Systematic Review

High Precision Use of Botulinum Toxin Type A (BONT-A) in Aesthetics Based on Muscle Atrophy, Is Muscular Architecture Reprogramming a Possibility? A Systematic Review of Literature on Muscle Atrophy after BoNT-A Injections

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Abstract: Improvements in Botulinum toxin type-A (BoNT-A) aesthetic treatments have been jeopardized by the simplistic statement: “BoNT-A treats wrinkles”. BoNT-A monotherapy relating to wrinkles is, at least, questionable. The BoNT-A mechanism of action is presynaptic cholinergic nerve terminals blockage, causing paralysis and subsequent muscle atrophy. Understanding the real BoNT-A mechanism of action clarifies misconceptions that impact the way scientific productions on the subject are designed, the way aesthetics treatments are proposed, and how limited the results are when the focus is only on wrinkle softening. We designed a systematic review on BoNT-A and muscle atrophy that could enlighten new approaches for aesthetics purposes. A systematic review, targeting articles investigating BoNT-A injection and its correlation to muscle atrophy in animals or humans, filtered 30 publications released before 15 May 2020 in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. Histologic analysis and histochemistry showed muscle atrophy with fibrosis, necrosis, and an increase in the number of perimysial fat cells in animal and human models; this was also confirmed by imaging studies. A significant muscle balance reduction of 18% to 60% after single or seriated BoNT-A injections were observed in 9 out of 10 animal studies. Genetic alterations related to muscle atrophy were analyzed by five studies and showed how much impact a single BoNT-A injection can cause on a molecular basis. Seriated or single BoNT-A muscle injections can cause real muscle atrophy on a short or long-term basis, in animal models and in humans. Theoretically, muscular architecture reprogramming is a possible new approach in aesthetics.

Keywords: botulinum toxins; type A; botox; muscular atrophy; muscle atrophy; wrinkles; facial lines; aesthetics; esthetics; muscular architecture reprogramming

Key Contribution: A systematic review of literature on muscle atrophy after BoNT-A injections.

1. Introduction

Botulinum toxin type A (BoNT-A) has been historically used for the aesthetic treatment of facial lines. Although there are an increasing number of on-label uses to treat a variety of disorders using BoNT-A, when it comes to aesthetics, all the on-label approvals refer to facial lines [1]. Currently BoNT-A is approved by the FDA for the aesthetic treatment of forehead, glabellar, and lateral canthal lines, while in some other countries, such as Brazil, the on-label aesthetic approval is more generic and permits BoNT-A injections all over the face to treat facial lines [2,3]. The main point is that all the aesthetic on-label approvals
concern facial lines only. Numerous published clinical trials objectify the improvement of facial lines after treatment with BoNT-A [4]. A multitude of articles aimed to compare the main brands of BoNT-A available on the market regarding the durability of the effect of softening wrinkles provided by these toxins [5]. Dose comparisons between BoNT-A brands generate misleading results because they are all different and are not interchangeable substances [6–8].

Despite differences in market brands, all currently marketed BoNT-A have one thing in common: a protein complex of 150 kDa composed of a heavy chain (HC, 100 kDa) linked via a disulfide bond to a light chain (LC, 50 kDa) [9–11]. After a BoNT-A injection, the simplified mechanism of action cascade can be described based on its biochemical structure [12–17] (Figure 1).

The whole cascade takes between 24 to 72 h to be completed after BoNT-A injection, and it is an irreversible process [18]. Once the SNAP-25 (synaptosomal-associated protein of 25 kDa) protein is inactivated, muscle contraction will only be reestablished after neuronal repair that depends on nerve sprouting and/or motor plate regeneration [19]. Although scientific evidence on this statement dates back to the 1970s [20], many still argue today about BoNT-A “durability” in relation to wrinkle control rather than studying the level of tissue damage caused by a BoNT-A injection and the time required for neuronal healing, as concerns aesthetics. The previous sentence is fundamental for the purpose of the new aesthetic approach of BoNT-A use in aesthetics that we intend to propose based on the real BoNT-A mechanism of action.

Many studies have demonstrated nerve terminal and nodal sprouting in the paralyzed nerves as early as two days after botulinum toxin injection [21,22]. Broadening the scope, studies on botulism have already provided a substrate to support the idea that the botulinum toxins durability for practical purposes is approximately 24 to 72 h and that the actual long-term effect of muscle paralysis depends only on nerve and muscle tissue regeneration processes. Treatment with antitoxin for patients with botulism, in order to be effective, should be started within 24 to 48 h of contamination, otherwise the already established neuronal chemical tissue injury is no longer reversible [23]. Once the disease is established by neuronal inability to release acetylcholine in the synaptic cleft of the neuromuscular junction, life support becomes essential, which is normally restricted to clinical care, with special attention to maintaining respiratory capacity, which requires mechanical ventilation for 2 to 6 months, until neuronal and muscular healing processes take place, restoring diaphragmatic and intercostal muscle function [24,25].

Studies addressing counter-terrorism measures suggest the use of antidotes against BoNT-A in the event of a mass attack using BoNT-A as a chemical weapon. Only 1 g
of BoNT-A in natura is capable of decimating 1 million humans, showing that it is a powerful and lethal toxin. All of the antidotes tested, even those capable of neuronal internalization, require concern regarding the therapeutic window, which must precede a chemical neuromuscular junction denervation of 24 to 72 h [24,26].

Understanding BoNT-A’s real mechanism of action makes it possible to identify some semantic misconceptions that have been repeated historically since its first use for aesthetic purposes and that directly impact the way scientific productions on the subject are designed, the way aesthetics treatments are proposed, and how limited the results are when the focus is only on wrinkles softening. Considering the statements above and the questions raised below (Table 1), we designed a systematic review on BoNT-A and muscle atrophy that could enlighten new approaches for aesthetics purposes.

Table 1. Questions that should be answered, based on the evidence, after reading this paper.

| Questions                                                                 | Answers |
|--------------------------------------------------------------------------|---------|
| Does the muscular impairment for contraction caused by BoNT-A really treat facial lines or causes muscle atrophy? | ?       |
| What is the relation of BoNT-A muscle injections and muscle atrophy in the long term? | ?       |
| Is it possible to modulate the level of muscle atrophy through time by using BoNT-A? | ?       |
| What if we used muscle atrophy caused by BoNT-A injections to optimize muscle architecture for facial aesthetic purposes? | ?       |
| What would it be like to reinterpret articles written in the last 30 years focused mainly on facial lines unveiling this concept of muscle atrophy? How many less subjective opportunities would arise? How classic BoNT-A injections techniques would be impacted? | ?       |

2. Aims

To conduct a systematic review of the literature regarding BoNT-A treatments and muscle atrophy that could support new perspectives in facial aesthetics and to propose a new reading for the aesthetic use of BoNT-A, no longer focusing on simple control of wrinkles and facial lines, but as a drug capable of selectively reprogramming long-term muscle strength and tonus through muscle atrophy. We will discuss the proposition that muscle architecture could be altered by creating areas of real atrophy—hyporesponsive or even irresponsive to acetylcholine stimuli for muscle contraction. The restoration of neuronal and muscular function would be based exclusively on the healing processes of these tissues.

3. Method

The present systematic review, targeting articles that investigate BoNT-A injections and its correlation to muscle atrophy in animals or humans, was conducted in a stepwise process for studies published before 15 May 2020 and in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [27]. The search strategy, the flow diagram of study selection, and the data extraction are detailed below, because the review was not registered. By the time our independent research group tried to register the review at PROSPERO in 2020, we had already started article extraction. After October 2019, PROSPERO only accepted earlier registration.

