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CURRENT OPINION

Pharmaceutical, Biological, and Clinical Properties of Botulinum Neurotoxin Type A Products

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Abstract Botulinum neurotoxin injections are a valuable treatment modality for many therapeutic indications and have revolutionized the field of aesthetic medicine so that they are the leading cosmetic procedure performed worldwide. Studies show that onabotulinumtoxinA, abobotulinumtoxinA, and incobotulinumtoxinA are comparable in terms of clinical efficacy. Differences between the products relate to the botulinum neurotoxin complexes, specific biological potency, and their immunogenicity. Protein complex size and molecular weight have no effect on biological activity, stability, distribution, or side effect profile. Complexing proteins and inactive toxin (toxoid) content increase the risk of neutralizing antibody formation, which can cause secondary treatment failure, particularly in chronic disorders that require frequent injections and long-term treatment. These attributes could lead to differences in therapeutic outcomes, and, given the widespread aesthetic use of these three neurotoxin products, physicians should be aware of how they differ to ensure their safe and effective use.

1 Introduction

Botulinum toxin is produced by anaerobic fermentation of the bacterium *Clostridium botulinum*. A number of different strains of *C. botulinum* have been identified, which produce eight immunologically distinct serotypes (type A–H) and consist of the botulinum neurotoxin complexed with a number of neurotoxin-associated proteins. Serotypes A and B have been developed for human use. The first approval of a botulinum neurotoxin was for the treatment of blepharospasm, hemifacial spasm, and strabismus in 1989. Since then, the number of commercial botulinum toxins and their uses has expanded for clinical as well as aesthetic indications, and botulinum toxin products are now licensed for a broad range of indications (with approved indications varying by country and product), including temporary improvement of dynamic facial lines, symptomatic relief of blepharospasm, cervical dystonia (spasmodic torticollis), and various forms of focal spasticity, management of severe hyperhidrosis, prophylaxis of headaches in adults with chronic migraine, and management of urinary incontinence due to spinal cord injury or multiple sclerosis [1–6].

Neurotoxin type A preparations are the most widely used worldwide and the only ones that are US FDA approved for aesthetic use. There are currently three leading botulinum neurotoxin type A (BoNT/A) products on the market in the Western hemisphere: onabotulinumtoxin A (ONA; Botox/Vistabel®, Allergan Inc., Irvine, CA, USA), abobotulinumtoxin A (ABO; Dysport®/Azzalure®, Ipsen, Paris, France), and incobotulinumtoxin A (INCO; Xeomin/Bocouture®, Merz Pharmaceuticals GmbH, Frankfurt, Germany). Recently published statistics from the International Society of Aesthetic Plastic Surgery show that BoNT/A injections are now the most popular of all cosmetic procedures worldwide, both surgical and non-surgical [7]. As a result of the growing popularity of BoNT/A injections among the general public, physicians from diverse specialties are integrating botulinum toxin injections into their practices. With three BoNT/A products now available for aesthetic use and a large population of potential patients with individual needs and preferences, it is important that practitioners are familiar with all aspects

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of the available preparations. The author of this paper has been involved in botulinum neurotoxin research since 1983 and was instrumental in the development of the latest BoNT/A product to reach the market. In this current opinion paper, the author highlights the similarities and differences between the currently available BoNT/A products in terms of their pharmaceutical and biological properties, and discusses why an understanding of these properties is important for optimal therapeutic use.

2 Clinical Comparisons

Several clinical studies in different indications (including, but not limited to, cervical dystonia, glabellar lines, crow's feet, and blepharospasm) have demonstrated comparable clinical efficacy of INCO compared with ONA, with a 1:1 conversion ratio between the two products [8–12]. The studies also reported very similar outcomes in terms of time to onset, time to waning, total duration of effect, and side effect profile.

Some publications give approximate conversion factors between preparations, with several reporting that the units of ONA and INCO are equivalent [13–17]. Recent evidence-based consensus reviews on BoNT/A applications in aesthetic medicine have summarized the evidence supporting a 1:1 dose relationship between ONA and INCO [18–20]. When converting between ABO and ONA, a ratio of 2–3 ABO units for 1 unit of ONA has been proposed in several publications [17, 21, 22]. A consensus review by Carruthers et al. [18] stated that, although no clear ONA:ABO conversion ratio has been established, a dose ratio of 1:2.5 may be assumed in aesthetic indications.

