The effect of Botulinum Toxin (A) on bone and supplying vasculature, from animal model to dental clinical application.

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Abstract

Background – Temporomandibular disorders (TMDs) are a complex group of disorders affecting the structural integrity of the temporomandibular joint and causing pain. Many treatment modalities are established, and others are being investigated for their efficiency in reducing TMD associated pain. One technique used to control the pain is intramuscular injections of botulinum toxin type A (BTX), a reversible neurotoxin inducing flaccid paralysis at the neuromuscular junction. BTX use in these conditions is relatively novel and few investigations into long term adverse effects exist. Of concern, BTX is used in preclinical models for investigating paralysis-induced bone loss. Since degenerative changes in hard tissues of the jaw are a concern in the progression of TMDs, it is critically important to understand potential effect of BTX use on bone health in TMD patients.

Objectives – The goal of the project is to investigate potential harmful effects of BTX on bone health in TMD patients using three complementary approaches: knowledge synthesis of available literature on the topic, assessment of preclinical animal model of BTX paralysis, and design of an observational clinical study for the response to BTX injections in a TMD population.

Methods – A comprehensive search in three databases was conducted to understand the current position of literature on effects of BTX on mandibular bone. Included articles were systematically reviewed and summarized. Quantitative data present in the literature was extracted and used to conduct a random effects meta-analysis. An established mouse model of BTX-induced hindlimb unloading was implemented to understand BTX-induced changes in bone and vascular compartments using imaging techniques including micro- computed tomography, laser doppler ultrasonography, and synchrotron radiation imaging. Bone and vascular outcomes were evaluated in BTX-injected and saline-injected animals. A protocol was designed for a prospective observational cohort study for individuals with TMD to monitor BTX-induced short-term changes

in muscle activity using electromyography and blood flow using ultrasound, and long-term changes in condylar bone structure using cone-beam CT.

Results – Systematic search identified six human and fourteen animal studies that assessed mandibular bone changes following BTX injection into masticatory muscles. In human mandibular bone, BTX caused decrease in cortical thickness with the highest effects in condyle. In animal models, significant decreases in bone cortical and trabecular indices were observed in BTX-injected animals. This study identified a need for more rigorous trials to draw a full picture of potential long-term adverse effects of BTX on bone. Based on the quantitative estimates obtained in this study, a future clinical study should recruit at least 50 patients and follow up for longer than 9 months for bone outcomes. Next, we used an established preclinical protocol for BTX-induced hindlimb paralysis in mice to examine potential short-term changes induced by BTX and their correlation with bone loss. We specifically focused on vascular changes, since the crosstalk between bone and blood or nerves has been recently suggested to play a role if unloading-induced bone loss. BTX injection in quadriceps and calf muscles lead to significant endocortical bone loss. Interestingly, BTX injection increased blood flow in femoral artery, while bone vascular porosity was reduced. This study demonstrates the novel effects of BTX on microcirculation and blood flow. Finally, a protocol was designed to examine BTX-induced bone and vascular changes in TMD patients. A prospective observational cohort study will follow individuals with TMD receiving BTX for pain management has received an approval from Institutional Review Board of McGill University.

Conclusions – This work contributed to better understanding of the detrimental effects of the neurotoxin on mandibular bone in pre-clinical animals and human participants and prepares the stage for the longitudinal clinical studies to provide clear insights into the safety of BTX use in dental clinic.

Résumé

Contexte – Les troubles temporo-mandibulaires (TTM) constituent un groupe complexe de troubles affectant l'intégrité de la structure de l'articulation temporo-mandibulaire, causant de la douleur. De nombreuses modalités de traitement sont établies et d'autres sont étudiées pour leur efficacité à réduire la douleur associée aux TTM. Une technique utilisée pour contrôler celle-ci est l'injection intramusculaire de toxine botulique de type A (BTX), une neurotoxine réversible induisant une paralysie relâchée au niveau de la jonction neuromusculaire. L'utilisation de la BTX dans ces conditions est relativement nouvelle et peu d'études ont été menées sur ses effets secondaires à long terme. Il est peut-être préoccupant que l'utilisation du BTX fasse partie des modèles précliniques pour étudier la perte osseuse induite par la paralysie. Étant donné que les changements dégénératifs dans les tissus durs de la mâchoire soient une préoccupation dans la progression des TTM, il est crucial de comprendre les effets potentiels de l'utilisation de la BTX sur la santé osseuse des patients atteints de TTM.

Objectifs – Le but du projet est d'étudier les effets nocifs potentiels de la BTX sur la santé osseuse des patients atteints de TTM en utilisant trois approches complémentaires : la synthèse des connaissances disponibles sur le sujet, l'évaluation d'un modèle animal préclinique de paralysie induite par la BTX et la conception d'une étude clinique observationnelle sur la réponse aux injections de BTX dans une population de TTM.

Méthodes – Une recherche complète dans trois bases de données a été effectuée pour mieux comprendre la position actuelle de la littérature sur les effets de la BTX sur l'os mandibulaire. Les articles inclus ont été systématiquement examinés et résumés. Les données quantitatives présentes dans la littérature ont été extraites et utilisées pour réaliser une méta-analyse à effets aléatoires. Un modèle murin établi de décharge des membres postérieurs induite par la BTX a été mis en œuvre pour comprendre les changements induits par la BTX dans les compartiments osseux et vasculaires en utilisant des techniques d'imagerie telles que la micro-tomographie,

l'ultrasonographie Doppler laser et l'imagerie par rayons synchrotron. Les résultats osseux et vasculaires ont été évalués chez les animaux injectés avec la BTX et ceux injectés avec une solution saline. Un protocole a été conçu pour une étude de cohorte observationnelle prospective chez les individus atteints de TTM afin de surveiller les changements à court terme induits par la BTX dans l'activité musculaire à l'aide de l'électromyographie et le flux sanguin à l'aide de l'échographie, et les changements à long terme dans la structure osseuse condylienne à l'aide d'un CT scan à faisceau conique.

Résultats – La recherche systématique a identifié six études humaines et quatorze études animales ayant évalué les changements dans l'os mandibulaire après injection de BTX dans les muscles masticateurs. Dans l'os mandibulaire humain, la BTX a entraîné une diminution de l'épaisseur corticale avec les effets les plus marqués au niveau du condyle. Dans les modèles animaux, de diminutions significatives des indices corticales et trabéculaires de l'os ont été observées chez les animaux injectés avec la BTX. Cette étude a identifié le besoin de réaliser des essais plus rigoureux pour obtenir un aperçu complet des effets secondaires potentiels à long terme de la BTX sur l'os. Sur la base des estimations quantitatives obtenues dans cette étude, cette future étude clinique devrait recruter au moins 50 patients ainsi que de suivre les résultats osseux pendant plus de 9 mois. Dans un autre ordre d'idées, nous avons utilisé un protocole préclinique établi pour la paralysie des membres postérieurs induite par la BTX chez les souris afin d'examiner les changements à court terme induits par la BTX et leur corrélation avec la perte osseuse. Nous nous sommes spécifiquement concentrés sur les changements vasculaires, puisque les interactions entre l'os, le sang et les nerfs ont récemment été suggérées comme ayant un rôle dans la perte osseuse induite par la décharge. L'injection de BTX dans les muscles quadriceps et du mollet a entraîné une perte osseuse endocorticale significative. Il est intéressant de noter que l'injection de BTX a augmenté le flux sanguin dans l'artère fémorale, tandis que la porosité vasculaire osseuse était réduite. Cette étude démontre les effets novateurs de la BTX sur la microcirculation et le flux sanguin. Enfin, un protocole a été conçu pour examiner les

changements osseux et vasculaires induits par la BTX chez les patients atteints de TTM. Une étude de cohorte observationnelle prospective suivra les individus atteints de TTM recevant de la BTX pour la gestion de la douleur ainsi qu'a reçu l'approbation du comité d'éthique de l'Université. Conclusions – Ce travail a pu contribuer à une meilleure compréhension des effets nocifs de la neurotoxine BTX sur l'os mandibulaire chez les animaux précliniques et les participants humains. Il peut également préparer le terrain pour des études cliniques longitudinales afin de fournir des informations claires sur la sécurité de l'utilisation de la BTX en clinique dentaire.

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Contribution

I highlight here the originality and contribution to knowledge and individual authors contributions for each individual work that is included in this thesis:

Manuscript 1: "*Adverse effect of botulinum toxin-A injections on mandibular bone: A systematic review and meta-analysis"* By: Mahmoud S. Moussa, Dona Bachour, and Svetlana V. Komarova

Contribution to knowledge: In collaboration with my co-authors, I conducted a systematic review and meta-analysis on the bony changes occurring in the mandible following botulinum toxin (A) injections. I provide qualitative assessment of studies conducted in animal and human mandible as well as identifying strength and gaps in the current published literature. I also conduct the first meta-analysis on this topic to ascertain the extent of bony changes in various mandibular regions such as the condyle, alveolar bone. Highlighting the potential unwanted changes in bones with BTX injection in orofacial musculature. This article is published in the Journal of Oral Rehabilitation.

Contribution of co-authors:

- **Mahmoud S. Moussa:** Conceptualization, screening, data extraction, meta-analysis, writing – original draft, writing – review and editing.
- **Dona Bachour:** Conceptualization, screening, data extraction, writing – review and editing.
- **Svetlana V. Komarova:** Conceptualization, writing – review and editing, supervision, funding acquisition.

Manuscript 2: "*Botulinum toxin (A) -induced bone loss is associated with increased blood flow and reduced vascular bone porosity*" By: Mahmoud S. Moussa, Taylor DeVet, Nadine Lebcir, Lorraine E. Chalifour, Bettina M. Willie, and Svetlana V. Komarova

Contribution to knowledge: In collaboration with my co-authors, I investigated the role of vasculature in botulinum toxin (A) toxin induced bone loss. I demonstrated that the toxin is capable of increasing blood flow locally in injected quadriceps muscle group. The increased flow did not sustain within the microvascular network in muscles and bone. I also showed that the induced bone loss resulted in bone vascular porosity reduction without altering the osteocyte network of bone. This work also highlights for the first time the diminished rearing observed in murine paralysis model. Highlighting the importance of neuromuscular function and the grave impact of dysfunction on bone structures.

Contribution of co-authors:

- **Mahmoud S. Moussa:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft, Writing - review & editing, approved the final version.
- **Taylor DeVet:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing - review & editing, approved the final version.
- **Nadine Lebcir:** Formal analysis, Investigation, Visualization, Writing - review & editing, approved the final version.
- **Lorraine E. Chalifour:** Formal analysis, Methodology, Project administration, Resources, Writing - review & editing, approved the final version.

- **Bettina M. Willie:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing - review & editing, approved the final version.
- **Svetlana V. Komarova:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Writing - review & editing, approved the final version.

Manuscript 3: "*Effects of botulinum toxin A on circulation and bone health in myofascial temporomandibular disorders: protocol for a prospective cohort study*" By: Mahmoud S. Moussa, Firoozeh Samim, Svetlana V. Komarova, Elizabeth A. Zimmermann

Contribution to knowledge: In collaboration with my co-authors, I constructed a prospective observational cohort study aimed at addressing the question of physiological changes accompanying botulinum toxin (A) injection. With much interest in myofascial pain and temporomandibular disorders research, there is an urgency to develop safe and effective treatments. There is currently a gap of knowledge regarding time dependent changes in tissues with repeated injections. I proposed the collection of data on various tissues using non-invasive imaging methods in a cohort of temporomandibular pain patients. Data collection of muscle, bone, and vasculature will be done prior to and at various time points following injection. This is the first study to assess the effect of botulinum toxin (A), a potent vasodilator, on the blood flow to muscles of mastication. Additionally, this study will address gaps in knowledge regarding previous investigations into bone related alterations. Institutional review board (IRB) approval has been granted for this study.

Contribution of co-authors:

- **Mahmoud S. Moussa:** Conceptualization, institutional review board protocol preparation, writing – drafted first version, writing – edited and revised.
- **Firoozeh Samim:** Conceptualization, institutional review board protocol preparation, writing – drafted first version, writing – edited and revised.
- **Svetlana V. Komarova:** Conceptualization, institutional review board protocol preparation, writing – drafted first version, writing – edited and revised.
- **Elizabeth A. Zimmermann:** Conceptualization, institutional review board protocol preparation, writing – drafted first version, writing – edited and revised.

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Abbreviations

- **TMD:** Temporomandibular disorders
- **TMJ:** Temporomandibular joint
- **BTX:** Botulinum toxin serotype (A)
- **FDA:** Food and Drug administration
- **RCT:** Randomized control trial
- **RDC/TMD:** Research diagnostic criteria for temporomandibular disorders
- **SNARE:** N-ethylmale-imide-sensitive factor-attachment protein receptor
- **VAS:** Visual analog scale
- **EMG:** Electromyography
- **CBCT:** Cone-Beam computed tomography
- **EC:** Endothelial cells
- **CI:** 95% confidence intervals
- **IC:** Internal control
- **BV.TV:** Bone volume to total tissue volume
- **Ct.Th:** Cotrical Thickness
- **Tb.Th:** Trabecular thickness
- **B.Ar/T.Ar:** Bone area to total tissue area
- **Ct.Ar/Tt.Ar:** Cortical area to total tissue area
- **Ma.Ar:** Marrow area

- **Tb.N:** Trabecular number
- **Tb.Sp:** Trabecular separation
- **EV/BV:** Eroded volume to bone volume
- **ES/BS:** Eroded surface to bone surface
- **MS/BS:** Mineralized surface to bone surface
- **MV/BV:** Mineralized volume to bone volume
- **VTI:** Velocity time integral
- **PV:** Peak velocity
- **LVmass:** Left ventricular mass
- **AoVTI**: Aortic velocity time integral.
- **AoCo**: Aortic cardiac output
- **VSa/BV**: Vascular surface area to bone volume
- **VV/BV**: Vascular volume to bone volume

1 Introduction

The oral cavity represents a unique mechanical loading environment, specifically, via action of muscles of mastication. Forces in this area are complex and result in loading of the temporomandibular joint's disc, condyle, and alveolar bone (Tuijt et al., 2010). These forces are dynamic, and moderate loading is important for healthy (re)modeling of the bone. Meanwhile, overloading is destructive and can play a role in osteoarthritis and degenerative joint diseases (de Souza et al., 2012). These destructive changes to the joint fall under the larger umbrella of temporomandibular disorders (TMDs), that affect as many as 5-12% of the general population (Plesh et al., 2011). TMDs are considered multifactorial and desired treatment outcomes such as pain relief can be at times difficult to achieve and maintain.

TMDs can arise from issues associated with hard tissues (arthralgia) and/or soft tissues (myalgia). Managing TMDs greatly depends on individual associated factors and the type of TMD present. Perhaps most commonly, oral stabilizing appliances are used with moderate success (Menchel et al., 2021). From a clinical standpoint, it is important to consider other forms of adjunctive therapy as part of a multimodal approach. Adjunctive therapy includes but is not limited to physiotherapy, jaw exercise, low level laser therapy, dietary modification (Dadjoo et al., 2022).

Notably, botulinum toxin type A (BTX) is also used in managing myofascial types of TMDs via intramuscular injections in the muscles of mastication. It can provide relief by interrupting the neuronal signaling pathways and reduce parafunctional habits such as

clenching of muscles and/or grinding of teeth (i.e. bruxism) (Chen et al., 2015). There has been considerable work on the efficacy of BTX in relieving TMD related pain, with systematic reviews finding inconclusive results (Di Francesco et al., 2022; Saini et al., 2024). In addition, there is limited work on the functional and structural effects exerted by BTX on the various tissue components of the joint.

Currently, the description of BTX adverse effects in the literature is limited and mostly comprised of minor events such as ecchymosis, bruising, pain, edema related (Jia et al., 2016), while potential long term adverse effects of repeated BTX use are not clear. Repeated injections can potentially predispose muscles to being in a state of disuse, where the mechanical forces are not sufficient in magnitude to maintain musculoskeletal health. This state of disuse over extended periods could have a negative effect on bone structures, which is not well addressed in the context of BTX, muscles of mastication and hard structures of the temporomandibular joint (TMJ).

Disuse is commonly studied in long bones given the relevance to other scenarios such as bone loss seen in extremities of spacefarers and long-term bed rest (Fu et al., 2021; Leblanc et al., 1990; Stavnichuk et al., 2020). Bones are complex organs that provide structural support for muscles and play many roles including withstanding mechanical forces and contributing to hematopoietic and endocrine functions (Clarke, 2008; Quarles, 2008). Additionally, our bones are constantly being (re)modeled to adapt to the environment surrounding us (Willie et al., 2020). Understanding the bone (re)modeling process is essential to formulating countermeasures for many pathological processes occurring in the human body.

(Re)modeling of bone is heavily reliant on mechanical forces and crosstalk with various other systems of the body. In conditions where there are alterations in gravitational forces (i.e. spaceflight) or muscle paralysis, there is a change in the amount of bone structure. Typically, this change would reflect a negative shift with higher amounts of resorption overall (Sievänen, 2010). Meanwhile, loading bones with physiologically acceptable forces results in overall bone formation (Guadalupe-Grau et al., 2009). Within the context of this thesis, different works will primarily address the absence of mechanical force (unloading) and its consequences in both long bones and irregular bones (i.e. mandible).

Research into bone loss due to disuse has been ongoing for decades. Currently, there is growing interest in understanding how the vascular and nervous systems contribute to the resulting changes in bone (Abeynayake et al., 2021; Yuan & Song, 2022). Specifically, the role of vasculature in bone (re)modeling and the importance of angiogenesis are well investigated in acute injuries like bone fracture but are less studied in more chronic scenarios like disuse.

Studying disuse in true unloading conditions such as spaceflight is expensive and not always attainable. To mimic this condition and the resultant bone loss, many preclinical models have been developed. The botulinum toxin model of disuse is an interesting model given its ability to achieve similar bone changes in a non-invasive manner and the potential to study a toxin that is increasingly used in clinical practice.

The objective of this thesis is to study the effect of BTX on the bone health of individuals with TMD and identify potential cross-links with different tissues, specifically

vasculature. **The first work in this thesis** explores the current knowledge on bone-related adverse effects of botulinum toxins in the mandible by conducting a systematic review and meta-analysis of the literature. By conducting the systematic review and meta-analysis I aim to provide quantitative estimate of the bone change and provide insightful knowledge for researchers planning to investigate this problem further. **The second work in this thesis** takes advantage of the established preclinical models investigating disuse-induced bone loss. I use the BTX model in mice to investigate the vascular alterations accompanying bone loss following injection. This study tested the hypothesis that BTX-induced neuromuscular paralysis will lead to a decreased flow in femoral and tibial arteries that will correlate with changes in bone structure and reduced osteocyte lacunar and vascular porosity in the tibia. **The third work in this thesis** builds on the lessons learnt from the first two studies to establish an observational cohort study of individuals with TMD and assess changes in orofacial vascular, muscle and bone physiology. This work will establish the extent of changes in orofacial tissues following BTX injections as well as pain outcomes to inform future guidelines for clinical use of BTX.

The knowledge produced in this thesis will guide clinicians and scientists in better formulating guidelines and dosage regimens for BTX use in TMD populations. This will also help TMD clinicians and patients become more fully informed on the potential positive and negative effects of BTX treatment, which will improve personalized care.

2 Review of the literature

2.1 Temporomandibular joint disorders

The temporomandibular joint (TMJ) is a unique fulcrum for jaw movement, as the only bilateral diarthrodial joint in the body and an essential component in everyday tasks (Bordoni & Varacallo, 2024). Temporomandibular joint disorders (TMDs) are a group of disorders affecting this joint causing morphological and functional deformities. The cost of addressing TMD issues in the United States has doubled in recent years (Schiffman et al., 2014). Although TMDs are the second most common cause of chronic pain, only a small number of people with TMD problems seek help (Murphy et al., 2013). Diagnostic criteria used for TMDs have evolved to include physical assessment, as well as psychosocial and pain-related assessments. These criteria have been proven to be reliable in both a clinical and research settings (Schiffman et al., 2010; Truelove et al., 2010). Nevertheless, diagnosing and managing TMDs remains challenging due to complexities of these disorders and the many conditions that mimic TMD symptoms (Gauer & Semidey, 2015).

TMDs can cause pain, difficulty in mouth opening and “clicking” of the joint. Many of these disorders are also associated with myofascial pain and can present with parafunctional habits, trauma, and unstable occlusion (Gonçalves et al., 2010; Huang & Rue, 2006; JANAL et al., 2008; Karibe et al., 2015). Degenerative changes in the joint area represent an undesired progression of TMDs which can cause deterioration of the joint components and further dysfunction. These degenerative changes can often be seen radiographically as a flattened fossa, articular eminence, or loss of condylar volume

(Tanaka et al., 2008). It is important to prevent TMD populations from reaching this stage of disease progression.

Controlling and managing TMDs usually start with non-invasive supportive methods (Dimitroulis, 1998; Gauer & Semidey, 2015). The goals of management include increasing jaw function and range of motion, decreasing pain and inflammation, and preventing any further degeneration of joint components. Some of the common methods used for controlling TMD pain include patient reassurance and education regarding their condition, trigger point injections, occlusal splint therapy, muscle relaxant use, low level laser therapy, acupuncture, physiotherapy, psychotherapy, transcutaneous electric nerve stimulation, therapeutic ultrasound, and botulinum toxin injections (Garrigós-Pedrón et al., 2019; Velly et al., 2022). More invasive methods can be employed and include direct injections into the joint space to lubricate and decrease inflammatory mediators (Iturriaga et al., 2017). In more severe cases, surgical interventions may be required, whether disc or total joint replacements being among these modalities (Tanaka et al., 2008).

Previously, intraoral appliances were most frequently recommended as a part of a TMD management plan (Velly et al., 2022). Appliances can help reduce pain and control parafunctional habits such as nocturnal clenching (Alkhutari et al., 2021). Literature has shown low to moderate pain alleviating effect for occlusal intraoral appliances in TMDs (Al-Moraissi et al., 2020; Zhang et al., 2020), with some not finding it more effective than other therapies or placebo treatments (Albagieh et al., 2023; Ram & Shah, 2021). Having additional adjunctive therapies as part of a multimodal approach can help manage more complex and refractory cases (Li & Leung, 2021).

Selecting the appropriate treatment is a challenge given the complexity of TMDs and the many aspects that we do not fully understand about these conditions. Nonetheless, the completed diagnostic criteria, physical examination, patient preferences, and necessary imaging should all be considered. Meticulous follow up and monitoring of patients is needed to ensure proper management. Although there is no clear superiority for one treatment, the use of botulinum toxins has become more prominent in recent years in TMDs associated with myofascial pain (Saini et al., 2024). In the coming sections botulinum toxin and the adverse effects that can arise from its use will be discussed.

2.2 Botulinum Toxins

Botulinum Toxin serotype A (BTX), also commonly known as Botox, is one of the most potent neurotoxins known to man (Pirazzini et al., 2017). It is a toxin secreted by anaerobic bacteria known as *Clostridium Botulinum*. Historically, this bacterium has been known to grow in poorly packaged foods, but with advances in technology this has become rare. The systemic effect of ingesting high amounts of this bacteria's toxin is known as botulism, which has a prevalence of 0.01 case of foodborne botulism per 100,000 persons in Canada between 2006-2021 (Harris et al., 2023). Today it is more frequent to find botulinum toxins in the form of a freeze- or vacuum- dried powder, which is then reconstituted for biomedical use. Commercially available products usually contain a protein load within the nanogram range greatly reducing the chance of toxicity (Pirazzini et al., 2017). BTX products were first used in scientific literature in the 1970's to treat strabismus (Scott et al., 2023). Since then, it has been approved by the FDA for use in various conditions across different disciplines (Padda & Tadi, 2024).

BTX particles are formed of a heavy and a light chain (100 kDa and 50 kDa respectively) and target nerve endings at neuromuscular junctions, for which they have high specificity. Once attached to the presynaptic terminal, the toxin's short chain enters the nerve ending and attaches to N-ethylmale-imide-sensitive factor-attachment protein receptor (SNARE) responsible for vesicle-membrane fusion and acetylcholine release (Nigam & Nigam, 2010). This in turn prevents action potential in muscle fibers and brings on a state of flaccid paralysis in the injected muscle. Flaccid paralysis is not permanent, and muscles regain function after a period that ranges from weeks in small animals to months in humans (Pirazzini et al., 2017).

Among the orofacial indications for BTX use is cervical dystonia characterized by involuntary muscle contractions of the shoulder and neck muscles. In these conditions, injections at doses ranging from 100-300 Units of Botox (Allergan, USA) are effective at relaxing muscles and improving patient conditions (Hallett et al., 2013). Other dystonia related applications include hemifacial spasms and blepharospasms (Guidubaldi et al., 2014; Hallett et al., 2013). Additionally, autonomic disorders such as sialorrhea and excessive salivation, can be addressed with BTX injection into salivary glands, blocking autonomic cholinergic endings and reducing salivary flow (Naumann et al., 2013). Another area of BTX use is cosmetic application, where it was shown to eliminate wrinkles around the eyes (crow's feet) and is approved for use in glabellar rhytids (Carruthers et al., 2016).

Indications are not limited to just spastic and myogenic conditions but BTX's effects have also been shown to reduce pain and sensitization in chronic diseases (Wheeler & Smith, 2013). The toxins antinociceptive effect rises from its ability to block neuropeptides and inflammatory mediators released via channels controlled by SNARE proteins on the

plasma membrane (Meng et al., 2014). Some authors have also reported a central nervous system effect via potential antero- or retro- grade axonal transport (Matak et al., 2012). A recent systematic review showed BTX to have therapeutic value in managing trigeminal neuralgia and suggested it should be considered as a pharmacological alternative to surgery (Morra et al., 2016). Additionally, with advances in bioengineering, the light chain of the BTX particle can be altered to bind more readily to specific types of nerve cells expanding the potential for the toxins use in the orofacial pain sphere (Masuyer et al., 2014). Given the complexity of temporomandibular disorders and the interplay of biological and psychosocial aspects, BTX can potentially provide relief for people with this condition. The next section discusses more in depth the current evidence for BTX use in TMDs.

2.3 Botulinum Toxin application in temporomandibular disorders

Many clinical trials and cohort studies in recent years have been conducted into the pain alleviating capacity of BTX in populations with myofascial TMD. In this section, I will discuss reviews that bring together findings across various controlled trials. In one systematic review identifying 11 randomized trials, pain scores were the primary outcome in 8 of the trials using a visual analog scale (VAS), while the remaining 3 did not report a primary outcome or used a questionnaire from the RDC/TMD axis II instead of a VAS (Patel et al., 2019). Most trials used saline injection as a control for BTX with the exception of few articles that used facial manipulation technique, low-level laser therapy, or conservative treatment (Chaurand et al., 2017; De Carli et al., 2016; Guarda-Nardini et

al., 2012). Overall, the systematic review favored BTX as a method of managing TMD pain through qualitative analysis of the primary outcome in these randomized trials, no meta-analysis was attempted (Patel et al., 2019).

Secondary outcomes included mouth opening, maximum biting force, and electromyography (EMG) associated bruxism events per hour (Patel et al., 2019). One trial showed reduction in muscle electromyography readings in BTX injected individuals at 14 days post injection (Kurtoglu et al., 2008). Interestingly EMG readings during clenching were lower in individuals with myofascial pain compared to control individuals at baseline (Kurtoglu et al., 2008). Another trial showed reduction in mean maximum biting force by 48.17kg in BTX injected participants (Zhang et al., 2016).

Two other systematic reviews and meta-analyses found that BTX significantly alleviates TMD pain after 1 month of injection but not at 3- or 6-months post-injection when compared to a placebo (Machado et al., 2020; Saini et al., 2024). BTX was also more effective than conventional therapy (i.e. behavioral management, occlusal splints) for up to 1-year post-injection but not more effective than facial manipulation techniques at 3 months (Machado et al., 2020). No differences were noted in maximum mouth opening by BTX as compared to placebo (Machado et al., 2020). Other systematic reviews and recent randomized clinical trials have also found no evidence supporting efficacy of BTX versus placebo or no treatment (Chen et al., 2015; Reeve et al., 2024).

Several limitations were identified by the studies discussed in this section including small sample sizes, low quality of evidence, failed previous treatments, lack of standardized treatment protocols. Thus, more investigations are still needed to clarify the

role of BTX in a dental setting and specifically in addressing myofascial TMD related pain. There is an additional urgency given the lack of defined guidelines for dosing and schedule of BTX use in these conditions making it difficult to examine the efficacy and safety of various doses. An interesting drawback in many of these studies is the modest reporting of adverse events following BTX injection, which will be expanded on in the coming section.