STEP 1—PubMed/MEDLINE and BVS (Biblioteca Virtual em Saúde) databases were explored using the following Medical Subject Headings (MeSH) entry terms: “Botulinum Toxin Type A” OR “Botulinum A Toxin” OR “Botulinum Neurotoxin A” OR “Botox” AND combined with the MeSH entry terms “Muscle Atrophy” OR “Muscular Atrophy” (Table 2). The overlapping studies were excluded in STEP 1.
Table 2. PubMed/MEDLINE and BVS (Biblioteca Virtual em Saúde) databases Search strategies.

| Four Search Strategies Used, Initially: |
|----------------------------------------|
| Search 1—PubMed/MEDLINE—((((BOTULINUM TOXIN TYPE A) OR (BOTULINUM A TOXIN)) OR (BOTULINUM NEUROTOXIN A)) OR (BOTOX)) AND (MUSCLE ATROPHY). |
| Search 2—PubMed/MEDLINE—((((BOTULINUM TOXIN TYPE A) OR (BOTULINUM A TOXIN)) OR (BOTULINUM NEUROTOXIN A)) OR (BOTOX)) AND (MUSCULAR ATROPHY). |
| Search 3—BVS—tw:((tw:(botulinum toxin type a)) OR (tw:(botulinum a toxin)) OR (tw:(botulinum neurotoxin a)) OR (tw:(botox)) AND (tw:(muscle atrophy))). |
| Search 4—BVS—tw:((tw:(botulinum toxin type a)) OR (tw:(botulinum a toxin)) OR (tw:(botulinum neurotoxin a)) OR (tw:(botox)) AND (tw:(muscular atrophy))). |

To encompass all possible missing studies that could not be retrieved from Searches 1–4, the preferred MeSH term entries “Botulinum Toxin Type A” and “Muscular Atrophy” were matched with all their alternative MeSH term entries listed below:

| Botulinum toxin type A | Muscular atrophy |
|------------------------|------------------|
| Clostridium Botulinum Toxin Type A | Atrophies, Muscular |
| Botulinum Toxin Type A | Atrophy, Muscular |
| Botulinum A Toxin | Muscular Atrophies |
| Toxin, Botulinum A | Atrophy, Muscle |
| Clostridium botulinum A Toxin | Atrophies, Muscle |
| Botulinum Neurotoxin A | Muscle Atrophies |
| Neurotoxin A, Botulinum | Muscle Atrophy |
| Meditoxin | Neurogenic Muscular Atrophy |
| Botox | Atrophies, Neurogenic Muscular |
| Neurinox | Atrophy, Neurogenic Muscular |
| Oculinum | Muscular Atrophies, Neurogenic |
| Vistabex | Muscular Atrophy, Neurogenic |
| Onabotulinumtoxin A | Neurogenic Muscular Atrophies |
| Onabotulinumtoxin A | Neurotrophic Muscular Atrophy |
| Vistabel | Atrophies, Neurotrophic Muscular |
| | Atrophy, Neurotrophic |
| | Muscular Atrophies, Neurotrophic |
| | Muscular Atrophy, Neurotrophic |
| | Neurotrophic Muscular Atrophies |

All the 15 alternative MeSH term entries for “Botulinum Toxin Type A” and all the 19 alternative MeSH term entries for “Muscle Atrophy” listed above were individually added to Search 1, Search 2, Search 3, and Search 4, one at a time, to check if any other study would be retrieved. No other search limits were added.

In STEP 2, the studies obtained in STEP 1 were screened by “title” and “abstract” by two independent researchers (A.D.N. and R.F.B.). Those not satisfying inclusion criteria or with exclusion criteria (Table 3) were excluded. The group of articles selected to proceed to the next step was determined through an interactive consensus process. Discrepancies were judged by a third reviewer (S.E.).
Table 3. Inclusion and exclusion criteria.

A study was considered eligible for data extraction if it fulfilled the criteria below:

- Human or animal striated skeletal muscle atrophy analysis after botulinum toxin type A injection(s), and
- Atrophy analyzed by imaging (ultrasonography (USG), nuclear magnetic resonance (NMR), computerized tomography (CT)), and/or by histological analysis and/or by biochemical analysis; and
- Minimal follow-up of 3 months, and
- The full manuscript was published in English.

In STEP 3, the full text of all the potential articles selected in STEP 2 were obtained and carefully read to screen for those whose purposes were in accordance with the aim of the present review.

In STEP 4, the eligible studies in STEP 3 were thoroughly read, and data for each study were extracted and analyzed according to a PICO-like structured reading (Table 4).

Table 4. PICO-like structured reading of the eligible studies and data collection.

| PICO-like structured reading of the eligible studies and data collection |
|-------------------------------------------------------------|
| Population/Problem (P) |
| Intervention (I) |
| Comparison group (C) |
| Outcomes (O) |

The following question was adopted to conduct data collection:

“Are botulinum toxin type A injections (I) related to muscle atrophy (O) of animal or humans (P), when compared to not injected subjects or muscles (C)?”

Detailed data were collected in two different groups (animal and human) to fulfill comparative tables, including: presence of a control group, population number, population age, health condition, muscle systems analyzed, BoNT-A number of injections and dose, muscle atrophy confirmation or not, muscle atrophy identification tool and correlated changes, follow-up, and muscle atrophy recovery.

The methodological quality of the articles included in the study was evaluated using a specific scale developed based on STROBE (Strengthening the Reporting of Observational studies in Epidemiology) principles [28]. Each item was categorized, and the maximum global score was set to 26 (Table 5).
Table 5. Quality analysis form used in the systematic review.

| Quality Analysis form Used in the Systematic Review. |
|-----------------------------------------------------|
| Q1 Is there in the abstract an explanation of what was done and found? |
| Q2 Is the scientific context clearly explained? |
| Q3 Are the objectives clearly stated? |
| Q4 Is the sampling size indicated? |
| Q5 If yes, is the sampling size statistically justified? |
| Q6 Are the characteristics of the subjects (height, weight, sex, healthy, or pathologic subject) described? |
| Q7 What is the design of the study? (0: retrospective study; 1: case study; 2: prospective study). |
| Q8 Is there a control group? (0: no, 1: contralateral member or nonrandomized control group, 2: randomized control group). |
| Q9 How long is the follow up? (0: ≥3 and <6 months; 1: ≥6 months and <1 year; 2: ≥1 year) |
| Q10 Is the reliability of the evaluation method clearly described? |
| Q11 Are the results interpretable? |
| Q12 Are the limitations of the study discussed? |
| Q13 Is the conclusion clearly stated? |

0: no description; 1: limited description; 2: good description.

4. Results

4.1. Selection of the Studies

From 191 articles initially identified after removing duplicates, thirty-five were deemed relevant after reading titles and abstracts. Thirty were included in the review (5 were excluded because they did not meet the selection criteria). Sixteen were animal studies and fourteen were human studies. The PRISMA Flow Diagram of Article Selection for Review is summarized in (Figure 2).
4.2. Quality of the Reviewed Articles

The quality of the reviewed articles was highly variable and is summed up in Table 6 [29–58]. Most studies, 28/30, were prospective ones, with 13 well-controlled and randomized, but this subgroup was only of animal studies. The descriptive quality of the experimental protocol results, as well as their interpretations and conclusions, were adequate in most studies. The follow-up ranged from 3 months to 4 years.

Table 6. Quality assessment. ** maximum global score = 26.