3 Structure and Mechanism of Action

Botulinum toxin consists of the 150 kDa neurotoxin itself and a set of neurotoxin-associated complexing proteins (NAPs), which together form high-molecular-weight progenitor complexes. All botulinum neurotoxin serotypes are synthesized as single chain proteins (150 kDa) that are proteolytically cleaved into di-chain proteins consisting of a 50 kDa light chain and a 100 kDa heavy chain, connected by a disulfide bond.

All three preparations of BoNT/A have a similar mechanism of action [23, 24]. The heavy chain binds to specific glycolipids, gangliosides (GT1b), and a specific cell surface receptor (SV2) on cholinergic nerve endings, enabling its uptake by endocytosis and promoting translocation of the light chain across the endosomal membrane and into the cytosolic compartment [24]. At the same time, the disulfide bond linking the two chains is reduced, allowing the light chain to diffuse freely in the cytosol. The light chain has proteolytic activity and, after internalization, binds with high specificity to a SNARE protein, which is subsequently cleaved. The target SNARE proteins vary among the different serotypes, but the BoNT/A serotype cleaves synaptosomal membrane-associated protein 25 kDa (SNAP-25). The cleavage of SNAP-25 prevents the fusion of the synaptic vesicle with the presynaptic membrane, thereby blocking the release of acetylcholine into the synaptic cleft [25, 26]. Depending on the target tissue, BoNT/A can block the cholinergic neuromuscular innervation of striated and smooth muscles or the cholinergic autonomic innervation of exocrine glands.

4 Molecular Weight and Complexing Proteins

Commercially available BoNT/A formulations contain different complements of NAPs, and therefore have different molecular weights and three-dimensional structures (Table 1) [27, 28]. Studies have shown that the complex composition of botulinum neurotoxins is specific to the method of growth and the method of purification [29, 30]. The complexes dissociate almost instantaneously on reconstitution of the lyophilized or vacuum-dried product [31] and have never been demonstrated in the vials of the commercial products.

The active protein in all commercially available products is the 150 kDa neurotoxin, the amino acid sequence of which is identical in ONA and INCO as they are both produced from the Hall strain for *C. Botulinum* type A [32]. The corresponding sequence for ABO has not been published, but is likely identical, because the manufacturer also uses a Hall strain [29].

INCO differs from ONA and ABO in that it is free from complexing proteins and consists of only the 150 kDa neurotoxin responsible for the therapeutic effect [32]. NAPs are not pharmacologically active on nerve terminals and consist of several hemagglutinins (HA) and a single non-toxic non-hemagglutinin (NTNH) [27, 33, 34]. Data support the role of these proteins in protecting the neurotoxin from acidic and proteolytic degradation in the digestive tract [35, 36]. The HAs play a key role in the oral toxicity of botulinum neurotoxin. By binding and disrupting the cell adhesion protein E-cadherin, they allow the toxin to pass through the intestinal epithelial barrier and enter the systemic circulation [37].

The botulinum neurotoxin-NAP progenitor complexes isolated from *C. botulinum* type A cultures adopt three sizes: 900, 500, and 300 kDa [38]. The complex size for ONA is 900 kDa [30] (Table 1). There is no information on the exact complex size of ABO, but data have shown that ABO complexing proteins are present as both full-length

Table 1 Comparison of botulinum neurotoxin type A formulations

Botulinum toxin type A	ABO	ONA	INCO
Brand name	Azzalure®, Dysport®	Botox®, Vistabel®	Xeomin®, Bocouture®
Approved aesthetic indication	Moderate to severe glabellar lines	Moderate to severe glabellar lines and crow's feet	Moderate to severe glabellar lines and crow's feet
Presentation	Freeze-dried (lyophilized) powder for reconstitution	Vacuum-dried powder for reconstitution	Freeze-dried (lyophilized) powder for reconstitution
Isolation process	Precipitation and chromatography	Precipitation	Precipitation and chromatography
Composition	*Clostridium botulinum toxin* type A; HA and non-HA proteins	*Clostridium botulinum toxin* type A; HA and non-HA proteins	*Clostridium botulinum toxin* type A
Excipients[a]	500 U vial: human serum albumin 125 µg; lactose 2.5 mg	100 U vial: human serum albumin 0.5 mg; NaCl 0.9 mg	100 U vial: human serum albumin 1 mg; sucrose 4.6 mg
Molecular weight (neurotoxin), kDa	Not published (150)	900 (150)	150
Approximate total clostridial protein content (ng per 100 U)	4.87	5.0	0.44
Neurotoxin protein load (ng neurotoxin per 100 U[a])	0.65	0.73	0.44
Specific neurotoxin potency (U/ng)	154	137	227
Shelf-life	2–8 °C 2 years	2–8 °C 2–3 years[b] (or freezer)	Room temperature 3–4 years[b]
Storage (post-reconstitution)	2–8 °C 4 h	2–8 °C 24 h	2–8 °C 24 h