2.4 Adverse events associated with Botulinum Toxin

There are nearly 3 million injections of botulinum toxins done a year making it the world's most popular cosmetic procedure (Yiannakopoulou, 2015). Some important aspects of BTX to consider include toxin concentration, and immunogenicity. As previously mentioned, BTX products available today only contain nanogram levels of the bacterial toxin, this is ultimately miniscule and could not cause severe reactions such as botulism (Pirazzini et al., 2017). However, researchers assessing concentration of toxin among three commercially available BTX products found higher concentrations of toxins in Dysport product (Field et al., 2018). Therefore, differences in BTX type used in individuals and trials can influence the outcomes and are important to consider for dosing regimens. For conditions where application of a BTX product might be repeated for long duration, proper documentation of BTX type and concentration is important.

BTX as a foreign substance to the body can induce an immune response that produces antibodies, reducing therapeutic efficacy (Benecke, 2012). Neutralizing antibodies block the direct effect of the toxin, while non-neutralizing antibodies are formed in response to complexing proteins that protect the toxin from degradation (Benecke,

2012). It has also been shown that higher dosing and repeated injections can contribute to increased antibody production (Dressler, 2004). Generally, a reduction in response over time may indicate a need to monitor neutralizing antibodies in patients expected to continue receiving injections. For individuals with allergic reactions to serotype A, they can be prescribed serotype B formulation (Myobloc). In these individuals, Botulinum toxin B is only temporarily effective as the host develops resistance due to cross-reactivity (Atassi, 2004).

Studies dedicated to adverse events are limited but multiple review articles have summarized reported events according to BTX use. In a review by Witmanowski, current knowledge of adverse effects is summarized within BTX cosmetic application. Adverse effects were classified into benign events and serious events (Witmanowski & Błochowiak, 2020). Benign events included ecchymosis, bruising, hematomas and usually did not require any intervention with bruising commonly occurring in 11-25% of facial rejuvenation patients (Vartanian & Dayan, 2005). While serious events in cosmetic use are rare as doses are much lower, they include dysphagia, muscle weakness, and severe allergic reactions. In 2005, the FDA reported 36 adverse events related to BTX use, mostly relating to dysphagia (Coté et al., 2005). Additionally, a meta-analysis conducted to assess adverse effects in injections for glabellar lines and crow's feet treatment with BTX found significantly higher incidence of adverse effects in BTX injections, most of which were mild (Jia et al., 2016). Thus, BTX presents a safe profile for use in cosmetics with mild adverse events.

When BTX is used in myofascial TMD, the dosing regimen is much higher than in cosmetic use. One study with a group of 20 people injected with 100 units of Botox

(Allergan) into masseter, temporalis, TMJ, and lateral pterygoid, found adverse events that included abnormal jaw appearance, and sensation of flushing (Blanco-Rueda et al., 2023). Interestingly, this study was conducted with unilateral injections and several patients also reported increased contralateral contracture and pain (Blanco-Rueda et al., 2023). In other reviews of BTX use in myofascial TMD, the most reported adverse events included temporary regional weakness, difficulty chewing and muscle atrophy, most commonly of the masseter muscle (De la Torre Canales et al., 2019; la Fleur & Adams, 2020). Overall, across most of the literature adverse effects are usually mild. Unfortunately, most studies only address short term and imminent issues that are self-reported.

2.5 Long term effects of Botulinum Toxin

Indeed, BTX has a reversible action which allows tissues to regain function after some time. However, whether tissues fully recover to pre-regimen level of function remains to be answered. In literature pertaining to humans, there is not enough evidence for long term follow up in TMDs. One area where data exists on long term follow up is in cerebral palsy, a lifelong condition characterized by involuntary muscle spasticity. In long term management of children affected by cerebral palsy, BTX is shown to be effective in reducing muscle tone and increasing range of motion (TEDROFF et al., 2009). This improvement in cerebral palsy related outcomes may dampen with multiple injections underscoring the diminishing effect over time related to potential immunogenicity (Fattal-Valevski et al., 2008). Adverse events to the injected muscles such as fibrosis or atrophy are not investigated in cerebral palsy studies (Multani et al., 2019).

Meanwhile, in animal literature there has been work investigating the loss and long-term recovery of BTX injected musculature. In rabbit masseter, one injection of BTX resulted in atrophy and reduced bone area in the condyle at 12 weeks post-injection (Rafferty et al., 2012). Hindlimb muscle mass was shown to remain lower than control animals at 6 months post-injection (Fortuna et al., 2011). This lack of muscle function and recovery could also be a driver for bone loss in injected areas (Grimston et al., 2007).

Musculature and bone are two systems that are closely related. Injections of high amount of BTX in muscles (i.e. masseter and temporalis) that cause loading of bony structures of the TMJ can have consequential effects on hard tissues (Balanta-Melo et al., 2019). To explore this associated link between BTX-injected muscles and how they could affect bone health, the next sections will first cover ground regarding bone physiology, what they are made of, how they maintain themselves, and how they interact with other tissues of the body.

2.6 Bone Biology

Bones are composed of 50-70% mineral content, 20-40% organic content composed primarily of type I collagen and non-collagenous proteins, and 5-10% water (Clarke, 2008). The collagen and mineral content of bone combine to build bone tissues that resist forces and give stiffness while also providing ductility and resilience. The water component can be bound to minerals or exist in a free form, the latter is able to flow freely in canaliculi of bone during loading events and can also be exchanged with minerals (Granke et al., 2015). This fluid in canaliculi is known as interstitial fluids and plays a role in bone biomechanical behavior (Fritton & Weinbaum, 2009).

These various microstructural components in bone form mineralized collagen fibrils which are arranged to form lamellae. In humans this arrangement is known as osteons which are separated by interstitial bone. Human osteons contain central haversian canals that encompass vessels and nerves supplying bone. These osteons are the building units for compact (cortical) bone that form the hard outer shell of bones and protect bone marrow compartments (Clarke, 2008; Reznikov et al., 2014). Trabecular bone is contained within cortical bone and play an important role in stress distribution.

Bones come in many shapes and sizes to best suit the needs of the region they are located in. Long bones contain many trabeculae at both ends and are shaped to withstand forces and give strength to the skeleton. Meanwhile, an irregular bone like the mandible is heavily corticated to support periodontal ligaments and teeth, and to provide strength (Clarke, 2008). Trabecular compartments exist in the mandible posteriorly and near the mandibular condyle, areas that are subject to high masticatory forces. These trabeculae will orient themselves to best withstand the types of mechanical forces exerted (van Eijden et al., 2006). In general, the bone structure of the skeleton does not take on a permanent shape but changes throughout life according to the environment.

There is a constant turnover of bone in humans that is led by bone cells and supported by other supplying structures, which together make up the bone remodeling unit. Osteoblasts are the main driver of bone formation and are derived from mesenchymal cells. They produce extracellular matrix and express important factors for mineralization such as alkaline phosphatase, as well as factors regulating bone resorption (Rutkovskiy et al., 2016). Osteoclasts are the main driver of bone resorption and are multinucleated cells of hematopoietic origin. RANKL and osteoprotegerin secreted by osteoblasts can

initiate or inhibit osteoclastogenesis respectively (McDonald et al., 2021). Lastly, osteocytes are the most abundant bone cells which rise from mature osteoblasts that become buried in their own matrix. Osteocytes play a crucial role in mechanosensation and orchestrating the continuous resorption and formation by osteoclasts and osteoblasts (Bonewald, 2011).

2.7 Bone Mechanobiology

Bone remains healthy by adapting to the forces it experiences (Willie et al., 2020). Forces acting on bone come from the force of gravity on the skeleton and the force of muscles attached to the skeleton. These loads are variable with change in environment, position, and movement. Harold Frost introduced the mechanostat theory that states that bone mass and arrangement are dictated by the bone cells responding to a varying mechanical stimulus (Frost, 1987). He suggested that above and below a predefined range, bone formation and resorption are activated respectively. While in an area dubbed the lazy or dead zone there is an equilibrium between cells and a steady rate of bone (re)modeling is maintained (Frost, 1987). He also suggested that in bone diseases such as osteogenesis imperfecta the ranges corresponding to his theory shift, which may explain the phenotype of hyper mineralized bone structure that has reduced qualities.

The (re)modeling process is a dynamic process relying on signaling between bone cells, which is heavily influenced by osteocytes. Additionally, osteocytes have been shown to have a direct resorptive capacity on its surroundings (Nakashima et al., 2011; Xiong et al., 2014). Osteocytes are dispersed in the bone matrix and interconnected via canals known as canaliculi. These canals are filled with interstitial fluids which deform osteocyte

surfaces under mechanical loads (Weinbaum et al., 2003). This load causes the release of intracellular Ca+ and ATP as well as its derivatives, that diffuse in tissue acting on various purinergic receptors (Dsouza et al., 2022; Mikolajewicz et al., 2019). Additional mechanosensors identified in osteocytes which induce cellular changes include gap junctions, voltage-gated ion channels, and the osteocyte cytoskeleton (Mikolajewicz et al., 2018). This complex process is known as mechanotransduction, where mechanical signals are translated to a biochemical signal that will induce a tissue level response from other cells known as effector cells.

The phases of mechanotransduction occur on different time scales and can take weeks to months for tissue adaptation to occur in bone (Turner et al., 2009). This process is also much faster in smaller sized animals (weeks) such as rodents in comparison to humans (months). A distinctive feature of human bones is the haversian canal and osteon system that is formed around vessels within cortical bone, this system is absent in rodents (Kerschnitzki et al., 2011; Moreno-Jiménez et al., 2020). While bone (re)modeling occurs on bone surfaces in some vertebrates such as rodents, in other vertebrates such as humans it occurs within the cortical bone around the osteons (Willie et al., 2020). As discussed, bone cells play a crucial role in (re)modeling and maintaining healthy bones, but they also exist in an environment surrounded by other tissues of different origins. These tissues include nerves and blood vessels which will be discussed further.

2.8 Vascular role in bone maintenance

In bones, blood vessels are essential for delivery of nutrients, oxygen, and carry waste away. Bones are highly vascularized tissues, receiving 10-15% of the cardiac output

(Tomlinson & Silva, 2013). Blood vessels are tubes lined by endothelial cells (EC), they have a semipermeable barrier to allow for nutrients, hormones and immune cells to enter tissues. ECs can also secrete growth factors and other signaling molecules, therefore they play a role in tissue morphogenesis (Ramasamy et al., 2015; Xiong et al., 2014). Much of the vascular system is a result of expansion of the already existing networks after birth through coordinated migration and proliferation of ECs. Blood vessel networks in different organs display different structural characteristics, for example in the kidneys, vessels have high fenestration allowing for better filtration (Kamba et al., 2006) and in the brain, ECs form a strong barrier to prevent harmful substances from entering (Bennett et al., 1959).

Within long bones, secretion of vascular endothelial growth factor- A (VEGF-A) by hypertrophic chondrocytes causes blood vessels to invade and form primary ossification centers along with osteoclasts and osteoprogenitors (Langen et al., 2017). In flat bones such as the calvaria, bone is formed by intramembranous ossification. In this method, condensation of mesenchymal cells into a sponge-like structure and secretion of VEGF-A promote osteogenic differentiation directly and is followed by invasion of blood vessels (Percival & Richtsmeier, 2013).

Long bones have three main routes for blood supply, these include the primary nutrient artery, the epiphyseal-metaphyseal vessels, and the periosteal arteries. Drainage occurs through a central vein in the diaphysis of long bones. In flat bones, the thickness seems to greatly dictate the architecture of this vascular network. Regional thickness smaller than 0.4 mm only possess a periosteal network with a large vessel connecting

both sides of the bone. As the thickness of the bone grows a more similar microvascular network to that of long bones is formed (Pannarale et al., 1997).

Recent literature in bone vascularity identified three subpopulations of ECs inside bone vascular networks. These subpopulations are mainly distinguished by their marker expression and functional characteristics. Type H ECs have high expression of both cell-adhesion molecule CD31 and sialo glycoprotein endomucin (Em). Type L ECs have low expression of these markers (Kusumbe et al., 2014). Type E ECs are similar to type H with high CD31 and relatively lower Em expression. Type E ECs are abundant during embryonic development and early postnatal life, they are replaced by type H vessels early on. With aging, type L vessels increase while number of type H decreases (Langen et al., 2017). Subpopulations of ECs seem to be localized to specific regions of the long bone, for example, type H are found in the metaphysis and endosteum of bone, while type L are found at the sinusoidal cavities of the diaphysis. Blood is proposed to flow in bones from arteries and arterioles into the type H vessels then to the type L sinusoidal network and finally to the large central vein (Ramasamy et al., 2016). Type H vessels have also been identified in the mandibular condyle correlating to areas of bone formation and representing an interesting target for promoting angiogenesis and regenerative therapeutics (Li et al., 2021).

ECs are also capable of supporting osteogenesis independent of blood flow. Specifically, type H vessels are usually found in close association to osteoprogenitors and are capable of secreting bone morphogenic proteins (BMP) and fibroblast growth factors (FGF) (Kusumbe et al., 2014). In fracture healing, type H vessels were shown to be the most abundant of all invading vessels at fracture healing site (Maes et al., 2010). Type H

vessels also play an important role in bone formation supported by findings in an aging experiment that pharmacologically induced maintenance of type H vessels resulting in higher bone density (Kusumbe et al., 2014).

The integrity of ECs and the vascular network is sustained by the continuous uninterrupted flow of blood through the system. The shear forces and stretch exerted by blood flow on the vessel walls and ECs cause cells to proliferate, migrate, and change shape (Campinho et al., 2020). Blood flow is best described as laminar flow confined to tubes and is equal to the difference of pressure gradient between two points divided by the resistance in the tube or vessel, this is known as Poiseuille's law. Diminished blood flow has been observed in conditions of disuse and aging (Colleran et al., 2000). The effect of BTX on the blood flow and the vascular network architecture has not been sufficiently addressed in the literature. Understanding those effects can provide more insight into BTX induced bone loss given the close relationship between bone and vasculature described in this section.

2.9 Nervous system contribution to bone

Nerves are important for skeletal development and adaptation. Early studies on skeletal nerves were interested in treating pain associated with surgical interventions. Many early denervation models in animals found little effect on bone mass but recently studies have identified abundant sensory, sympathetic, and parasympathetic nerve terminals ending in bone (Tomlinson et al., 2020). Suggesting a closer relationship between nerves and bone may exist than what was initially thought.

Sensory nerves are responsible for proprioception, nociception, recognizing changes in temperatures, and non-painful stimuli (Jones & Smith, 2014; Julius & Basbaum, 2001). Generally, innervation is dense in the areas of the periosteum and marrow space and is higher in areas of bone remodeling (Sayilekshmy et al., 2019). In bone, all the myelinated and unmyelinated sensory nerves express neurotrophic receptor tyrosine kinase type 1 which has high affinity for nerve growth factor (NGF) (Mantyh, 2014). NGF is also expressed by osteoblasts and acts directly on nerve axons in bone stimulating other nociceptive pathways. Due to its action, NGF and its antibody anti-NGF have been studied extensively as a potential target for reducing skeletal pain, with promising results in fracture healing and cancer-related pains respectively (Jimenez-Andrade et al., 2011; Wang et al., 2006).

Other sensory neuropeptides include calcitonin gene-related peptide (CGRP) which has a bone anabolic function through stimulation of Wnt signaling in osteoblasts (Mrak et al., 2010). CGRP also inhibits osteoclast differentiation and function (Elefteriou, 2005). Targeting these neuropeptides may be more effective with local drug delivery systems since they are widely present outside bone as well (Tomlinson et al., 2020). Substance P (SP) is another example of a neuropeptide released by skeletal sensory nerves. It has the ability to increase both resorption and formation with a heavier influence on formation, leading to impaired material and structural properties (Niedermair et al., 2014).

The autonomic nervous system is composed of sympathetic nerves that release norepinephrine and activate alpha- and beta- adrenergic receptors, and parasympathetic nerves that release acetylcholine and activate nicotinic and muscarinic receptors.

Sympathetic nerves play a role in circadian rhythm and regulation of activation of formation and resorption by bone cells (Tomlinson et al., 2020). In bone, these nerves are often associated with vascular structures and studies staining these nerves show their spiral morphology around blood vessels (Tabarowski et al., 1996). Both sympathetic and parasympathetic nerves have opposite effects on bone mass with sympathetic causing decrease through stimulating resorption and inhibiting formation (Bajayo et al., 2012).

In disuse conditions such as spinal cord injury bone loss is rapid and may indicate that other factors aside from disuse osteoporosis are driving this response. This is supported by findings in unilateral sciatic nerve transection experiment, where bone loss was observed in denervated limb and the contralateral limb non-affected limb (Monzem et al., 2021). Other findings suggest that sensory nerve downregulation can cause bone loss and cause the bone environment to be less sensitive to mechanical stimulation (Tomlinson et al., 2020). Sympathetic nerves activation seemingly reduces bone mass but does not cause further loss in unloading models (Kondo et al., 2005). Therefore, the nervous system, particularly peripheral sympathetic and sensory nerves, plays a direct and indirect role in bone regulation and presents multiple targets for potential therapeutics in bone diseases. In this section as well as the previous we discussed how other tissues can play a role in bone regulation and homeostasis. Using various preclinical models it is possible to study such interactions in great depth. In the next section I cover the various established models for unloading induced bone loss.

2.10 Experimental models of paralysis/unloading

Conditions of disuse are known to cause bone degradation and loss due to absence of mechanical stimulation. To study the effects of prolonged disuse and better characterize the pathways involved, there are multiple animal models that exist to mimic the effect of bone loss. Their aim is generally to either eliminate the ground forces or eliminate muscular forces. These models include different methods that range in their invasiveness from irreversible neurectomies to casting of hindlimbs. In a recent systematic review characterizing the different animal disuse models, the most used animals were rats (59%) followed by mice (30%). Tibia bones (68%) were the most frequently analyzed bone followed by femurs (61%) with less frequently studied regions including ulna (2%) and mandible (2%) (Brent et al., 2021).

Hindlimb unloading is the most common model representing about half the literature on disuse models. Botulinum toxin A (BTX) injections represent 9% of disuse studies and is arguably the least invasive of all methods presented. BTX injections are an established disuse model that causes rapid bone loss which exceeds other models such as hindlimb unloading (Ellman et al., 2014). Similar to BTX, neurectomies induce high levels of bone deterioration which exceeds that seen in less extreme models such as peripheral nerve injury and hindlimb unloading. Extreme bone loss presenting with BTX and neurectomies are similar to age-related changes in bone (Buettmann et al., 2022).

Studying the various system interactions with bone tissue in unloading is heavily centered around the disuse model of hindlimb unloading. This may not be the case for models with different mechanisms of action such disruption of neuronal signaling and may

also not represent all scenarios such as aging. Further investigations are needed to distinguish the differences in system interactions across various models.

2.11 Conclusion

Throughout this literature review I've covered TMDs, a common pain disorder affecting a significant portion of the general population, and BTX, one of the conservative approaches used in managing myofascial types of TMDs. I then introduced and summarized the action of BTX and it's uses in general biomedical practice as well as in myofascial TMDs specifically. Myofascial TMDs are the second most common cause of chronic pain and understandably much of the literature in managing this condition is interested in alleviating pain and measuring pain outcomes with various modalities. As a toxin with a potential effect on many tissues and with the lack of guidelines more research is needed into all aspects of this toxin. Studying the adverse effects associated with BTX use is warranted to help establish proper practice guidelines and avoid any unwanted consequences. Of interest to me is the potential biomechanical consequences to bone health. Therefore, I would like to investigate this potential adverse effect in TMD populations receiving BTX. I would do so by conducted comprehensive systematic search of the literature and conducting a meta-analysis where applicable.

Additionally, these consequences to bone may take a long time to manifest as discussed in an earlier section. Musculoskeletal health and maintenance is a fascinating topic that is of importance to various scenarios and also overlaps with the potential repeated use of BTX in TMDs. To give greater context I covered in this literature review the aspects of bone biology, mechanobiology and different crosstalk between bone and

other systems. This crosstalk is a novel aspect which is of growing interest to researchers. Using BTX as a model to induce musculoskeletal deterioration I would investigate the crosstalk with vasculature. I would like to monitor the temporal changes in blood flow after BTX injection in mice and characterize the bone and vascular architecture to identify potential correlations.

Lastly, I would construct a clinical study to build on the knowledge to be obtained from these studies. This clinical study will provide us with knowledge of the physiological changes accompanying BTX injections in humans. There is currently a substantial gap in knowledge on this topic compared to the literature investigating pain relief. In tandem with studies on pain alleviating capacity of BTX the community can work towards safe guidelines and proper monitoring of the accompanying changes with BTX.

3 Adverse effect of botulinum toxin-A injections on mandibular bone: A systematic review and meta-analysis

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3.1 Abstract

Introduction: Botulinum toxin-A (BTX) is a potent neurotoxin that is emerging in the scope of dental practice for its ability to temporarily paralyse musculature and reduce hyperfunction. This may be desirable in diseases/disorders associated with hyperactive muscles such as the muscles of mastication, most implicated in painful temporomandibular disorders (TMDs). The use of BTX extends beyond its indications with off-label use in TMD's and other conditions, while potential adverse effects remain understudied. BTX is well-established hindlimb paralysis model in animals leading to significant bone loss with underlying mechanisms remaining unclear. The objective of this study is to systematically review the literature for articles investigating changes in mandibular bone following BTX injections and meta-analyse available data on re-ported bone outcomes. **Methods**: Comprehensive search of Medline, Embase and Web of Science retrieved934 articles. Following the screening process, 36 articles in animals and humans were included for quantitative synthesis. Articles in human individuals (6) and three different animal species (14) presented mandibular bone outcomes that were included in the meta-analysis. **Results**: The masseter and temporalis muscles were frequently injected across all species. In humans, we observe a decrease of about 6% in cortical thickness of mandibular regions following BTX injection with no evident changes in either volume or density of bone structures. In animals, bone loss in the condylar region is significantly high in both cortical and trabecular compartments. **Discussion**: Our analysis supports the concept of BTX-induced bone-loss model in animal mandibles. Further, bone loss might be confined to the cortical compartments in humans. Most studies did not address the reality of repeated injections and excessive dosing, which

occur due to the reversible action of BTX. More rigorous trials are needed to draw a full picture of potential long-term adverse effects on bone.

3.2 Introduction

Botulinum toxin (A) (BTX) is one of the most potent neurotoxins known to humans.[1] When injected into musculature, BTX mainly targets the nerve endings at the neuromuscular junction.[2] Once inside the motor endplate, BTX short chains cleave to the synaptosomal-associated protein SNAP-25 which is a key component of SNARE protein complex responsible for neurotransmitter exocytosis. This leads to blocking transmission of action potential and flaccid paralysis of muscles.[3,4] This can be a beneficial outcome in conditions characterized by muscular spasms, hyperactivity and hypertrophy. The Food and Drug Administration (FDA) has approved using BTX for cervical dystonia, hemifacial spasm and chronic migraines.[5] BTX is also used extensively in cosmetics, with current FDA approval for treating glabellar rhytids.[6] There are nearly 3 million injections of BTX done per year, making it the world's most popular cosmeticprocedure.[7]

The use of BTX is not limited to FDA- approved indications, with recent literature suggesting BTX can provide a beneficial therapeutic effect in off-label treatment of painful temporomandibular disorders (TMDs).[8] TMDs are several disorders involving the temporomandibular joint and supporting structures such as the muscles of mastication. TMDs are highly prevalent in the general population (5%– 12%)and significantly impact quality of life and daily function of affectedindividuals.[9] Aetiology of these heterogenous disorders remains unclear, and evidence suggests multifactorial origins which may involve psychosocial, pathophysiological and anatomical factors, as well as joint and

muscle trauma.[10] The OPPERA study also proposed a heuristic model with two intermediate phenotypes of psychological distress and pain amplification that influence TMDs development.[11] TMDs may present with intra-articular issues, myofascial involvement or both, and diagnosis primarily relies on comprehensive patient history and clinical examinations.[12] Weak evidence has been accumulated for BTX effectiveness in reducing TMD related pain.[13] However, results remain conflicting due to weaker study designs, with more precise randomized control trials ongoing to test BTX clinical effectiveness in TMDs.

In preclinical studies, BTX injections in hindlimbs are an established experimental animal model to study disuse- induced boneloss.[14,15] To maintain healthy bones, mechanical loads from muscular contraction and gravitational forces are required. The forces falling on bone are sensed by cells known as osteocytes that signal other bone cells including osteoblasts and osteoclasts to form and resorb bone, respectively, maintaining a steady state of bone remodelling.[16] Disrupting this remodelling process by removal of forces will induce bone structure deterioration. Additionally, the orofacial region has a unique biomechanical environment which includes a complex muscular and ligament system supporting the jaws' hard tissue, and occlusal forces between teeth, impacting the non-weight-bearing bones. Preclinical studies suggest BTX injections can lead to bone loss in this region.[14,17]

Clinical adverse effects of BTX injections in the orofacial region are understudied,[18] with literature typically reporting on acute reactions to BTX or injection techniques.[19] Given the importance of maintaining healthy bone structure for long-term oral health, we

aimed to systematically review and quantitatively synthesize information for mandibular bone changes following BTX injections in humans and experimental animals.

3.3 Methods

This review was carried out in compliance with the Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) statement (PRISMA Checklist is available in the Supporting information S1). The review protocol was registered in an international prospective register of systematic reviews (PROSPERO, CRD42022349881).

3.3.1 Search strategy

A comprehensive search combining two major concepts of mandibular bone and botulinum toxin (A) was applied to three databases (Medline, Embase and Web of Science) in May 2022 and repeated in December 2022 to include any newly published material. Search results were uploaded and deduplicated using Rayyan software on-line,[20] and screened by two independent reviewers (MSM and DB). Inclusion criteria for initial screening required articles to include BTX as an exposure/treatment and discuss any region or whole of mandibular hard tissues. No limitations were placed on time of publication, language or species. Next, full-text screening was performed to identify articles providing quantitative data on bone parameters following BTX administration.

3.3.2 Data extraction

Information extracted from articles included first author, year of publication, country, study design (humans), species (animals), age, sex, diagnosis (humans), study groups, BTX

dose, injected muscles, injection regimen, follow up/experimental length, method of outcome acquisition and types of bone outcomes. For measured bone outcomes, we extracted sample sizes for each BTX and control groups, regions of interest, time point at which measures were taken, mean values (μ_{btxj}) along with corresponding variance measure.

3.3.3 Study level outcomes

For human studies, a comparator of either baseline value (μ_{basej}) of individual receiving BTX or separate group of control individuals (μ_{ccj}) were used. For animals, we used either values measured internally at non-injected contralateral site or external control animals. In certain cases when data were made available but could not be used due to lack of reporting on measure type or variance, authors were contacted for further elucidation. Study level outcomes (θ_j) were calculated as normalized mean differences and converted to percentage to facilitate reporting using the following equation (1):

$$\theta j = \frac{\mu btxj - \mu basej}{\mu basej} \times 100\ \% \qquad (1)$$

In human studies, when different regions of mandibular bone were reported, the outcomes for each site within the same study were compiled as unweighted means. Different groups of participants within the same study were treated as independent groups.

3.3.4 Meta-analysis

To conduct the meta-analysis we used the random-effects model to calculate global effect sizes ($\hat{\theta}$) and corresponding standard error (SE) using the DerSimonian Laird τ^2 estimator.[21,22] Meta-analysis model and forest plot construction were performed using the Metafor package in R software.[23] Confidence intervals (CI) were calculated as $95\%\ \mathrm{CI} = \hat{\theta} \pm z_{(1-\alpha/2)} \times SE(\hat{\theta}) = \hat{\theta} \pm 1.96 \times SE(\hat{\theta})$.

3.3.5 Heterogeneity and bias

Selected articles were evaluated for bias using a quality assessment tool tailored for human and animal studies, with maximum obtain-able score of 10 points. Heterogeneity was reported as I^2 and H^2. Funnel plot asymmetry and single study omission analysis were done to assess publication bias in the largest datasets available in humans and experimental animals.

3.3.6 Additional analyses

To assess the influence of covariates, we conducted meta-regression analysis on experiment duration in the largest animal dataset (BV.TV) and performed subgroup analysis for outcomes in different bone regions in the human data and condylar subregions in animal data.

