influence muscle remodeling. While all spastic muscles exhibit some degree of atrophy, stiffness, and connective tissue accumulation, the magnitude and distribution of these changes are pathology-specific, emphasizing the need to tailor assessment and intervention strategies to the underlying neurological condition.

9.2 Mechanical consequences: stiffness and altered passive behavior

Alterations in skeletal spastic muscle structure following neurological injury are expected to translate into significant changes in the mechanical behavior of the muscle–tendon unit. Beyond neural hyperexcitability, spastic muscles are characterized by marked modifications in their passive mechanical properties, which may contribute substantially to movement limitations, joint resistance, HR and functional impairments. In this section, we discuss the mechanical consequences of muscle alterations in neurological spastic populations, with a particular focus on increased passive stiffness and altered viscoelastic behavior.

One of the most consistently reported mechanical features of spastic muscles is an increase in passive stiffness. Passive stiffness refers to the resistance of a muscle–tendon unit to lengthening in the absence of active neural contraction. This passive stiffness reflects the intrinsic properties (such as elasticity and viscosity) of the muscle and surrounding connective tissues. It emerges from the combined contributions of muscle fibers, extracellular matrix, aponeuroses, and tendon, and determines the force required to stretch a muscle through its range of motion. Understanding passive stiffness is critical, as it directly affects joint mobility, movement efficiency, and the risk of contracture, independently of neural activation.

9.2.1 Pathophysiological progression of muscle remodeling in spasticity

Accumulating evidence suggests that increased passive stiffness in spastic muscles may represent an early and potentially reversible stage of a progressive remodeling process that can lead to muscle fibrosis. In the early phases following neurological injury, elevated passive stiffness may primarily reflect changes in muscle tone, altered sarcomeric properties, and increased

baseline tension (264,265). At this stage, mechanical alterations remain largely functional and may still be modulated through rehabilitation, stretching, and activity-dependent interventions (264). Over time, however, chronic exposure to altered loading conditions, reduced voluntary activation, and persistent muscle shortening may promote extracellular matrix remodeling and collagen accumulation (265). This fibrotic transformation progressively shifts passive stiffness from a sarcomere-dominated mechanism toward an extracellular matrix–dominated mechanism, resulting in a more rigid and less adaptable tissue. As fibrosis advances, muscle compliance decreases further, limiting longitudinal growth of muscle fibers and reducing the capacity for sarcomere addition in series (266). In the later stages, these structural changes lead to a state of marked and persistent mechanical stiffness characterized by fixed architectural constraints within muscle tissue, where stiffness is no longer primarily explained by neural factors or reversible viscoelastic adaptations. At this stage, the muscle–tendon unit exhibits substantial structural and compositional remodeling, including increased connective tissue content and reduced extensibility, suggesting a progressive shift from reversible functional stiffness toward more permanent morphological and mechanical alterations (266).

An illuminating example of how advanced imaging can bridge structural and mechanical aspects of muscle stiffness is provided by Menon *et al.* (252), who used T1ρ MRI to quantify intramuscular glycosaminoglycan content in post-stroke patients. This study reported elevated T1ρ values in the biceps of patients with chronic post-stroke stiffness compared with healthy controls, suggesting increased glycosaminoglycan content, particularly hyaluronan accumulation within the extracellular matrix of stiff muscles.

Interestingly, no significant differences were observed in the triceps brachii, highlighting that muscle remodeling following neurological injury is not homogeneous and that different muscles may exhibit distinct structural and mechanical trajectories. Importantly, following intramuscular hyaluronidase treatment, which degrades hyaluronan, T1 ρ values decreased, providing imaging evidence in support of the hyaluronan hypothesis of muscle stiffness. Although this study did not directly demonstrate a clinical reduction in muscle stiffness, it provides important imaging evidence for molecular alterations within the extracellular matrix that may contribute to increased tissue viscosity and HR. These findings emphasize the complexity of spastic muscle remodeling and suggest that passive stiffness cannot be attributed to a single uniform mechanism across muscles or individuals.

Beyond its mechanical implications, this line of research also opens promising therapeutic perspectives. By identifying extracellular matrix components such as hyaluronan as potential contributors to muscle stiffness, advanced imaging approaches suggest new treatment targets that go beyond neuromuscular transmission. In this context, pharmacological interventions aimed at modifying connective tissue properties, such as enzymatic degradation of glycosaminoglycans, may represent valuable complementary strategies to current treatments like BoNT-A injections. Such approaches could specifically address the mechanical and structural components of spasticity, which are not directly targeted by neurotoxin-based therapies, and may help prevent or slow the progression from reversible stiffness to irreversible fibrosis and contracture. Raghavan *et al.* (267) reported that intramuscular injections of hyaluronidase led to increased passive and active joint movement and reduced muscle stiffness in patients with spasticity of cerebral origin.

Importantly, the beneficial effects were maintained for 3 months of follow-up, suggesting that targeting extracellular matrix components can induce sustained improvements in muscle mechanical behavior.

These findings are particularly relevant to the present thesis because they illustrate a biochemical mechanism potentially underlying the early stages of mechanical alteration, preceding or contributing to fibrotic remodeling.

Excess hyaluronan in the extracellular matrix can increase tissue viscosity and resistance to deformation, offering a plausible link between molecular changes and the elevated passive stiffness quantified by SWE in spastic muscles. The use of advanced MRI techniques like T1 ρ mapping therefore complements ultrasound elastography by providing non-invasive insights into the molecular and structural environment of muscle tissue, supporting a more integrated understanding of how mechanical stiffness evolves toward fibrosis and, ultimately, fixed contracture in neurological populations (Figure 16).

Collectively, these findings highlight that spasticity is not merely a neural phenomenon but a driver of secondary peripheral adaptations. Chronic muscle overactivity and altered mechanical loading create a muscle environment prone to remodeling, impacting architecture, composition, and mechanical properties. Recognizing these changes is essential when interpreting muscle assessments in spastic patients, as they contribute to the non-neural component of hyper-resistance and may influence the response to interventions such as BoNT-A injections. In this context, imaging techniques capable of evaluating muscle mechanical properties *in vivo* may provide valuable insights into the progression of muscle remodeling and its functional consequences. Among these approaches, SWE has emerged as a promising tool for the quantitative assessment of muscle stiffness in neurological populations.