ABO abobotulinumtoxin A, *HA* hemagglutinin, *INCO* incobotulinumtoxin A, *ONA* onabotulinumtoxin A

[a] Units of measurement for the three commercially available BoNT/A preparations are proprietary to each manufacturer and are not interchangeable

[b] Depending on the number of units

proteins and as a succession of fragments [29]. As most of the NTNH is truncated in ABO, one can infer that there is little or no 500 kD and no 900 kDa complex, and that the 300 kDa complex is probably the most abundant.

Importantly, it has been argued that molecular weight or protein complex size do not affect biological activity and pharmacological properties, as the BoNT/A neurotoxin rapidly dissociates from the complexing proteins (if present) after dilution, drying, and reconstitution of the preparation, with ≥85 % of neurotoxin present in the 150 kDa free form prior to injection into target tissues [31].

5 Botulinum Neurotoxin Type A Manufacturing Process and Reconstitution

The precise details of manufacturing processes are proprietary, but product purification involves precipitation as a first step for each commercially available preparation. ONA is purified by repeated precipitation and redissolution, whereas ABO is produced by purification using column chromatography [39]. During the manufacture of INCO, the complexing proteins are removed. This is performed in a series of chromatographic steps to minimize the risk of inactive toxin content, and thus limit possible denaturation, degradation, and loss of biological activity [40]. The three commercial preparations of BoNT/A discussed in this paper are supplied in either vacuum-dried or lyophilized (freeze-dried) form (Table 1). Excipients (NaCl or sucrose or lactose, and human serum albumin) are added to minimize the risk of product inactivation during this process and during long-term storage. ONA is diluted in a solution containing NaCl and albumin prior to vacuum-drying, which has been proposed to negatively impact on neurotoxin activity and may be responsible for its toxoid (inactive neurotoxin) content [32, 41]. From a clinical perspective, the implication is that inactive neurotoxin would not be taken up by nerve cells, but could be recognized by the immune system.

Practitioners should be aware that suboptimal reconstitution of BoNT/A preparations can diminish their efficacy [42, 43]. The complexing proteins dissociate almost completely from the neurotoxin following reconstitution with saline before injection into the target. The pH of the saline used for their reconstitution has been reported to vary between pH 4.5 and 7.0 [44], which provides a slightly acidic solution because the products are not buffered. A low pH can cause a stinging sensation reported by sensitive patients, but this is true for all products.

6 Potency per Unit Weight of Toxin Protein

For safety and efficacy reasons, it is important for BoNT/A biological activity to be accurately determined. The biological potency of BoNT/A drugs is based on the determination of the median lethal dose of toxin/neurotoxin after intraperitoneal injection in mice (median lethal dose [LD50] assay) [3, 6]. On this basis, 1 unit of toxin is defined as one mouse LD50, i.e. the dose of toxin/neurotoxin capable of killing 50 % of a group of mice. Product dose for treating patients is determined by each manufacturer's LD50 potency assay results [45]. These assays use different in-house diluents and standards, so the unit of measurement for the three commercially available BoNT/A preparations is proprietary to each manufacturer [21]. This precludes direct comparisons of potency between products [29, 46–48] and highlights the importance of clinical head-to-head studies for comparing different BoNT/A products and their respective conversion ratios. Nevertheless, labeled potency for ONA and INCO is identical, with a 1:1 conversion ratio between the products [6, 46], and several studies have reported clinical equipotency for these agents [8–10]. Measurement of ONA and INCO in the same LD50 assay using diluent as in clinical setting conditions showed equivalent potency [13]. As required by governmental agencies, the LD50 assay is being replaced by more humane, cell-based assays, which must be cross-validated against the LD50 assay to provide the same potency result. Each manufacturer is developing their own proprietary cell-based assay.