3.4 Results

3.4.1 Overview of relevant literature

The systematic search (Supporting information S2) of studies examining mandibular bone following BTX injection was conducted in Medline, Embase and Web of Science and retrieved 934 candidate articles. We also searched google scholar and clinical reports published online, from where no additional sources were found. Following screening by two independent reviewers (MSM and DB), 48 articles describing the effect of BTX on mandibular bone were included, of which 36 contained quantitative data. After analysis of included studies, articles of cephalometric growth were excluded, leaving 20 articles (6 humans and 14 animals) with quantitative data on changes in mandibular bone microarchitecture following BTX (Figure 1A). In human studies, 96% of participants were female; most participants (86%) were treated for myofascial TMDs, the next most common treatment cause was cosmetic facial contouring. (Figure 1B). Animal studies demonstrated an even distribution of sex and were performed in rabbits, rats and mice (Figure 1C). The quality assessment using a 10- point questionnaire (Supporting information S3) resulted in 3 of 6 human papers and 8 of 14 animal papers scoring at 7 or higher (Figure 1D).

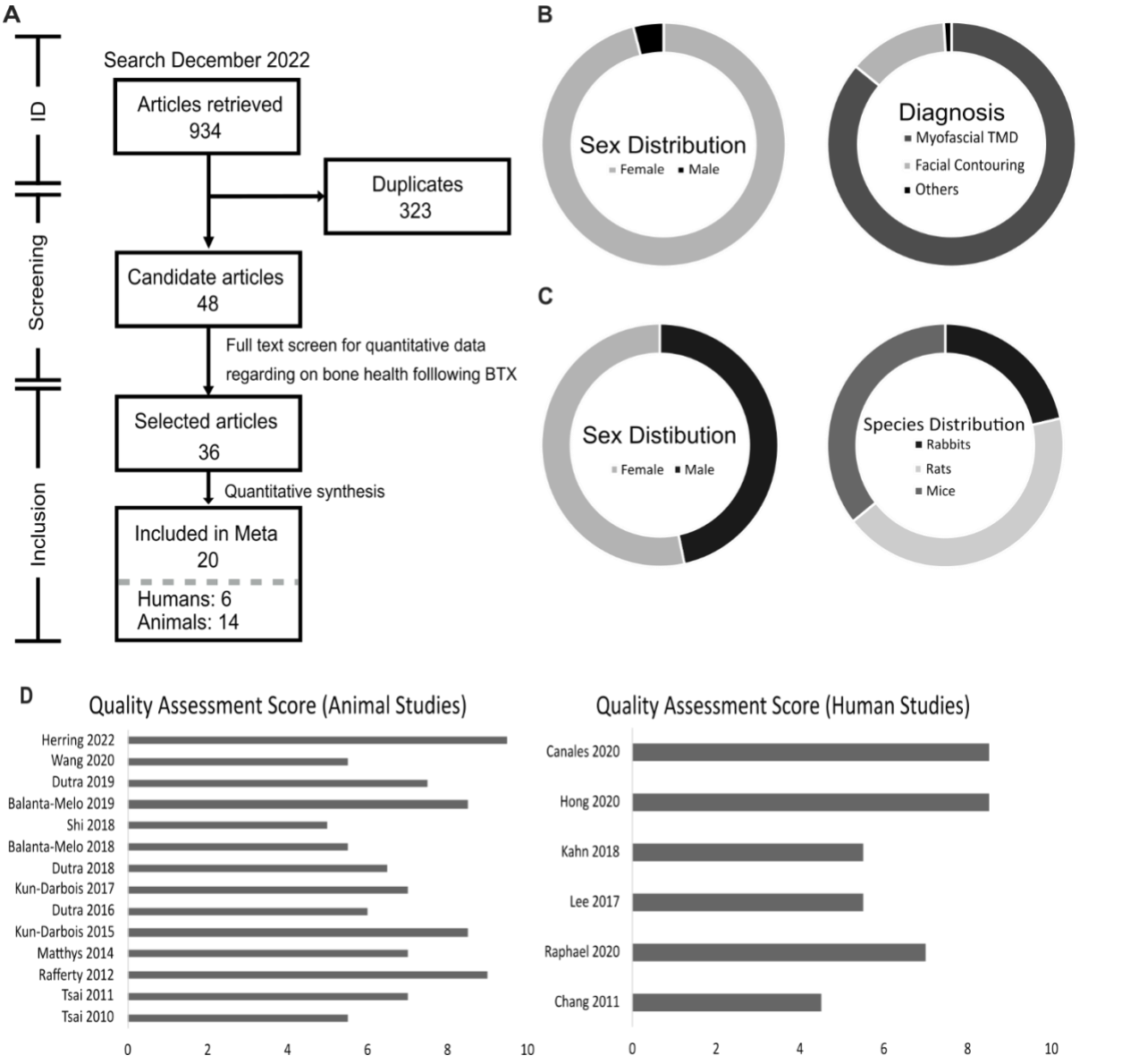

Figure 1. Information flow and characteristics of included studies. A) Prisma diagram of the information flow in the project. B) Characteristics of human participants. C) Characteristics of experimental animals. D) Quality assessment results for both human and animal studies.

3.4.2 Human literature

Included studies in humans came from over four continents, covering a vast demographic representation (Table 1). Four studies were prospective cohorts,[24–27] one randomized control trial[28] and one retrospective cohort receiving BTX prior to study start.[29] Three studies reported multiple cohorts of patients, which we included as independent datasets:

one study used three varying doses of BTX,[28] one study had two groups receiving either one or two injections,[26] and one study reported the findings in young and post-menopausal women.[25] In addition, one study reported a combination of oral appliances (OA) and BTX treatment in addition to BTX treatment alone.[25] Four of six studies included a control group;[24,25,28,29] however, one of them did not report the values in control participants.[24] All studies except for retrospective cohort provided baseline values for reported outcomes prior to BTX administration. Thus, we identified 11 groups of participants with baseline values and 4 groups with controls participants. The age of participants was 26.9–55.3 years of age and doses administered varied from 50 units to 200 units of the same product (Botox, onabotulinumtoxinA), one study administering 240 units of a different product (Dysport, abobotulinumtoxinA). All studies except the retrospective cohort reported bilateral injections in the masseter (6/6) and/or temporalis (4/6) muscles. Bone changes were assessed at 3, 6 or 12 months following BTX injection, and regions of interest were the mandibular condyle, coronoid process and angle/ramus.

Table 1: Human participant studies characteristics

Author	Country	Study Design	Age	Groups (n)	Total BTX units and distribution	Bone Acquisition Time Points	Outcome included in Meta-analysis
Kahn, A. (2018)	France	Cohort (Prospective)	31.5	CTL (6), BTX (12)	Total: 100 (M: 60, T: 40)	Baseline 12 months	Condylar Ct.Th
De la Torre Canales, G. (2020)	Brazil	Randomized Control Trial	36.8	OA, BTX-L, BTX-M, BTX-H, CTL (20 each)	Total: 80, 140, 200 (M: 60,100,150, T: 20, 40, 50)	Baseline 3 months	Condylar and Coronoid BV
Lee, H. (2017)	South Korea	Cohort (Prospective)	28.5	BTX (10), BTX*2 (10)	50, 100 (M: 50, 100)	Baseline 6 months	Mandibular Angle BV
Raphael, K. (2020)	United States	Cohort (Retrospective)	41.2	CTL (44), BTX (35)	Not assigned (Retrospective)	Within 12 months following BTX	Condylar and Alveolar BD, Condylar BV
Chang, C. (2010)	Taiwan	Cohort (Prospective)	28.4	BTX (10)	240 (M: 240)	Baseline 3 months	Mandibular Volume, Ramus Ct.Th
Hong, S. (2020)*	South Korea	Cohort (Prospective)	26.9 & 55.3	CTL (12), OA (22), BTX (23), OA & BTX (20)	180 (M: 100, T: 80)	Baseline 12 months	Condylar, Coronoid, and Ramus Ct.Th and BD

Table Legend: Botulinum Toxin treated group (BTX); low dose (BTX-L), medium dose (BTX-M), High dose (BTX-H), Oral Appliance treatment (OA), Controls (CTL), Bone Volume (BV), Bone Density (BD), and Cortical Thickness (Ct.Th), Masseter (M) and Temporalis (T)
*Two study populations (Young, Post-Menopausal)
**Average values based on participant dosing over past year

3.4.3 Animal literature

Animal studies included 14 studies with 3 species (rabbits (3/14),[30–32] mice (5/14)[33–37] and rats (6/14)[38–43] investigated by seven different research groups (Table 2). Ages of animals varied (4–20 weeks old) with many performed in young animals. Most studies injected BTX unilaterally (11/14), with randomization of injected side done in rabbit studies. Studies used contralateral mandibular sites in the same experimental animals as internal controls, and external control animals by comparing changes in BTX- injected side to

contralateral, non-injected control (4/14) or vehicle-injected control (10/14) side. Dosing in animal studies was reliant on animal body weight and ranged for a single injection from (0.3–0.2 U) in mice, (2–7.5 U) in rats and (10 U) in rabbits. The masseter was injected in all included studies with three studies injecting equal doses in masseter and temporalis.[38,39] Length of experiments varied in studies (2–36 weeks) with 4 weeks following injection being the most common time point (Table 2). Only female rabbits were studied; while male and female rodents were examined, no study compared them directly.

Table 2: Animal studies characterization and design

Author & Year	Species	Strain	Age (Week)	Sex	Groups (nTotal)	Dose (U)	Botox Injected Muscle	Study Length (Months)
Dutra, E. (2016)	Mice	Col10a1 on CD1	5	F/M (9/4)	Injected/Internal Controls (13)	0.3	RM	1
Dutra, E. (2018)	Mice	C57Bl/6J	6	Female	CTL (8) BTX (8)	0.3	RM	1
Dutra, E. (2019)	Mice	C57Bl/6J	6	Female	CTL (16) BTX (16)*	0.3	RM	1
Balanta-Melo, J. (2018)	Mice	BALB/c	8	Male	CTL (5) BTX (11)	0.2	RM	0.5
Balanta-Melo, J. (2019)	Mice	BALB/c	9	Male	CTL (10) BTX (8)	0.2	RM	0.5
Tsai, C. (2010)	Rats	Sprague-Dawley	8	Male	Injected/Internal Controls (10)	7.5	LM	3
Tsai, C. (2011)	Rats	Long-Evans	4	Male	CTL (15) BTX (45)*	4	BM, BT	1.5
Kun-Darbois, J.D. (2015)	Rats	Sprague-Dawley	18	Male	CTL (6) BTX (9)	2	RM, RT	1
Kun-Darbois, J.D. (2017)	Rats	Sprague-Dawley	18	Male	CTL (4) BTX (7)	2	RM, RT	1
Shi, Z. (2018)	Rats	Sprague-Dawley	5	Female	CTL (n=20), BTX (n=20)	4	BM	1
Wang, Z. (2020)	Rats	Sprague-Dawley	4	Female	CTL (8) BTX (16)*	6 and 18	BM	2.5
Rafferty, K. (2012)	Rabbits	NZ white	20	Female	CTL (n=20) BTX (n=21)*	10	Random UM	1, 3
Matthys, T. (2014)	Rabbits	NZ white	20	Female	CTL (n=19) BTX (n=31)	10	Random UM	1, 3
Herring, S. (2022)	Rabbits	NZ white	20	Female	CTL (n=5) BTX (n=13)	30	Random UM	9

Table Legend: Control group (CTL), Botulinum Toxin group (BTX), Right Masseter (RM), Left Masseter (LM), Bilateral Masseter (BM), Right Temporalis (RT), Bilateral Temporalis (BT), and Unilateral Masseter (UM). *Indicates multiple experimental BTX groups

3.4.4 Meta-analysis of human bone outcomes

The largest datasets available for human participants were bone volume and cortical thickness, each measured in six independent groups. Meta-analysis of bone volume changes at 3 (4/6) or 6 (2/6) months compared to baseline value did not demonstrate significant changes (−2.36%; [−11.82, 7.09], p-Value = .6) for BTX-treated participants (Figure 2A); however, this measure had very high variability. Cortical thickness in human mandibles demonstrated a significant decrease (−6.34%; [−10.25, −2.42], p-Value = .001) (Figure 2B) at 12 (5/6) or 3(1/6) months following BTX injections. Comprehensive analysis of the cortical thickness in specific regions was not possible because data for different regions were reported in one paper only.[25] Nevertheless, when we combined five condyle datasets from two studies(−12.82%; [−20.31, −5.33]),[24,25] four coronoid datasets from a single study (−4.66%; [−8.64, −0.68]),[25] and five mandibular ramus datasets from two studies (−1%; [−4.59, 2.59]),[25,27] the data suggested that cortical thickness may be most affected in the condylar region.

We also meta-analysed the changes in bone density in BTX-injected individuals compared to non- injected participants. We combined data from the retrospective study,[29] in which the amount of BTX received and follow-up time varied with participants' treatment plan, and data for two groups of different ages from the Hong et al study, that reported baseline and untreated control groups. We observed a trend of decreasing bone density in BTX-injected participants compared to control participants (−4.43%; [−9.15, 0.29], p-Value = .06) (Figure 2C).

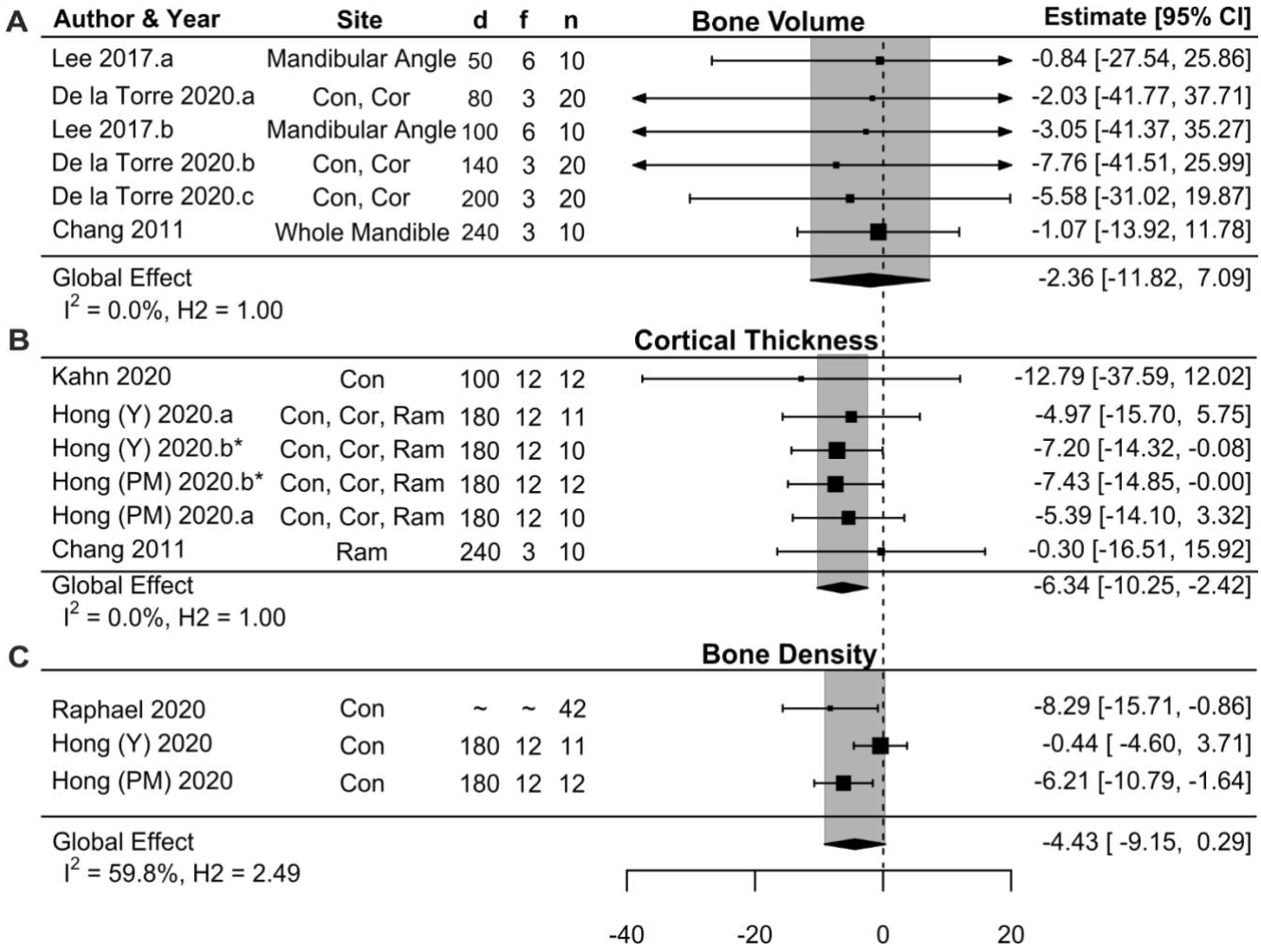

Figure 2. BTX-induced changes in bone parameters in human participants. (A-C) Forest plots of percentage changes from baseline in (A) volume, (B) cortical thickness, and (C) density of mandibular bone following BTX injection in masticatory muscles. Indicated are the studies used for analysis, the bone sites examined, the cumulative BTX- dose received in units by each participant (d), time to follow up in months (f) and the sample size of BTX-injected group (n). Squares/lines: study effect size (%) and 95% confidence interval (CI), the square size is proportional to the study weight. Diamonds/bands: global effect sizes and CI. Heterogeneity statistics I2 and H2 are reported. *Indicates sample in which combination therapy of BTX and oral appliance was used. (~) doses and follow up are less accurate due to the retrospective nature of this study. Different ages defined as young (Y) and post-menopausal (PM).

3.4.5 Meta-analysis of animal bone outcomes

The largest dataset available in animal studies was bone volume to tissue volume (BV.TV) of the mandibular condyle, containing 9 datasets with internal contralateral control (IC), and 11 datasets with control of sham- or non- injected animals (CTL). Compared to IC, BV.TV demonstrated a significant reduction in bone volume of BTX-injected side

compared to non-injected side (−9.69%; [−11.85, −7.53], p < .001) (Figure 3A). When compared to BV.TV of CTL, BTX- injected animals showed more bone loss with a wider CI (−14.08% [−22.93; −5.23], p-Value = .001) (Figure 3A). Subgrouping by species demonstrated overlapping effect sizes with larger variance in reported outcomes in rat studies compared to rabbits and mice. Changes in bone cortical thickness (Ct.Th) of two mandibular regions assessed in five datasets were significant in BTX-injected bone and similar compared to IC (−13.55% [−18.97; −8.12], p < .0001) or CTL (−14.25% [−22.24; −6.26],p-Value = .0005) (Figure 3B). Subgrouping by species reveal similar cortical loss between rabbit and rodent with higher CI in the former.

Analysis of bone area to tissue area (B.Ar/T.Ar, 5 datasets) and trabecular thickness (Tb.Th, 5 datasets compared to IC, 6 com-pared to CTL) of subchondral bone showed B.Ar/T.Ar decrease by(−38.51% [−51.66; −25.37], p-Value = .0001) in BTX-injected sides compared to IC and by (−29.56% [−45.86; −13.27], p-Value = .0004) compared to CTL animals (Figure 4A). Tb.Th decreased by (−14.63% [−22.75; −6.52], p-Value = .0004) compared to IC and by (−18.99% [−31.43; −6.55], p-Value = .0028) compared to CTL (Figure 4B).

To further analyse the differences in trabecular and cortical bone, we sub-grouped BV.TV data by subregion of condylar bone in any animal species, and we found similar BTX-induced bone loss in subchondral (region containing only trabecular bone) and in total condyle (region containing both cortical and trabecular bone) (Figure S4). We also examined the association between effect size and experiment duration but found no significant correlation (Figure S5).

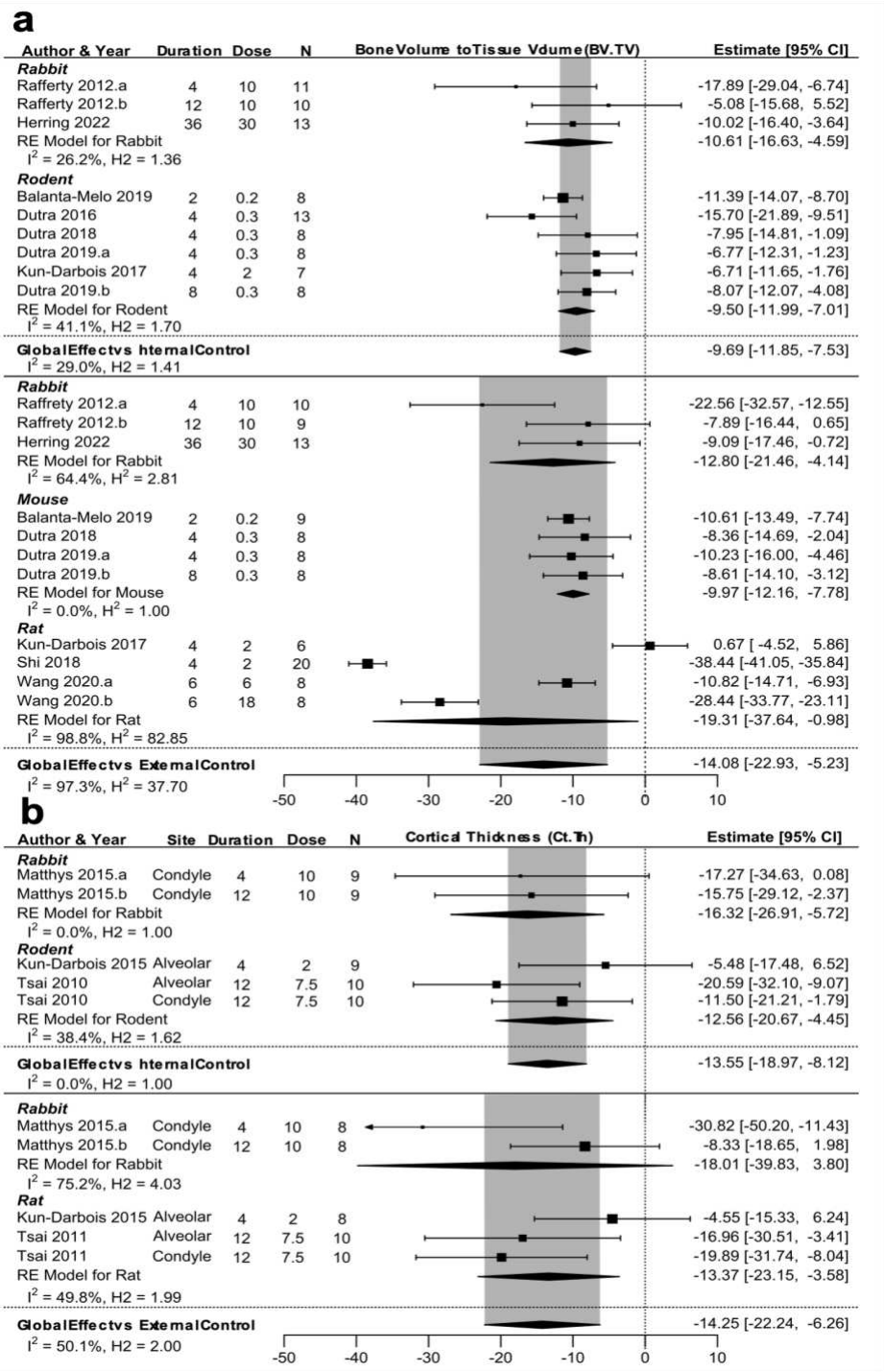

Figure 3. BTX-induced changes in condylar volume (BV.TV) and cortical thickness (Ct.Th) in animal models. Forest plots of percentage change in (A) BV.TV and (B) Ct.Th in animal models receiving BTX injections, compared to internal control side of the mandible (upper) and external control animals (lower). Indicated are studies, duration of experiment post injection, sample size of injected group (n) of injected animals, and region of mandibular Ct.Th measure. Squares and lines represent study effect size (%) and 95% confidence interval (CI), sizes of squares rely on individual study variance. Heterogeneity statistics such as I^2 and H^2 presented.

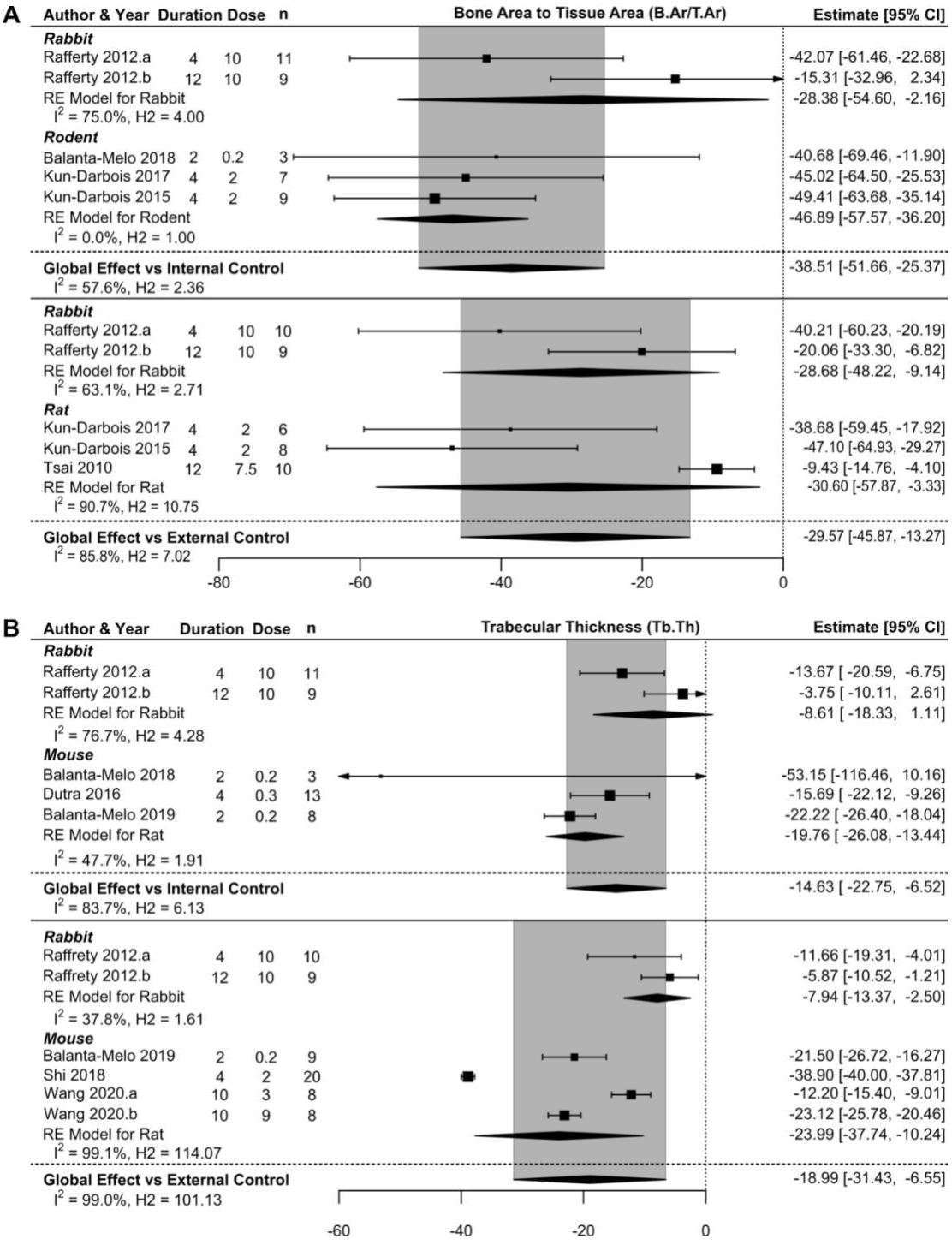

Figure 4. BTX-induced changes in subchondral bone in mandible of animal models. Forest plots of changes in (A) trabecular bone area (B.Ar/T.Ar) and (B) trabecular thickness (Tb.Th) in animal models receiving BTX injections, compared to internal control side condyles (upper) and external control animals (lower). Indicated are studies, length of experiment post injection, and sample size (n) of injected animals. Squares and lines represent study effect size (%) and 95% confidence interval (CI), sizes of squares rely on individual study variance. Heterogeneity statistics such as I2 and H2 presented.