| Study               | Q1 | Q2 | Q3 | Q4 | Q5 | Q6 | Q7 | Q8 | Q9 | Q10 | Q11 | Q12 | Q13 | TOTAL ** |
|---------------------|----|----|----|----|----|----|----|----|----|-----|-----|-----|-----|--------|
| Borodic (1992) [29] | 1  | 2  | 2  | 2  | 0  | 1  | 2  | 1  | 2  | 1   | 1   | 0   | 2   | 17    |
| Hamjian (1994) [30] | 1  | 1  | 1  | 2  | 0  | 2  | 2  | 1  | 0  | 2   | 1   | 0   | 1   | 14    |
| Ansved (1997) [31]  | 2  | 2  | 2  | 2  | 0  | 2  | 1  | 1  | 2  | 1   | 1   | 0   | 1   | 17    |
| Fanucci (2001) [32] | 2  | 2  | 2  | 2  | 0  | 2  | 2  | 1  | 0  | 2   | 2   | 0   | 2   | 19    |
| To (2001) [33]      | 2  | 2  | 2  | 2  | 0  | 2  | 2  | 1  | 2  | 1   | 2   | 0   | 2   | 20    |
| Kim (2005) [34]     | 2  | 2  | 1  | 2  | 0  | 2  | 2  | 0  | 1  | 2   | 2   | 0   | 2   | 18    |
| Shen (2006) [35]    | 2  | 2  | 2  | 2  | 0  | 2  | 2  | 2  | 1  | 2   | 2   | 0   | 2   | 21    |
| Singer (2006) [36]  | 2  | 2  | 2  | 2  | 0  | 2  | 2  | 0  | 1  | 1   | 2   | 2   | 2   | 20    |
| Herzog (2007) [37]  | 2  | 2  | 2  | 2  | 0  | 2  | 2  | 2  | 1  | 2   | 2   | 0   | 2   | 23    |
| Frick (2007) [38]   | 2  | 2  | 1  | 2  | 0  | 2  | 2  | 2  | 0  | 1   | 2   | 1   | 2   | 19    |
| Kwon (2007) [39]    | 2  | 2  | 2  | 2  | 0  | 2  | 2  | 2  | 1  | 2   | 2   | 1   | 2   | 22    |
| Lee (2007) [40]     | 2  | 2  | 2  | 2  | 0  | 2  | 2  | 0  | 2  | 1   | 2   | 0   | 2   | 19    |
| Schroeder (2009) [41]| 2  | 2  | 2  | 2  | 0  | 1  | 2  | 1  | 2  | 1   | 2   | 0   | 2   | 19    |
| Babuccu (2009) [42] | 2  | 2  | 2  | 2  | 0  | 2  | 2  | 2  | 0  | 2   | 2   | 0   | 2   | 20    |
| Tsai (2010) [43]    | 2  | 2  | 1  | 2  | 0  | 1  | 2  | 1  | 2  | 1   | 1   | 1   | 2   | 18    |
| Fortuna (2011) [44] | 2  | 2  | 2  | 2  | 0  | 2  | 2  | 2  | 1  | 1   | 1   | 1   | 2   | 20    |
| Fortuna (2013a) [45]| 2  | 2  | 2  | 2  | 0  | 1  | 2  | 2  | 1  | 2   | 2   | 1   | 2   | 20    |
| Van Campenhout (2013) [46]| 2  | 2  | 2  | 2  | 0  | 1  | 2  | 0  | 1  | 2   | 2   | 2   | 2   | 20   |
| Koerte (2013) [47]  | 2  | 2  | 2  | 2  | 0  | 2  | 2  | 1  | 2  | 1   | 2   | 0   | 1   | 19    |
| Fortuna (2013b) [48]| 2  | 2  | 2  | 2  | 0  | 2  | 2  | 2  | 1  | 2   | 2   | 1   | 2   | 22    |
| Mukund (2014) [49]  | 1  | 2  | 2  | 2  | 0  | 2  | 2  | 1  | 2  | 2   | 1   | 2   | 1   | 21    |
| Fortuna (2015) [50] | 2  | 2  | 2  | 2  | 0  | 2  | 2  | 2  | 2  | 2   | 0   | 2   | 2   | 22    |
| Caron (2015) [51]   | 2  | 2  | 2  | 2  | 0  | 2  | 2  | 2  | 1  | 2   | 0   | 1   | 2   | 20    |
| Valentine (2016) [52]| 2  | 2  | 2  | 2  | 0  | 2  | 2  | 1  | 2  | 1   | 2   | 1   | 1   | 20    |
| Li (2016) [53]      | 1  | 1  | 2  | 2  | 0  | 2  | 1  | 0  | 2  | 0   | 1   | 0   | 1   | 13     |
| Kocaelli (2016) [54]| 2  | 2  | 2  | 2  | 0  | 2  | 2  | 2  | 0  | 2   | 1   | 2   | 1   | 21    |
| Hart (2017) [55]    | 2  | 2  | 2  | 2  | 0  | 2  | 2  | 2  | 2  | 1   | 2   | 1   | 2   | 22    |
| Han (2018) [56]     | 2  | 2  | 2  | 2  | 0  | 2  | 2  | 0  | 1  | 1   | 2   | 1   | 1   | 18    |
| Alexander (2018) [57]| 2  | 2  | 2  | 2  | 0  | 2  | 2  | 1  | 1  | 2   | 2   | 2   | 2   | 22    |
| Lima (2018) [58]    | 2  | 2  | 2  | 2  | 0  | 2  | 2  | 2  | 0  | 1   | 2   | 0   | 2   | 19    |
4.3. Literature Analysis

A general overview of the population type of the 30 studies is summarized in Table 7. All Animal studies had good quality control groups. Human studies, on the other hand, lacked control groups or had poor quality control groups.

Most animal studies used mature healthy animals. Human studies, on the other hand, used very heterogeneous subjects in relation to age (varying from children to 91-year-old adults) and health status.

Overall, there were very few studies regarding the facial mimetic musculature in humans—only two: Borodic (1992) [29] and Koerte (2013) [47]. The facial masticatory musculature represented mainly by the masseter muscle were studied in three human studies: To (2001) [33], Kim (2005) [34], Lee (2007) [40]; and three animal studies: Kwon (2007) [39], Babuccu (2009) [42], Kocaelli (2016) [54].

Numerical heterogenic population samples (from 1 to 383 subjects) and qualitative heterogenic samples, more specifically in human studies (healthy and subjects with different muscle disorders), were observed.

There was also heterogenic BoNT-A dose, BoNT-A brand types used in the studies and follow-up period, summarized in Table 8.

The methodological variability among the small number of studies made it mandatory to conduct an extensive evaluation based on the identification of muscle atrophy after BoNT-A injections registered separately via different tools in animal or human studies. The general findings are summarized in Section 4.3.1. (Animal Studies) and Section 4.3.2. (Human Studies), below.

4.3.1. Animal Studies

Muscle Balance

Muscle balance was measured in 10 out of 16 animal studies to evaluate muscle atrophy. Significant muscle balance reduction after seriated BoNT-A injections and after one single BoNT-A injection were observed in 9 out of 10 studies. The reduction varied from 18%, Fortuna (2013b) [48], to 60%, Fortuna (2011) [44], and there was a BoNT-A dose dependency/interval of injection association identified by Herzog (2007) [37], Frick (2007) [38], Tsai (2010) [43], Fortuna (2011) [44], Fortuna (2013a) [45], Fortuna (2013b) [48], and Caron (2015) [51]. The higher the dose, the higher the muscle balance reduction. Long intervals between injections permitted partial muscle balance recovery. Only Fortuna (2015) [50] found no muscle balance alterations after 6 months of injection (Table 9).

Optical and Electron Microscopy

Hystologic (optical and electron microscopy) analysis and histochemistry showed profound muscle structure changes in animal models, such as sarcomere distortion, decrease in myofibrillar diameters, and myofibrilloyisis/myonecrosis—Babuccu (2009) [42], Tsai (2010) [43], Kocaelli (2016) [54]. Significant reduction of percentage of contractile material—Frick (2007) [38], Fortuna (2011) [44], Fortuna (2013a) [45], Fortuna (2013b) [48], Fortuna (2015) [50]. Replacement of contractile fibers with fat, fatty infiltration, and increased collagen fibers forming perimysium—Herzog (2007) [37], Fortuna (2011) [44], Kocaelli (2016) [54] (Table 10).

Imaging

Kwon (2007) [39] showed a computed tomography (CT) scan rabbit masseter muscle volume reduction of up to 18.41% (±3.15) after 6 months of a BoNT-A injection. Magnetic resonance imaging (MRI) was used in monkeys by Han (2018) [56] and showed significant paraspinal muscles atrophy after BoNT-A injections (Table 11).