The respective amounts of neurotoxin per 100 U, measured using a high-sensitivity enzyme-linked immunosorbent assay (ELISA) technique, were 0.73 ng for ONA, 0.65 ng for ABO, and 0.44 ng for INCO (Table 1) [32]. The specific neurotoxin potency or biological activity (U) per mass of neurotoxin protein was calculated based on the overall mean concentration of BoNT/A neurotoxin, giving INCO the highest specific biological activity (U/ng neurotoxin) at 227 U/ng compared with 137 U/ng for ONA and 154 U/ng for ABO [32, 46]. INCO contains no other clostridial proteins, and, therefore, the specific biologic potency relative to the total foreign protein is 227 U/ng. As the reported clostridial protein content per 100 U of ONA is 5 ng and of ABO is 4.35 ng, the equivalent specific biologic potency relative to the total foreign-protein load for onabotulinumtoxinA is 20 U/ng and for ABO is 115 U/ng. Thus, the foreign-protein load delivered per unit of INCO is lower than that for both ONA and ABO.

The units of ABO are different from those of ONA and INCO. However, comparing ONA and INCO, which have demonstrated similar clinical activity [32], the findings suggest that 0.44 ng of INCO has the same biological activity as 0.73 ng of ONA. It is hypothesized that part of the neurotoxin in ONA may be inactive or denatured due to the specific vacuum-drying process used in the manufacture of the final drug [32, 49].

7 Spread and Diffusion

Discussions on neurotoxin spread and diffusion are hampered by inconsistent use of terminology. Spread occurs when the injected molecule travels from the original injection site, for example as a result of injection technique, volume of injection, or needle size. In contrast, diffusion indicates the passive movement of neurotoxin toxin along a concentration gradient within the target tissue beyond its original injection site [50].

Precise localization of neurotoxin is required to produce the desired clinical results. Temporary disfigurement or functional impairment can occur if the neurotoxin diffuses into adjacent muscle. Aoki et al. [51] proposed that different diffusion characteristics were attributed to protein complex size and pharmacological properties, whereby the high-molecular-weight toxin complex of ONA would limit tissue distribution and explain reported differences in side effects favoring ONA over ABO [51]. However, more recent studies, which have compared diffusion of BoNT/A products by measuring the size of anhidrotic halos following injection of identical volumes into the forehead of patients, suggest that this is not the case. A comparison of ONA and ABO, using dose ratios of 1:2.5, 1:3, and 1:4, showed that the area of anhidrosis was larger with ABO in 93 % of comparisons at all dose ratios and identical injection volumes [52]. A separate study, which used a dose ratio of 1:2.5, observed no significant differences between the mean size of halos produced by the two products [53]. There were no differences in product diffusion when the same dose was injected with the same technique. A comparison of INCO with ONA showed no difference in the size of the anhidrotic area produced following injection of 5 U of INCO versus 5 U of ONA on either side of the forehead after 6 weeks and 6 months. Importantly, the adverse event profile in the pivotal head-to-head studies did not show any difference between INCO and ONA [8–10]. While containment of diffusion is a desirable goal [54], data show that the presence of complexing proteins in the pharmaceutical preparation does not reduce migration of the neurotoxin [55].

The underlying reason for the lack of difference in diffusion is because the neurotoxin is already dissociated from the complexing proteins after reconstitution of a vial prior to injection into target tissues, and migrates alone in the injected tissue [31].

8 Stability

In the commercial formulations evaluated in this paper, human serum albumin (HSA) is required to stabilize the BoNT/A products, with ABO having the lowest content of all (Table 1) [1–6]. The low amount of HSA in ABO could at least partly explain why not all the neurotoxin in ABO is bioavailable depending on the concentration of the HSA in the injected volume [56]. According to respective product labels, ABO has a shelf life of 2 years at 2–8 °C, ONA can be stored for 2 or 3 years at 2–8 °C (depending on the number of units) or in the freezer, and INCO has a shelf life of 3 or 4 years at room temperature. After reconstitution, ONA and INCO are stable for 24 h at 2–8 °C, and ABO is stable for 4 h at 2–8 °C [1–6]. The prolonged shelf life and less stringent temperature restrictions displayed by INCO (Table 1) suggest that complexing proteins are not required for BoNT/A stability [57]. Among the three leading available BoNT/A products, INCO is the only botulinum product that is stable in lyophilized form for up to 4 years at room temperature, whereas ONA and ABO products must be stored refrigerated [58]. In a stress stability study, INCO survived storage at temperatures as high as 60 °C for 1 month without loss of potency [57].