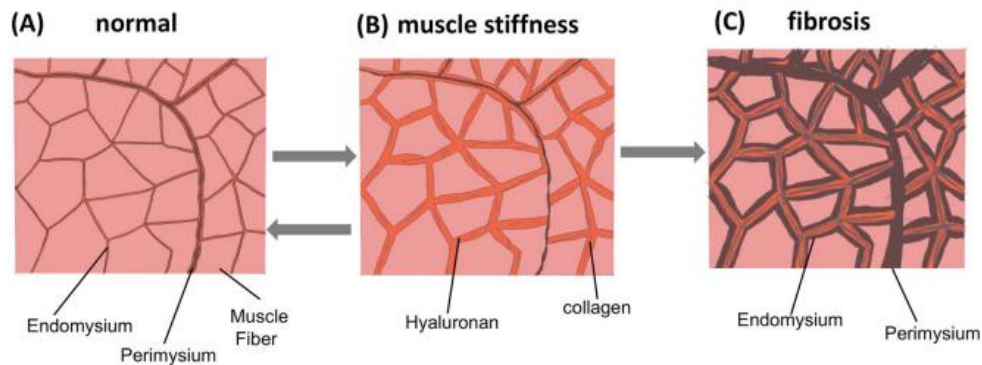


Figure 16. Model of the evolution of muscle biochemical/structural changes post stroke. (A) Shows a crosssection of a normal skeletal muscle, (B) shows the evolution of muscle stiffness following post-stroke paralysis and immobility that results in accumulation of hyaluronan in the extracellular space, increasing the viscosity of the ECM, and causing the muscle fibers to stick together (C) shows that continued inflammation, immobility and muscle stiffness initiates fibrosis leading to the replacement of hyaluronan by collagen, thickened endomysium and perimysium, and muscle fiber atrophy. Note that while (C) depicting fibrosis is an irreversible stage, data from literature suggests that the processes between stages depicted in (A) and (B) are reversible and may represent a potential therapeutic target. Reproduced from Menon *et al* (252).

9.2.2 Assessing muscle stiffness through shear wave elastography

Imaging-based methods such as SWE provide non-invasive estimates of tissue elastic stiffness at the level of individual muscles. Although SWE has emerged as a promising non-invasive tool to quantify muscle stiffness *in vivo*, its use in neurological populations is still associated with important methodological limitations. To date, there is no standardized acquisition protocol for muscle elastography, particularly regarding patient positioning, joint angle, and muscle activation. Measurements are highly dependent on muscle state, as SWV varies with muscle length, and passive/active tension.

This lack of standardization likely contributes to the heterogeneity observed across studies, both in terms of absolute stiffness values and in reported group differences between spastic and non-spastic muscles. Consequently, comparisons between studies should be made with caution, and elastography results should always be interpreted in light of the specific experimental conditions under which they were obtained. These methodological issues highlight the need for preliminary validation studies aimed at optimizing and harmonizing elastography protocols in neurological contexts.

This was the rationale underlying our third study (chapter 6) (215) of the present thesis, which specifically sought to identify the most valid and reliable measurement conditions, particularly with respect to patient positioning and muscle length, in order to improve the robustness and interpretability of SWE assessments in spastic muscles. Measurements were performed on both the affected and less-affected lower limbs with participants lying in a prone position under three standardized conditions: at rest with the ankle in a neutral position, during passive dorsiflexion, and during isometric contraction. Each condition was repeated three times in order to assess measurement consistency and reliability across muscle states. This experimental design allowed us to evaluate how muscle length and activation modulate elastography-derived stiffness, and to identify the most valid and reproducible conditions for assessing muscle mechanical properties in individuals with spasticity.

Based on the analysis of data from 30 individuals with HR of the gastrocnemius muscles, our findings suggest that SWE measurements obtained in the ankle neutral resting position are the most reliable and may represent the most valid condition for assessing passive muscle stiffness in neurological population.

In the ankle neutral resting position, a significant side effect was observed, with higher SWV in the affected spastic gastrocnemius muscles compared with the less-affected muscles, reflecting increased elastic stiffness.

Consistent with our findings, several authors (178,179) observed significantly increased elastic stiffness in the spastic GM and GL muscles relative to the unaffected side in individuals with stroke. Similar alterations have also been reported in studies focusing on upper-limb muscles (180,268). This objective increase in muscle stiffness is in line with HR typically perceived during clinical examination and suggests that SWE captures a meaningful mechanical correlate of spastic muscle behavior.

An important and still debated issue concerns the relationship between clinical “spasticity” scales, such as the MAS and the MTS, and quantitative measures of muscle stiffness obtained with elastography.

MAS and MTS are widely used in clinical practice to assess resistance to passive movement, yet they rely on subjective ratings and integrate multiple components, including neural reflex activity, intrinsic muscle stiffness, joint structures, and examiner-dependent factors. As a result, these scales cannot distinguish neural from non-neural components. If one of the components is predominant, it will be overlooked by those clinical scales. Therefore, MAS and MTS may not be sufficiently sensitive to detect changes in muscle elastic stiffness, which predominantly reflect the non-neural component of the spasticity syndrome. The relative contribution of neural and non-neural factors to resistance against passive movement likely varies across individuals, depending on the stage of the condition and the degree of structural muscle remodeling.

