SAPIENZA
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# Evaluation of the preventive and therapeutic administration of an inhibitor of the Thioredoxin - Thioredoxin Reductase (Trx-TrxR) enzyme complex (Ebselen) in human botulism


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**Dottorato in Advances in infectious diseases, microbiology, legal medicine and public health sciences**

**XXXVIII Cycle**

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A.A. 2022-2023

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# ABSTRACT

**Background:** Botulism is a severe neuroparalytic disease caused by botulinum neurotoxins (BoNTs), which act in the cytosol of nerve cells by specifically cleaving proteins of the SNARE complex, thereby blocking the release of acetylcholine contained in synaptic vesicles. This results in flaccid neuroparalysis that can lead to death due to respiratory failure. BoNTs have been classified by the Centers for Disease Control and Prevention (CDC) in Atlanta as category A agents with potential bioterrorism use. To date, no adequate therapies are available to prevent the effects of BoNTs, and the only available treatment is post-exposure, based on the administration of serotype-specific antisera. However, these antisera can induce individual hypersensitivity reactions and require long preparation times. The study of the molecular mechanism of action of botulinum toxins has enabled the identification of new strategies for the development of effective pharmacological approaches. Preclinical studies indicate that the reduction of the disulfide bond is mediated by the Thioredoxin–Thioredoxin Reductase (Trx–TrxR) redox system located on the cytosolic surface of synaptic vesicles. The identification of this mechanism has led to the discovery of inhibitors of Trx and TrxR, particularly Ebselen, which has been shown to be highly effective in preventing paralysis caused by BoNTs. For this reason, the Trx–TrxR system represents a novel potential therapeutic target for future drug development.

**Methods:** The project included preclinical evidence with translational and clinical development activities. In particular, preclinical studies demonstrating the efficacy of Ebselen in preventing BoNT-induced neuroparalysis were used to support clinical translation. Based on this evidence, a protocol was designed for a randomized, double-blind, placebo-controlled phase I/II clinical trial in healthy volunteers, using a human challenge model based on the injection of botulinum neurotoxin type A (BoNT/A) into the extensor digitorum brevis (EDB) muscle. Pharmacodynamic effects will be assessed through electrophysiological measurements of the compound muscle action potential (CMAP). To enable the initiation of the clinical trial, the project included

evaluation of the active pharmaceutical ingredient (API) and production of the investigational medicinal product (IMP) in compliance with Good Manufacturing Practice (GMP). In addition, regulatory and ethical aspects were addressed in accordance with Good Clinical Practice (GCP), the European Clinical Trials Regulation (EU No. 536/2014), and CTIS requirements, including preparation for submission to the Ethics Committee. To ensure proper study conduct, a Contract Research Organization (CRO) and a clinical center authorized to conduct Phase I clinical trials in humans were involved.

**Results:** The efficacy of Ebselen in inhibiting BoNT-induced neuroparalysis across different serotypes through blockade of the Trx–TrxR system is supported by preclinical studies. Furthermore, previous clinical studies have demonstrated a favorable safety and tolerability profile in humans. These data represent a key element, as they reduce translational uncertainty and support the feasibility of a clinical trial in this new indication. The clinical trial protocol was successfully developed, integrating safety, pharmacokinetic, and exploratory pharmacodynamic endpoints within an adaptive design. The EDB muscle is confirmed to be reproducible and sensitive for detecting pharmacodynamic effects in early-phase studies; moreover, CMAP is suitable for the electrophysiological assessment of neuromuscular transmission in the EDB. Based on these premises, the regulatory submission process to the competent authorities has been initiated to obtain authorization to start the phase I/II clinical trial in healthy volunteers.

**Conclusions:** This study demonstrates the feasibility of translating mechanistic knowledge on BoNT activation into early clinical development of a novel "host-directed" therapeutic strategy. Ebselen represents a promising candidate for the treatment of botulism, with potential advantages over current antitoxin-based therapies, including broader applicability across different BoNT serotypes. Furthermore, this project provides a comprehensive translational framework for the development of medical countermeasures against high-impact neurotoxins.