9 Immunogenicity

Immunogenicity refers to the ability of a protein product to elicit antibody formation. As with any therapeutic protein, botulinum toxin is regarded as foreign by the host and therefore has the potential to induce an immune response, particularly with repeated administration. This can lead to the development of neutralizing antibodies that may or may not result in secondary treatment failure. Overall, BoNT/A products exhibit lower clinically detectable levels of antibodies than do other approved biologic products [59]. The development of neutralizing antibodies is more common in therapeutic indications, where doses tend to be much larger, but they are increasingly been reported in patients receiving botulinum toxin for aesthetic treatment along with cases of secondary non-responsiveness [60–64]. A number of factors can impact the immunogenicity of botulinum neurotoxins, including product-related factors such as the manufacturing process, the antigenic protein load, and the presence of complexing proteins, as well as treatment-related factors such as the overall toxin dose, booster injections, and prior exposure.

A distinguishing feature among the commercially available neurotoxins is the presence or absence of complexing proteins. NAPs do not play a role in toxin-induced blockade of cholinergic transmission and, until recently, were thought to be just a group of passive bystanders when injected for therapeutic and aesthetic uses. However, several lines of evidence that have examined the fate and possible interactions of NAPs with patient tissues after intramuscular injection suggest this may not be the case and that the presence of complexing proteins might be clinically relevant [65–67].

Preclinical data have shown that, in contrast to ONA and ABO, INCO does not lead to the production of neutralizing antibodies following repeated injections into New Zealand white rabbits [65]. Kukreja et al. [66] measured the immunological reactivity of BoNT/A in its purified and complex forms and demonstrated that BoNT/A with complexing proteins (including HA-33) triggered a stronger immune response than the purified 150 kDa neurotoxoid alone. HAs are known to act as adjuvants [68, 69] and can bind and activate dendritic cells, which play a key role in early phases of the immune response. In particular, HA-33 is the largest component of the complexing proteins and a major immunoreactive protein in the BoNT/A complex [70, 71].

That complexing proteins can induce an inflammatory response has recently been demonstrated in a human neuroblastoma cell line (SH-SY5Y) [67]. While pure BoNT/A, BoNT/A complex, and NAPs all bound to the SH-SY5Y neuronal cells, the BoNTA complex and NAPs additionally bound to lymphoblasts and fibroblasts. Furthermore, pure BoNT/A did not affect inflammatory cytokine release, whereas the BoNT/A complex and NAPs increased the release of multiple inflammatory cytokines. Moreover, the cytokines induced by the BoNTA complex and by NAPs alone varied, suggesting that the different structure of BoNT/A complex induces significantly differential host response in human neuronal cells.

The clinical implication is that complexing proteins are immunogenic and can elicit an immune reaction against BoNT/A [67]. However, antibody titers required to cause resistance to botulinum toxin have not been defined and immune responses can differ between patients. Furthermore, variability in the reported prevalence of neutralizing antibodies and treatment failure can be attributed to study design, administered doses, indication, assay methodology, timing of serum sample testing, and treatment history [72, 73]. Not all immune responses preclude the biological therapy from being clinically effective. Only antibodies that bind botulinum toxin in a manner that neutralizes its biological activity sufficiently will attenuate its effect on the neuromuscular junction. Thus, the formation of antibodies may have no effect on treatment or may result in partial or complete clinical unresponsiveness to botulinum toxin type A [74, 75]. However, there is a risk that antibody titers will increase with further injections, which might have a booster effect. Today's cosmetic patients start their aesthetic treatments at increasingly younger ages and not

only for a single indication, resulting in an increased frequency of neurotoxin use, as well as a larger total amount of neurotoxin use over a lifetime.