Molecular Biology

Direct and indirect muscle atrophy identification via molecular biology was studied and is detailed in Tables 12 and 13.
**Table 7. Systematic review—Summary table of the results (PART 1).**

| Author (Year) | Human/Animal | Control Group | Age (Number) | Health Condition |
|---------------|--------------|---------------|--------------|------------------|
| Borodic (1992) [29] | Human | Yes | 56–91 years | 14 | Blepharospasm/Meige’s disease |
| Hamjian (1994) [30] | Human | Contralateral muscle | 25–49 years | 10 | Healthy |
| Ansved (1997) [31] | Human | Yes | 32–54 years | 22 | Cervical dystonia |
| Fanucci (2001) [32] | Human | Contraleteral Muscle | 29–54 years | 30 | Piriformis muscle syndrome (PMS) |
| To (2001) [33] | Human | Yes | 16–32 years | 15 | Masseteric muscle hypertrophy |
| Kim (2005) [34] | Human | No | Teenagers—40s | 383 | Masseteric muscle hypertrophy |
| Shen (2006) [35] | Animal (Sprague-Dawley rats) | Yes | 1 month | 56 | Healthy |
| Singer (2006) [36] | Human | No | 16–40 years | 8 | Chronic anterior knee pain and related disability |
| Herzog (2007) [37] | Animal (New Zealand white rabbits) | Yes | 1 year | 25 | Healthy |
| Frick (2007) [38] | Animal (Sprague-Dawley rats) | Contralateral muscle | Mature | 39 | Healthy |
| Kwon (2007) [39] | Animal (New Zealand rabbits) | Yes | 4 weeks | 21 | Healthy |
| Lee (2007) [40] | Human | No | 20–29 years | 10 | Healthy (square face) |
| Schroeder (2009) [41] | Human | Contralateral muscle | 31–47 years | 2 | Healthy |
| Babuccu (2009) [42] | Animal (Wistar rats) | Yes | 15-day-old | 49 | Healthy |
| Tsai (2010) [43] | CD® (SD) IGS rats | Contralateral muscle | Mature | 60 | Healthy |
| Fortuna (2011) [44] | Animal (New Zealand White rabbits) | Yes | 1 year | 20 | Healthy |
| Fortuna (2013a) [45] | Animal (New Zealand White rabbits) | Yes | Mature | 17 | Healthy |
Table 7. Cont.

| Author (Year)          | Human/Animal          | Control Group | Age     | Population (Number) | Health Condition                                      |
|------------------------|-----------------------|---------------|---------|---------------------|-------------------------------------------------------|
| Van Campenhout (2013)  | Human                 | No            | Children| 7                   | Cerebral palsy (symmetric spastic diplegia)            |
| Koerte (2013)          | Human                 | Yes           | 34–50 years | 4                   | Healthy                                               |
| Fortuna (2013b)        | Animal (New Zealand White rabbits) | Yes | 1 year | 27                   | Healthy                                               |
| Mukund (2014)          | Animal (Harlan Sprague-Dawley rats) | No  | 3 months | 20                   | Healthy                                               |
| Fortuna (2015)         | Animal (New Zealand White rabbits) | No | 1 year | 23                   | Healthy                                               |
| Caron (2015)           | Animal (Sprague-Dawley rats) | Yes | Mature  | 27                   | Healthy                                               |
| Valentine (2016)       | Human                 | Different muscle same participant | 6–16 years | 10                   | Cerebral palsy                                        |
| Li (2016)              | Human                 | No            | 40–59 years | 3                   | Strabismus                                            |
| Kocaelli (2016)        | Animal (Sprague-Dawley rats) | Yes | 5–6 months | 30                   | Healthy                                               |
| Hart (2017)            | Animal (New Zealand White rabbits) | No | 1 year | 22                   | Healthy                                               |
| Han (2018)             | Animal (Cynomolgus monkey—Macaca fascicularis) | No | 9 years | 1                   | Healthy                                               |
| Alexander (2018)       | Human                 | Baseline status same participant | 5–13 years | 11                   | Cerebral palsy                                        |
| Lima (2018)            | Animal (Wistar rats)  | Yes           | 10-week-old | 50                   | Healthy                                               |

Systematic review—Summary table of the results (PART 1). Human studies Animal studies.
Table 8. Systematic review—Summary table of the results (PART 2).

| Author (Year)       | BoNT-A Number of Injections and Dose                                                                 | Follow-Up |
|---------------------|------------------------------------------------------------------------------------------------------|-----------|
| Borodic (1992) [29] | 2–19 injections over 1–5.5 years. Dose?                                                             | 1–52 weeks|
| Hamjian (1994) [30] | 1 injection. Dose 10 units of BoNT-A (Oculinum®) #                                                  | 0–100 days|
| Ansved (1997) [31]  | Number? 2–4 years of treatment. Mean cumulative dose 2.815 units of BoNT-A                          | 2–4 years |
| Fanucci (2001) [32] | 1 or 2 injections. Dose 200 units of BoNT-A (Botox®) ##                                            | 0–3 months|
| To (2001) [33]      | 1 or 2 injections. Dose 100–300 units of BoNT-A (Dysport®) ### per side                             | 0–1 year  |
| Kim (2005) [34]     | 1 or 2 injections. Dose 100–140 units of BoNT-A (Dysport®) ### per side                            | 0–2 years |
| Shen (2006) [35]    | 1 injection. Dose 6 units/kg body weight of BoNT-A (Botox®) ##                                      | 0–360 days|
| Singer (2006) [36]  | 1 injection. Dose 300–500 units of BoNT-A (Dysport®) ###                                            | 0–24 weeks|
| Herzog (2007) [37]  | 1–6 injections over 6 months. Dose 3.5 units/kg body weight of BoNT-A (Botox®) ### per injection    | 1–6 months|
| Frick (2007) [38]   | 1 injection. Dose 0.625 units or 2.5 units or 10 units/kg body weight of BoNT-A (Botox®) ##         | 128 days  |
| Kwon (2007) [39]    | 1 injection. Dose 5–15 units of BoNT-A                                                             | 4–24 weeks|
| Lee (2007) [40]     | 1 injection. Dose 25 units of BoNT-A (Botox®) ##                                                  | 0–12 months|
| Schroeder (2009) [41]| 1 injection. Dose 75 units of BoNT-A (Xeomin®) ####                                                | 3–12 months|
| Babuccu (2009) [42] | 1 injection. Dose 0.4 units BoNT-A (Botox®) #### per muscle                                       | 4 months  |
| Tsai (2010) [43]    | 1 or 2 injections. Dose 2.5 ng of BoNT-A (Botox®) ## per side (single injection group) or (two injections group full dose—30 weeks apart) or (two injections group half dose—30 weeks apart) | 1–58 weeks|
| Fortuna (2011) [44] | 1 or 3 or 6 monthly injections. Dose 3.5 units/Kg of BoNT-A (Botox®) ### per muscle group, per side, per month | 1–6 months|
| Fortuna (2013a) [45]| 6 monthly injections. Dose 3.5 units/Kg of BoNT-A (Botox®) ### per muscle group, per side, per month | 6 months  |
| Van Campenhout (2013) [46]| 1 injection. Dose 2 units/Kg/psoas muscle of BoNT-A (Botox®) ##                                  | 0–6 months|
| Koerte (2013) [47]  | 1 injection. Dose 20 units of BoNT-A (Botox®) ##                                                  | 0–12 months|
| Fortuna (2013b) [48]| 6 monthly injections. Dose 3.5 units/Kg of BoNT-A (Botox®) ### per muscle group, per side, per month | 6–12 months|
Table 8. Cont.

| Author (Year) | Bont-A Number of Injections and Dose | Follow-Up |
|---------------|-------------------------------------|-----------|
| Mukund (2014) [49] | 1 injection. Dose 6 units/Kg of Bont-A (BoNT-A®) ** per side | 1–52 weeks |
| Fortuna (2015) [50] | 1, 2, or 3 injections (every 3 months). Dose 3.5 units/Kg of BoNT-A (Botox®) **** per muscle group, per side, per injection | 6–12 months |
| Caron (2015) [51] | 1 injection. Dose 15 units/Kg of BoNT-A (Dysport®) ####### per side | 12–40 months |
| Valentine (2016) [52] | 1–15 injections. Dose 2–6 units/Kg of BoNT-A (Botox®) ### per side | 3.5 months–3 years |
| Li (2016) [53] | 1–2 injections. Dose 3.75–7.5 units of BoNT-A (Botox®) ### per side | 6–18 months |
| Kocaelli (2016) [54] | 1 injection. Dose 6 units/Kg of BoNT-A (Botox®) ** per side | 12 weeks |
| Hart (2017) [55] | 1, 2, or 3 injections (every 3 months). Dose 3.5 units/Kg of BoNT-A (Botox®) **** per muscle, per side | 6–12 months |
| Han (2018) [56] | 10 (one injection every two weeks). Dose 2 units/Kg of BoNT-A (Nabota®) ############ | 0–21 weeks |
| Alexander (2018) [57] | 1 injection. Dose 1.4–4.8 units/Kg of BoNT-A (Botox®) ## per side | 0–25 weeks |
| Lima (2018) [58] | 1 injection. Dose 5 units of BoNT-A (Dysport®) ### per side | 12 weeks |

Human studies Animal studies # (Oculinum®)—Allergan Corp., Irvine, CA. ## (Botox®)—Allergan Corp., Irvine, CA. ### (Dysport®) Ipsen Ltd., Slough, United Kingdom. #### (Botox®) Allergan Inc., Toronto, Ont., Canada. #### (Xeomin®) Merz Pharma, Germany. ###### (BoNT-A) Allergan Pharmaceuticals, Ireland. ####### (Dysport®) Ipsen Ltd., Boulogne-Billancourt, France. ****** (Nabota®) Daewoong Pharmaceutical Hwaseong, Korea.