In line with this interpretation, no significant correlations between MAS or MTS scores and SWV were observed in our third study of the present thesis (chapter 6). Our findings are consistent with several previous studies reporting weak or absent associations between clinical “spasticity” scales and elastography-derived stiffness measures in spastic lower limb (178) and upper limb (180,182,189,202,269) muscles of stroke survivors. A study by Alibiglou and colleagues (270) investigated the relationship between clinical assessments of spasticity and objective neuromechanical measurements following stroke. Using advanced system identification techniques, they quantified the dynamic stiffness of the joints, and separated its muscular (intrinsic) and reflex components of the elbow and ankle and examined their correlations with the MAS. Similarly, the study found no significant correlation between MAS scores and either intrinsic or reflex stiffness measures, indicating that changes in mechanical properties of muscle–tendon units are not consistently captured by this clinical scale. On the other hand, in the systematic review of Miller *et al.* (40) evaluating the reliability and validity of ultrasound elastography in neurological populations, convergent validity was demonstrated by strong correlations between stiffness and clinical measures of spasticity (n=5). However, 3 studies did not find any significant correlation. In the same way, the MAS correlated with ultrasound elastography stiffness in 10 studies and did not correlate in 4 studies in the systematic review of Roots *et al* (205). While some investigations have found moderate associations in specific muscle groups or experimental conditions, the overall relationship remains inconsistent across populations and methodologies.

This suggests that clinical scales and elastography capture overlapping but distinct aspects of spastic muscle behavior: MAS and MTS primarily reflect global resistance to movement, whereas elastography provides a localized and tissue-specific estimate of passive mechanical properties. These discrepancies highlight the limitations of relying solely on clinical scales to characterize muscle mechanical alterations and support the added value of quantitative imaging tools.

In this context, elastography should not be viewed as a replacement for clinical assessment, but rather as a complementary technique capable of disentangling the mechanical component of spasticity from its neural expression.

9.3 Functional and rehabilitation implications of spasticity-related muscle structural and mechanical alterations

Structural alterations observed in spastic muscles, whether in the presence or absence of botulinum toxin treatment—such as reduced fascicle length, decreased muscle thickness, connective tissue accumulation, and fatty infiltration—have important consequences for motor function. From a functional perspective, a reduction in muscle thickness is commonly interpreted as a marker of muscle atrophy and decreased force-generating capacity. Similarly, a shortening of fascicle length reflects a reduction in the number of sarcomeres in series, which may limit muscle excursion, reduce contraction velocity, and ultimately impair the ability to generate force across the full range of joint motion. Together, these structural alterations are likely to contribute to reduced functional performance and increased joint stiffness in individuals with spasticity (271).

Furthermore, fatty infiltration represents a major determinant of muscle dysfunction in chronic neurological conditions (272). By replacing contractile tissue with adipose tissue, fatty infiltration directly reduces the force-generating capacity of muscles and alters their mechanical efficiency. Even when muscle volume is preserved, increased intramuscular fat compromises muscle quality by decreasing the proportion of active contractile elements, leading to lower specific force and impaired voluntary motor performance. From a mechanical perspective, fatty infiltration also contributes to increased passive stiffness and altered viscoelastic behavior of the muscle–tendon unit, particularly when associated with connective tissue accumulation and fibrosis. Functionally, high levels of intramuscular fat have been associated with reduced walking speed, increased fatigue, and poorer rehabilitation outcomes, suggesting that fatty infiltration not only reflects structural degeneration but also represents a key barrier to functional recovery (272). In the most advanced stages of chronic spasticity-related remodeling, these structural and mechanical changes may culminate in joint contracture, defined as a permanent limitation of joint range of motion resulting from structural shortening of muscles, tendons, and periarticular connective tissues.

While neural overactivity may contribute to maintaining muscles in shortened positions, increasing evidence suggests that contractures primarily reflect long-term mechanical and structural changes, including loss of sarcomeres in series, increased collagen content, fibrosis, and reduced tissue extensibility (273). In such cases, conservative treatments become largely ineffective at restoring muscle length and joint mobility. Management often relies on orthopedic surgical interventions, such as muscle–tendon lengthening or release procedures, aimed at mechanically correcting the contracture.

The frequent need for surgical treatment at this stage underscores the irreversible nature of late structural and mechanical adaptations and further supports the concept of a progressive transition from increased stiffness to contracture. From a clinical standpoint, this emphasizes the importance of early identification and management of muscle mechanical alterations, before irreversible tissue remodeling necessitates surgical correction.

These structural and mechanical changes highlight the limits of therapeutic approaches that exclusively target neural hyperexcitability. While interventions such as BoNT-A injections effectively reduce the neural component of spasticity, they may not be sufficient to restore function when substantial non-neural muscle adaptations are already present. In such cases, improvements in muscle tone may not translate into meaningful functional gains if muscle architecture and passive mechanical properties remain altered. From a rehabilitation perspective, these observations emphasize the need to integrate pharmacological treatments with interventions specifically addressing muscle structure and mechanical behavior.

Rehabilitation strategies should therefore combine approaches targeting neural overactivity with interventions promoting muscle lengthening, strength, and preservation of muscle quality to mitigate potential deleterious changes and optimize functional outcomes. Distinguishing between neural and non-neural components of HR is therefore crucial to better tailor rehabilitation interventions, especially regarding the prescription of adjunctive and/or multimodal therapies.

Addition of a secondary pharmacologic or non-pharmacologic treatment to optimize the efficacy of the initial modality, is considered an adjunctive therapy. It is time-specific and requires the added therapy be initiated within a specific period to enhance the primary treatment. In our context, adjunctive therapy refers to non-pharmacologic interventions (e.g. casting) applied in combination with BoNT-A injections to enhance clinical and functional outcomes (59). Multimodal therapy is an integrated, patient-centric program of pharmacologic and non-pharmacologic strategies utilized in a concurrent/integrated or sequential manner to enhance the overall treatment effect. Moreover, within a multimodal approach, adjunctive therapy can be used to help enhance the treatment effect of one specific modality (59).

A group consensus statement led by Francisco *et al.* (274) emphasized that BoNT-A should systematically be followed by a structured physiotherapy program and that adjunctive therapies should primarily aim to optimize functional recovery. Consistent with these recommendations, Schillebeeckx *et al.* (275) conducted an international survey among rehabilitation specialists and reported that self-rehabilitation (including passive stretching and active exercises), and splinting were the most frequently prescribed adjunctive interventions following BoNT-A injections. However, despite their widespread use, high-level evidence demonstrating the superiority of specific adjunctive strategies in improving functional outcomes remains limited (276). In the following section, selected interventions are discussed as illustrative examples. This non-exhaustive overview does not aim to review all available adjunctive therapies, but rather to provide a mechanistic perspective on how these interventions may act on muscle structure and mechanical properties, thereby influencing the non-neural component of hyper-resistance.