3.4.6 Assessment of heterogeneity and bias

When BTX effect was compared to baseline values in humans or IC in animals, we observed lower overall heterogeneity statistics (I2 and H2), relative to similar comparisons to CTL. In animal studies, outcomes compared to IC had effect sizes and confidence intervals smaller than outcomes compared to CTL, suggesting that BTX effect may be masked by high biological and/or measurement variability in outcome measures. We further analysed individual participant data for 12 participants provided in one human study,[24] by (a) calculating the normalized mean difference between the group means before and after treatment or (b) calculating the normalized mean difference for each participant and averaging the effect sizes among participants. While the overall effect size was similar in these scenarios (−12.8% vs. −14.0%), the standard error was 12.6% and 3.5% in the first and second scenarios, respectively. Assuming that measurement errors are consistent within a single study, the biological variability among participants likely drives higher variability estimates. Single study exclusion analysis found that one study[40] in the CTL studies data pool significantly influenced residual heterogeneity, while no study in IC data pool affected heterogeneity (Figure S5). Funnel plot analysis of the largest animal datasets (BV.TV) showed more asymmetry in CTL datasets than in IC (Figure S5). Funnel plot analysis for bone cortical thickness and volume in human participants suggested lack of bias, although the number of studies was very limited (Figure S6).

3.5 Discussion

We systematically reviewed and quantitatively synthesized the literature on mandibular bone changes following BTX injection into masticatory muscles in humans and

experimental animals. In human mandibular bone, BTX caused a decrease in cortical thickness with the highest effects in condyle as well as a trend of decreased bone density. In animal models, significant decreases in BV/TV, Ct.Th, B.Ar/T.Ar and Tb.Th were observed in BTX-injected animals whether com-parison was made to contralateral sites or control animals. Human literature was more limited than animal literature, with greater variability in study designs and reported outcomes. Both in humans and experimental animals, secondary analysis for the effect of sex, age, BTX dose and time to follow-up was not possible, suggesting significant knowledge gaps requiring further investigations. Meta-analysis performed in this study provides quantitative estimates for BTX-induced bone loss in the mandible that can be used for planning future studies. Moreover, based on our analysis, we identified and described below several considerations for planning future studies on the effect of BTX on bone health.

3.5.1 Sample size

We estimated the change in cortical bone thickness of human adults receiving BTX as −6.34% with a standard error of 1.9%. Given this information, sample size calculations[44] recommends recruiting at least 50 individuals for BTX injection group to detect this change in cortical bone with a type I error of 0.05 and power of 80%. When we compared the effect of BTX to baseline values in the same participant, the variance was lower, suggesting that study power would improve with such design. We applied similar calculations using changes in BV.TV observed in rodents or rabbits. We used subgroup effects for species from our BV.TV dataset to account for physiological differences such as presence of haversian systems in rabbit but not rodents. Using at least 10 animals per

group in rodent studies and 25 animals per group in rabbit studies is recommended. These estimates demonstrate that most studies performed to date are underpowered.

3.5.2 Demographics

Based on the published data, it was not feasible to assess the impact of age or sex on bony changes following BTX injection. Only one study separated participants by age and demonstrated higher losses in older patient population.[25] Most animal studies were conducted on skeletally immature animals, reducing translational power of pre-clinical studies, and age- dependent differences have not been studied. It has been established that humans and rodents share similar patterns of age-related bone loss.[45] In humans and animals, ageing leads to trabecular bone loss following skeletal maturity.[45] With further ageing, in 50- to 80-year-old humans, cortical compartments of bone exhibit higher rates of bone loss than already diminished trabecular compartments, becoming thinner and more porous.[46] Future studies for human participants should reflect on cortical and trabecular structures as a function of age when planning study outcomes. It is important, if possible, to include a non-injected control group in older participants to assess the degree of age- related mandibular bone loss with time. A direct comparison of BTX-induced bone loss in animals of different ages would provide useful translational data.

Our study also demonstrates a significant gap in knowledge of the effect of sex and gender on BTX-induced bone loss. A notice-able proportion of participants were also seeking facial aesthetic contouring, suggesting an unexamined potential role of gender. Painful TMDs are more prevalent in women; however, given the general prevalence of painful TMD,[47] reasonably large number of affected men can be anticipated to be

indicated for such treatment, suggesting the need to understand whether sex- related differences exist. Preclinical studies can provide such evidence; however, even though outcomes for both male and female animals were previously reported, no direct comparison was made in any of the studies. The widespread cosmetic use of BTX, societal views on anti-ageing practices and potential marketing for younger women give certain urgency in better understanding any harmful effects of BTX injections, and potential sex- and gender-related differences.

3.5.3 Dose and follow-up time

Studies included in this meta-analysis used highly variable BTX doses(50–250 U/injection in humans and 1–2 U/100 g in animals) and follow-up times (3–12 months in humans and 2–36 weeks in animals).Repeated injections of BTX are necessary as the muscle paralysis action of BTX,[5] even though it is not yet fully established if repeated injections are needed for BTX-induced pain relief.[48] In preclinical rat hindlimb study, no difference was found in trabecular bone changes 8 weeks after one and two injections.[49] In rat mandible study, a more significant impact on bone mass was observed after three BTX injections compared to a single injection.[41] Moreover, it is important to distinguish the neuromuscular effect of BTX from its pain alleviating ability. In a recent clinical study, a single injection of BTX lead to a significant reduction in self-perceived pain, as measured by visual analog scale and pain pressure threshold, up to 6 years afterinjection.[48] Thus, detailed dose- and time- dependence of BTX effects on bone and pain needs to be investigated in future pre-clinical studies. Bone remodelling is a slow process, with recommended follow-up every 2 years for bone mineral density in diseases like osteoporosis.[50] Remodelling episodes take about 2 weeks in rodents, a little over 2 months

in rabbits, and can take up to 6–9 months inhumans.[51] Therefore, studies should allow sufficient time for at least one remodelling episode before analysing bone changes. Given the rapid bone turnover in experimental animals, preclinical studies designed to examine the effect of BTX regimen (e.g. dose/injection, number of injections and interval between injections) on bone over time would be translationally valuable. In human studies, it is recommended to follow- up individuals for >9 months and use a conservative approach in selecting BTX dose (~80 U based on the efficacy reported in a randomized clinical trial by De la Torre Canales studying effect of various BTX doses on myofascial pain).

3.5.4 Site dependent characteristics

Most animal parameters included in our analysis were measured at the condyle, except for Ct.Th which included measurements of alveolar bone. All human sites measurements were done in the posterior mandible corresponding to insertion sites of muscles of mastication. The direction of forces exerted by muscles impacts the orientation of bone microarchitecture as demonstrated by differences in ratio of trabecular to cortical bone and different degrees of anisotropy.[52] This is evident in complex structures such as the condyle, known for complex biomechanical loads that include compressive and regional tensile forces.[53] Two studies from our analysis[24,29] suggest a decrease in density and thickness of anterior part of the condyle. In preclinical studies, bone loss was observed on all surfaces of condyle, which may be explained by species-specific masticatory patterns, muscle size and function, and bone morphology.[53] Moreover, higher resolution imaging using micro- CT can only be performed in preclinical studies and is not feasible in clinical settings. In our study, the main method of bone acquisition was micro-CT for pre-clinical studies and cone beam CT (CBCT) or conventional CT techniques for human

participants. While CBCT with higher resolution gives sharper images for trabecular analysis, it will also increase the scanning time and effective radiation dose received by participants. Nevertheless, current CBCT imaging techniques using a voxel size of 0.3 mm or less were shown to be suitable for trabecular analysis.[54]

Limitations of our review arise from low number of studies which limited our analysis on human bone outcomes and secondary analysis in animal datasets. Study designs varied in human studies, and only one RCT has been conducted thus far. Lack of standardization of site and parameters may introduce bias in human estimates of bone loss. Due to the low number of studies, we combined a variety of BTX doses and follow-up times in our analysis, which limits the specificity of our quantitative estimates. Cosmetic facial use of BTX is becoming more common and is performed by a variety of practitioners. Even though facial muscles differ from muscles of mastication in their insertion and function, long-term BTX use may still induce changes in surrounding bone tissue; therefore, studies are needed to account for adverse effects of repeated BTX use in this population.

3.5.5 Overall conclusions

Our study demonstrated that the neurotoxin BTX has detrimental effects on mandibular bone in preclinical animals and human participants. The correlation of bone loss with BTX dosage and frequency of administration remains unknown. Further longitudinal studies are required to provide a clear understanding of this phenomenon in human populations. In the meantime, clinicians should consider lower doses when possible and follow-up individuals for changes in bony structures in repeated injections over long periods.

3.6 Author Contributions

All authors contributed to study conception and design. MSM and DB performed screening, data extraction and curation; MSM performed meta-analysis; MSM wrote the first draft; MSM, DB and SVK critically edited the manuscript. All authors gave their final approval and agreed to be accountable for all aspects of the work.

3.7 Acknowledgments

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3.8 Conflict of Interest Statement

None of the authors have any perceived or actual conflicts of interest to report.

3.9 Peer Review

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/joor.13590.

3.10 Data Availability Statement

Data collected for this study are available upon reasonable request to authors.

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3.12 Supplementary Material

S1. PRISMA Checklist:

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3,4
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	4
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	5
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	5
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	5
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	S2
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	5,6
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	5,6
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	5,6

Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	6,7
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	6
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	6
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	6, S3
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	6
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	7
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	7,8, (Table 1,2)
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	Figure 1
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	Figure 2,3,4
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	Fig 2,3,4
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	11
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	9,10,11
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	12
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	13-16
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	16
FUNDING			

Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	1

S2. Search Strategy

Searched in EMBASE (May 22, 2022, and December 15, 2022)

1 exp mandible/
2 Mandib*.mp.
3 exp temporomandibular joint/
4 Mandibular head.mp.
5 mandibular condyle.mp. or exp mandible condyle/
6 subchondral bone.mp. or exp subchondral bone/
7 Alveolar bone.mp. or exp alveolar bone/
8 1 or 2 or 3 or 4 or 5 or 6 or 7
9 Botulinum Toxin.mp. or exp botulinum toxin/
10 exp botulinum toxin A/
11 BOTOX.mp.
12 9 or 10 or 11
13 8 and 12

These searches were then transcribed for two other databases, Medline and Web of Science.

S3. Quality checklist

Quality score checklist for included articles:
(Total of 10)

1) Clear description of participant characteristics; in humans, age, sex, and diagnosis (1) and in animals, age, sex, and strain (1)
2) Do authors specify reasoning of sample size choice (1)
3) Were studies randomized in human participants (0.5) or allocation of animals to groups (0.5) while describing the method of randomization/allocation (0.5).
4) Do studies properly account for control samples:
   - In human participants, provides baseline value or external control value (0.5) or both (1)
   - In animals, if study provides internal control (0.5) or external control (0.5) or both (1)
5) Clear description of dose administered to participants (0.5) and reason for choice of dose (1)
6) Clearly indicate all outcome measurement units (including type of data spread) (1)
7) Adequate follow up of participants to observe bony changes in respective species (1)
8) All data concerning outcomes were presented in tables (1) or in graph (0) form.

9) Measurement techniques used are clearly indicated (0.5) and in regions of interest clearly defined (1)
10) Study limitations are acknowledged and discussed by authors (1)

Figure S4. Random effects model for BV.TV dataset of experimental animals with subgrouping by condylar subregion.

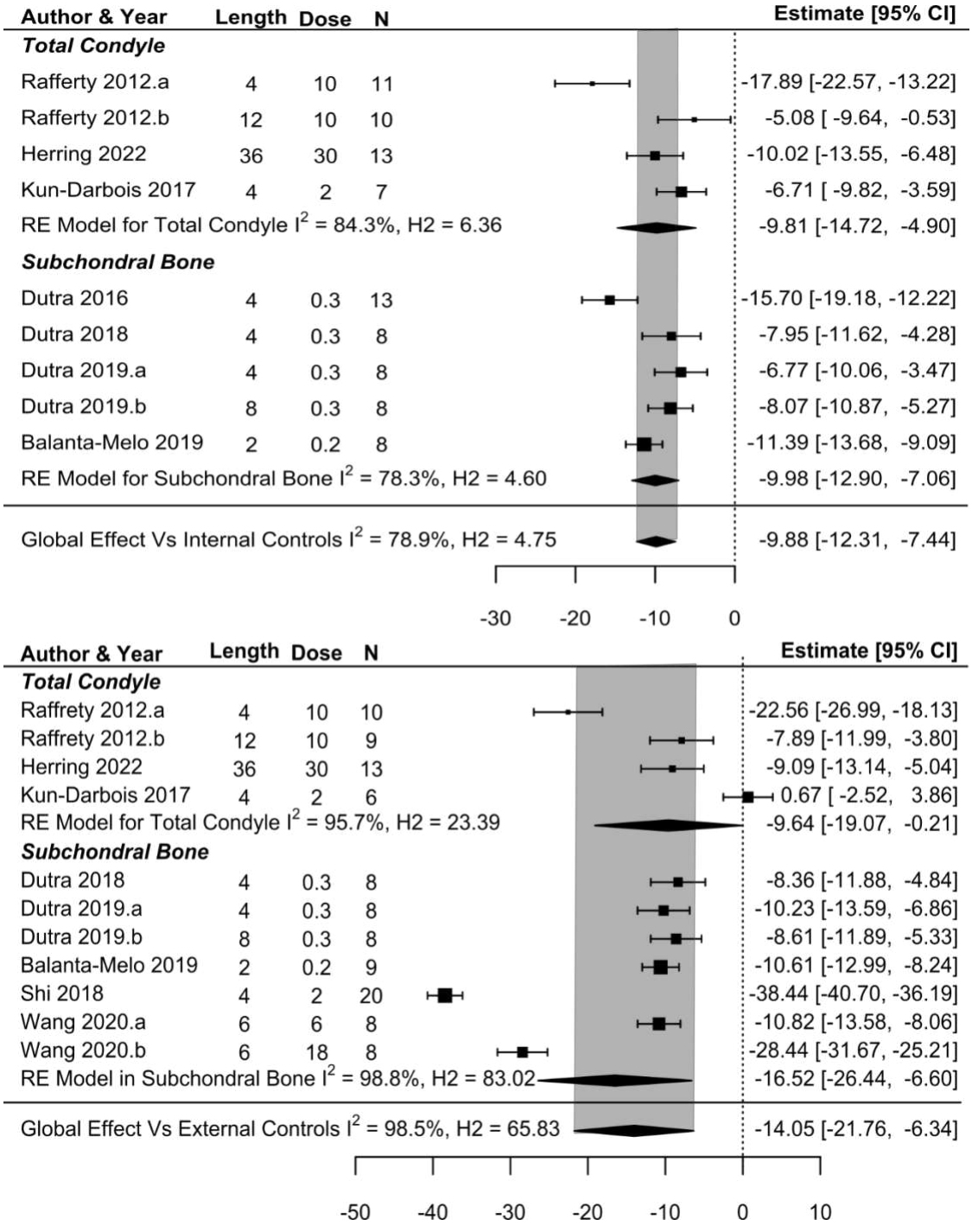


Figure S5. Heterogeneity and sensitivity analysis for BV.TV dataset in animal studies *Top*: meta-regression of experimental time to observed outcomes; *Middle*: leave-one-out test for residual heterogeneity; *Bottom:* funnel plot asymmetry for BTX-injected animals compared to **(a)** external control animals (CTL) and **(b)** contralateral site in same animals (IC).

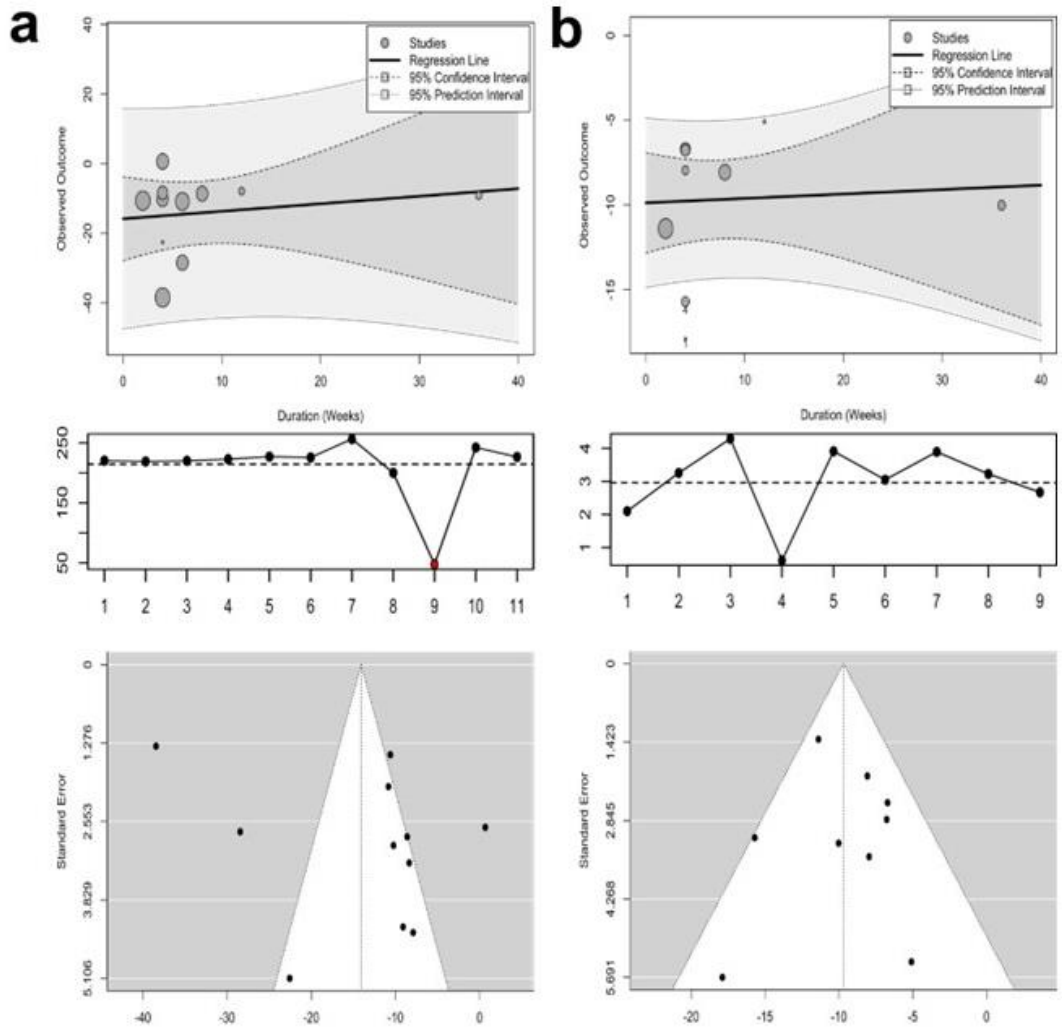
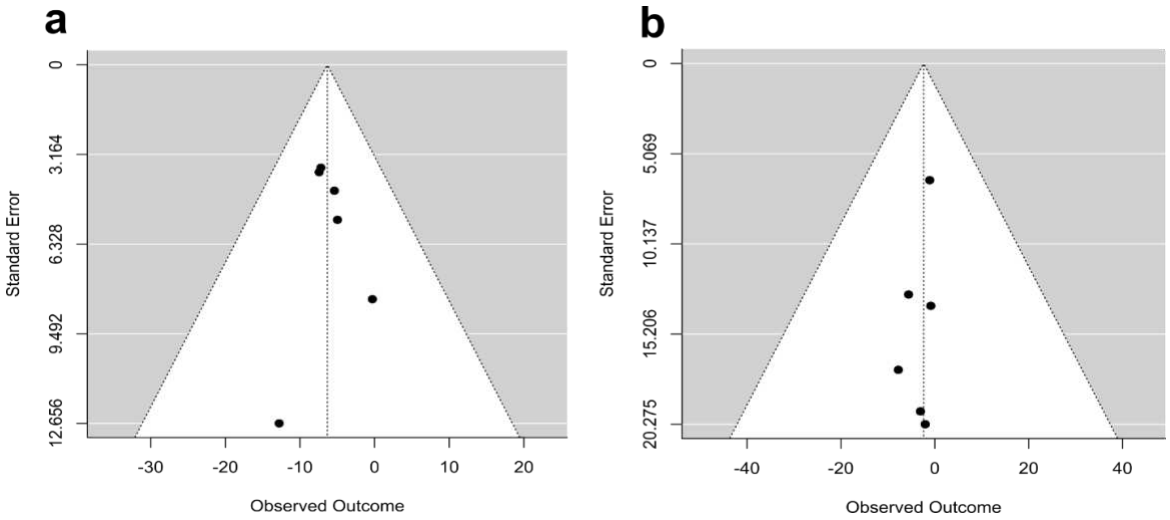


Figure S6. Funnel plot asymmetry analysis of **(a)** bone cortical thickness and **(b)** volume of human participant data



Bridging Statement

In Chapter 3, I show that BTX has a detrimental effect on mandibular bone structure in both humans and animals. The evidence suggests multiple discrepancies in conducted human studies such as the large variation in doses, too short of follow up time for bone, and small sample sizes in conducted studies. Additionally, limitations in imaging technologies and concerns regarding radiation exposure restrict analysis of smaller bone structures such as trabeculae in the human mandible. Cortical thickness represents a more reliable measurement in cone beam tomography scans. For this parameter we note a negative change over time following BTX injection in individuals.

Meanwhile, animals studies demonstrated more consistent changes following BTX injection. The bone loss in animals demonstrated a smaller confidence interval when comparing injected to contralateral side of the mandible. These findings suggest reliability of animal BTX models in replicating disuse induced loss. In the next chapter we engage the BTX model in hindlimbs of mice to study the adaptation of vasculature with bone loss. Using ultrasonography provides a unique opportunity to study time dependent changes in blood flow as opposed to end-of-experiment imaging using microspheres. Using this technique in mandibles of smaller animals is challenging given the size of vasculature, and maintaining anesthesia while imaging as opposed to main nutrient arteries of the hindlimb.

4 Botulinum toxin (A) -induced bone loss is associated with increased blood flow and reduced vascular bone porosity

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4.1 Abstract

Disuse-induced bone loss is a common consequence of spaceflight and prolonged bed rest. Intraosseous blood vessel volume and number is decreased in rodents after sciatic nerve resection as well as reduced femoral and tibial perfusion and blood flow to the femoral shaft and marrow after hindlimb unloading. However, it is unclear if alterations in blood flow contribute to botulinum toxin (BTX)-induced bone loss. The objective of this study was to assess patterns of tibial bone loss and alterations in blood flow in murine hindlimbs following BTX injection. We hypothesize that blood flow to the affected hindlimb will diminish along with bone mass and structure. Skeletally mature C57Bl/6J female were injected with BTX (n=15) or vehicle (n=14). Paralysis was confirmed using digit abduction, wire hang tests and activity analysis. In vivo microCT and ex vivo synchrotron tomography was used to assess bone mass, microstructure, (re)modeling, as well as vascular and lacunar porosity. Blood flow in the hindlimbs and cardiac structure/function was monitored by echocardiography. After three weeks, BTX-injected tibiae had 16% lower cortical thickness, and 66% lower trabecular bone volume fraction compared to baseline. Timelapse morphometry showed bone loss was predominantly at endocortical surfaces. Bone loss in the contralateral limb coincided with reduced rearing capability of BTX injected animals compared to vehicle controls. Vascular porosity thickness and surface area was reduced, but there was no change in lacunar properties due to BTX. In vivo ultrasound demonstrated increased velocity time integral for blood flow in femoral and popliteal arteries, but not in saphenous. BTX led to significant bone loss in hindlimbs, while increasing blood flow in femoral and popliteal arteries and decreasing vascular

porosity. The vascular response to BTX differs from what has been observed in other hindlimb unloading models, which could be attributed to neuromuscular dysfunction.

4.2 Introduction

Bone (re)modeling is a life-long process driven by the mechanical forces exerted on bone through gravity and muscular contractions via tendons and ligaments. In an altered gravitational environment such as microgravity, bone loss occurs in the absence of these mechanical forces.[1,2] Other conditions involving disuse include paralysis and long-term bed rest, which also cause decreases in bone mineral density.[3] Mechanical forces vital to bone homeostasis are sensed by osteocytes, which act as the primary mechanosensor in bone. Osteocytes, the most abundant cell in bone, are embedded in the bone tissue within lacunae. They orchestrate bone (re)modeling by signalling to bone-forming osteoblasts and bone-resorbing osteoclasts.[4] It is well accepted that interstitial fluid flow in the vast osteocyte lacunar canalicular network is essential for mechanosensing by osteocytes and for delivery of nutrients to bone cells driving the anabolic changes.[5] Additionally, bones are highly vascularized with multiple routes of entry/exit for blood and a complex network of trans-cortical vessels that interface with the interstitial fluids of bone.[6]

Blood is delivered to the hindlimb via a main conduit femoral artery, which bifurcates proximal to the knee into saphenous artery, supplying superficial musculature and tissue of the medial hindlimb, and popliteal artery, supplying the tibia and deeper tissues.[7] Blood flow in the marrow space drives interstitial fluids into the osteocyte lacunar canalicular network.[8] In the bone cortex, the blood vessels occupying the vascular porosity of bone serve as site of exchange for nutrients, oxygen, and waste products with

the surrounding osteocyte network.[8] Thus, adequate perfusion and flow of blood is essential for bone (re)modeling and reduced vascularization is reported in populations affected by disuse osteopenia and ageing.[9]

Multiple animal models which either remove ground reaction forces or eliminate muscular forces acting on bone seek to replicate the deteriorative musculoskeletal changes in disuse conditions. The most used is the hindlimb suspension model, developed by Morey-Holton at NASA, aims to mimic microgravity.[10] Other disuse models include cast immobilization, chemical denervation (botulinum toxin A, (BTX)), and surgical denervation to mimic paralysis and prolonged bed rest.[11] Numerous studies have shown the degree of bone loss is variable across these different models of disuse.[12] The advantage of using a BTX model is that it is non-invasive, well-controlled and provides an easy access for imaging the affected limb in vivo. Although the BTX model has often been used to examine bone loss, the effects on bone vascularity have not been investigated. In the skin, several studies have shown that Botox leads to improved perfusion and causes vasodilation in blood vessels.[13] Similarly, the reduced blood flow found after femoral artery occlusion of Sprague-Dawley rats was mitigated in a dose dependent fashion with BTX perivascular injections.[14] Likewise, direct injections of BTX to the femoral artery also resulted in significant vasodilation.[15] However, changes to bone structure and bone vascularity were not investigated. Other preclinical studies examined bone and blood flow in hindlimb suspension models in rats and mice. In 6-month-old male Sprague-Dawley rats, reduced femoral and tibial perfusion and blood flow to the femoral shaft and marrow were observed.[16] In 10-week-old male C57BL/6N mice, hindlimb-unloading was associated with reduction in bone formation and the number of type H

vessels.[17] In another method of unloading, spinal cord injury and sciatic nerve resection showed decreased intraosseous blood vessel volume and number in 9-week-old male C57BL/6 mice.[18] Thus, decreased vascular flow has been implicated as a potential contributing factor in disuse-induced bone loss, but whether this is also a factor in BTX models remains unclear.

Since blood flow drives bone interstitial fluid transport and pressure, and thus subsequent osteocyte mechanosensation, studies have tried to characterize osteocyte lacunar properties such as density, volume or porosity in models of muscle disuse. After space flight and in animals which underwent sciatic neurectomy, significant decreases in lacunar volume were detected.[19,20] While conversely, a more recent study found no changes in osteocyte lacunar density at room or thermoneutral temperatures in a hindlimb unloading model.[21] Alterations in the osteocyte lacunae following BTX injection in rats revealed conflicting results. Whereas Bach-Gansmo *et al*. found no difference in the osteocyte lacunar volume of BTX-injected versus control animals[22], Gatti *et al*. reported reduced osteocyte lacunar density in the BTX-injected group as well as higher vascular porosity.[23] The contrasting results may be due to different bone regions and/or the age of animal subjects under study.

In our study, we used a single BTX injection in the mouse hindlimb musculature to induce flaccid paralysis and quantified blood flow and bone loss. We hypothesized that the vasculature supplying the hindlimb and tibia would have decreased flow in arterioles in response to BTX-induced unloading. Further, we hypothesized changes in bone structure and reduced osteocyte lacunar and vascular porosity in the tibia would develop. The objectives of the study were to assess patterns of BTX-induced changes in (1) bone

morphology, (2) osteocyte lacunar properties, (3) blood flow in hindlimb arteries, and (4) morphology of blood vessels of the hindlimb and the intraosseous vascular porosity.

4.3 Methods

4.3.1 Animals

All animal use in this project was approved by the McGill Animal Compliance Office as well as the Facility Animal Care Committees at the Shriner's Hospital for Children (SHC) and Jewish General Hospital (JGH) (AUP#2020-8192). Twenty-nine, 26-week-old female C57BL/6 J mice (Jackson Laboratories, Bar Harbor, ME, USA) were acclimatized in the animal facility at SHC. Mice were housed together up to five animals per cage, with food and water access ad-libitum.