Table 9. Animal studies—Muscle balance.

| Muscle Atrophy Identification Tool | BoNT-A Number of Injections and Dose | Follow-Up |
|-----------------------------------|-------------------------------------|-----------|
| Heneg (2007) [37] Quadriceps Femoris 25 New Zealand White Rabbits | 1 injection. Dose 6 units/Kg of BoNT-A (Botox®) ** per side | 1–52 weeks |
| Frick (2007) [38] Tibialis 39 Sprague-Dawley Rats | 1 injection. Dose 6 units/Kg of BoNT-A (Botox®) ** per side | 12 weeks |
| Rebecca (2009) [42] Masticator and Temporalis 40 Wistar Rats | 1 injection. Dose 1.4–4.8 units/Kg of BoNT-A (Botox®) ## per side | 0–25 weeks |
| Tai (2010) [43] Captopril in rats 60 CT®/SD® IGS Rats | 1 injection. Dose 5 units of BoNT-A (Dysport®) ### per side | 12 weeks |
| Fortuna (2013a) [44] Quadriceps Femoris 20 New Zealand White Rabbits | 1 injection. Dose 6 units/Kg of BoNT-A (Botox®) ** per side | 12 weeks |
| Fortuna (2013b) [45] Quadriceps Femoris 27 New Zealand White Rabbits | 1 injection. Dose 6 units/Kg of BoNT-A (Botox®) ** per side | 12 weeks |
| Fortuna (2015) [46] Quadriceps Femoris 23 New Zealand White Rabbits | 1 injection. Dose 6 units/Kg of BoNT-A (Botox®) ** per side | 12 weeks |
| Caron (2015) [51] Captopril 27 Sprague-Dawley Rats | 1 injection. Dose 5 units of BoNT-A (Dysport®) ### per side | 12 weeks |
| Lima (2018) [58] Quadriceps Femoris 60 Wistar Rats | 1 injection. Dose 5 units of BoNT-A (Dysport®) ### per side | 12 weeks |

| Muscle Atrophy (immediately post-neuromuscular muscle harvest under general anesthesia) | |
|-----------------------------------|-----------------------------|
| Wet muscle mass | Wet muscle mass | Wet muscle mass | Wet muscle mass | Wet muscle mass | Wet muscle mass | Wet muscle mass | Wet muscle mass |
| Mean percent loss of muscle mass of 30% at 1 month and 40% at 6 months. | Significant (p < 0.001) decrease of 30% in (group 1) and 35% in (group 2) units. | Significantly diminished (p < 0.001). | No recovery. | Reduction of 10.7% (±3.8) at 56 weeks after a single BoNT-A injection. | Significant atrophy (p < 0.001). | Mean quadriceps femoris muscle mass reduction of 45% (1 month group), 60% (5 months group), and 50% (6 months group). | No recovery. | Reduction of 52% (p < 0.001) at 6 months of repeated monthly BoNT-A injection and a sustained reduction of 15% (p < 0.01) at 4 months after the last BoNT-A injection. | No alteration at 6 months. | Significantly lower weight (p < 0.001) at 12 days post BoNT-A injection. | Significantly lower weight (p < 0.001) at 12 days post BoNT-A injection. | Significantly lower weight (p < 0.001) at 12 days post BoNT-A injection. | No alteration at 6 months. | Significant reduction of 37% (p < 0.001). |
**Table 10. Animal studies—Hystologic (optical and electron microscopy) analysis and histochemistry.**

| Muscle Atrophy Identification Tool | Animal(s) | Studies | Muscle structure (qualitative) | Muscle structure (percentage of contractile material) | Histologic analysis (optical microscopy)/ histochemistry | Muscle ultrastructure | Histologic analysis (electron microscopy)/ histochemistry |
|-----------------------------------|-----------|---------|-------------------------------|-----------------------------|-----------------------------------------------------|---------------------|------------------------------------------------------|
| Herzog (2007) [37] Quadriceps Femoris 25 New Zealand White Rabbits | | | Replacement of contractile fibers with fat. | | Fatty infiltration at 3 and 6 months (increased). No recovery. | | |
| Frick (2007) [38] Tibialis 30 Sprague-Dawley Rats | | | | | Increase in the collagen fibers forming perimysium around the striated muscle cells at 12 weeks. | | |
| Babuccu (2009) [42] Mastication and Temporadis 49 Wistar Rats | | | | | | | |
| Tsai (2010) [43] Gastrocnemius 60 CD (SD) IGS Rats | | | | | | | |
| Fortuna (2011) [44] Quadriceps Femoris 20 New Zealand White Rabbits | | | | | | | |
| Fortuna (2013a) [45] Quadriceps Femoris 20 New Zealand White Rabbits | | | | | | | |
| Fortuna (2013b) [48] Quadriceps Femoris 27 New Zealand White Rabbits | | | | | | | |
| Kocaelli (2016) [54] Masseter and Gluteal 30 Sprague-Dawley Rats | | | | | | | |

Muscle structure (qualitative)

- Herzog (2007) [37]: Replacement of contractile fibers with fat.
- Frick (2007) [38]: Significant decrease at 128. No recovery at day 128.
- Babuccu (2009) [42]: Fatty infiltration at 3 and 6 months (increased).
- Tsai (2010) [43]: Reduction of 36.1% (±16.9), (p < 0.001) at 6 months after 1 BoNT-A injection.
- Fortuna (2011) [44]: Reduction of 40.8% (±6.0), at 6 months after 1 BoNT-A injection.
- Fortuna (2013a) [45]: Reduction of 37.5% (±6.1), at 6 months after 2 BoNT-A injection.
- Fortuna (2013b) [48]: Reduction of 40.1% (±11.8), at 6 months after 3 BoNT-A injection.
- Kocaelli (2016) [54]: Stratification degree of the muscle, nucleus internalization, multinucleation, myofibril diameter, and myonecrosis compatible with muscle atrophy. No recovery at 4 months.

Muscle structure (percentage of contractile material)

- Herzog (2007) [37]: No recovery at day 128.
- Frick (2007) [38]: Significant reduced (p < 0.05). 8 months group: for 45% (±9.2) vastus lateralis, for 70% (±4.0) rectus femoris, for 78% (±4.2) vastus medialis. No recovery.
- Babuccu (2009) [42]: Reduction of 36.1% (±16.9), (p < 0.001). No recovery.
- Tsai (2010) [43]: Significant reduced (p < 0.05), for 43% (±7.2) vastus lateralis, for 70% (±4.0) rectus femoris, for 78% (±4.2) vastus medialis. No recovery.
- Fortuna (2011) [44]: Reduction of 40.8% (±6.0), at 6 months after 1 BoNT-A injection, reduction of 37.5% (±6.5), at 6 months after 2 BoNT-A injection, reduction of 40.1% (±11.8), at 6 months after 3 BoNT-A injection. No recovery.
- Fortuna (2013a) [45]: Reduction of 37.5% (±6.1), at 6 months after 2 BoNT-A injection.
- Fortuna (2013b) [48]: Reduction of 40.1% (±11.8), at 6 months after 3 BoNT-A injection.
- Kocaelli (2016) [54]: Significant (p < 0.001). decrease of diameters of muscle fibers in bundles and fascicles at 12 weeks.