Stretching interventions, whether passive or active, are traditionally prescribed to increase joint range of motion and prevent contracture formation. At the tissue level, prolonged or repeated stretching is thought to induce adaptations within the muscle–tendon unit, including increased muscle extensibility, potential addition of sarcomeres in series, and remodeling of the extracellular matrix (277). Although the extent of true structural adaptation remains debated, stretching may reduce passive stiffness by modifying viscoelastic properties of connective tissue and altering collagen alignment, thereby improving the passive length–tension relationship. However, evidence from a Cochrane review published in 2017 suggests that these expected mechanical benefits may not translate into clinically meaningful improvements (278). In people with neurological conditions, it was uncertain whether stretch had clinically important short-term effects on pain (SMD 0.2; 95% CI -0.1 to 0.5; 5 studies with 174 participants) or activity limitations (SMD 0.2; 95% CI -0.1 to 0.5; 8 studies with 247 participants). No trials examined the short-term effects of stretch on quality of life or participation restrictions in people with neurological conditions. These findings highlight a discrepancy between the proposed biomechanical effects of stretching at the tissue level and its limited impact on clinically relevant outcomes in neurological populations.

Similarly, casting and splinting aim to maintain muscles in prolonged stretched positions, thereby applying sustained mechanical loading to shortened muscle–tendon units. This prolonged stretch may promote connective tissue remodeling and limit the progression of contractures by influencing collagen turnover and muscle extensibility.

However, the effectiveness of these approaches likely depends on timing, duration, and patient compliance, and their long-term structural impact remains insufficiently documented (266–268).

Progressive resistance training and active exercise may also influence non-neural muscle properties by counteracting disuse-related atrophy and promoting muscle hypertrophy and improved muscle quality. By increasing muscle cross-sectional area and stimulating neuromuscular activation, strength training may partially restore force-generating capacity and reduce the relative proportion of non-contractile tissue within spastic muscles. In this context, active interventions are particularly relevant for addressing fatty infiltration and muscle degeneration associated with chronic spasticity (279).

In the same way, functional electrical stimulation (FES) is widely used in neurorehabilitation to enhance voluntary movement and prevent disuse-related muscle atrophy. By delivering controlled electrical impulses to targeted muscles, FES induces repeated contractions that mimic active movement, promoting both neural activation and muscle engagement. Mechanistically, FES may influence the non-neural component of hyper-resistance by maintaining or increasing muscle cross-sectional area, reducing disuse atrophy, and potentially limiting fatty infiltration. Repeated contractions also generate cyclic mechanical loading on the muscle–tendon unit, which may improve muscle extensibility, maintain sarcomere length, and modulate the viscoelastic properties of connective tissue. In this way, FES provides a dual benefit: it addresses neural activation deficits while also mitigating structural and mechanical deterioration that contribute to passive stiffness (280,282).

Extracorporeal shockwave therapy (ESWT) has recently gained interest as a potential treatment for spasticity, particularly in chronic conditions. Picelli *et al.* (221) compared the combined structural (as measured by conventional US and SWE) and clinical effects of BoNT-A and ESWT versus BoNT-A alone on the spastic muscles of children with CP. At 1-month, significant differences in the injected muscles percentage of stiffness and the MAS were found between groups supporting the hypothesis that the combined effects of BoNT-A and ESWT derive from their respective action on neurological and non-neural components in spastic muscles. Although its precise mechanisms remain incompletely understood, ESWT is hypothesized to act on neural and on non-neural components by inducing mechanical and biological effects within soft tissues. Shock waves are thought to produce a “neurological” effect by inducing enzymatic and nonenzymatic nitric oxide synthesis, which induces modulation of neurotransmission at the neuromuscular junctions (221). Furthermore, ESWT has been suggested to have a direct non-neural effect through modulation of connective tissue stiffness, disruption of fibrotic tissue, stimulation of local blood flow, and activation of cellular pathways involved in tissue remodeling (283).

Altogether, these observations suggest that adjunctive therapies may not simply enhance the clinical effect of BoNT-A through functional practice, but may also directly target the biomechanical substrate of spasticity by modifying muscle and connective tissue properties. Finally, it is important to note that many interventions considered as adjunctive therapies such as electrical stimulation therapies, strengthening, and active exercise are independently effective and recognized as core components of best practice in neurorehabilitation (284).

International consensus supports their systematic integration within multimodal management programs for spasticity. Ultimately, the choice of adjunctive procedures should be guided by individual impairments, functional objectives, and the relative contribution of neural and non-neural mechanisms, reinforcing the need for comprehensive and mechanistically informed assessment frameworks (284).

Chapter 10. Stroke-Induced Changes in Skeletal Muscle Properties

Recovery after stroke is a dynamic and time-dependent process (Figure 17), with distinct biological and clinical phases that reflect not only neural plasticity and functional restitution but also progressive adaptations of peripheral tissues such as skeletal muscle. The Stroke Recovery and Rehabilitation Roundtable has worked toward standardized definitions of these post-stroke recovery phases to facilitate research and clinical practice (285). According to consensus definitions, the acute phase typically spans the first 1–7 days after stroke onset and is dominated by medical stabilization, prevention of secondary complications, and the initiation of early rehabilitation when safe. The early subacute phase covers approximately 7 days to 3 months, a period during which spontaneous neural recovery and neuroplasticity are particularly active, and during which many of the most prominent gains in motor function are observed. A late subacute phase extends from around 3 to 6 months, during which recovery continues but generally at a slower rate. After about 6 months, the stroke enters a chronic phase, where spontaneous functional improvements tend to plateau and long-term adaptations and compensatory strategies become more prominent.