The prevalence of patients developing neutralizing antibodies after long-term treatment with ONA or ABO is dependent on the condition being treated and thus treatment dose, with incidence rates ranging from 0.3 to 6 % [72, 76–82]. To date, there has been only one case of antibody-induced therapy failure with INCO. This occurred in a patient with progressive hereditary juvenile-onset generalized dystonia whose immune system had already been sensitized by pretreatment with ABO for 15 years [83], supporting the hypothesis of reduced immunogenicity with INCO [84]. Furthermore, a prospective blinded study in 37 cervical dystonia patients previously treated with ONA or ABO who developed neutralizing antibodies and partial secondary non-responsiveness, reported that continuous treatment with INCO every 3 months for 48 months did not result in an increase in neutralizing antibody titer [85]. Despite a transient increase in ten patients in the first 24 months, neutralizing antibodies in fact declined significantly below the initial titer in 84 % of patients ($P < 0.001$), and 62 % of patients became seronegative.

In addition to selecting a product with a low risk of antigenicity, it is important to establish good practice to minimize the risk of neutralizing antibodies. Studies of BoNT/A formulations containing complexing proteins suggest that higher dosing frequency, short treatment intervals, and greater number of injections may increase the likelihood of their development [75, 86–88]. Most experts currently recommend using the smallest dose that achieves the desired clinical effect, avoiding booster injections, and waiting at least 3 months between treatments.

10 Conclusions

The repetitive contraction and activity of the muscles involved in facial expression is a major factor in the formation of lines and wrinkles, especially in the forehead and around the eyes. Botulinum toxin blocks presynaptic acetylcholine release, thus preventing the nerve impulses responsible for muscle contraction, and can be used to treat all wrinkles that are the result of normal facial movement. Practitioners currently have a choice of three BoNT/A products for the treatment of facial lines. As of 2014, ONA and INCO share the same aesthetic indications: the temporary improvement in the appearance of moderate to severe glabellar lines and crow's feet lines (the latter indication is approved in Europe, but not yet in the USA) in adults younger than 65 years of age [2, 3]. ABO currently only has aesthetic approval for the treatment of moderate to severe glabellar lines [4]. However, all three products are effectively used off-label for a number of other aesthetic indications.

ONA and INCO have comparable efficacy, with a 1:1 conversion ratio, and have demonstrated therapeutic equivalence in different indications, including cervical dystonia, blepharospasm, glabellar lines, and crow's feet. The ONA to ABO conversion ratio is approximately 1:2.5.

All three preparations have similar mechanisms of action. For storage stability and convenience of handling, BoNT/A products are formulated as either lyophilized (ABO and INCO) or vacuum-dried powders (ONA). Any one of the pH, temperature, formulation, and concentration range conditions required to lyophilize or vacuum dry a botulinum toxin into a format ready for reconstitution by a physician can increase the likelihood of inactivated toxoid proteins that may be immunogenic. Of the three products, ONA is the only one dissolved in a solution containing NaCl prior to drying, which has been proposed as a potential explanation for its toxoid content [32, 41]. The presence of inactive botulinum toxin molecules in a clinical preparation will contribute to the overall protein load of the preparation without contributing to its clinical efficacy.

The major difference between the three products relates to the presence or absence of complexing proteins. INCO consists of only the pure neurotoxin and contains no other clostridial proteins. Its foreign-protein load delivered per unit of toxin is lower than that for both ONA and ABO. Complexing proteins are not required for the effectiveness of BoNT/A preparations for injection. They are not required for the stability of BoNT/A preparations, nor do they limit their diffusion, and a definitive need for the presence of NAPs in therapeutic and aesthetic indications has not been established. Until recently, much of the information surrounding NAPs was speculative, but data are beginning to emerge that show that complexing proteins, and in particular HAs, can trigger an immune response.

The therapeutic benefits of BoNT/A are not permanent, and periodic injections are necessary. While immunogenicity may not yet be a major issue in aesthetic indications because of the low doses used, the concern is that it may become one in subjects receiving frequent dosing over a prolonged period; for example, an individual who begins treatment for glabellar lines and crow's feet at 30 years of age and who receives repeat injections several times a year over the next 35 years. Given the lack of therapeutic effect of NAPs for therapeutic and aesthetic indications, clinical strategies to reduce or eliminate neutralizing antibody development and secondary treatment failure are warranted and include using the lowest effective dose, with the longest acceptable interval between injections.

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