Histologic analysis (optical microscopy)/ histochemistry

- Herzog (2007) [37]: No recovery at day 128.
- Frick (2007) [38]: Significant reduced (p < 0.05). 8 months group: for 45% (±9.2) vastus lateralis, for 70% (±4.0) rectus femoris, for 78% (±4.2) vastus medialis. No recovery.
- Babuccu (2009) [42]: Reduction of 36.1% (±16.9), (p < 0.001). No recovery.
- Tsai (2010) [43]: Significant reduced (p < 0.05), for 43% (±7.2) vastus lateralis, for 70% (±4.0) rectus femoris, for 78% (±4.2) vastus medialis. No recovery.
- Fortuna (2011) [44]: Reduction of 40.8% (±6.0), at 6 months after 1 BoNT-A injection, reduction of 37.5% (±6.5), at 6 months after 2 BoNT-A injection, reduction of 40.1% (±11.8), at 6 months after 3 BoNT-A injection. No recovery.
- Fortuna (2013a) [45]: Reduction of 37.5% (±6.1), at 6 months after 2 BoNT-A injection.
- Fortuna (2013b) [48]: Reduction of 40.1% (±11.8), at 6 months after 3 BoNT-A injection.
- Kocaelli (2016) [54]: Significant (p < 0.001). decrease of diameters of muscle fibers in bundles and fascicles at 12 weeks.

Muscle ultrastructure

- Herzog (2007) [37]: Sarcomere distortion (mild destruction at 8 weeks).
- Frick (2007) [38]: Partial recovery at 26 weeks.
- Babuccu (2009) [42]: Myofilaments atrophic changes characterized by: decrease in myofilament diameter and myofilbrillolysis, dilatations in the terminal cisternae and T-tubules, disorganized Z bands, vacuolar appearance as a result of dilatation in the sarcoplasmic reticulum cisternae and mitochondria swelling.
- Tsai (2010) [43]: Sarcomere distortion (mild destruction at 8 weeks).
- Fortuna (2011) [44]: Partial recovery at 26 weeks.
- Fortuna (2013a) [45]: Partial recovery at 26 weeks.
- Fortuna (2013b) [48]: Partial recovery at 26 weeks.
- Kocaelli (2016) [54]: Partial recovery at 26 weeks.
Table 11. Animal studies—Imaging.

| Muscle Atrophy Identification Tool | Kwon (2007) [39] Medicine 21 New Zealand Rabbits | Han (2018) [56] Paraspinal 01 Cynomolgus Monkey—Macaca Fascicularis |
|------------------------------------|--------------------------------------------------|------------------------------------------------------------------|
| Magnetic resonance imaging (MRI)   | Muscle cross-sectional areas at T12–L1, L1–L2, L2–L3, L3–L4 and L4–L5 levels | Muscle cross-sectional areas at T12–L1, L1–L2, L2–L3, L3–L4, and L4–L5 levels |
| Computed tomography (CT) scan      | Muscle volume                                     | Muscle volume                                                    |
|                                   | Reduction of 19.72% (±4.80) in Group 2 and        | Reduction of 19.72% (±4.80) in Group 2 and                       |
|                                   | of 21.34% (±5.37) in Group 3 at 8 weeks.          | of 21.34% (±5.37) in Group 3 at 8 weeks.                         |
|                                   | Reduction of 13.76% (±5.34) in Group 2 and        | Reduction of 13.76% (±5.34) in Group 2 and                       |
|                                   | of 18.41% (±3.15) in Group 3 at 24 weeks.         | of 18.41% (±3.15) in Group 3 at 24 weeks.                        |
|                                   | Partial recovery at 24 weeks.                     | Partial recovery at 24 weeks.                                    |

Table 12. Animal studies—Direct and indirect muscle atrophy identification via molecular biology.

| Molecular Biology Alterations                                                                 | Articles |
|---------------------------------------------------------------------------------------------|----------|
| Upregulation of proapoptotic: anti-apoptotic protein ratio (Bax:Bcl-2) ratio significantly had an 83.3 fold increase, peak at 4 weeks. | Tsai (2010) [43]. |
| Muscle substitution for adipose tissue determined by adipocyte-related molecules upregulation of adiponectin (APN), Leptin, adipocyte binding protein 2 (AP2), and adipogenic lineage marker upregulation of peroxisome proliferator-activated receptor γ (PPARγ). The APN, Leptin, AP2, and PPARγ were significantly upregulated after BoNT-A injections. | Hart (2017) [55]. |
| Muscle atrophy inferred via molecular biology in regard to upregulation of Transforming Growth Factor-beta TGF-β; upregulation of Nuclear Factor-kappaB (NF-κB); upregulation of p53/Cell cycle control; upregulation of Inhibitor of DNA binding (ID) proteins—Id1, Id2, Id3, Id4, and muscle RING-finger protein-1 (MRF1) upregulation. | Mukund (2014) [49]. Fortuna (2015) [50]. |
| Muscle atrophy and muscle atrophy recovery response indirectly identified via NMJ restoration (muscle-specific receptor tyrosine kinase (MuSK) upregulation, nicotinic acetylcholine receptor (nAChR) upregulation), protection against muscle cell apoptosis (P21 protein upregulation), myogenesis modulation/muscle regeneration (insulin-like growth factor-1 (IGF-1) upregulation, myogenin upregulation, and mitogen-activated protein kinase (MAPK) upregulation. | Shen (2006) [35]. Mukund (2014) [49]. Fortuna (2015) [50]. |
Table 13. Animal studies—Molecular biology.

| Muscle Atrophy Identification Tool | Shen (2008) [35] Gastrocnemius 56 Sprague-Dawley Rats | Tsai (2010) [43] Gastrocnemius 60 CT17 (SD) IGS Rats | Maukund (2014) [49] Tibialis Anterior 20 Sprague-Dawley Rats | Fortuna (2010) [39] Quadriceps Femoris 23 New Zealand White Rabbits | Hart (2017) [55] Quadriceps Femoris 22 New Zealand White Rabbits |
|-----------------------------------|----------------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| Indirect atrophy identification via upregulation of gene and molecule expression signaling neuromuscular junction (NMJ) restoration, protection against muscle cell apoptosis, myogenesis modulation/muscle regeneration. | | | | | |
| | NMJ restoration | NMJ restoration | NMJ restoration | NMJ restoration | NMJ restoration |
| Muscle-specific receptor tyrosine kinase (MuSR) | | | | | |
| Nicotinic acetylcholine receptor (nAChR) significant upregulation (p < 0.05) from day 3 to day 60 | | | | | |
| Myogenin significant upregulation (p < 0.05) from day 3 to day 14 | | | | | |
| | Protection against muscle cell apoptosis | Protection against muscle cell apoptosis | Protection against muscle cell apoptosis | Protection against muscle cell apoptosis | Protection against muscle cell apoptosis |
| Protection atrophy identification via muscle substitution for | | | | | |
| Adipocytokine-related molecules upregulation of | | | | | |
| Adiponectin (APN), Leptin, adipocyte binding protein 2 (AP2), and adipogenic lineage marker upregulation of peroxisome proliferator-activated receptor γ (PPARγ) | | | | | |
| | Muscle-specific receptor tyrosine kinase (MuSR) | | | | |
| | Nicotinic acetylcholine receptor (nAChR) significant upregulation (p < 0.05) from day 3 to day 60 | | | | |
| | Myogenin significant upregulation (p < 0.05) from day 3 to day 90 | | | | |
| | Insulin-like growth factor-1 (IGF-1) significant upregulation (p < 0.05) from day 3 to day 60 | | | | |
| | Myogenesis modulation/muscle regeneration | | | | |
| | Insulin-like growth factor-1 (IGF-1) significant upregulation (p < 0.05) at 4 months | | | | |
| | Recovery not evaluated | | | | |
| | Insulin-like growth factor-1 (IGF-1) significant upregulation (p < 0.05) (at 6 months) | | | | |
| | Recovery not evaluated | | | | |
| | Insulin-like growth factor-1 (IGF-1) significant upregulation (p < 0.05) (at 6 months after 3 BoNT-A injections) | | | | |
| | Recovery not evaluated | | | | |
| | Insulin-like growth factor-1 (IGF-1) significantly upregulated (p < 0.05) (at 6 months) | | | | |
| Protection atrophy identification via muscle substitution for | | | | | |
| Adipose tissue | | | | | |
| | Adipocytokine-related molecules upregulation of | | | | |
| | Adiponectin (APN), Leptin, adipocyte binding protein 2 (AP2), and adipogenic lineage marker upregulation of peroxisome proliferator-activated receptor γ (PPARγ) | | | | |
| | Insulin-like growth factor-1 (IGF-1) significantly upregulated (p < 0.05) (at 6 months) | | | | |
| | Recovery not evaluated | | | | |
| | Insulin-like growth factor-1 (IGF-1) significantly upregulated (p < 0.05) (at 6 months after 3 BoNT-A injections) | | | | |
| | Recovery not evaluated | | | | |