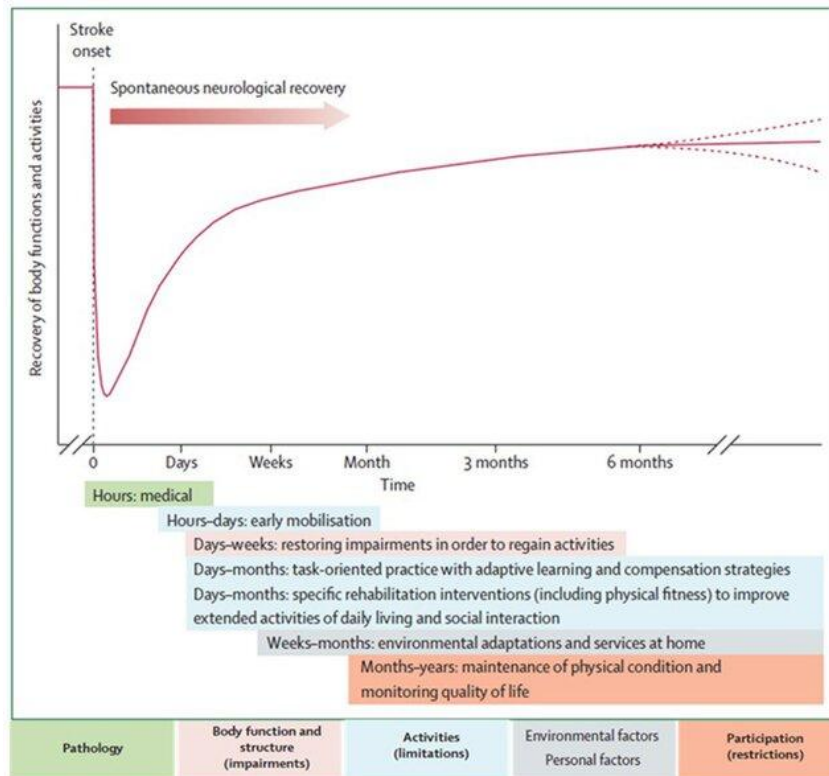


Figure 17. Pattern of recovery after stroke with timing of intervention strategies. The neurological recovery after stroke displays a nonlinear, logarithmic pattern. The greater part of recovery is reported to take place in the first three months following stroke. Reproduced from Hatem *et al.* (286).

These phases not only describe patterns of neurological recovery, but also frame the timeline over which muscle structure and mechanics may change after stroke. Indeed, beyond the effects of therapeutic interventions and the clinical syndrome of spasticity, the neurological lesion itself may directly induce early and specific alterations in skeletal muscle properties. Stroke represents a sudden disruption of supraspinal motor control, leading not only to neural deficits but also to profound changes in muscle activation, loading conditions, and sensorimotor integration.

Even in the absence of clinically detectable spasticity, these factors are likely to initiate a cascade of structural and mechanical adaptations within the muscle tissue. In the acute phase, reduced activation and unloading of paretic muscles begin immediately, setting the stage for early muscle atrophy and alterations in viscoelastic properties. During the subacute period, when spontaneous recovery and rehabilitation efforts are most intensive, both neural and non-neural factors (e.g., changes in muscle protein turnover, connective tissue remodeling) interact to shape muscle quality and function. In the chronic phase, cumulative effects of persistent weakness, compensatory movement patterns, disuse, and altered loading further influence muscle architecture, composition, and stiffness. Integrating these temporal recovery stages into the study of muscle adaptations helps disentangle primary lesion-related changes from those that evolve under the influence of disuse, spasticity, medical complications, and rehabilitation exposures.

It is also important to acknowledge that skeletal muscle is rarely a “healthy baseline” tissue at the time of stroke onset. In many patients, muscle structure and mechanical properties may already be altered prior to the neurological event as a result of pre-existing physiological and pathological conditions. Ageing, in particular, plays a major role, as stroke predominantly affects older individuals. Sarcopenia associated with ageing is characterized by a progressive loss of muscle mass and strength, preferential atrophy of type II fibers, motor unit remodeling, and increased intramuscular fat and connective tissue infiltration, all of which contribute to reduced muscle quality and increased passive stiffness (287). In addition, malnutrition, which is highly prevalent in elderly and chronically ill populations, promotes muscle protein

catabolism, impairs muscle regeneration, and accelerates muscle wasting (288). Other frequent comorbidities in stroke populations, such as physical inactivity, diabetes, chronic inflammation, cardiovascular disease, and prior periods of immobilization or hospitalization, are also associated with adverse muscle adaptations, including mitochondrial dysfunction, increased fibrosis, and reduced mechanical compliance (289,290).

Furthermore, muscle and tendon properties may also be influenced by chronic exposure to pharmacological agents commonly prescribed in stroke populations. Several drug classes are known to modulate muscle tone, metabolic activity, and exercise capacity, thereby indirectly affecting muscle structure and mechanical behavior. For instance, β -blockers, widely used for the management of hypertension and cardiovascular disease, reduce sympathetic drive and heart rate, which may limit exercise tolerance and physical activity levels, ultimately promoting muscle deconditioning (291). Moreover, β -blockers have been shown to alter skeletal muscle perfusion, oxidative capacity, and fiber type distribution, potentially contributing to reduced endurance and increased fatigability (291). Other pharmacological treatments, such as corticosteroids, statins, or sedative agents, may further exacerbate muscle atrophy, impair mitochondrial function, or increase susceptibility to myopathic changes (292,293). Altogether, these pharmacological factors represent an additional layer of pre-existing muscle vulnerability that may shape the baseline muscle phenotype upon which stroke-related alterations subsequently develop. Consequently, post-stroke muscle alterations should be interpreted not only as a direct consequence of the neurological lesion, but also as the superimposition of stroke-induced remodeling on a muscle tissue that is often already structurally and mechanically compromised before the event.