# 1. INTRODUCTION

In Italy, botulism has been included among the diseases requiring immediate notification due to the potential serious public health implications of mass poisoning. Outbreaks of human and animal botulism, which sometimes involve many individuals per episode, are increasing in Italy.

Human botulism is generally associated with serotypes /A, /B, /E, and rarely /F, while no cases of human poisoning have been reported for serotypes /C and /D, but primarily cases of animal and avian botulism. To date, no cases of botulism associated with serotype /G have been identified [1].

Many BoNT subtypes are poorly neutralized by the corresponding antiserum. This poses a serious public health problem, as not all new types of BoNTs can be neutralized with the available antisera [2,3].

Furthermore, it must be kept in mind that the heptavalent antiserum against seven BoNT(A-G) serotypes produced in horses (HBAPTA supplied by CDC in the USA and in Italy by the Pavia Poison Control Center) must be injected within a few hours of intoxication to be effective [2,3]. In order to identify an effective antidote against the BoNT variants that cause human botulism and active both pre-treatment and, in the hours, following exposure, studies on animal models have been completed and are still ongoing that allow the identification of essential targets in the BoNT intoxication process that may be pharmacologically valid. Through an approach defined as "drug repurposing", it is essential to proceed with the identification of candidate anti-botulism drugs through appropriate screening of libraries of chemical compounds or through the evaluation of the inhibitory activity of already known compounds. This doctoral project aimed to evaluate the potential of Ebselen, a TrxR inhibitor, as a novel therapeutic agent against botulinum neurotoxins and to translate preclinical findings into early-phase clinical development through the design and implementation of a Phase I/II clinical trial.

## 1.1 BOTULISM: CLINICAL OVERVIEW AND EPIDEMIOLOGY

The World Health Organization (WHO) classifies botulism into several forms, including foodborne, infant, wound, inhalational, adult intestinal, and iatrogenic botulism. All of these conditions are potentially life-threatening and require urgent medical attention. Notably, there is no evidence of person-to-person transmission of botulism [4,5].

Regardless of the route of exposure, the clinical presentation is largely consistent and is characterized by an afebrile, descending flaccid paralysis. If left untreated, this condition can become fatal due to involvement of the respiratory muscles, ultimately leading to respiratory failure and death. The incubation period varies depending on both the quantity of toxin exposure and the mode of contamination [6].

Early manifestations typically involve cranial nerve dysfunction, including symptoms such as diplopia, blurred vision, vertigo, dysarthria, dysphagia, dysphonia, and dryness or discomfort of the mouth and throat. Fixed mydriasis may also be observed, usually in the absence of sensory impairment [2]. As the condition progresses, patients may develop generalized muscle weakness, ptosis, and respiratory distress due to paralysis of the respiratory musculature. Despite severe neuromuscular impairment, cognitive function remains intact even in advanced stages, as BoNTs do not cross the blood–brain barrier [6].

## 1.2 BOTULINUM NEUROTOXINS (BoNTs): STRUCTURE AND MECHANISM OF ACTION

Underlying the pharmaceutical development, subject of our study, is the identification and in-depth characterization of the molecular structure and mechanism of action of botulinum neurotoxins (BoNTs), which represent the key biological target of the study. BoNTs are encoded by *bont* genes, which may be located on chromosomal DNA, plasmids, or bacteriophages. The *bont* gene is consistently found adjacent to the non-toxic non-haemagglutinin (*ntnha*) gene. BoNTs are released as part of progenitor toxin

complexes (PTCs), in association with NTNHA and, in some cases, additional neurotoxin-associated proteins (NAPs), forming the so-called M complex. This structural organization limits the exposure of the neurotoxin to external degrading factors, suggesting that NTNHA plays a protective role by shielding BoNT from proteolytic and chemical damage [7].

The *bont* and *ntnha* genes are located near genes encoding either haemagglutinin proteins or OrfX proteins. These components interact with the BoNT–NTNHA complex and are also believed to contribute to toxin stability and protection [7].

From a structural standpoint, all BoNT serotypes consist of two polypeptide chains linked by a conserved disulfide bond: a heavy chain (H, ~100 kDa) and a light chain (L, ~50 kDa) [8]. Functionally, the heavy chain is divided into two domains: the C-terminal domain (HC), responsible for binding to the plasma membrane of motor neurons, and the N-terminal domain (HN), which facilitates translocation by forming a channel that allows the light chain to enter the cytoplasm. Following reduction of the interchain disulfide bond, the light chain—representing the catalytic portion of the toxin—becomes active [7].