4.3.2 Experimental Design

All twenty-nine animals in this study were randomly assigned to either botulinum toxin (BTX) or vehicle injection group. A cohort of fifteen animals (BTX (8), vehicle (7)) were allocated to investigate bone (micro-CT imaging, synchrotron vascular and lacunar network imaging) and hindlimb vascular (ultrasonography of main arteries, contrast agent perfusion) changes. The right and left tibiae were assessed by *in vivo* microCT 3 days before BTX injection at SHC. After the *in vivo* microCT scan, mice were transported to the JGH for ultrasonographic imaging of blood flow. Transported animals were allowed 48 hours for acclimatization and then measurements for cardiac structure/function were performed on days -1, 9, and 19, and assessments of left and right hindlimb blood flow were performed immediately before the injection on day 0, and on days 2, 5, 7, 13, and 15 after the injection (Fig. 1A). Mice were then transported back to SHC on day 21 and *in*

vivo microCT scans of both tibiae were collected. Mice were then euthanized and perfused with the Microfil contrast agent (Flow Tek Inc., USA) via left ventricular perfusion. Whole hind limbs with the soft tissue intact were dissected and imaged using *ex-vivo* microCT. Tibia bones were dissected and preserved in 70% ethanol preparatory for imaging for lacunar network properties at a synchrotron facility (European Synchrotron Radiation Facility, Grenoble, France).

A separate cohort of fourteen animals (BTX (7), Vehicle (7)) remained housed at SHC animal facility for activity (Open Field confined locomotion behaviour) monitoring. Activity testing was performed before injection on day 0 and repeated on days 2, 3, 4, 8, 9, 10, 11, 12, 16, 18, 20 (Fig. 1A).

4.3.3 Botulinum Toxin Injections

Vacuum sealed BTX (Botox, Allergan, USA) was diluted in 10 ml of saline to a concentration of 10 U/ml. On day 0, the BTX cohort received a single injection of 2 units per 100-gram body weight divided equally between calf and quadricep muscle groups. The vehicle cohort received a saline injection using the same injection protocol.[24] Following injection, all animals were monitored for body weight and degree of paralysis daily during the first week then bi-daily for the remainder of the study. Degree of paralysis was evaluated using two tests (1) hindlimb abduction test, which involves inducing a startle response in mice by picking them up from the tail and observing the abduction of toes. Scoring was done based on a previously established grading system.[25] and (2) wire hang test, which assess the ability of animal to use injected limbs to hang from cage

wiring.[26] Scores from each test were combined to assess the onset and recovery from BTX-induced paralysis.

4.3.4 In vivo microCT imaging

In vivo microCT imaging (Skyscan 1276, Bruker; 70 kVp, 57 µA, 0.3° rotation step, 0.5 mm Al filter) was used to collect 8µm isometric voxel resolution images of tibiae at baseline (day -3) and immediately before euthanasia (day 21). Animals were anesthetized with isoflurane (2% in 0.6 L/min O_2) during scans and kept immobile using a custom 3D-printed mouse bed. Both tibiae were captured, and each scan took about 30mins. Tibia projection scans were 3D reconstructed using NRecon (Bruker, Belgium) software prior to analysis. The two volumes of interest included the tibial mid-diaphysis (extending 5% of the bone length at midline) and proximal tibial metaphysis (100 microns below the growth plate, extending 10% of the bone's length). For the cortical bone of the mid-diaphysis region a global threshold of 601 mgHA/cm^3 was used, and for the proximal tibial metaphysis a global threshold of 564.36 mgHA/cm^3 was used for cortical bone and 348.29 mgHA/cm^3 was used for trabeculae. Both regions were analyzed for bone morphometric parameters (bone volume fraction, BV/TV; cortical thickness, Ct.Th; marrow area, Ma.Ar; cortical area, Ct.Ar; trabecular thickness, Tb.Th; trabecular number, Tb.N; trabecular separation, Tb.Sp) using XamFlow software (V 1.8.8.0 Lucid Concepts AG, Zurich Switzerland). A custom workflow was created following a prior CTAn protocol used for calculating bone morphometric parameters.[27] Results were validated across the two software programs to ensure consistent results using a separate dataset. Cortical bone in both regions were analyzed, as well as trabecular bone present in the proximal metaphyseal region.

4.3.5 In vivo ultrasound imaging

Ultrasound acquisition was done using a 40 MHz transducer (VEVO-3100, VisualSonics) placed over a shaved hindlimb with the animal lying on bed parallel to the ground with a 0% tilt to avoid gravity induced fluid shifts.[28] Timepoints for acquisition included once prior to injection, with five subsequent measurements on days 2, 5, 7, 13, and 15 following injections. Animals were anesthetized (3% isoflurane, 2L/min O2) and an infrared lamp was used to maintain body temperature at approximately 37 °C. Additionally, heart rate was maintained at 500-550 beats per minute during acquisitions. Data on blood flow was collected for both injected (left) and non-injected limbs (right) of both BTX and vehicle groups. The acquisition of blood flow was done by placing the transducer above and parallel to the femoral, popliteal, and saphenous arteries. Each artery was assessed for velocity time integral (VTI), a measure of blood flow equivalent to the area under the velocity time curve of each pulse and denotes the distance travelled by blood within the artery. Other measurements derived from this curve include mean velocity (mm/s), mean gradient (mmHg), peak velocity (mm/s), peak gradient (mmHg), and femoral root diameter (mm). Additionally, echocardiography was done at baseline and repeated twice after BTX injection (day 9 and 19), (experimental time points shown in Figure 1.A).[29]

4.3.6 Microfil contrast agent

At the end of the experimental time (day 21), animals were euthanized using anaesthetic and CO2 overdose. Animals were immediately perfused using a peristaltic pump with 50mL of warm (37℃) heparinized saline (100 U heparin/mL) through the left ventricle to maintain vessel integrity and prevent blood clotting. This was followed by perfusion of 10% neutrally buffered formalin for fixation. Lastly, a 10 mL mixture of MicroFil yellow

contrast agent (Flow Tek Inc., USA) and curing agent was prepared and introduced to the vasculature as previously described.30 The contrast agent is lead-based and allows for the visualization of blood vessels using computed tomography. Following perfusion, cadavers were placed at 4°C overnight to allow for complete curing of contrast agent. Whole hindlimbs were dissected and stored in 70% ethanol. Dissected tibia with soft tissue intact were imaged using ex-vivo microCT (Skyscan 1272, 70 kVp, 138 µA, 0.4° rotation step, 0.05 mm Al filter) to collect 5µm isometric voxel resolution images of the entire mouse hindlimb using batch scanning. Reconstructed images were imported into XamFlow software (Lucid concepts, Zurich Switzerland) and a custom workflow was used to quantify the volume of Microfil filled vessels. This was done by using Otsu segmentation to select all bone and perfused vasculature as they are similar densities. The largest object was labeled and removed from (i.e. tibia and fibula) while maintaining the perfused vessels in the volume of interest. Any regions of bone that remain were interactively segmented out to leave behind only the Microfil filled vessels of the hindlimb. The total volume of the hindlimb was calculated by segmenting the soft tissue and bone from the background and filling any small gaps in the selected region. Analysis was done for the volume of the filled vasculature along the length of the tibia. Normalization of vessel volume was performed according to total hindlimb volume as BTX injected animals display significant deterioration of soft tissue volume.[31]

4.3.7 Activity monitoring

A 3D-camera mounted on an open field arena (Bioseb, Vitrolles, France) (40cm x 40cm x 40 cm) was used for capturing movement and rearing activity in the mice. Prior to acquisition, animals were acclimated in the Open Field boxes for one hour per day for two

weeks. Recordings were taken for a 15-minute period at approximately the same time of day. Animals were left to roam freely for 30 minutes before commencing recording to allow for dissipation of stress related to handling and change of environment. Data on total distance traveled, average speed were acquired from recordings at baseline (day -1) and daily afterwards for the first two weeks (Figure 1A). Additionally, the behavior of rearing (up righting of the animal) was assessed using the 3D functionality of the monitoring camera which counted the amount of rears performed in the 15-minute period.

4.3.8 MicroCT-based timelapse morphometry

The same two volumes of interest analyzed for the static microCT analysis were subsequently analyzed using timelapse histomorphometry to assess the surface area and volume of newly formed and resorbed bone during the 21-day period. For both regions, the day 21 image was registered using the Elastix image registration python library, integrated into the Xamflow software. Timelapse histomorphometry was performed using a previously defined MatLab script (https://github.com/BWillieLab/Timelapse-Morphometry) which was also integrated into XamFlow software.[32,33] Parameters analyzed per group include the erosion volume and surface fraction (EV/BV, ES/BS), and mineralizing volume and surface fraction (MV/BV, MS/BS).

4.3.9 Synchrotron radiation imaging of osteocyte lacunar and vascular porosity

Dissected hindlimbs were sealed in 70% ethanol vapour in 5ml Eppendorf tubes and stabilized using a dense foam containing excess ethanol. Samples were scanned at BM05 at the European Synchrotron Radiation Facility (Grenoble, France). Images were

acquired using an 82keV energy with a propagation distance of 1.4um at two volumes of interest, corresponding to those analyzed using microCT. Images were reconstructed using Tomwer (ESRF, Grenoble, France), with a phase retrieval delta beta ratio of 400 and a Munch ring removal algorithm. This resulted in images with an isometric voxel size of 1.4um. Samples were then processed using an XamFlow workflow as previously described.[34] The bone was divided into the 4 primary sub-regions corresponding to the anterior, medial, lateral and posterior quadrants, based on strain patterns during habitual loading that occur due to the large curvature of murine tibiae.[35] The following lacunar properties were measured: number density, angle, volume and surface area as well as various shape parameters for the full volume of interest as well as sub-regions. The following vascular canal properties were measured: volume, surface area, number, separation as well as porosity. In addition, we also measured total larger porosity, and validated basic microCT measurements as previously defined.

4.3.10 Statistical analysis

Statistical analysis was performed using SAS software (version 9.4, SAS Institute Inc.). The effect of BTX injection (BTX, Vehicle) and effect on limbs (Injected, Contralateral) and the interaction between these two terms was assessed with two-way ANOVA for values in all conducted analysis with two time points. More specifically, ultrasound data was assessed using a three-way repeated measure ANOVA to assess the effect of injection (BTX, Vehicle), limb (Injected, Contralateral), and repeated days (0 to 15) as well as presence of interactions among these variables. For all ANOVA analyses Kramer-Tukey post hoc tests were performed for comparisons among groups. Paired T-tests were used to compare injected and contralateral limbs within BTX-injected mice. A p-

value of <0.05 was considered significant. Data is presented as mean ± standard deviation in tables and graphs. In the main text, the data is presented as percent changes, which were calculated as (*time point – baseline) / baseline * 100*, the mean ± standard deviation values for all reported parameters are provided in Supplementary Tables 1-9.

4.4 Results

4.4.1 BTX induced local paralysis reduced rearing in the mice

To assess and quantify the degree of BTX-induced paralysis, we combined digit abduction and wire hang test scores (Fig. 1B). All vehicle injected animals scored 0 on both digit abduction and wire hang, while the BTX-injected group scored between 3 and 7 on paralysis tests (Fig. 1B), indicating that peak paralysis occurred between days 2 and 6 post injection. After this, affected animals slowly regained function. By day 21 only one BTX-injected animal recovered fully (score 0) with others scoring an average of 2.7 (Fig 1B). Using a 3D-mounted camera to assess movement, we did not observe significant differences in the total distance travelled (Fig. 1C left), activity duration (Fig. 1C middle) or mean speed (not shown) between BTX injected and vehicle injected animals at baseline, peak paralysis (days 2 to 6), or recovery (day 7 to 21). Interestingly, rearing activity was significantly reduced in BTX injected animals (Fig. 1C right). This lack of rearing was evident at peak paralysis and persisted during the recovery period. After 21 days, BTX-injected animals lost an average of 11.7 ± 3.3 % of their body weight, while vehicle-injected animals gained 1.7 ± 3.9 %.

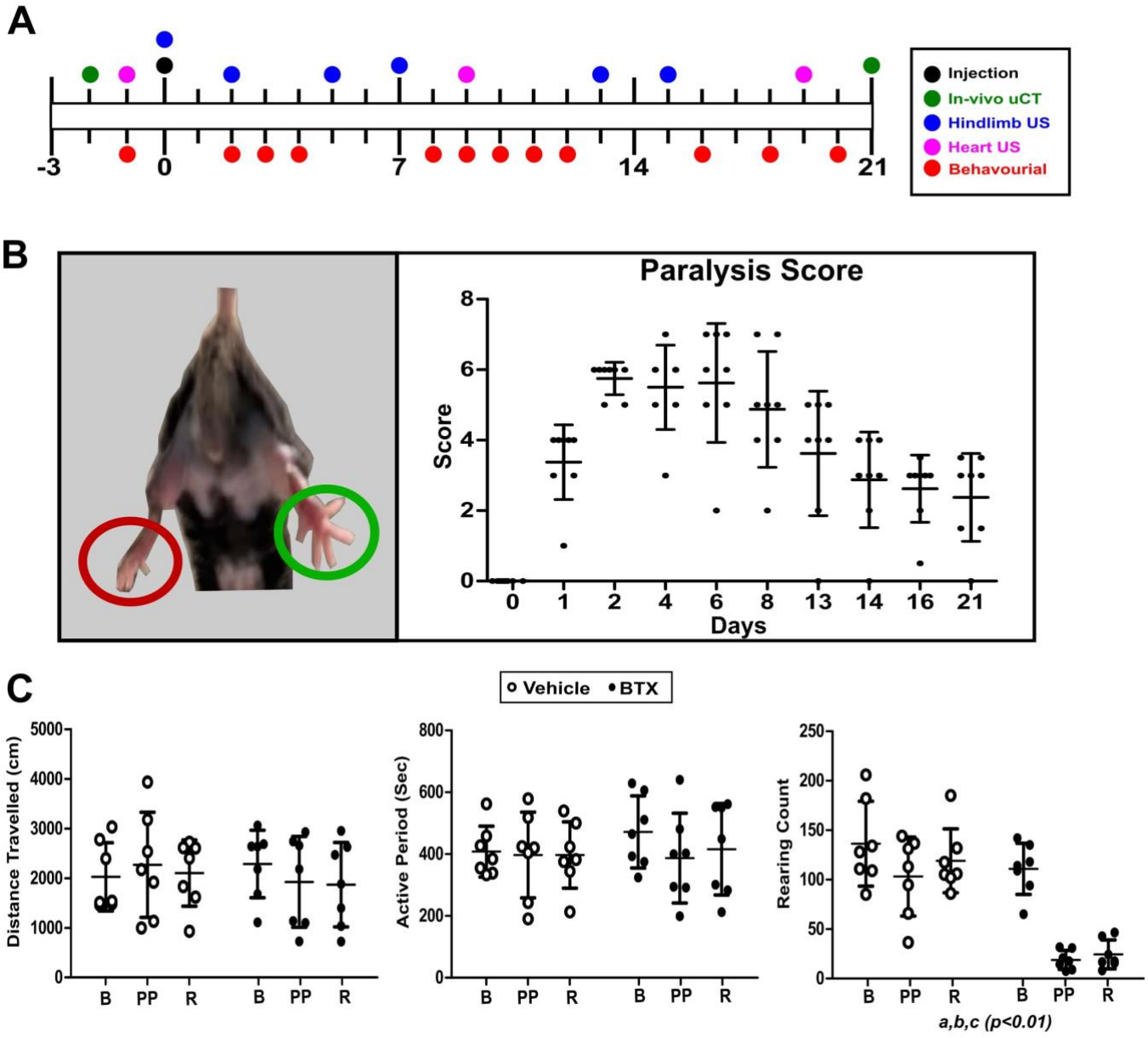


Figure 5. BTX-induced paralysis and changes in locomotion. (A) Experimental timeline denoting all procedures carried out over the 3-week unloading period. (B) Paralysis score of BTX-injected animals. B, left: an example of the digit abduction test with lack of abduction in BTX-injected (red circle) limb and contralateral (green circle) limb, B, right: average combined scores for digit abduction and wire hanging in BTX-injected animals, all vehicle-injected animals scored 0. (C) Open field assessment of average distance travelled, active period, and rearing counts before the injection [B], at peak paralysis [PP, days 2-6], and at recovery period [R, day 8-20] in BTX- (black circles) and vehicle- (open circles) injected mice.

4.4.2 BTX induces extensive tibial bone loss in hindlimbs

To examine the effect of BTX-induced paralysis on tibial bone structure, injected and contralateral tibias in BTX- and vehicle-injected animals were scanned using in vivo microCT (8 μm voxel size). Changes between baseline (3 days before the injection) and end of experiment (21 days after the injection) were used for analysis and graphical presentation. At the mid-diaphysis in the BTX group, the injected and contralateral limbs exhibited a loss of 15% and 9% in cortical area to tissue area respectively, 17% and 8% cortical bone thickness (Fig 2A). In contrast to these losses, marrow area increased 23% in injected and 15 % in contralateral limb of BTX group (Fig. 2A). The injected and contralateral limbs of the vehicle group exhibited little change at 21 days from baseline (Fig. 2A). Similarly, at the cortical bone of the proximal metaphysis region of BTX injected and contralateral limbs, Ct.Ar/Tt.Ar was reduced by 11% and 9% and Ma.Ar increased by 12% and 8% in injected and contralateral BTX limbs respectively, while these measures were stable in the vehicle group (Fig. 2B). An effect of treatment (BTX versus Vehicle; ANOVA term: a) was evident in all cortical parameters assessed at both tibial regions (a, $p < 0.01$) by two-way ANOVA, and Tukey-Kramer post-hoc analysis showed significant differences between BTX- and vehicle-injected animals (Fig. 2A, B).

Trabecular bone in proximal tibia metaphysis was adversely affected by BTX injection into hindlimb musculature. Trabecular bone volume per tissue volume (Tb.BV/TV) was reduced at 21 days compared to baseline in BTX group in both injected and contralateral limbs by 66% and 51%, while bone loss was also measured in the vehicle group, due to physiological aging (Fig. 2C). Changes in trabecular properties were most evident with trabecular thickness (Tb.Th), which decreased in BTX group by 9% and

4% in injected and contralateral limbs) but increased in vehicle group limbs by10 and 13%. Trabecular separation (Tb.Sp) and trabecular number (Tb.N) were not different between groups (Fig. 2C). Thus, morphological assessment of tibiae in BTX injected mice demonstrates bone loss in both injected and contralateral limbs, causing deterioration at both diaphysis and metaphysis regions of the bone.

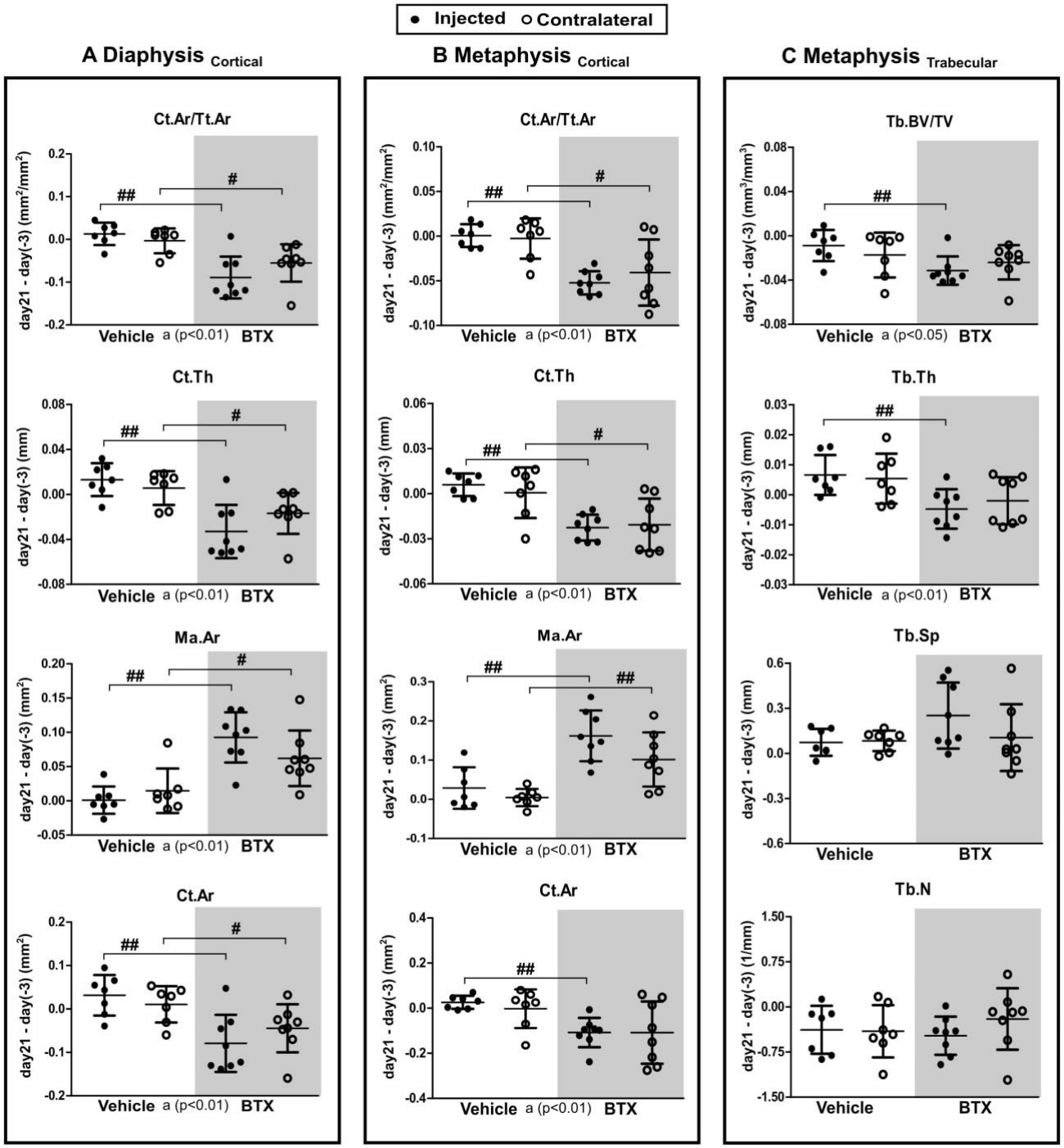


Figure 6. BTX-induced bone loss. (A) Tibial mid-diaphysis, and (B) trabecular proximal metaphysis, and (C) cortical proximal metaphysis regions were imaged 3 days before and 21 days after the BTX injection. Cortical parameters (Ct.Ar/Tt.Ar, Ct.Th, Ma.Ar, Ct.Ar) and Trabecular parameters (Tb.BV/TV, Tb.Th, Tb.Sp, Tb.N) were assessed and the changes between timepoints in injected (black circles) and contralateral (white circles) bones in BTX (open area) and vehicle-injected (shaded area) mice were calculated. Data presented as means ± SD; ANOVA main effects: (a) Treatment; (b) Limb; (c) Interaction. # (# $p<0.05$; ## $p<0.01$) indicates significance based on Tukey-Kramer post-hoc test.

4.4.3 BTX-induced bone loss occurred endocortically

To spatially characterize BTX-induced bone loss, microCT-based timelapse morphometry was performed by registering the reconstructed scans from baseline and day 21 and assessing changes on periosteal and endocortical surfaces of the tibia. At the mid-diaphysis, bone resorption covered most of the endocortical surface in the BTX injected limb, while both resorption and formation were evident at the periosteal surface (Fig 3A). The endocortical surface of BTX group demonstrated greater eroding volume, normalized to bone volume (EV/BV) which was 10-fold greater than EV/BV in vehicle injected limbs and eroding surface normalized to bone surface (ES/BS) was 3 fold the ES/BS in vehicle injected limb (Fig. 3A). In the BTX group contralateral limbs, EV/BV and ES/BS was nearly double that of vehicle contralateral (Fig. 3A). Meanwhile, Bone formation at the mid-diaphysis was almost non-existent on the endocortical surface in the BTX-injected limb, mineralizing volume fraction (MV/BV) and mineralizing surface fraction (MS/BS) was 96% less than that observed in vehicle injected limb and suppressed in the contralateral limb compared to its counterpart (80% less than vehicle contralateral limb) (Fig. 3A).

Cortical bone of the proximal metaphysis demonstrated more erosive changes in the BTX group (Supp. Fig. 1). At the endocortical surface, BTX group EV/BV was 5-fold greater than vehicle injected limb and ES/BS was 1.3-fold higher compared to vehicle injected limb, meanwhile mineralization was suppressed compared to vehicle animals (Supp. Fig. 1). On the periosteal surface, erosion was greater in the BTX group than vehicle, while the mineralization indices were similar (Fig. 3B). Trabecular bone erosion in the proximal metaphysis, Tb.EV/BV was also greater in the BTX group compared to the vehicle group (Fig. 3B). When assessed by two-way ANOVA, an effect of treatment

(BTX vs Vehicle; ANOVA term: a) was evident for all endocortical parameters at both tibial regions, as well as for bone resorption at the periosteal surface of the proximal metaphysis ($p < 0.05$), with Tukey-Kramer post-hoc analysis showing significant differences between BTX- and vehicle-injected animals (Fig. 3A, B). Taken together, 3D-time-lapse morphometry demonstrates that bone deterioration occurred predominantly at the endocortical surface of the tibia.

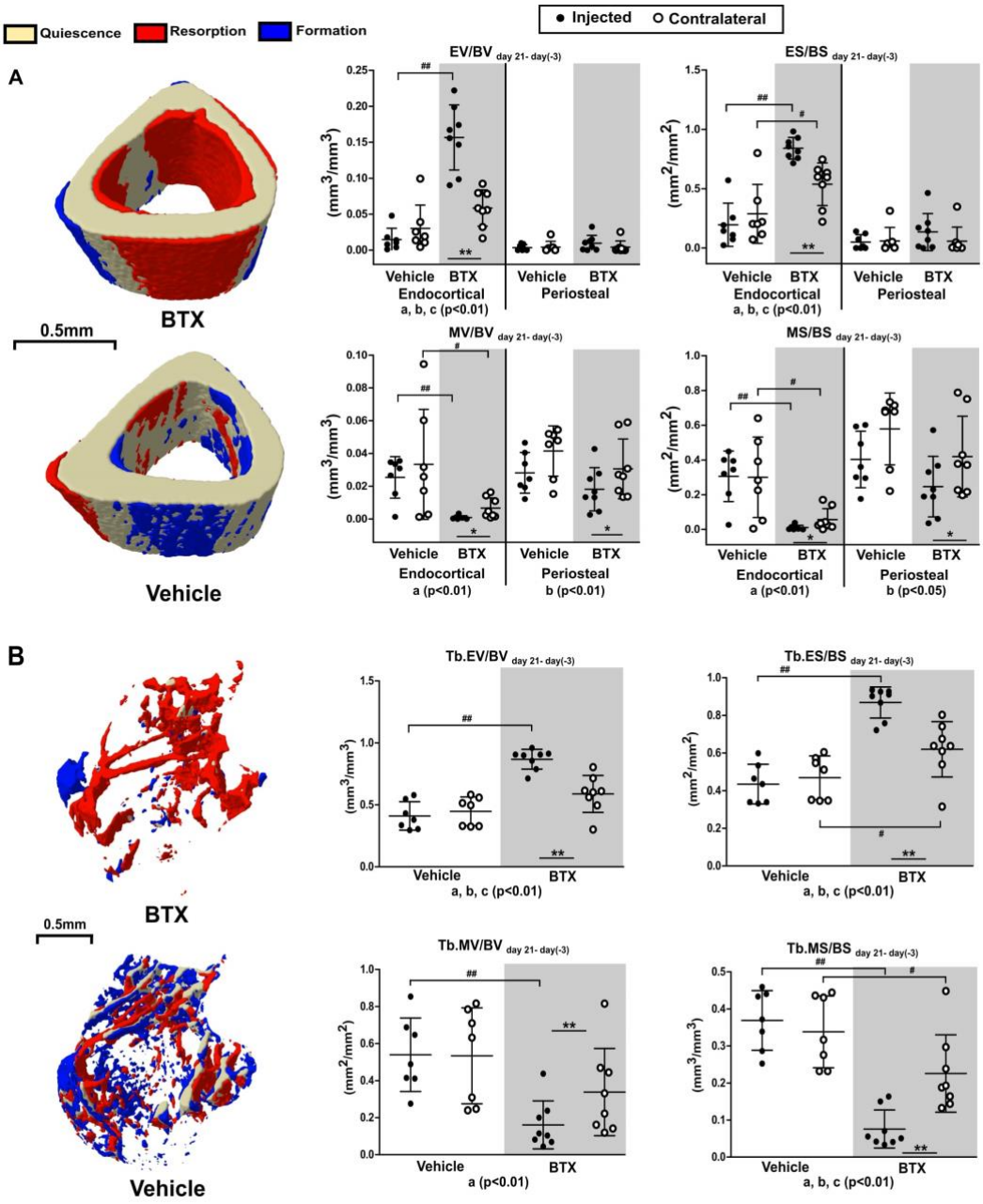


Figure 7. 3D-registered micro-CT based time-lapse. Cortical bone of (A) the tibial mid-diaphysis and (B) trabecular proximal metaphysis morphometry. Illustration (left); bone (re)modeling in BTX-injected and vehicle-injected limbs, quiescent (yellow), formed (blue), and resorbed (red) bone on both endocortical and periosteal surfaces. Graphs (right); endocortical and periosteal eroding volume fraction (EV/BV), eroding bone surface (ES/BS), and mineralizing volume fraction (MV/BV), mineralizing bone surface (MS/BS) for injected (black circles) and contralateral (white circles) bones in BTX- (open area) and vehicle-injected (shaded area) animals. Data presented as means ± SD; ANOVA main effects: (a) Treatment; (b) Limb; (c) Interaction. # (# $p<0.05$; ## $p<0.01$) indicates significance based on Tukey-Kramer post-hoc test. Asterisk indicates significance based on differences between injected and contralateral limbs (paired t-test) (* $p<0.05$; ** $p<0.01$)

4.4.4 Blood flow alterations after BTX-injection

To assess temporal patterns in the mouse hindlimb blood flow, in vivo ultrasound imaging was conducted prior to injection and on days 2, 5, 7 and 13 and 15 following injections. Three main arteries (femoral, saphenous, popliteal) were imaged at approximately the same location along the vessel's length in all animals (Fig. 4A). Once located, multiple pulse waves were recorded, and flow parameters measured (Fig. 4A). Data from various days were combined into three periods of paralysis identified in behavioral study (Fig. 1B), and labels are representative of baseline (day 0), peak paralysis (day 2, 5, 7), and recovery (day 13 and 15).