10.1 Primary structural muscle adaptations following stroke

An emerging body of literature has begun to address the methods by which post-stroke muscle structural changes can be quantified, a necessary step for accurately characterizing stroke-induced remodeling. A recent systematic review by Li *et al.* examined the reliability of ultrasound-based measurements of skeletal muscle architecture in individuals after stroke (52). They reported moderate-to-excellent reliability for key parameters such as muscle thickness, pennation angle, cross-sectional area, muscle volume, and muscle length across various muscles including the gastrocnemius muscle. This review highlights that quantitative imaging modalities can robustly capture structural differences between paretic and non-paretic muscles, supporting their use in longitudinal studies of post-stroke muscle adaptations.

Recent longitudinal work has started to elucidate the earliest structural muscle changes after stroke, even before the emergence of spasticity. For example, the systematic review by Beckwée *et al.* investigated muscle volume in the acute and early subacute phases following first-ever stroke, using ultrasonographic measures of muscle thickness and CSA, as well as lean muscle mass assessment through DEXA (216). They observed that significant declines in muscle thickness occur within the first weeks after stroke onset. Importantly, these alterations were evident as early as 1–2 weeks post-stroke, suggesting that intrinsic muscle remodeling begins rapidly after the neurological insult, likely driven by reduced voluntary activation and mechanical unloading. However, several important limitations emerge from the current literature. Although 6 studies have evaluated muscle thickness in the acute phase, only 1 study has assessed CSA and only 1 has measured lean muscle mass, leaving other critical architectural parameters—such as muscle

length, pennation angle and fat fraction—virtually unexamined in the early post-stroke period. This highlights a substantial gap in knowledge regarding the complete characterization of muscle structural changes in the acute phase. Furthermore, while studies included a longitudinal follow-up, these typically stopped at 1 month post-stroke, preventing a comprehensive understanding of how muscle structure evolves through the late subacute and chronic phases.

Longer-term longitudinal studies are therefore warranted as chronic investigations are essential to understand the persistence and potential progression of muscle remodeling. A recent systematic review published in 2023 evaluated the structural muscle changes up to one year after post-stroke hemiplegia (217). Four studies have used DEXA to quantify fat and lean mass in the lower limbs after stroke. Overall, these studies report that lean mass decreases as early as 3 weeks post-stroke, highlighting the rapid onset of muscle wasting following the neurological insult (294). Importantly, these alterations differ according to functional status and limb side. In non-ambulatory patients, a significant reduction in lower limb lean mass is observed during the early subacute phase on both the paretic and non-paretic sides, suggesting a global effect of inactivity. In contrast, ambulatory patients do not exhibit a significant decrease in lower limb lean mass during the same period (295). In the chronic phase, however, a more asymmetrical pattern emerges, with a preferential reduction in lean mass on the paretic side, while the non-paretic limb remains stable or even shows increased lean mass, potentially reflecting compensatory overuse (294,296).

Regarding fat mass, DEXA-based studies indicate a progressive increase from the late subacute phase into the chronic phase, with greater fat accumulation in non-ambulatory patients, further emphasizing the influence of mobility and mechanical loading on post-stroke muscle composition (295,297).

Overall, these observations underscore the critical need for longitudinal quantitative imaging approaches that begin in the acute phase and extend through the chronic phase. Such approaches would allow for a comprehensive characterization of the dynamic evolution of muscle atrophy, architectural remodeling, and compositional changes in the paretic limb, independent of spasticity, and would capture the full trajectory of post-stroke muscle remodeling across all stages of recovery.

10.2 Beyond spasticity: primary stroke-related changes in muscle stiffness

A recent systematic review synthesizing the available evidence on post-stroke muscle stiffness measured by ultrasound elastography highlights several important features relevant to this thesis (205). Roots *et al.* reviewed 29 studies using both strain and shear wave elastography to quantify muscle stiffness in stroke survivors and consistently found that paretic muscles exhibit higher stiffness compared to both the non-paretic limb and healthy controls across multiple muscle groups, including the medial gastrocnemius. Ultrasound elastography measures also varied with joint angle and introducing a passive stretch or muscle activation, indicating that passive muscle properties are influenced by muscle length and loading.

However, a closer examination of the studies included in the systematic review by Roots *et al.* further highlights the methodological limitations of the existing literature. Among the 29 studies analyzed, only 3 specifically included patients without clinically established spasticity, while the vast majority focused on populations presenting with moderate to severe spasticity. This imbalance severely limits the ability to disentangle the respective contributions of intrinsic muscle tissue remodeling and neural hyperexcitability to the observed increases in muscle stiffness. Consequently, although the review provides robust evidence that paretic muscles are stiffer than non-paretic muscles in chronic stroke populations, it remains unclear to what extent these findings reflect stroke-induced primary muscle adaptations rather than secondary changes related to spasticity. Furthermore, the authors specifically note that there is a pronounced gap in the literature regarding stiffness assessments in the acute phase after stroke and a lack of longitudinal data tracing the natural development of increased stiffness over time. This gap underscores the relevance of our longitudinal design, which aims to capture early and progressive changes in muscle mechanical properties from 2 weeks to 6 months post-stroke.

Part IV General conclusions

A general overview of the findings of this thesis

This thesis seeks to bridge gaps in current knowledge and contribute to a better understanding of post-stroke spasticity syndrome, focusing on both the structural and mechanical alterations of calf muscles, as well as the impact of BoNT-A injections. Across the studies presented, several consistent patterns emerge.

Firstly, stroke itself appears to induce early alterations in skeletal muscle properties, even before the development of clinically established spastic muscle overactivity. The findings of this thesis support the idea that muscle remodeling begins rapidly after the neurological lesion and is not solely a secondary consequence of spastic muscle overactivity or its treatment. In particular, changes in muscle composition may occur before detectable alterations in muscle morphology or passive mechanical stiffness, suggesting that early post-stroke muscle adaptations initially develop at a microstructural level. However, the exact timing and progression of these different alterations remain incompletely understood. The current literature still lacks studies initiated in the acute phase after stroke and combining detailed quantitative imaging with longitudinal follow-up extending beyond the first weeks of recovery. The results of this thesis therefore reinforce the importance of future studies combining early multimodal muscle assessment with longitudinal monitoring extending over at least the first 6 months post-stroke. Such approaches are essential to better characterize the natural history of post-stroke muscle remodeling and to disentangle the respective influences of the neurological lesion, spasticity, disuse, and therapeutic interventions.