Molecular biology (Real-Time Quantitative Polymerase Chain Reaction (qPCR), and/or Microarray Data Analysis, and/or Western blot analysis)
4.3.2. Human Studies
Optical and Electron Microscopy

Histologic (optical and electron microscopy) analysis and histochemistry showed results in humans similar to those found in animal models. Muscle atrophy (atrophic muscle fibers, myofibrillar disorganization, fibrosis, necrosis, and increase of the number of perimysial fat cells) were well-documented by Kim (2005) [34], Schroeder (2009) [41], Valentine (2016) [52], and Li (2016) [53]. The Orbicularis oculi muscle showed that the morphometric measurements of muscle fibers reduced, with an irregular diameter at 3 months after BoNT-A injections, \( p < 0.05 \). Ansved (1997) [31] showed a mean diameter reduction of type IIB striated muscle fibers (Vastus lateralis) of 19.6\% after 2–4 years of BoNT-A treatment \( p < 0.05 \). Partial recovery of the changes described above were seen in some articles (Table 14).

Table 14. Human studies—Histologic (optical and electron microscopy) analysis and histochemistry.

| Identification Tool | Muscle Atrophy | Muscle Ultrastructure | Morphometric Measurements of Muscle Fibers | Morphometric Measurements of Muscle Fibers | Morphometric Measurements of Muscle Fibers |
|---------------------|----------------|----------------------|------------------------------------------|------------------------------------------|------------------------------------------|
| Borodic (1992) [29] | Orbicularis Oculi 14 | Histologic analysis (optical microscopy)/ histochemistry | Reduced and irregular diameter at 3 months \( p < 0.05 \). Partial recovery at 6 months. | Muscle ultrastructure | Reduced muscle atrophy, necrosis, and hyaline degeneration at 4 months. |
| Ansved (1997) [31] | Vastus Lateralis (Non-Target Muscle) 22 | Muscle structure | Mean diameter reduction of type IIB fibers of 19.6\% after 2–4 years of BoNT-A treatment, \( p < 0.05 \). | Muscle ultrastructure | Muscle atrophy and mild increase of the number of perimysial fat cells. Muscle fiber area reduction of 24\% at 12 months. Partial recovery at 12 months. |
| Kim (2005) [34] | Masseter 305 | Morphometric measurements of muscle fibers | Muscle structure | Muscle ultrastructure | Fibrosis with no identifiable muscle fibers. |
| Schroeder (2009) [41] | Gastrocnemius 2 | Muscle structure | Muscle structure | Muscle ultrastructure | Muscle ultrastructure |
| Valentine (2016) [52] | Gastrocnemius 10 | Muscle structure | Muscle ultrastructure | Muscle ultrastructure | |
| Li (2016) [53] | Medial Rectus (Extraocular Muscle) 3 | Muscle structure | Muscle ultrastructure | Muscle ultrastructure | |

Imaging

All the 10 human studies that evaluated images to measure muscle atrophy after BoNT-A treatments showed signs of muscle atrophy, irrespective of the technology used: ultrasound, MRI, CT scan, or cephalometry. Muscle atrophy was registered in the short term (42 days to 3 months) and in the long term (up to 2 years). No full recovery was identified (Table 15).
| Muscle Atrophy Identification Tool | Muscle Thickness | Muscle Thickness | Muscle Thickness | Muscle Thickness | Muscle Thickness | Muscle Thickness | Muscle Thickness | Muscle Thickness | Muscle Thickness |
|-----------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Hamjian (1994) [30]               | Decrease of 16% | Median decrease  | Average decrease | Muscle thickness | Muscle thickness | Muscle thickness | Muscle thickness | Muscle thickness | Muscle thickness |
| Essecro Digitatum 10              | at peak (day 42) | of 30%-35% at peak (5 months) and 13.4% (1 year), (p < 0.01). Partial recovery 2 years. | of 31% (5 months after BoNT-A injection), (p not calculated). Partial recovery 2 years. | 17 of 24 |
| Fanucci (2001) [32]              | (day 42), (p < 0.05) | Recovery (Partial? Complete?) 100 days. | | | | | | | |
| Tu (2001) [33]                   | Recovery (Partial? Complete?) 100 days. | | | | | | | | |
| Fanucci (2001) [32]              | | | | | | | | | |
| Masseter 15                      | | | | | | | | | |
| Hamjian (1994) [30]              | | | | | | | | | |
| Masseter 15                      | | | | | | | | | |
| Koo (2001) [34]                  | | | | | | | | | |
| Masseter 15                      | | | | | | | | | |
| Kim (2005) [36]                  | | | | | | | | | |
| Masseter 15                      | | | | | | | | | |
| Koerte (2001) [36]               | | | | | | | | | |
| Procerus 4                       | | | | | | | | | |
| Lee (2007) [40]                  | | | | | | | | | |
| Masseter 15                      | | | | | | | | | |
| Schroeder (2009) [41]            | | | | | | | | | |
| Gastrocnemius 2                  | | | | | | | | | |
| Singer (2006) [42]               | Median decrease of 30.9% at peak (3 months) and 13.4% (1 year), (p < 0.001). Partial recovery 1 year. |
| Psoas 7                          | | | | | | | | | |
| Lee (2007) [40]                  | | | | | | | | | |
| Masseter 15                      | | | | | | | | | |
| Schroeder (2009) [41]            | | | | | | | | | |
| Gastrocnemius 2                  | | | | | | | | | |
| Koerte (2001) [47]               | | | | | | | | | |
| Procerus 4                       | | | | | | | | | |
| Koerte (2001) [47]               | | | | | | | | | |
| Procerus 4                       | | | | | | | | | |
| Koerte (2001) [47]               | | | | | | | | | |
| Procerus 4                       | | | | | | | | | |
| Koerte (2001) [47]               | | | | | | | | | |
| Procerus 4                       | | | | | | | | | |
| Koerte (2001) [47]               | | | | | | | | | |
| Procerus 4                       | | | | | | | | | |
| Koerte (2001) [47]               | | | | | | | | | |
| Procerus 4                       | | | | | | | | | |
| Koerte (2001) [47]               | | | | | | | | | |
| Procerus 4                       | | | | | | | | | |
| Koerte (2001) [47]               | | | | | | | | | |
| Procerus 4                       | | | | | | | | | |
| Koerte (2001) [47]               | | | | | | | | | |
| Procerus 4                       | | | | | | | | | |
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| Koerte (2001) [47]               | | | | | | | | | |
| Procerus 4                       | | | | | | | | | |
| Koerte (2001) [47]               | | | | | | | | | |
| Procerus 4                       | | | | | | | | | |
| Koerte (2001) [47]               | | | | | | | | | |
| Procerus 4                       | | | | | | | | | |
| Koerte (2001) [47]               | | | | | | | | | |
| Procerus 4                       | | | | | | | | | |
| Koerte (2001) [47]               | | | | | | | | | |
| Procerus 4                       | | | | | | | | | |
| Koerte (2001) [47]               | | | | | | | | | |
| Procerus 4                       | | | | | | | | | |
| Koerte (2001) [47]               | | | | | | | | | |
| Procerus 4                       | | | | | | | | | |
| Koerte (2001) [47]               | | | | | | | | | |
| Procerus 4                       | | | | | | | | | |
| Koerte (2001) [47]               | | | | | | | | | |
| Procerus 4                       | | | | | | | | | |
| Koerte (2001) [47]               | | | | | | | | | |
| Procerus 4                       | | | | | | | | | |
| Koerte (2001) [47]               | | | | | | | | | |
| Procerus 4                       | | | | | | | | | |
| Koerte (2001) [47]               | | | | | | | | | |
| Procerus 4                       | | | | | | | | | |
5. Discussion