The femoral artery is the main conduit artery supplying the hindlimb. At baseline, there were no differences in the VTI in the femoral artery in the limbs of BTX (Injected 6.12 ± 0.66 mm, Contralateral 6.89 ± 1.62 mm) and vehicle (Injected 6.57 ±1.07 mm, Contralateral 7.07 ± 0.83mm) injected groups (Fig. 4B). During peak paralysis (days 2, 5, and 7 post-injection), BTX injected animals exhibited increased VTI in the injected limb by 20% from baseline and decreased VTI in the contralateral limb by 29% from baseline with significant differences between both BTX limbs ($p<0.01$). In contrast, the VTI in the femoral artery in vehicle animals decreased in both vehicle limbs from baseline by 9% and 20% in injected and contralateral limbs respectively (Fig. 4B). During recovery period (day 13, 15 post-injection), femoral artery VTIs in BTX animals remained significantly lower in the contralateral limb (-35% difference from baseline) but normalized in the injected limb (3% difference from baseline). The vehicle group limbs during this recovery period were 16-25% lower than their baseline values (Fig. 4B). Similar alterations over time were noted in other parameters of femoral flow, including peak velocity (PV), which

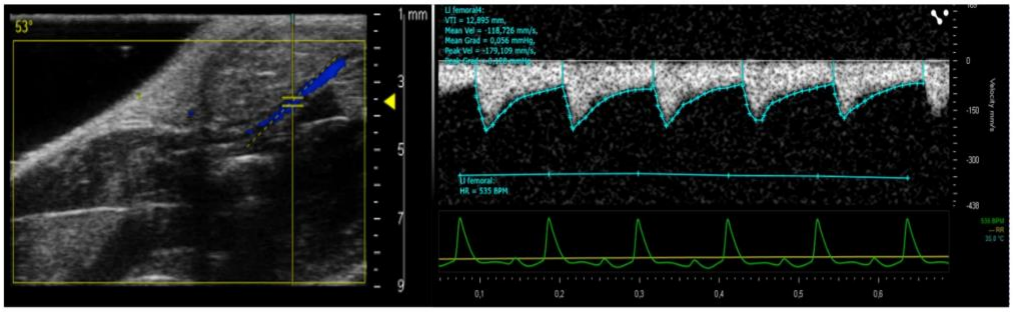
was elevated by 17% in BTX-injected limb compared to a 20% decrease from baseline in contralateral limb at peak paralysis. In vehicle-injected animals, PV at baseline, peak paralysis, and recovery did not show differences between injected and contralateral (Fig. 4B). When assessed by three-way repeated measures ANOVA, there was a significant effect of the period (ANOVA term: c) on which measures are taken (c, $p<0.05$). Additionally, interactions were found between the limb (Injected Vs. Contralateral; ANOVA term: b) and period (ANOVA term: c) (b*c, $p<0.01$) and between the treatment and limb (a*b, $p<0.05$) in both VTI and PV datasets, however post-hoc Tukey-Kramer analysis identified significant differences between BTX and vehicle animals for VTI but not PV. Paired T-test analysis demonstrated significant differences between injected and contralateral limbs in the BTX group, but not the vehicle group (Fig. 4B). Therefore, within the BTX injected animal, the injected limb saw elevated blood flow opposed by a lower flow in the contralateral limb, meanwhile, both vehicle limbs exhibited similar flow over time.

The popliteal and saphenous arteries arise from femoral bifurcation. The popliteal artery is responsible for deep muscle and tibial bone supply, while saphenous artery supplies superficial medial musculature and skin of the hindlimb.[7] At baseline, VTI and PV were similar in BTX and vehicle groups in popliteal (Fig. 4C) and saphenous (Fig. 4D) arteries. During peak paralysis, the effect of BTX injection was evident for VTI, but not for PV in popliteal artery, while these parameters were similar in saphenous artery. At recovery, the VTI in popliteal and saphenous arteries were affected in BTX-injected animals. Specifically, in the popliteal artery like the femoral artery, VTI was lower in the contralateral limb than the injected limb of BTX group (Fig. 4C). While in the saphenous

artery, the contralateral limb had a higher VTI than the injected limb (Fig. 4D). When assessed by three-way repeated measures ANOVA, there was a significant effect of day/period on which measures are taken (c, $p<0.01$) for all parameters. In the popliteal VTI dataset, a significant effect of limb as well as interaction between limb and time were evident (b, b*c, $p<0.05$). Paired T-test showed significant differences between injected and contralateral limbs in the BTX group at peak paralysis (VTI) and recovery (VTI, PV). The blood flow in both arteries seemingly did not respond similarly to BTX as the femoral artery section, with higher flow continuing from femoral to popliteal arteries.

Additionally, both groups underwent echocardiography prior to injection and at two timepoints after injection (day 9, 19). At baseline there were no differences between groups, except for left ventricular mass (LVmass), which was higher in vehicle group ($p<0.05$) (Supp. Fig. 2). At day 9, there were no differences between groups, except for relative wall thickness (RWT) which was higher in the vehicle group (0.533 ± 0.056) compared to BTX group (0.478 ± 0.049) ($p<0.05$) and the left ventricular posterior wall (LVPW) which was higher in the vehicle group (0.849 ± 0.08) compared to BTX group (0.746 ± 0.08) ($p<0.05$) (Supplemental Fig. 2). At day 19, changes were seen at the level of aorta, where aortic velocity time integral (AoVTI), aortic cardiac output (AoCO), and cardiac index were all higher in the BTX group ($p<0.05$) (Supplemental Fig. 2). Additionally, at this time point LVmass was no longer statistically significant between groups but remained different when indexed to tibia length.

A



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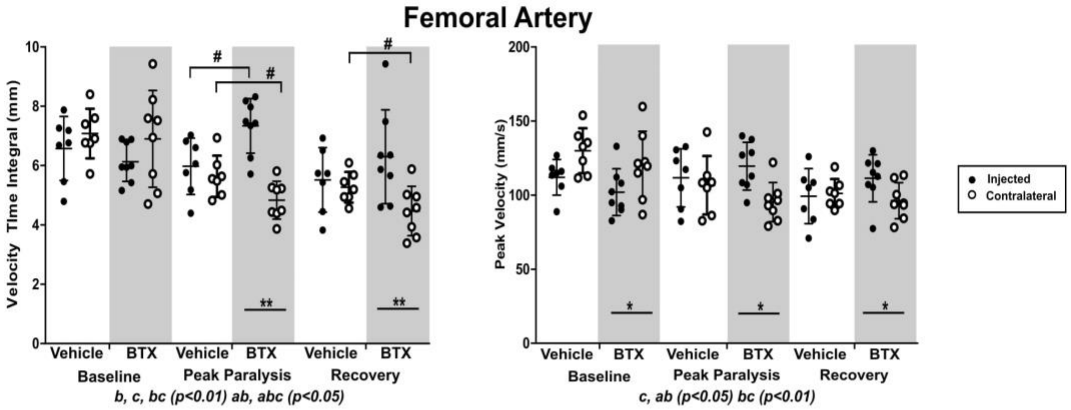
Femoral Artery
Velocity Time Integral (mm)
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BTX
Baseline
Peak Paralysis
Recovery
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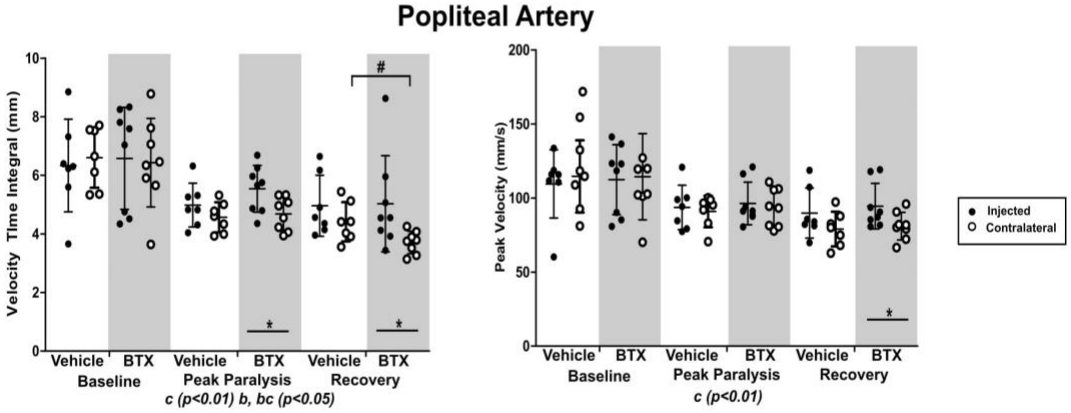
c, ab (p<0.05) bc (p<0.01)

C


Popliteal Artery
Velocity Time Integral (mm)
Peak Velocity (mm/s)
Vehicle
BTX
Baseline
Peak Paralysis
Recovery
Injected
Contralateral
c (p<0.01) b, bc (p<0.05)
c (p<0.01)

D

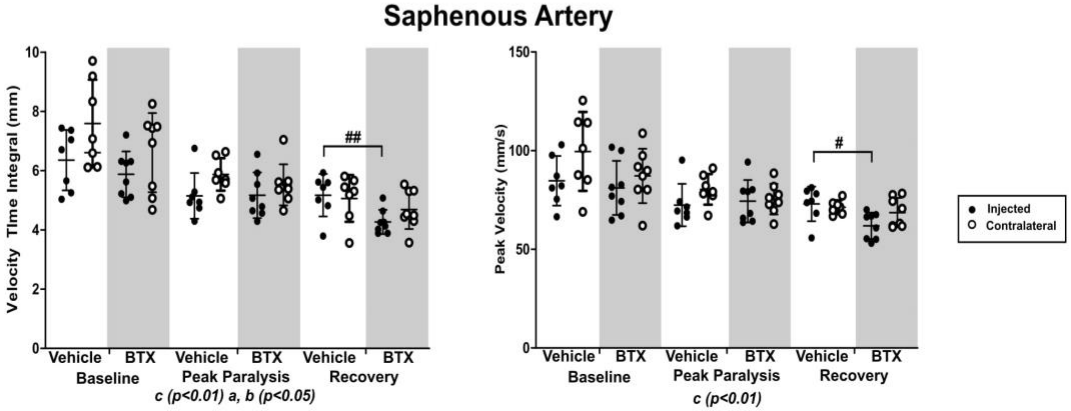

Saphenous Artery
Velocity Time Integral (mm)
Peak Velocity (mm/s)
Vehicle
BTX
Baseline
Peak Paralysis
Recovery
Injected
Contralateral
c (p<0.01) a, b (p<0.05)
c (p<0.01)

Figure 8. Blood flow of main hindlimb arteries. (A) location of femoral artery (blue) in animal hindlimb as visualized under ultrasound probe (40MHz) and the corresponding flow averaged over multiple cycles. (B) The velocity time integral (VTI) and peak velocity (PV) of blood in three main arteries (femoral, saphenous, and popliteal) during distinct unloading windows (baseline, peak paralysis, and recovery) for injected (black circles) and contralateral (white circles) bones in BTX- (open area) and vehicle-injected (shaded area) animals. Data presented as means ± SD; ANOVA main effects: (a) Treatment; (b) Limb; (c) Period. # (# $p<0.05$; ## $p<0.01$) indicates significance based on Tukey-Kramer post-hoc test. Asterisk indicates significance based on differences between injected and contralateral limbs (paired t-test) (* $p<0.05$; ** $p<0.01$)

4.4.5 Vessel architecture in the hindlimb

Lead-based contrast agent (MicroFil) was injected at euthanasia to fill the vascular network of the hindlimbs. The tissue between the proximal and distal ends of the tibia was included in the volume of interest, and all MicroFil-perfused vessels between these points were assessed with microCT in BTX- and vehicle-injected limbs (Fig. 5A). Twenty-one days after injection, the blood vessel volume in BTX-injected limbs was 31% lower than in the vehicle-injected limbs ($p<0.05$) (Fig 5B). However, the limb's total volume (including hard and soft tissues) in the same region of interest was 43% less in BTX-injected limb than in vehicle-injected limbs ($p<0.01$) (Fig 5C). Normalized vessel volume per total hindlimb tissue volume was similar between the groups (Fig 5D).

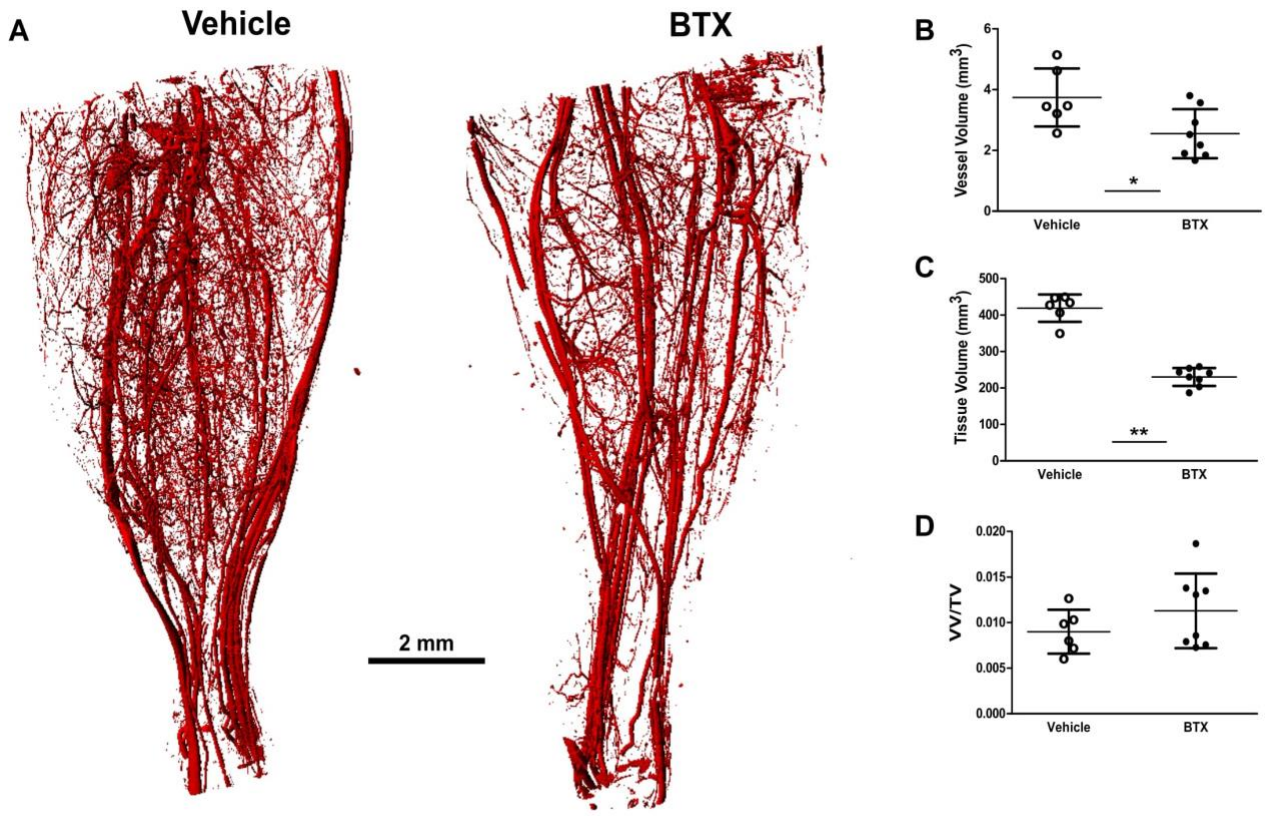


Figure 9. Contrast agent infused vessels.(A) Reconstructed 3D representation of microfil filled vessels in hindlimb of vehicle and BTX-injected limbs. (B) The volume of filled vessels, total tissues, and ratio of both in BTX-injected (black circles) and vehicle-injected (white circles) hindlimbs. Data presented as means ± SD; Asterisk indicates significance based on differences between groups (unpaired t-test) (* $p<0.05$; ** $p<0.01$*)*

4.4.6 Lacunar and vascular bone porosity

To visualize bone osteocyte lacunar and vascular porosity porosities, BTX and vehicle injected limbs were imaged using synchrotron radiation at the mid-diaphysis and proximal metaphysis regions of BTX- and vehicle- injected tibia. In each region, the lacunar and vascular porosities were analyzed for the full volume of bone and among four quadrants (anterior, posterior, medial, and lateral) (Fig. 6A, Fig. 7A). Lacunar properties of the whole volume and four quadrants did not differ between BTX- and vehicle-injected limbs in the mid-diaphysis (Fig. 6B). In contrast, vascular surface area to bone volume (VSa/BV) and

vascular volume to bone volume (VV/BV) were significantly altered after BTX injection. In the mid-diaphysis, BTX injected limbs had a lower VSa/BV by 22% and lower VV/BV by 24% compared to vehicle group's ($p<0.05$) (Fig. 6C). This difference was most pronounced in the posterior quadrant of the mid-diaphysis, which displayed diminished vascular porosity in BTX injected animals (50% lower than vehicle injected limb posterior quadrant) (Fig. 6C).

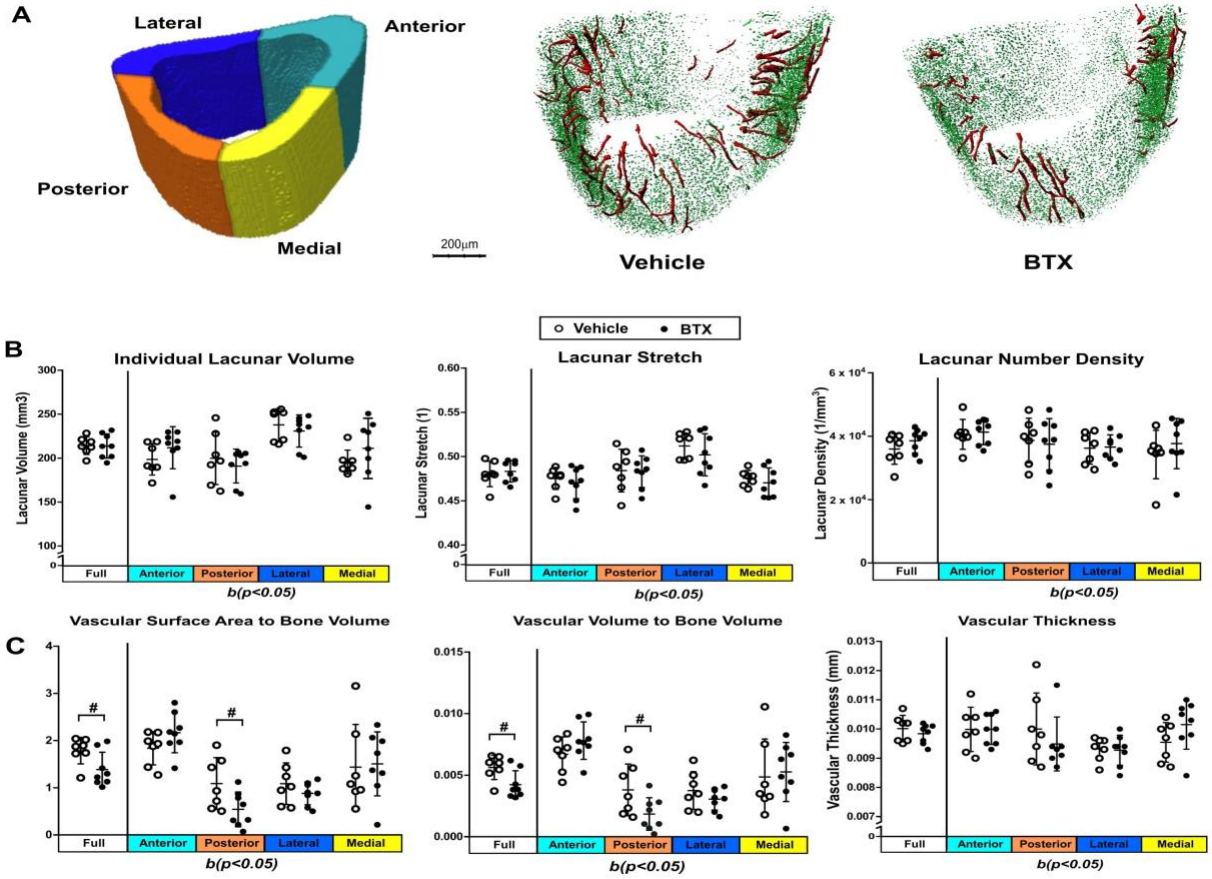


Figure 10. Osteocyte lacunar and vascular network in tibia mid-diaphysis. (A) Cortical bone segmentation into four quadrants (Anterior, Posterior, Lateral, Medial) from the center of mass of each sample and 3-D reconstruction of osteocytes (green) and vascular pores (red). (B) Lacunar properties (volume, stretch, density) between vehicle-injected (open circle) and BTX- (closed circle) injected animals in full volume and 4 color-coded quadrants. (C) Comparison of vascular pore properties (surface area to bone area, volume to bone area, thickness) in vehicle-injected (open circle) and BTX-injected (closed circle) animals in full volume and 4 color-coded quadrants. Data presented as means ± SD; ANOVA main effects: (a) Treatment; (b) Quadrant; (c) Interaction. # (# $p<0.05$; ## $p<0.01$) indicates significance based on Tukey-Kramer post-hoc test.

In the proximal metaphysis, whole lacunar stretch was higher in the BTX group compared to vehicle group ($p<0.05$) while lacunar density and volume did not differ (Fig. 7B). Within the quadrants, the anterior quadrant was different between groups with regards to lacunar stretch and lacunar number density (Fig. 7B). Vascular porosity parameters, specifically VV/BV and VSa/BV, were lower by 21% and 17% respectively in BTX-injected animals compared to vehicle animals (Fig. 7C). Similarly, quadrant specific changes were observed in the anterior quadrant of the proximal metaphysis (Fig. 7C).

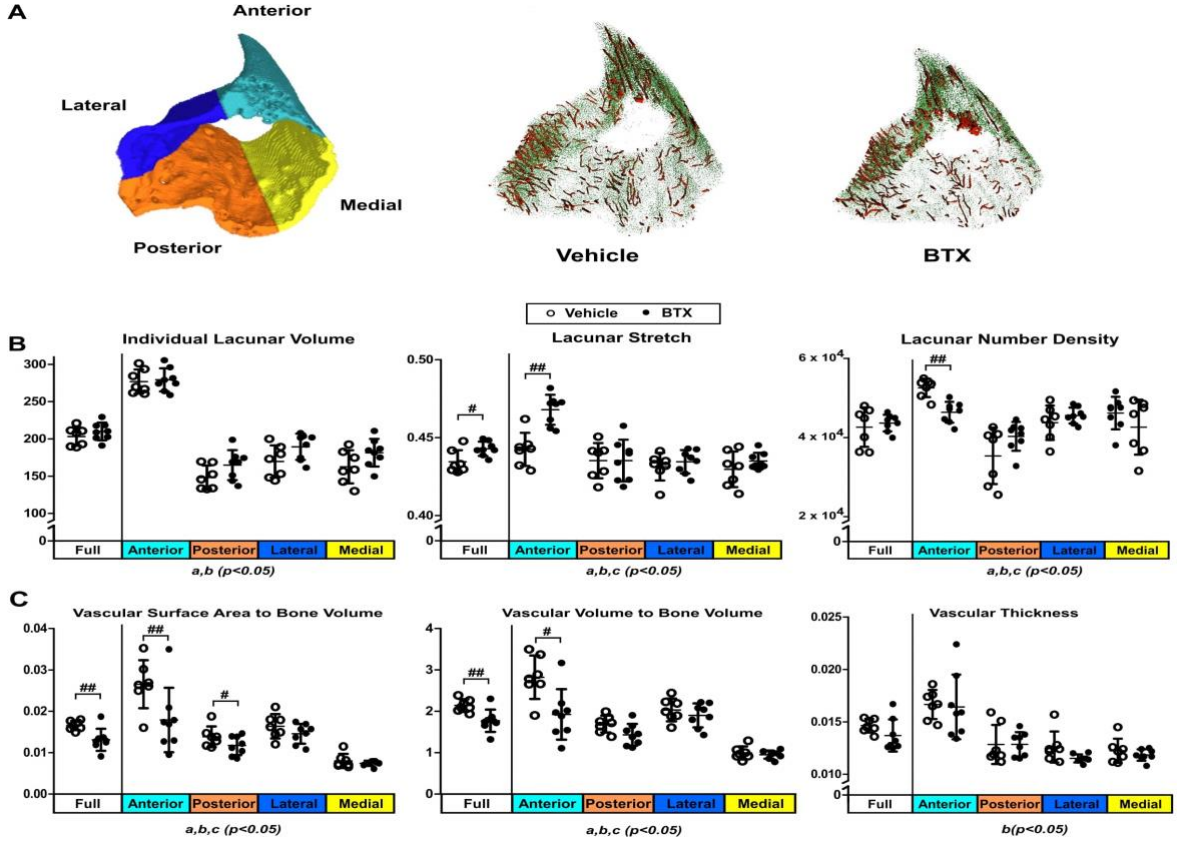


Figure 11. Osteocyte lacunar and vascular network in tibia proximal metaphysis. (A) Cortical bone segmentation into four quadrants (Anterior, Posterior, Lateral, Medial) from the center of mass of each sample and 3-D reconstruction of osteocytes (green) and vascular pores (red) in a vehicle and BTX sample. (B) Lacunar properties (volume, stretch, density) between vehicle-injected (open circle) and BTX-injected (closed circle) mice in full volume and 4 color-coded quadrants. (C) Comparison of vascular pore properties (surface area to bone area, volume to bone area, thickness) in vehicle-injected (open circle) and BTX-injected (closed circle) mice in full volume and 4 color-coded quadrants. Data presented as means ± SD; ANOVA main effects: (a) Treatment; (b) Quadrant; (c) Interaction. # (# $p<0.05$; ## $p<0.01$) indicates significance based on Tukey-Kramer post-hoc test.