Importantly, the findings also suggest these early muscle adaptations do not occur uniformly across muscles or patients. Rather, they seem to depend on muscle-specific characteristics, such as functional role and architecture, as well as on the patient's ambulatory status and associated mechanical loading conditions.

Secondly, spastic muscle overactivity, structural alterations and mechanical changes appear to evolve dynamically in the post-stroke period. Beyond its neural origin, spastic muscle overactivity induces progressive changes in muscle structure, composition, and mechanical properties. These include muscle atrophy, fascicle shortening, fatty infiltration, and increased connective tissue, although findings remain heterogeneous across studies and muscles. At the microscopic level, alterations in muscle fibers and extracellular matrix components further contribute to these changes. Importantly, these adaptations are muscle-specific and influenced by factors such as muscle architecture, fiber type composition, and patterns of activation.

These structural changes translate into altered mechanical behavior, notably increased passive stiffness, which plays a major role in functional impairment. Passive stiffness appears to evolve along a continuum, from early and potentially reversible alterations to irreversible contracture. This progression highlights the importance of early intervention. The results of this thesis confirm that muscle stiffness can be objectively quantified using elastography, although methodological challenges remain, and its relationship with clinical scales such as MAS or MTS is inconsistent, reflecting the multifactorial nature of spasticity.

These findings emphasize that spastic muscle overactivity should not be considered solely as a neural disorder but as a condition involving complex interactions between neural and peripheral mechanisms. Structural and mechanical muscle alterations contribute significantly to functional limitations and may reduce the effectiveness of treatments targeting neural overactivity alone. This underscores the need for comprehensive, multimodal management approaches that address both neural and non-neural components of the spasticity syndrome.

Thirdly, BoNT-A treatment effectively reduces neural overactivity and transiently decreases muscle tone. However, BoNT-A injections, whether administered once or repeatedly, may have complex effects on muscle structure and passive mechanical properties. The findings of this thesis indicate that frequent BoNT-A administration could contribute to muscle atrophy and altered muscle quality, emphasizing the importance of carefully considering injection timing, dosing, and the selection of muscles to target. In this context, BoNT-A should no longer be considered as a neutral intervention acting solely on neural mechanisms, but rather as a factor capable of reshaping muscle structure and mechanics. This has major implications for the interpretation of muscle changes observed in spastic populations, as some of these changes may be iatrogenic rather than intrinsic to the neurological condition. Disentangling treatment-related muscle adaptations from lesion-related and spasticity-related effects therefore represents a critical step toward a more accurate understanding of muscle plasticity in neurorehabilitation. Importantly, the structural muscle changes observed after BoNT-A injections should be interpreted within the context of its primary mechanism of action.

By inducing a temporary and reversible chemodenervation, BoNT-A inevitably reproduces, to a certain extent, a state of reduced neuromuscular activation, which is known to influence muscle trophicity and composition. From this perspective, the presence of muscle atrophy or alterations in muscle quality should not necessarily be interpreted as unexpected or pathological findings, but rather as a biological consequence of the intended pharmacological effect.

More importantly, these structural and imaging findings must be weighed against the established clinical benefits of BoNT-A treatment. A large body of evidence has demonstrated improvements in spasticity-related outcomes, including reductions in MAS scores, as well as gains in functional performance, goal attainment (GAS), ease of care, and patient-reported quality of life. These clinical improvements remain the primary therapeutic objective in the management of post-stroke spasticity. Therefore, while understanding potential muscle-level effects is essential for optimizing long-term treatment strategies, they must be interpreted in parallel with the functional benefits that underpin the clinical value of BoNT-A injections.

Overall, these findings highlight the intricate interplay between neural and structural factors in post-stroke spasticity and underscore the need for individualized, multimodal approaches. While neural overactivity can be effectively modulated with BoNT-A, preserving muscle structure and function requires integrated strategies combining early intervention, physical therapy, and physiologically informed management of repeated injections.

Implications for clinical practice

The findings of this thesis suggest important considerations for the clinical management of post-stroke spastic muscle overactivity, particularly regarding the timing and repetition of BoNT-A injections. Increasing evidence supports the benefit of early intervention following stroke, with several studies indicating that initiating BoNT-A treatment in the early stages may lead to better outcomes in terms of spasticity reduction (faster and greater reduction in spasticity associated with greater time to reach re-injection criteria) (285–287), improved motor recovery (244), and reduced contracture development (301,302). Early treatment may therefore help to preserve muscle properties or limiting structural muscle changes which may be a key factor in reducing long-term disability and improving functional outcomes. However, while early injections appear beneficial, the optimal strategy for repeated administrations remains less clearly defined. Botulinum toxin is known to induce temporary muscle tone reduction, but the findings of this thesis suggests that repeated injections may contribute to muscle atrophy and alterations in muscle quality and passive mechanical properties, particularly if administered too frequently. In this context, a more cautious approach to reinjection timing may be warranted. Rather than systematic or fixed-interval injections, clinical decision-making could benefit from a more individualized approach, taking into account muscle recovery, changes in mechanical properties, and functional goals. Allowing sufficient time for muscle recovery between injections may help to limit potential deleterious structural effects while maintaining clinical efficacy. Overall, these considerations highlight the need to move toward a more tailored and physiologically informed use of BoNT-A, balancing early intervention with careful long-term management of muscle health.

These structural changes also have important implications for rehabilitation. Interventions aimed solely at reducing neural hyperactivity, such as botulinum toxin injections, may not fully restore functional capacity if underlying muscle architecture is altered. Rehabilitation strategies should therefore combine approaches targeting neural overactivity with interventions promoting muscle lengthening, strengthening, and maintenance of muscle quality, such as splinting, progressive resistance training, and functional task practice. Understanding the interplay between spasticity, muscle adaptations, and function is essential for optimizing treatment timing and modality, particularly in populations with lifelong or chronic spasticity.