The use of BoNT-A for cosmetic purposes is a fast-growing procedure, with more than six million treatments performed by plastic surgeons in the year 2018 alone [59]. Despite this significant number, we believe that improvements in BoNT-A aesthetic treatments have been jeopardized by the famous, but simplistic, statement used by the media, patients, and doctors: “BoNT-A treats wrinkles”. BoNT-A monotherapy relating to wrinkles is, at least, questionable. The BoNT-A mechanism of action is presynaptic cholinergic nerve terminals blockage by inhibition of the release of acetylcholine, causing paralysis and subsequent functional denervated muscle atrophy to some degree [60]. It is important to keep in mind that wrinkles have a multitude of causes, besides muscle contraction, and that treatments of wrinkles based only on the use of BTX-A have poor quality results in the long term [61]. Rohrich (2007) [62] brilliantly demonstrated modern topographic anatomic studies proving the relationship between wrinkles and underlying structures other than muscles, such as arteries, veins, nerves, and septa of fat compartments [62].

The use of BTX-A was first studied by Scott (1973) [63] for the treatment of strabismus by pharmacologic weakening the extraocular muscles [33]. The first described use of the toxin in aesthetic circumstances was by Clark and Berris (1989) [64], but it still carried out the essence of the BoNT-A mechanism of action based on muscle paralysis and atrophy [64]. At some point during the 1990s, Carruthers and Carruthers [65] began to use botulinum toxin type A in full-scale treatments for aesthetic purposes. Since then, the aesthetic focus regarding the use of BoNT-A moved towards removing wrinkles only [65]—a shift in the medical literature on BoTN-A for aesthetics purposes that has persisted until today. We are not underestimating the importance of Carruthers and many other authors that previously studied the use of BoNT-A in aesthetics but, as mentioned above, we intend to provide the aesthetic use of BoNT-A a new perspective. The real mechanism of actions of BoNT-A for aesthetic purposes have been forgotten, to a level where recent publications still focus on the fact that muscle paralysis and muscle atrophy is a complication of the “wrinkle treatment” capacity of BoNT-A instead of its expected effect [66–68].

This systematic review can shed new light on aesthetic BoNT-A treatments basing itself on old, but scientifically correct, concepts of striated muscle contraction physiology, muscle hypertrophy, and muscle atrophy—basic concepts of muscle physiology from reference physiology medical books such as the Guyton and Hall Textbook of Medical Physiology [69].

The results of this systematic review showed evidence that seriated or single BoNT-A muscle injections can cause real atrophy on a short or long-term basis, in animal models and in humans, in skeletal striated muscles of the limbs, facial masticatory muscles, and facial mimetic muscles. Due to only limited good quality data being available, we included animal model studies and human studies, but we know that data extrapolation from animal model studies to humans are, at least, naïve. The sensitivity of animals to BoNT-A has been known for many years to be less than that perceived in humans [70]. There are even differences in sensitivity between rats and mice [71]. On this basis, animal studies must be carefully designed and carefully analyzed, or they cannot be interpreted with respect to human effects [72]. Here we will discuss the results of this systematic review, making clear distinction between animal model studies and human studies (Figure 3).
Figure 3. Animal model studies results—Discussion overview. This finding might be of clinical relevance, because muscle volume measured using non-invasive imaging techniques (MRI, ultrasound) are sometimes used to approximate muscle mass in patient populations to determine progression of a disease or success of a treatment intervention—Damiano and Moreau (2008) [73]. Structural integrity and functional properties of muscles, rather than muscle mass or volume, might be more appropriate outcome measures to determine disease progression or aesthetics intervention effects.
Increasing the number of injections did not produce additional loss in muscle strength and contractile material, as one might have suspected, suggesting that most of the muscle damage effects of BTX-A injection into muscles are caused by the first injection, or that the recovery period between injections was sufficient for partial recovery, thereby offsetting the potential damage induced by each injection.

Genetic alterations related to muscle atrophy/recovery through molecular biology were analyzed by five studies and showed how much impact a single BoNT-A injection can cause on a molecular basis. Mukund (2014) [49] realized that the direct action of BTX-A in skeletal muscle is relatively rapid, inducing dramatic transcriptional adaptation at one week and activating genes in competing pathways of repair and atrophy by gene-related impaired mitochondrial biogenesis.

Much like the findings of animal studies, human studies have also clearly shown atrophy in different muscle types after BTX-A injections. All six human studies that evaluated muscle histology showed atrophy, and when muscle recovery was assessed, there was no full recovery—Borodic (1992) [29] and Schroeder (2009) [41]. Bringing this idea into the context of facial aesthetics, the treatment of the Orbicularis oculi muscle, for example, with BTX-A sporadic injections could atrophy this muscle, but serial and controlled treatments could really maintain a certain degree of atrophy capable of allowing a smile with more open eyes, less caudal traction vector in the cranial part of this muscle postponing gravitational aging, and even give less contribution to the formation of the famous periorbital wrinkles, this time, as a secondary effect. Extrapolations of the powerful tool of muscle atrophy control through time using BTX-A injections could change completely the way BTX-A is used for aesthetic purposes. Dosages, injection intervals, and target muscles would be different from the patterns used nowadays. Instead of planning BTX-A injections to treat wrinkles, a modern anatomy understanding of the facial mimetic muscles as described by Boggio (2017) [74] would be of unparallel importance for aesthetic treatment planning [74]. New approaches for facial aesthetic treatments using BoNT-A could be completely based on mimetic facial muscle interactions and focused on reducing the activity of muscles that enhance gravitational aging (facial depressor muscles), such as the platysma muscle, for example, and preserving antigravitational muscles (elevator facial muscles), such as the frontalis (Figure 4).

After analyzing the results of this paper, we can attempt to answer the questions raised in the introduction (Table 16).

Table 16. Possible and plausible evidence-based answers for the questions raised in the introduction.

| Questions                                                                 | Answers                                                                 |
|--------------------------------------------------------------------------|-------------------------------------------------------------------------|
| Does the muscular impairment for contraction caused by BoNT-A really treat facial lines or cause muscle atrophy? | Muscle atrophy occurs after BoNT-A injections. Facial lines are, only in part, treated by BoNT-A injections. |
| What is the relationship between BoNT-A muscle injections and muscle atrophy in the long term? | Muscles tend to maintain atrophy or have partially recover after BoNT-A injections. |
| Is it possible to modulate the level of muscle atrophy through time by using BoNT-A? | At least theoretically it is, and further studies could help us master this new frontier in facial aesthetics. |
| What if we used muscle atrophy caused by BoNT-A injections to optimize muscle architecture for facial aesthetic purposes? | It seems smart to use the atrophy after BoNT-A injections as a tool for aesthetic purposes instead of the old idea of an adverse event. |
| What would it be like to reinterpret articles written in the last 30 years that focused mainly on facial lines unveiling this concept of muscle atrophy? How many less subjective opportunities would arise? How would classic BoNT-A injections techniques would be impacted? | We are sure that understanding BoNT-A as a muscle atrophy tool for aesthetic purposes will bring us to new readings of previous articles and shed new light on future treatments. |
6. Conclusions

This systematic review showed evidence that seriated or single BoNT-A muscle injections can cause real muscle atrophy on a short or long-term basis, in animal models and in humans, in skeletal striated muscles of the limbs, facial masticatory muscles, and facial mimetic muscles. Theoretically, muscular architecture reprogramming is a possible new approach in aesthetics. Depressor facial muscles could be targeted to have some degree of atrophy with BoNT-A injections, while elevator facial muscles could be spared to some degree to maintain antigravitational traction forces and facilitate a lift effect.

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