4.5 Discussion

Our study aimed to understand vascular and bone adaptation in the BTX model of mechanical unloading. We observed distinct vascular and bone changes in the BTX-injected and contralateral limbs of skeletally mature (26-week-old) female mice. In the BTX-injected limb, we identified a significant negative impact on tibila bone structure 3-weeks post-injection. Simultaneously, the BTX-injection was associated with an increase in blood flow in the femoral artery and to a smaller degree in the popliteal artery. Despite this increased flow in the main hindlimb arteries of BTX-injected limbs, bone vascular porosity was significantly diminished suggesting deterioration of bone microvasculature. We noted that BTX-induced surface (endocortical > periosteal) and site (metaphysis > diaphysis) dependant changes in bone morphology as well as quadrant (e.g. anterior, posterior, medial, lateral) dependant changes in osteocyte network of bone. In BTX-injected mice, the contralateral, non-injected limb also demonstrated changes in bone and blood flow. This limb demonstrated a temporal decrease of blood flow in the main hindlimb arteries and concurrent bone loss over the 3-week experiment. Examining the physiologic mechanical loading environment in both groups, we noted similar running speed and distance for BTX-injected and control mice, which may suggest higher mechanical loads on the contralateral limb during ambulation due to paralysis of the injected limb. Importantly, BTX-injected mice demonstrated sustained impairment in behavioural rearing, reducing greatly the frequency of vertical ground forces in both limbs. This suggests that rearing behaviour may play a major role in bone biomechanics in murine models. In summary, BTX-induced paralysis resulted in skeletal and vascular

changes in the injected limb due to neuromuscular blockade and altered activity patterns, while in the contralateral limb changes may be due to altered activity patterns alone.

In the BTX injected animal we observe a body weight loss of more than 10% from baseline. Previous studies observing similar weight loss hypothesize that it may be related to acute starvation accompanied by metabolic acidosis in BTX-injected animals.[26] In our study the lack of rearing may also contribute to the inability to access food commonly found above wiring near roof of the cage which requires animals to rear in order to reach the food. In animals losing significant weight fortified nutrition provided at the floor of the cage was able to mitigate some of this rapid weight loss. The observed bone degradation was induced by BTX-mediated blockage of the release of neurotransmitters from peripheral nerve endings in muscle and the subsequent paralysis.[36] A recent scoping review outlining the extent of osteosarcopenia in BTX-treated animal models suggests a comparable trabecular BV/TV reduction (46-80%) in studies with similar experimental design as ours.[37] Several studies directly compared BTX-induced bone loss to other models of mechanical unloading. BTX-induced bone loss was found to be higher than control in the hindlimb suspension model, and combining both methods resulted in a more deleterious effect.[26] Comparison of different models of paralysis suggests higher bone loss following BTX injection than peripheral nerve injury group [31], but similar bone deterioration following complete sciatic neurectomies as in BTX model.[12] Thus, compared to ambulatory impairment only, disrupting neuronal signalling appears to have an additional negative effect on bone structure, which corresponds to the degree of impairment.

Bone loss in the contralateral limb was described in other BTX studies carried out with similar conditions, although the cause was not elucidated.[26,38] We suggest that this effect is due to the reduced rearing ability of BTX-injected animals compared to vehicle controls. Rearing, the frequent up-righting of rodents in their cage, likely accounts for higher vertical loads on the hindlimb. Though this activity was not previously examined in BTX studies, differences between injected and contralateral limb ground reaction forces have been assessed.[31,39] One study reported no change in the contralateral ground reaction force following BTX to compensate for reduced ground reaction force in the injected limb.[31] Another study measured increased peak ground reaction force in the contralateral limb at 5 days post injection, which returned to control levels by day 10.[39] These studies demonstrated sustained reduction of ground reaction forces for BTX-injected limb, but either no change[31] or a relatively small transient increase [39] in the ground reaction forces for the contralateral limb. The lack of rearing behaviour could also affect the orthostatic response in these animals and subsequently blood flow. In physiological conditions, up-righting results in blood redistribution to the lower part of the body followed by active adaptation through vasoconstriction.[40] After actual and simulated spaceflight, multiple studies have shown a decreased vasoconstrictor responsiveness in hindlimb vessels.[41,42] Thus, changes in biomechanical environment and blood flow associated with rearing may potentially underlie the observed bone loss in the contralateral limbs of BTX-injected animals. The effect of rearing alone needs to be further investigated in its role in bone and vascular maintenance.

We report an increase in femoral artery blood flow in BTX-injected limbs, which was also detected in the popliteal artery to a lesser extent, but not in the saphenous artery.

This finding is consistent with previous studies demonstrating that BTX prevents the release of norepinephrine from presynaptic nerve endings in vascular smooth muscle thereby leading to dose-dependent vasodilation.[43] The differences in degree of changes in three arteries may be explained by localized action of BTX. Based on previous experimental and clinical use, the toxin's spread is dose dependant with 15-30 mm diffusion with 1U injections.[44] Given that our site of injection, the quadriceps muscle, is directly adjacent to the femoral artery while the bifurcation of this artery is at the distal end of the muscle [7,45], this likely explains the higher effect on flow in the femoral artery. Since vasculature flow is an important component of bone homeostasis and a prerequisite for new bone formation[46], it would be expected that the vasculature would adapt to diminished mechanical loading. Several studies found that the decrease in blood flow following hindlimb suspension is due to attenuated endothelial-dependant vasodilation in the femoral artery.[47,48] Similarly, after sciatic nerve injury, blood flow in the femur is reduced at 2 weeks.[49] In conditions of microgravity (i.e. spaceflight) associated with reduced hindlimb bone mass, a cephalic fluid shift exists resulting in less blood flow to the hindlimbs.[50] In contrast to these models, BTX-injection increased blood flow in the femoral artery, which, nonetheless, did not mitigate BTX-induced muscle atrophy or bone loss.

Although blood flow was increased in the femoral artery, we observed reduced bone vascular porosity in BTX-injected limbs, while the changes in lacuna-canalicular network were minimal. Currently, multiple reports provide conflicting results in adaptation of lacunar and vascular pores in BTX-injected hindlimbs. In long term follow up of BTX-injected rats, femoral lacunar density and volume did not differ between injected,

contralateral, and control limbs.[22] Another study assessing the proximal metaphysis in rats, lacunar density in the whole cortex was reduced after BTX injection.[23] These differences could be explained by the different regions analysed across studies, age of the animals and target muscles of both studies. Our study to our knowledge is the first to explore these lacunar alterations in the mouse model and no one has previously examined vascular porosity of bone after BTX injection. A potential increase in bone vascular porosity after hindlimb suspension or sciatic nerve resection could further explain the coincident decrease in blood flow for these conditions, in contrast to what we have observed after BTX injection: decreased bone vascular porosity and increased blood flow. However, further studies are needed to characterize vascular porosity after hindlimb suspension or sciatic nerve resection. These data suggest complex changes in blood flow and vascularization following BTX injection, supporting the importance of crosstalk between neuronal signalling and mechanical forces in bone maintenance.

Our study demonstrates that the BTX model presents with unique combination of changes in muscular, bone and vascular environment of hind limbs. We report unique macro- and micro-vascular adaptation in BTX-injected limbs. Other experimental models demonstrate bone loss following interventions that include mechanical unloading, including hind limb suspension, peripheral nerve injury or neurectomy, casting and BTX injection. Comparing the degree and timing of changes in different physiological compartments observed in these models will allow reconstructing the pathological sequelae leading to bone loss, thus providing novel approaches to developing countermeasures for spaceflight crew and patients affected by paralysis.

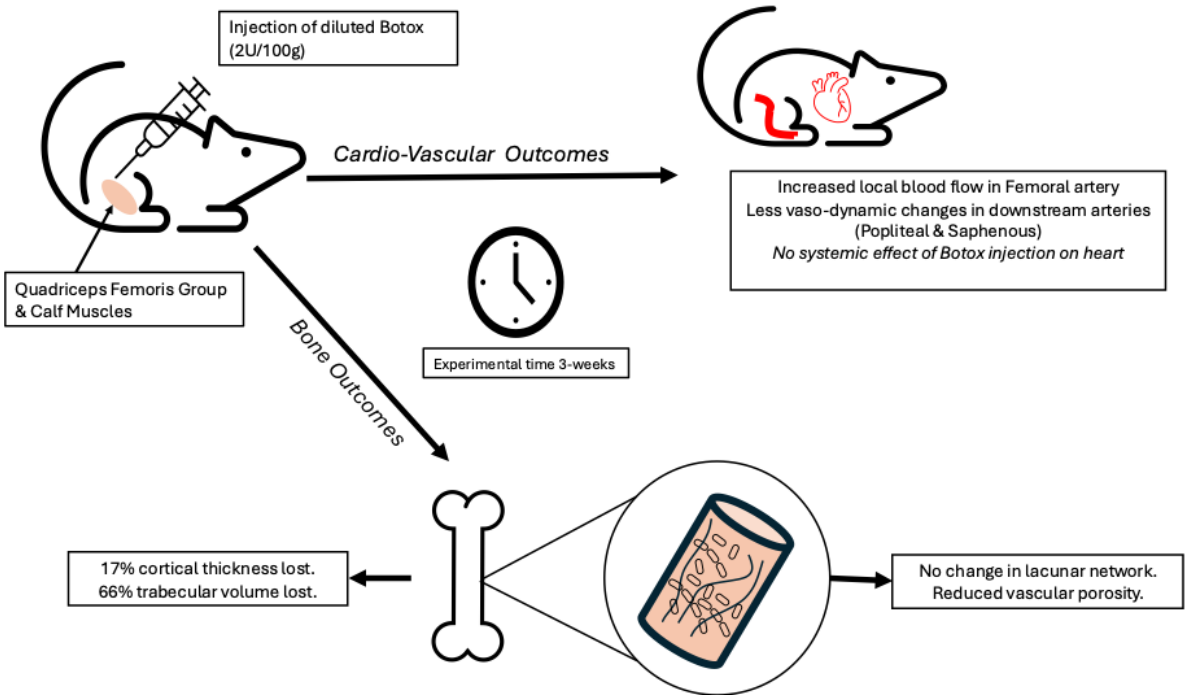


Figure 12. Graphical summary of both bone- and cardio-vascular- related outcomes in BTX-injected animals at 3-weeks post-injection.

4.6 Acknowledgements

We are grateful to our ultrasonography technician, Veronique Michaud (Lady Davis Institute for Medical Research, Jewish General Hospital, Montréal, Canada) for ultrasound acquisition of hindlimb and cardiovascular blood flow and data procurement throughout the experiment.

4.7 Conflict of Interests

None of the authors have any perceived or actual conflicts of interest to report.

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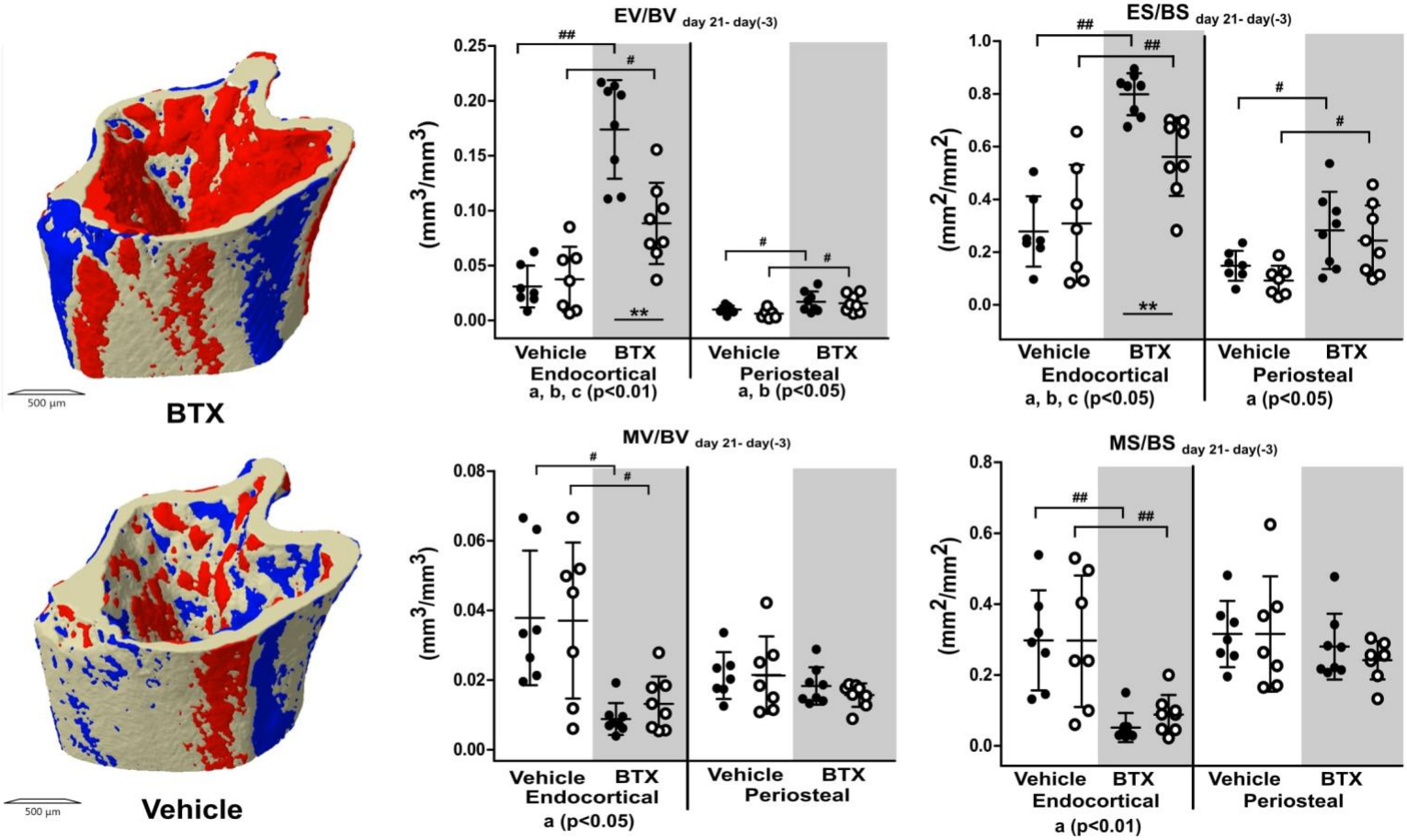
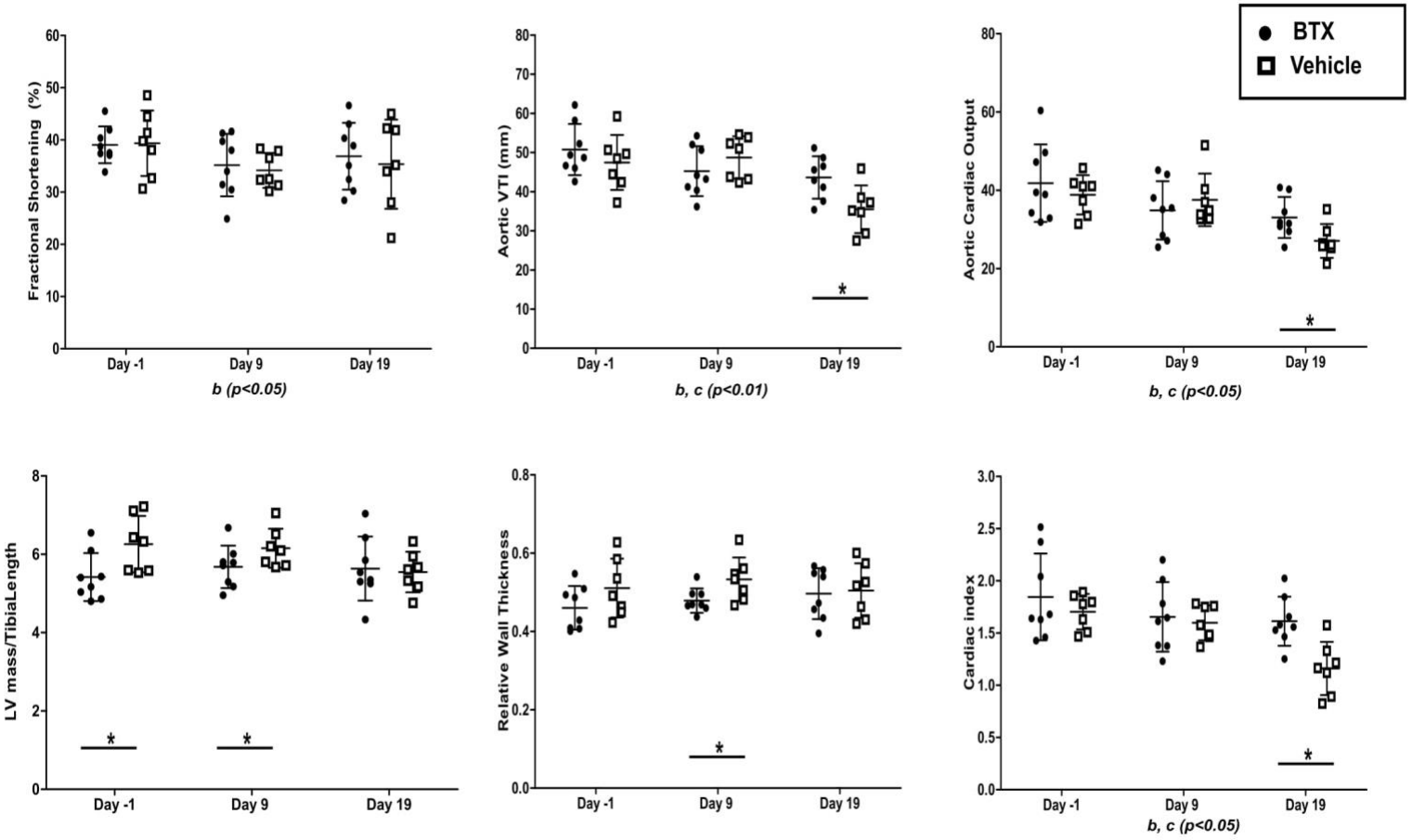
4.9 Supplementary material



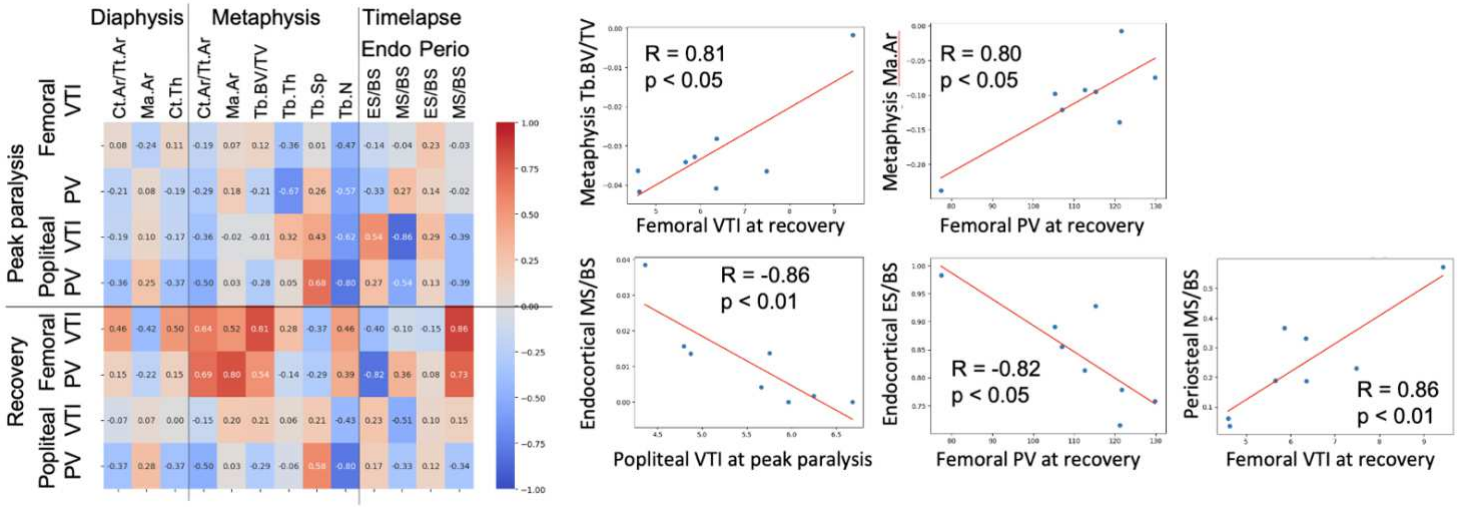
12 Supplemental Figure 1: 3D timelapse of cortical proximal metaphysis. Showing the erosive and formative changes in this region between both groups. Illustration (left); bone (re)modeling in BTX-injected and vehicle-injected limbs, quiescent (yellow), formed (blue), and resorbed (red) bone on both endocortical and periosteal surfaces. Graphs (right); endocortical and periosteal eroding volume fraction (EV/BV), eroding bone surface (ES/BS), and mineralizing volume fraction (MV/BV), mineralizing bone surface (MS/BS) for injected (black circles) and contralateral (white circles) bones in BTX- (open area) and vehicle-injected (shaded area) animals. Data presented as means ± SD; ANOVA main effects: (a) Treatment; (b) Limb; (c) Interaction. # (# p<0.05; ## p<0.01) indicates significance based on Tukey-Kramer post-hoc test. Asterisk indicates significance based on differences between injected and contralateral limbs (paired t-test) (* p<0.05; ** p<0.01)



13 Supplemental Figure 2: Echocardiography in both vehicle and BTX groups. Changes in heart echocardiography from baseline (day -1), midpoint (day 9), and end (day 19) between BTX- and vehicle-injected animals. Outcomes analyzed include aortic (Aortic VTI, Aortic Cardiac Output, Cardiac Index), pulmonary (VTI), left ventricle (LV mass, LV mass indexed to tibia length), relative wall thickness, and fractional shortening. ANOVA terms: (a) Treatment; (b) Time; (c) Interaction. Asterisk indicate significance in unpaired t-test between groups (* $p<0.05$; ** $p<0.01$)

Bridging Statement

In Chapter 3 and 4, I demonstrate that neuromuscular dysfunction can predispose to bone loss. In Chapter 4, I also present data showing the complex vascular response in the mouse hindlimb arteries following BTX injection. The application of toxin in injected quadricep muscle group induced higher femoral artery flow in the week following injection but did not elicit a similar response in downstream artery, suggesting a local effect of the toxin. In addition, the BTX injection resulted in reduced microvasculature structure of the bone blood vessels and in musculature. Importantly, investigating the correlation between bone and vascular alterations demonstrates that changes in vascular flow correlate with changes in bone structure and cellular activities (Figure below). This led me to hypothesize that in individuals receiving BTX in muscles of mastication changes in vascular flow precede and may predict changes bone morphology. In the following chapter we establish a clinical study to monitor the BTX-induced vascular changes in humans with TMD, and correlate them with changes of mandibular bone structures.



Legend: Correlation analysis of vascular outcomes at various time periods (peak paralysis, recovery) and location (femoral, popliteal) with bone outcomes at various regions (metaphysis, diaphysis) and surfaces (endosteal, periosteal). Presented as heatmap (left) of significantly altered outcomes and significant correlations (right).

5 Effects of botulinum toxin A on circulation and bone health in myofascial temporomandibular disorders: protocol for a prospective cohort study.

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Keywords: Botulinum toxin, Temporomandibular disorders, Doppler ultrasonography, Cone-beam computed tomography, Electromyography

Manuscript in preparation

5.1 Abstract

Temporomandibular disorders are complex conditions with a need for multimodal management approaches. Botulinum toxin is currently an off-label management option for myofascial temporomandibular disorders. In order to establish sound guidelines for use of this toxin in temporomandibular disorders, we must comprehensively investigate its therapeutic and adverse effects. With substantial literature currently available on the therapeutic effects of botulinum toxins, we focus our efforts in this study to address adverse effects to biological tissues. We hypothesize that muscle paralysis caused by botulinum toxin will have a deleterious effect on bone structure in the temporomandibular joint. This change may be accompanied by alterations in other tissues involved in the biomechanical maintenance of bone such as vasculature and muscles. We propose an observational prospective cohort study for individuals with myofascial temporomandibular disorders. A group of 50 individuals anticipated to receive their first botulinum toxin injection will be recruited to the study. Imaging of bone (CBCT), vasculature (doppler ultrasound), and muscles (electromyography) will be conducted at baseline, 2-4 weeks, and one year post injection. Additionally, demographic and pain-associated disability data will be collected for reporting and exploratory analysis. Student's t-test will be conducted on data at timepoints to establish statistical significance form baseline values. Correlation analysis will be conducted to assess whether vascular alterations correlate with the amount of exhibited bone changes in individuals. Ultimately, this cohort study will address the gaps in knowledge previously identified in studies assessing bony changes in individuals receiving botulinum toxin in the orofacial region. This study will also provide

comprehensive and valuable data for future studies into biological alterations with toxin use and for developing guidelines in clinical practice.

5.2 Background

Temporomandibular disorders (TMDs) are a group of painful joint disorders associated with the hard tissue components (arthralgia), the muscles of mastication (myalgia), and/or headaches affecting 5 to 12% of the general population [1, 2]. Muscle paralysis agents, such as botulinum toxin A (BTX), are used as an off-label treatment for myofascial type TMD pain [3]. While muscle paralysis agents may be able to reduce myofascial TMD associated pain, they also reduce functional loading (unloading) on bones leading to deterioration [4]. The long term effects of repeated injections of muscle paralysis agents on musculoskeletal health in the head and neck are unknown.

Vascular supply and oxygenation of muscles of mastication in TMD patients is compromised compared to control individuals [5]. Vasculature is an integral component to bone remodeling and has been associated with unloading induced bone loss [6]. Bone is a slow adapting tissue which could take up to 9 months for a single bone remodeling event to occur [7]. During this time multiple BTX injection could theoretically be injected to improve TMD associated pain. Identifying mediators and correlations for unloading induced bone loss can help identify patients at risk of long term deterioration with repeated injections.

Multiple muscles are involved in applying mechanical forces to the disc and joint area. Specifically, two large muscles that close the mandible are the masseter and temporalis muscles [8]. When these muscles contract they apply mechanical loads, within

physiological limits these loads are important for keeping the TMJ region healthy [9]. Pathological overloading can be destructive to tissues and contribute to multiple disorders of the joint such as disc dislocation [10]. On the other hand, under loading of tissues, such as the use of soft diets to reduce masticatory forces, leads to negative impact on jaw bone metabolism [11].

Multiple systematic reviews and RCTs have investigated the therapeutic effect of BTX in painful TMD conditions [12, 13]. Findings currently indicate conflicting results on BTX efficacy with drawbacks such as quality of evidence and heterogeneity, limiting interpretation of results [14, 15]. Understandably the primary outcome for most studies was pain reduction usually measured by a visual analog scale, the long term direct or indirect effects on mandibular bone, muscles or blood supply were not investigated. This is important given that the effects of BTX are transient and additional injections over a period of time (years) may be needed. While little is known about the effect of BTX injections on human bone structure [16], in experimental animals BTX injection in muscles of mastication leads to bone loss with similar dosing (BTX unit per gram of muscle weight) between animal models and human studies of TMJs [17].

To our knowledge, no study has previously investigated the vascular response to BTX injections in the head and neck region. Additionally, tissue level response of bone to BTX induced flaccid paralysis remains an understudied area [16, 18]. Understanding these responses is important to optimize treatment of TMDs with BTX (doses, injection intervals) and to mitigate any potential deleterious effects of the toxin on surrounding tissues. The research question proposed in this study is “In individuals with myofascial TMD, does BTX injection lead to decreased bone structure after one year from baseline

which correlates with vascular adaptation?" We hypothesize that individuals receiving BTX in the masseter and/or temporal muscles for TMDs will have reduced bone structure in the mandibular condyle after 12 months as well as reduced muscle activity and vascular supply at various midway timepoints.

5.3 Methods

5.3.1 Study design

The study will be conducted in accordance with the ethical principles stated in the Declaration of Helsinki (2013) and was approved by the McGill University Institutional Review board (#23-08-008). This study is a prospective, single cohort, observational study in an outpatient clinic setting at a university affiliated hospital (Montreal General Hospital, McGill University Health Center, Montreal, Canada). During the timeline of the study, each participant will be asked to attend three appointments, and each visit should not exceed 1-1.5 hour excluding the consent process and time allocated to standard care procedures. These appointments include, 1) baseline measurement collection, 2) Follow up for vascular and muscle measures (2-4 weeks following injection), and 3) Follow up for bone measures (12 months following injection).

5.3.2 Study population

Inclusion – All adults 21 years and older will be given the opportunity to participate, both male and females regardless of gender, ethnicity, social status, and/or income. Participants presenting to the orofacial pain clinic at the McGill University Health Center

(MUHC) with a positive diagnosis for TMD with myofascial involvement and have been prescribed a first time BTX treatment regimen will be included in this study.

Exclusion – In the following situations potential participants will be excluded: history of previous BTX injections in muscles of mastication, as potential cumulative effects of repeated injections is unaddressed. History of uncontrolled hypertension, temporal vasculitis, or other vascular diseases affecting blood flow. Participants with bone metabolic disorders such as osteoporosis, osteomalacia, hyperparathyroidism that affect can affect bone morphology. Participants that during the past year have taken medication altering blood flow, or bone metabolism such as bisphosphonates, antiresorptive medication. Prior or anticipated surgical intervention for participants TMD. History of jaw facture or reconstructive surgery of the maxillofacial complex. Participants missing all molar teeth or are completely edentulous.