Perspectives and future research

The findings of this thesis raise several important questions for future research in the assessment and management of post-stroke spasticity syndrome.

First, further improving our understanding of the respective contributions of neural and non-neural components to hyper-resistance, as well as identifying the most appropriate methods to assess them, remains essential for optimizing patient management. In this context, the integration of emerging technologies—such as biomechanical measurements and advanced imaging techniques—represents a particularly promising avenue. Among these, instrumented spasticity assessment (ISA), as developed by Bar-On *et al.* (57) and further investigated by Schillebeeckx *et al.* in a post-stroke population (303), offers a relevant and clinically applicable approach.

ISA enables the decomposition of neural and non-neural contributions to joint hyper-resistance through the simultaneous acquisition of biomechanical and electrophysiological data. Specifically, the system combines inertial measurement units (including gyroscopes and accelerometers) to quantify joint angles and angular velocity, thereby estimating muscle lengthening velocity. In parallel, surface electromyography records the activity of agonist and antagonist muscles, while a six-degrees-of-freedom force sensor measures the torque applied at the joint, reflecting resistance to passive stretch. Measurements are performed during both low- and high-velocity stretches, allowing for a more comprehensive characterization of spastic muscle behavior. One of the main advantages of such a tool lies in its ease of use and portability, enabling its implementation across various clinical settings and supporting the development of individualized therapy plans.

Second, further advancements in the assessment of muscle structure are needed to better characterize post-stroke muscle alterations and their evolution over time. Both magnetic MRI and ultrasound techniques offer valuable and complementary insights into muscle morphology and composition.

MRI remains the gold standard for the *in vivo* evaluation of muscle volume, fat infiltration, and architectural changes. Recent developments such as quantitative MRI, Dixon imaging, and T2 mapping enable a more precise characterization of tissue composition and muscle quality. In addition, emerging approaches such as DTI and tractography may provide further insight into muscle architecture and fiber organization by enabling the reconstruction of muscle fiber orientation and integrity *in vivo* (304).

This could be particularly valuable for detecting subtle alterations in muscle microstructure following stroke, such as changes in fiber orientation, fascicle organization, or early increases in connective tissue, potentially at earlier stages than conventional imaging (305). Magnetic resonance elastography also holds promise for the non-invasive assessment of tissue stiffness, with the advantage of providing a more global and depth-independent evaluation of mechanical properties compared to ultrasound elastography, which is more operator-dependent and limited to superficial regions (306,307). However, despite its strengths, MRI presents several important limitations, including high cost, limited accessibility, lack of portability, and long acquisition times, which may restrict its use in routine clinical practice. Furthermore, the post-processing of MRI data remains time-consuming. During this thesis, a substantial amount of time was dedicated to muscle segmentation, even when using semi-automated software. This highlights the need for the development and validation of fully automated segmentation tools, which would facilitate the analysis of larger datasets and enable more comprehensive investigations across multiple muscles.

Ultrasound, on the other hand, represents a more accessible and clinically feasible tool, with growing potential for bedside assessment. Advances in ultrasound imaging, including SWE, allow for the quantification of muscle stiffness, while conventional B-mode imaging can be used to assess muscle thickness, fascicle length, and pennation angle. Additionally, emerging techniques such as ultrasound-based texture analysis may provide indirect markers of muscle quality and fibrosis (247). Furthermore, 3D free-hand ultrasound extends conventional 2D imaging by reconstructing three-dimensional datasets of muscles and tendons, allowing direct quantification of muscle volume, length, thickness, and echo intensity.

As mentioned previously, Schillebeeckx *et al.* demonstrated that 3DfUS is a reliable method for quantifying tibialis anterior muscle structure (volume, length) and composition (echo-intensity) in chronic stroke survivors (54). Moreover, 3DfUS has proven reliable for assessing medial gastrocnemius muscle and tendon properties (muscle thickness, muscle length, tendon length) in patients with stroke (56). Given its portability, lower cost, and real-time capabilities, 3DfUS could play a key role in the longitudinal monitoring of muscle changes and in guiding individualized treatment strategies.

Thirdly, the present longitudinal study evaluating spastic calf muscle structure using MRI following repeated BoNT-A injections did not allow us to distinguish between 2 treatment strategies: injecting a larger number of muscles involved in the spasticity pattern with lower doses per muscle, versus administering higher doses to a smaller number of more specifically targeted muscles. This distinction remains important and should be specifically addressed in future studies. Moreover, in order to strengthen evidence-based practice for BoNT-A treatment, a randomized control trial investigating 3 cycles of high-dose BoNT-A injections should be conducted. Such a study would help differentiate between cumulative effects and the specific impact of higher dosing, thereby providing valuable insights for both clinical decision-making and national reimbursement policies, particularly within the Belgian healthcare system. Another key issue concerns the long-term effects of repeated botulinum toxin injections. Although short-term benefits are well documented, their potential role in promoting muscle properties alterations remains controversial. Longitudinal studies are therefore essential to clarify these effects and guide clinical practice.

Finally, a longitudinal study is currently underway to investigate the natural evolution of muscle structure and stiffness following stroke. Such a design is particularly relevant, as longitudinal approaches can provide valuable insights into the progression of muscle changes over time, thereby improving the understanding of causal relationships and clinical trajectories. Indeed, the temporal evolution of post-stroke muscle alterations remains insufficiently understood, and identifying critical time windows during which interventions may be most effective could significantly enhance rehabilitation strategies. Ideally, this study aims to recruit a large and heterogeneous sample in order to enable subgroup analyses based on level of muscle weakness, compensation strategies such as disuse or overuse, the development, timing and severity of spasticity, as well as the use and timing of BoNT-A injections. This would allow for a more detailed characterization of how different clinical pathways influence muscle properties, and to distinguish between the respective effects of natural recovery, spasticity onset, and therapeutic interventions on muscle structure and passive mechanical behavior.

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