5.3.3 Sample size

Based on the combined outcomes of previous datasets investigating bone differences in humans receiving BTX [18] and the primary outcome of interest in our study (i.e. cortical bone thickness) a power analysis was conducted to determine the appropriate sample size. We found that in previous studies the response within each subject group was normally distributed with standard error 1.39%. If the true difference in the experimental means from baseline to follow up time is 6.34% difference and assuming a Type I error equal to 5%, and a Type II error of 20%, this study will need to recruit 50 participants to be able to reject the null hypothesis. Given the chance that there will be dropouts,

researchers will consider the admission of more participants on a rolling basis past the initial recruitment period.

5.3.4 Screening and recruitment

Individuals attending the outpatient orofacial pain clinic, who have previously consented to use of their data for research purposes, will have their medical history reviewed by clinician and research team to identify individuals fulfilling the inclusion criteria previously described. Following which potential participants will be contacted via phone to inquire about their interest in joining the study. Pre-screened individuals with upcoming appointments for BTX injections will be contacted within the first 6 months of the study and informed of the possibility to partake in this study. Should pre-screened individuals express interest in participating, study staff will verify that they meet the inclusion/exclusion criteria. Following verification, they will be provided with electronic copies of the study consent form to allow them enough time to review all aspects of the study.

5.3.5 Study instruments

Cone beam computed tomography (CBCT) – CBCT has recently emerged as a cost- and dose- effective method of imaging osseous structures of the orofacial region in comparison to traditional CT methods. [19] This study will use an iCat FLX CBCT machine to collect scans of temporomandibular joint region. Images will be saved in Digital Imaging and Communications in Medicine (DICOM) format. CBCT scans will be collected at baseline and 12 months following injection. Regions of interest will include both right and left mandibular condyle, articular eminence and will be reconstructed in planes parallel

and perpendicular to the long axis of each condyle. Cortical bone will be segmented from trabecular regions using a custom-made script in XamFlow software (https://github.com/BWillieLab/TMJAnalysis). The following parameters will be measured in 3D: mean cortical bone thickness (primary outcome), condylar bone volume, joint space volume, trabecular thickness, trabecular number, and trabecular separation. [20] Raw values will be exported into a spreadsheet and percent differences from baseline calculated for each participant. Potential issues may arise from quality of CBCT resolution that may hinder analysis of trabecular bone. To mitigate this a flood-fill operation to eliminate trabecular porosity or fractal analysis may alternatively be carried out [30, 31].

Ultrasound color doppler – Ultrasound technology represents a non-invasive method for imaging musculature as well as blood vessel and nerve structures in the orofacial region. [21] Specifically, doppler mode can quantify the flow of blood in specific arteries and veins. A portable ultrasound machine (Vscan air CL, GE) will be used by trained personnel to assess the diameter and blood flow in the superficial temporal artery and the masseteric branch of the facial artery as previously described. [22, 23] These measurements will be collected while the participant's blood pressure is monitored to minimize bias in outcome. Ultrasound transducer will be set at angles according to direction of blood flow and in a longitudinal orientation to best assess blood flow. [24] Recording will provide data for the peak systolic velocity, end diastolic velocity, time-averaged average velocity, and flow volume by measuring vessel diameter. [25] The measurements will be repeated twice to assess repeatability with recording at rest for 30 seconds and at maximum voluntary contraction 30 seconds with 5 minutes of rest in between.

Surface electromyography (sEMG) – sEMG is a commonly used validated tool in measuring muscle activity. It can detect electrical potential arising from contracting muscle. [26] Using double sided tape, sEMG sensors will be placed bilaterally on the masseter and anterior temporalis, such that the sensor is parallel to the muscle fibers and positioned over the belly of the muscle. Measurements will be collected for 3 cycles of maximal voluntary clenching and rest (5 seconds each) for a total of 30 seconds of recording. This will be repeated with 3 cycles of maximum clenching using a cotton roll as a comparator with good inter- and intra-individual repeatability [27]. During the recordings noise will be kept to a minimum in the room and participants will be instructed to avoid sudden movements of the head or jaw. Using the raw sEMG amplitude vs time data we will extract relevant parameters such as maximum root mean square (RMS) normalized to maximum voluntary contraction and measures of asymmetry using a cotton roll and in intercuspal position.

Questionnaires – Participants will be asked to complete a questionnaire inquiring about a participant's history of previous BTX use (in muscles other than the masseter and temporalis) as systemic and/or cumulative dose effect have not been explored in previous studies, but case report has previously reported serious adverse effects on bone structures with excessive dosing. [28] Three orofacial pain questionnaires from the DC/TMD axis I/II (symptoms questionnaire (14 items), chronic pain scale (8 items), and GAD-7 (7 items)) will be used for exploratory analysis for correlations between disease severity, pain, and psychological factors (e.g. anxiety) with main outcomes [2]. Additionally, a questionnaire on demographics such as age, sex, and gender based on current recommendations [29] and this information will be collected from individuals.

Women have been shown to have higher prevalence of myogenous TMDs [30]. This study is currently not powered to assess those differences in sex or gender, but information will be collected and reported for future consideration.

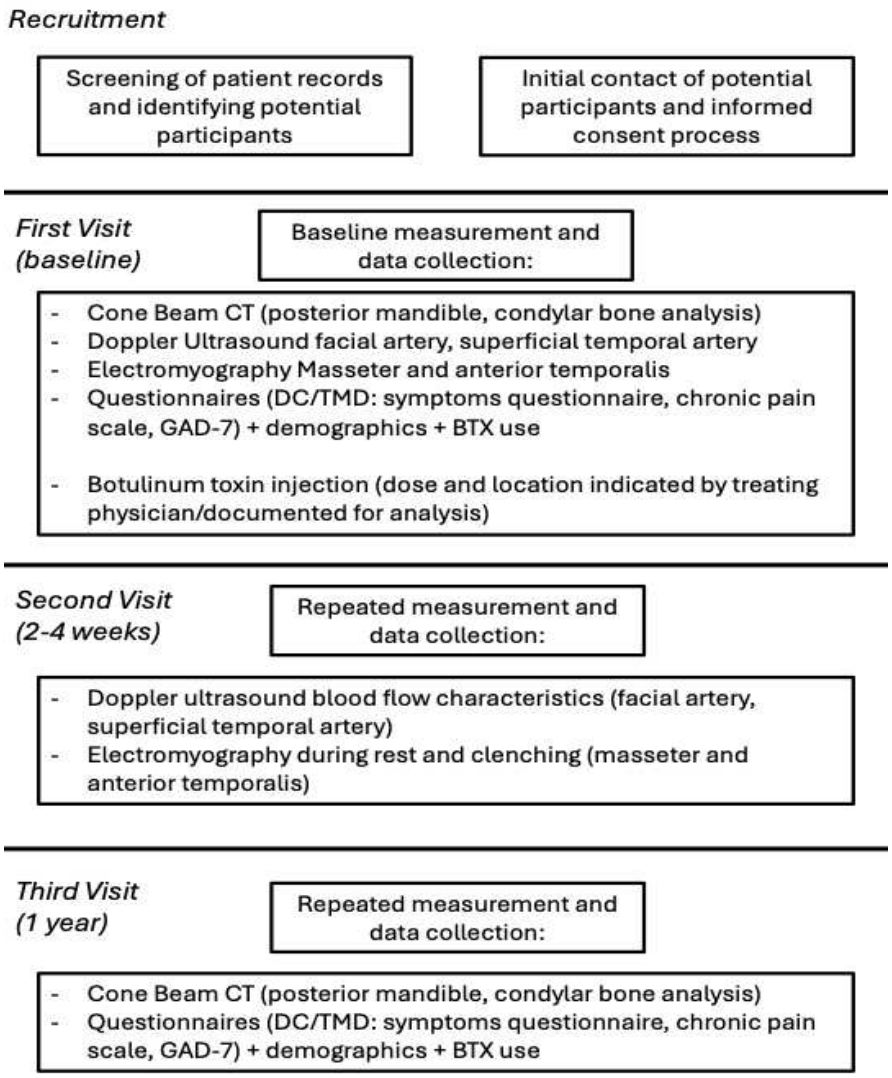

Figure 14. Flowchart of study process.

5.3.6 Data management

Participants will be given a unique code that does not directly or indirectly identify their personal information. This code will be used on all forms of paper and digital files created

throughout the study. Digitization of all questionnaire and paper records will be done and stored on an institution-approved cloud service. No physical records will exist outside the participant's file at the outpatient clinic in the Montreal General Hospital. Data related to the study participants will be retained for 7 years.

5.3.7 Statistical analysis

The primary outcome of the study is percent difference in cortical thickness from baseline to 1 year. Secondary outcomes in the study include changes in other bone morphometry parameters, muscle activity and blood flow over time, and pain-related outcomes. All data will be presented as mean and standard deviation for each datapoint with multiple repetitions. Paired student T-test will be used to assess statistical significance in post-injection data as compared to baseline measure. Similar analysis will be done for both the average RMS from sEMG and blood flow parameters from doppler ultrasound at both time points. A correlation analysis will be conducted with the CBCT data and the doppler/sEMG data to identify if shorter term changes in blood flow or muscle activity can predict the changes that happen in bone in our cohort. Additionally, demographic and pain-related outcomes from various DC/TMD sources will be reported in tabular format and exploratory analysis will be conducted using this data. This will be done through stratification of participants into various groups by identifiable cohort characteristics.

5.4 Discussion

Botulinum toxin injections are currently used as a management for myofascial TMDs [3]. Given the need for repetitive injection regimen in some cases, potential long term adverse effects need to be considered. In much of the literature, adverse effects are often self-reported or within a short window after injection [15,18]. Understanding the long term implications of BTX to tissues will ensure clinicians offer the best treatment and follow up for their patients.

This study will consolidate data on various tissue level responses to BTX use in the same cohort of individuals, which has not been attempted to this level in other studies. Given the complexity of TMDs and the biological and psychosocial implications, there is a need to be more selective with management plans. This study will help in highlighting the biological implications and also exploring the psychosocial aspects of individuals with myofascial TMDs. This study will help formulate future guidelines on BTX use by identifying individuals who respond positively to treatment and those at risk of complications.

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5.6 Supplementary Material

Institutional Review Board Approval

McGill | Faculty of Medicine and Health Sciences | Faculté de médecine et des sciences de la santé

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May 1, 2024

Dr. Elizabeth Zimmermann
Faculty of Dental Medicine and Dental Health Sciences
3640 University
Montreal, Quebec H3A 0C7

Info-Ed/eRAP File Number: **23-08-008** **IRB Internal Study Number:** **A10-M49-23A**

Study Title: *Effect of Botulinum Toxin A on Mandibular Circulation and Bone Health in Myofascial Temporo-mandibular Disorders*

Principal Investigator: Elizabeth Zimmermann

Student Investigator: Mahmoud Moussa

Dear Dr. Zimmermann,

At a full Board meeting on October 16, 2023, the Faculty of Medicine Institutional Review Board examined the above-referenced research project and considered the experimental procedures acceptable and in adherence to the ethical requirements for research involving human subjects.

Final ethics approval was granted on May 1, 2024. **The ethics certificate is valid until October 15, 2024**.

The following documents were reviewed and approved:

- Initial submission and notification, dated August 8, 2023
- Scientific Review, dated August 3, 2023
- Study Protocol, dated March 22, 2024
- French and English Informed Consent, IRB dated May1, 2024
- French and English Questionnaire on previous use of Botulinum Toxins, dated March 22, 2024

The Faculty of Medicine Institutional Review Board (IRB) is a registered University IRB working under the published guidelines of the Tri-Council Policy Statement 2, in compliance with the Plan d'action ministériel en éthique de la recherche et en intégrité scientifique (MSSS, 1998), and the Food and Drugs Act (17 June 2001); and acts in accordance with the U.S. Code of Federal Regulations that govern research on human subjects (**FWA 00004545**). The IRB working procedures are consistent with internationally accepted principles of good clinical practice.

The Principal Investigator is required to immediately notify the Institutional Review Board Office, via amendment or progress report, of:

- Any significant changes to the research project and the reason for that change, including an indication of ethical implications (if any);
- Serious Adverse Effects experienced by participants and the action taken to address those effects;
- Any other unforeseen events or unanticipated developments that merit notification;
- The inability of the Principal Investigator to continue in her/his role, or any other change in research personnel involved in the project;
- A delay of more than 12 months in the commencement of the research project, and;
- Termination or closure of the research project.

The Principal Investigator is required to submit an annual progress report (continuing review application) on the anniversary of the date of the initial approval (or see the date of expiration).

The Faculty of Medicine IRB may conduct an audit of the research project at any time.

If the research project involves multiple study sites, the Principal Investigator is required to report all IRB approvals and approved study documents to the appropriate Research Ethics Office (REO) or delegated authority for the participating study sites. Appropriate authorization from each study site must be obtained before the study recruitment and/or testing can begin at that site. Research funds linked to this research project may be withheld and/or the study data may be revoked if the Principal Investigator fails to comply with this requirement. A copy of the study site authorization should be submitted the IRB Office.

It is the Principal Investigator's responsibility to ensure that all researchers associated with this project are aware of the conditions of approval and which documents have been approved.

The McGill IRB wishes you and your colleagues every success in your research.

Kind regards,

Roberta M. Palmour, PhD
Chair
Institutional Review Board

Please quote the IRB Study Number and title in all correspondence.

cc: Associate Dean, FMHS Research
A10-M49-23A (23-08-008)

6 Discussion

Physiological considerations for choosing BTX for TMD treatment

6.1 Introduction

Temporomandibular disorders can be highly debilitating. Unfortunately, not many individuals with TMD signs seek help when signs first appear. The journey for many patients is long and often leads to psychological distress that has negative impacts on quality of life and can deter from reaching specialist care (Durham et al., 2011). Pain related disability is the primary factor for seeking care and individual characteristics such catastrophizing, attitudes, and pain threshold can impact patients taking action (Ilgunas et al., 2023; Rollman et al., 2013). Among treatments available, BTX had the least patient related satisfaction compared to surgical interventions (Rodrigues et al., 2023). Despite other studies finding BTX to improve quality of life (Villa et al., 2019) and satisfaction (Meral et al., 2021), the lack of established guidelines for BTX use in TMDs increases variability in study designs and outcomes and makes interpretation of such results difficult.

In addition to establishing dosing and follow up regimens for BTX use in myofascial TMD, there should be meticulous selection of individuals for BTX management. This selection should follow a similar biopsychosocial model used in TMD diagnosis, where

both the state of biological tissues of the individual and the psychosocial aspects should be taken into consideration. In this section, I will cover more in depth the biological considerations for choosing BTX as in intervention by considering insights from preclinical and clinical literature.

6.2 Differences in various physiological tissues in TMD

Individuals with TMD can present with variations in biological tissues as compared to healthy individuals. Electromyography studies show significant differences in masseter and temporalis muscle activity during rest and chewing between TMD and non-TMD groups (Lauriti et al., 2014; Politti et al., 2016). Masseter and sternocleidomastoid muscles thickness have been shown to be less in TMD groups compared to non-TMD, with little differences in temporalis muscle thickness (Lee & Chon, 2021; Strini et al., 2013). When combining bruxism with TMD we see a significant increase in the muscle thickness (Lee et al., 2024), suggesting a role parafunctional habits can play in muscle physiology of individuals with TMD. Nevertheless, current data is heterogenous likely due to variability in methods and sampling used in various studies assessing electromyographic findings (Pelai et al., 2023).

Some studies evaluating the osteoarthritic changes in bone structures of the joint space qualitatively found no differences in TMD and non-TMD groups (Al-Ekrish et al., 2015; Shahidi et al., 2018). When assessing quantitatively the individual components of the joint area, the roof of the glenoid fossa was observed to be thicker and having more discontinuity in TMD patients (Khojastepour et al., 2019). An increase in bone density with decreased dimensional changes has also been noted in TMD patients compared to non-

TMD groups (Chang et al., 2022; Eisazadeh et al., 2021). Additionally, the incidence of degenerative changes such as irregularities, osteophytes, flattening in the condyles were higher in TMD groups and in older individuals (Talaat et al., 2016). When considering parafunctional habits, there is evidence of condylar degenerative changes in bruxers (Casazza et al., 2023) which may be due to the forces loading the TMJ exceeding what is physiologically tolerable. In orthodontic cases without parafunctional habits, high occlusal forces resulted in increased morphology at the lateral and posterior condyle (Kurusu et al., 2009). Interestingly, biting force which translates to loads in the TMJ have been noted to be lower in TMD and bruxer groups compared to control (Koc et al., 2010). This is attributed to increased symptoms and dysfunction often associated with TMDs compared to non-TMD individuals (Pizolato et al., 2007).

The vascular supply to muscle can be assessed directly by using doppler ultrasonography on vessels supplying muscles (Ariji et al., 2001) and by measuring tissue oxygenation using near-infrared spectroscopy (Suzuki et al., 2016). In healthy individuals with high parafunctional habits masseter oxygen saturation during maximum voluntary clenching was found to be 5 times lower than low parafunctional individuals potentially contributing to TMD development (Shah et al., 2019). In individuals with established TMD diagnosis, masseter muscles exhibit less oxygen extraction and low values of oxyhemoglobin as compared to non-TMD individuals (Ferreira et al., 2017; Puel et al., 2023). Masseter oxygenation was increased in TMD group following dry needling technique as compared to sham intervention (Macedo et al., 2023). Meanwhile, ultrasound doppler has been used extensively in the masseter muscle with most outcomes evaluating the muscle thickness and sonographic appearance

(De Nordenflycht et al.). Some studies used doppler mode to assess the number of vessels in masseter muscles pre- and post- splint therapy and to assess the flow in various arteries feeding the masseter muscle in individuals with hemangiomas and inflammation (Aldemir et al., 2013).

This section thus far demonstrates the complex biological changes which can occur in various tissues in individuals with TMD. Most of these investigations in muscle, vascularity and bone can be achieved with non-invasive methods and contribute to better understanding of an individual's condition. Previous chapters in this thesis demonstrated an effect of BTX on some of these tissues which should be taken into account. In the rest of this discussion the effect of BTX in each tissue in preclinical and clinical studies will be addressed as well as current gaps in knowledge.

6.3 Muscular consideration for BTX management

In preclinical studies, muscle atrophy is a well-documented concern for animals receiving BTX. In rabbits monthly quadricep BTX injections at 1, 3, and 6 months periods resulted in significant reduction of muscle mass and contractility in both injected and contralateral limbs at all time points (Fortuna et al., 2011). Despite muscle mass being the same at 3 and 6 months, animals at 6 months exhibited high amounts of fat deposition in muscle (Fortuna et al., 2011). Further investigations into muscle atrophy found that a single injection of BTX can have lingering effect for up to one year in preclinical models (Mathevon et al., 2015). In the animal masseter, one injection of BTX induced histomorphometric changes such as smaller muscle diameter, increase in connective tissue and quantity of myocyte nuclei up to 3 months post injection (Ramos et al., 2024).

Although recovery is attainable following injection, it is not certain that full recovery is achieved (Rafferty et al., 2012). In the muscles of mastication there are similarities between the amount of BTX used per muscle size in animals and humans (Balanta-Melo et al., 2019). Therefore, preclinical studies warrant the investigation into potential muscular atrophy in humans receiving BTX.

In humans, there is a lack of follow up of muscle outcomes in long-term use of BTX. In cosmetic application there has been studies into the “hourglass” deformity caused by temporal muscle injection for recontouring (Durand et al., 2016). In cosmetic application this may represent a wanted change that was noted to be more permanent with repeated injections as electromyographic data suggest smaller muscle size persist following regain of function (Durand et al., 2016). More specifically in TMD populations, masseter and anterior temporalis thickness was significantly reduced 3 months after a single BTX injection and returned to pre-injection level at 6 years post-injection (De la Torre Canales et al., 2022). Masseter muscle activity was most affected at 2 weeks following a single injection and had not fully recovered at 4 months as measured by electromyography (Sitnikova et al., 2022). Additionally, contralateral effects have been noted in humans receiving long term unilateral injections for cervical dystonia in the sternocleidomastoid muscle (Erdal et al., 1999).

As previously discussed, individuals with TMD already have an affected masticatory apparatus which can be characterized by reduced bite force and muscle activity due to symptoms and dysfunction. Muscles of mastication are regularly evaluated clinically by palpation but little imaging is done in a clinical setting for these muscles. Given the current literature in preclinical models as well as the significantly reduced

hindlimb cross-sectional areas seen in Chapter 4 after BTX injection, there should be careful investigation into the status of the muscles to be injected repeatedly to avoid any unnecessary atrophy or weakness in muscles which may be affected by the already existing TMD condition.

6.4 Bony consideration for BTX management

Bone and muscles are highly intertwined and bone relies on the muscular forces for maintenance as discussed in Chapter 2. Also, previously discussed is the effect that reducing muscle forces will have on bone microarchitecture with various models of bone unloading. In Chapter 4, I demonstrated the significant endocortical bone loss occurring in mice hindlimb following single BTX injection. Seemingly, this bone loss is of higher severity than in other unloading models (i.e. hindlimb unloading) underscoring the increased bone loss when unloading is combined with neuromuscular dysfunction (Buettmann et al., 2022). Recently, similar amounts of bone loss was reported in animals receiving repeated injection of BTX as after a single injection (Dechaufour et al., 2024), while another study found higher amounts of bone loss with 3 injections as compared to 1 injection (Wang et al., 2020). As seen in Chapter 3, most animal studies are conducted with single injection regimen in the muscles of mastication and further studies are needed to verify the resultant bone loss of repeated injection regimen.

In Chapter 3, I synthesize the literature for the changes in mandibular bone structure following BTX in humans. Most of the meta-analyzed parameters in humans demonstrated wide confidence intervals due to high heterogeneity in studies. Cortical thickness was affected by single BTX injection resulting in 6% loss from baseline. One

study in healthy individuals found the thickness of masseter and number of occluding teeth to correlate with the mandibular alveolar bone mass (Jonasson & Kiliaridis, 2004). To my knowledge, no study has attempted to correlate the change in condylar bone with altered muscle function in individuals with TMD, which is an outcome of interest to be addressed by studies designed in Chapter 5.

The TMJ is complex and proper imaging is essential for diagnosis and follow up. Cone beam computed tomography (CBCT) allows for 3-D visualization of mandibular bone, although 2-D panoramic X-rays are commonly used in diagnosing TMDs. CBCT has been shown to provide superior reliability in detecting condylar erosion than panoramic imaging (Honey et al., 2007). Assessment of patients with CBCT led to change in diagnosis and management in more than half of the patients who were initially assessed using clinical exam and 2-D panoramic imaging (Whyte et al., 2021). Using CBCT is valuable in visualizing the different planes of the condyle which show specific changes as seen in studies using BTX management for TMD (Kahn et al., 2020). Although magnetic resonance imaging (MRI) provide the most information regarding both hard and soft tissues of TMJ region, they are costly and may not be needed in all cases. With the rise in CBCT use in dental practice and the limited radiation in comparison to traditional computed tomography, TMD patients with signs of degenerative changes and receiving BTX would benefit from yearly monitoring with CBCT scans.

6.5 Vascular consideration for BTX management

In most cases BTX is injected as an intramuscular injection with some clinicians using it around different tissues such as perivascular injections to increase blood flow. Preclinical

application of BTX in the perivascular space resulted in dose- and time- dependent vasodilation of the femoral artery (Arnold et al., 2009; Hu et al., 2019). Although, these studies demonstrate that BTX can induce localized vasodilation, they did not assess the changes in muscle and bone morphology. It would be interesting to discover whether localized perivascular BTX injections near bone nutrient arteries could increase bone mass. One study using intramuscular BTX injection in rabbit masseter demonstrated significantly higher blood flow with concurrent muscle weight loss (Matic et al., 2007). Thus, with the ability to locally infiltrate muscles BTX can also affect vessels coursing through injected muscles. This is a potential explanation for the increased localized flow observed in the femoral arteries of the BTX group in Chapter 4.

The previously mentioned perivascular injection of BTX is clinically beneficial for individuals with vasospastic conditions such as Raynaud's syndrome (Neumeister, 2010). While vasculature and blood flow changes with intramuscular BTX injections in larger muscles such as the masseter remain largely unknown, one case report of BTX for masseteric hypertrophy reported venous malformation (Choi et al., 2010). One study into adverse effects of BTX for muscles of mastication reported flushing sensation in multiple patients (Blanco-Rueda et al., 2023), suggesting that there may indeed be vascular effects following BTX injection that are not yet fully characterized in TMD population. As summarized previously in this discussion, blood flow has been well investigated in an indirect fashion by using near infrared spectroscopy to assess oxygenation of muscles of mastication. It is not known whether the oxygenation of tissues improve due to BTXs vasodilatory capacity. Thus, there remains a significant gap in knowledge regarding vascular flow in individuals with TMD and if BTX can play a therapeutic role by targeting

vasculature. This is an area where studies designed in Chapter 5 will provide new context to these gaps in knowledge.

6.6 Neuronal consideration for BTX management

BTX primarily acts on nerves but the action is not limited to motor or sympathetic neurons but also can target sensory neurons. In preclinical studies, BTX injection into the whisker pad of rats with formalin-induced facial pain had antinociceptive effects, and axonal transport to the medullary dorsal horn was observed 3 days following injection(Matak et al., 2011). Transient receptor potential vanilloid type I (TRPV1) expression which coincided with neuropathic pain induction in rats can be attenuated with peritoneal BTX injection and lead to increase of pain threshold (Xiao et al., 2013). These studies demonstrate that BTX may have an antinociceptive effect through direct (peripheral) and indirect (central) sensitization (Park & Chung, 2018).

In humans, this antinociceptive effect of BTX is relatively novel and has been explored in few neuropathic pain conditions. Some case series have demonstrated its effect in sciatic nerve injury related pain (Han et al., 2016; Jabbari et al., 2003). Other applications included multiple sclerosis, complex regional pain syndrome, post-stroke shoulder pain (Park & Chung, 2018). In TMD and myofascial pain the trigeminal nerve plasticity is affected and abnormalities have been noted in magnetic resonance imaging of individuals with these conditions (Moayedi & Hodaie, 2019). The proposed mechanism for BTX action in central nervous desensitization is the blockade of glutamate, calcitonin gene related peptide and substance P (Tang et al., 2020). Imaging and mechanistic

studies following BTX injection would improve our understanding of its antinociceptive effect in chronic TMD.

All in all, BTX is a dynamic substance that can influence multiple systems. Additional research is needed to characterize this influence and identify clinically beneficial changes which may provide new treatment targets in complex conditions such as TMDs.

7 Conclusion

This thesis investigated the adverse effects and physiological changes induced by intramuscular BTX injection. We identify the current reported extent of bony change in the mandible of individuals with TMD after BTX management. Through this work we highlight the lack of standardized investigations and methods across studies. We also identify an additional effect on vasculature and blood flow in BTX injected muscles. The potential contribution of vasculature to bone loss is documented in other conditions of disuse but is not well established with BTX models of disuse. Additionally, the vascular alterations with BTX have not been identified in individuals with TMD. Given the limited knowledge of biological consequences and the lack of standardized guidelines for BTX management there is a need for stronger clinical studies. We established the ground work for prospective cohort study to assess biological changes prior to first injection and up to one year after injection.

Few limitations have risen throughout the execution of this work and vary according to the chapter. In our meta-analysis of mandibular changes due to BTX application, there is wide variance in the dose, follow up time in human participants that restricted additional analysis and contributed to observed heterogeneity. Nevertheless, establishing a baseline and highlighting current advancement in this field is important for guiding future research. In our pre-clinical study of the effect of BTX on vascular flow, most notably the region of interest is different (hindlimb vs. mandible), which may reduce the translatability of the findings to human mandibles. Despite this apparent disconnect, this study offers a unique approach to examining bone and vasculature, providing in depth analysis of lacunar and vascular networks. The identified changes in vasculature may not

be directly translatable to the mandible and orofacial region but warrants their exploration in this region. Lastly, the proposed clinical study may be limited in a traditional sense by the lack of control participants and by the potential bias in sex and gender of individuals that will be recruited to the study, which may limit identifying such sex related differences. Given the current limited knowledge on the topic of BTX and adverse effects this study with its larger sample size will provide insight into the changes observed and could help extrapolate findings to future larger studies on the topic.

The knowledge from this thesis highlights the need for regular monitoring of biological tissues in cases requiring long-term injection regimens in addition to pain related outcomes. This is necessary until a consensus is reached regarding the definitive role of BTX in these conditions.

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