The light chain exhibits zinc-dependent endopeptidase activity and specifically cleaves SNARE (Soluble NSF Attachment Protein Receptor) proteins within neuronal cells, which are essential for neurotransmitter release [9,10]. (Figure 1)

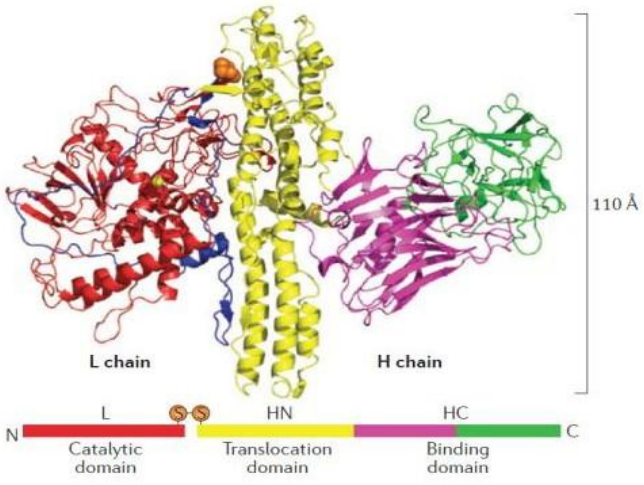


***Figure 1.*** *Structure of BoNT/A (from Ref. [5])*

BoNT can enter the organism through ingestion, inhalation, or infected wounds. Once absorbed, it is distributed via the bloodstream to various tissues. Due to its molecular size, the toxin cannot cross the blood–brain barrier. Although it may undergo retrograde axonal transport to the central nervous system (CNS) [4], its primary site of action remains the peripheral nervous system, particularly at neuromuscular junctions, where it inhibits neurotransmission and induces paralysis [7].

The mechanism of action of BoNT involves a multistep process culminating in the inhibition of acetylcholine release from presynaptic nerve terminals through the cleavage of SNARE proteins. Initially, the toxin binds with high specificity and affinity to cholinergic nerve endings of both skeletal and autonomic systems. This interaction occurs via a dual-receptor mechanism: first, BoNT binds to polysialogangliosides (PSGs), promoting its accumulation in unmyelinated regions; subsequently, it interacts with a second receptor, typically a luminal domain of synaptic vesicle (SV) proteins specific to each serotype [11].

Following receptor binding, the toxin is internalized into synaptic vesicles. The acidification of the vesicle lumen, mediated by ATP-dependent proton pumps, generates a pH gradient that triggers conformational changes in the toxin and the vesicle membrane. This acidic environment allows the heavy chain to mediate the translocation of the partially unfolded light chain into the cytosol. Once in the cytoplasm, the light chain refolds, dissociates after reduction of the disulfide bond, and cleaves its specific SNARE targets [8,12]. The reduction of the interchain disulfide bond is mediated by the Trx–TrxR redox system, which is localized on the cytosolic surface of synaptic vesicles [13-16]. This reduction releases the light chain (L), which then exerts its activity by specifically cleaving SNARE complex proteins through proteolysis, thereby inhibiting the release of acetylcholine stored within synaptic vesicles. This results in a flaccid neuroparalysis, which is characteristic of botulism [17,8], and persists for the duration of the light chain's activity within the neuron.

BoNT-induced paralysis results from the inhibition of acetylcholine release at the neuromuscular junction, caused by the proteolytic cleavage of SNARE complex proteins, including VAMP/synaptobrevin, syntaxin, and SNAP-25. These proteins are essential for synaptic vesicle fusion with the presynaptic membrane and subsequent neurotransmitter release into the synaptic cleft, which normally triggers muscle contraction [7].

Different BoNT serotypes target distinct SNARE proteins: serotypes B, D, F, and G cleave VAMP/synaptobrevin, whereas serotypes A, E, and C cleave SNAP-25, with serotype C also targeting syntaxin [18] (Figure 2).

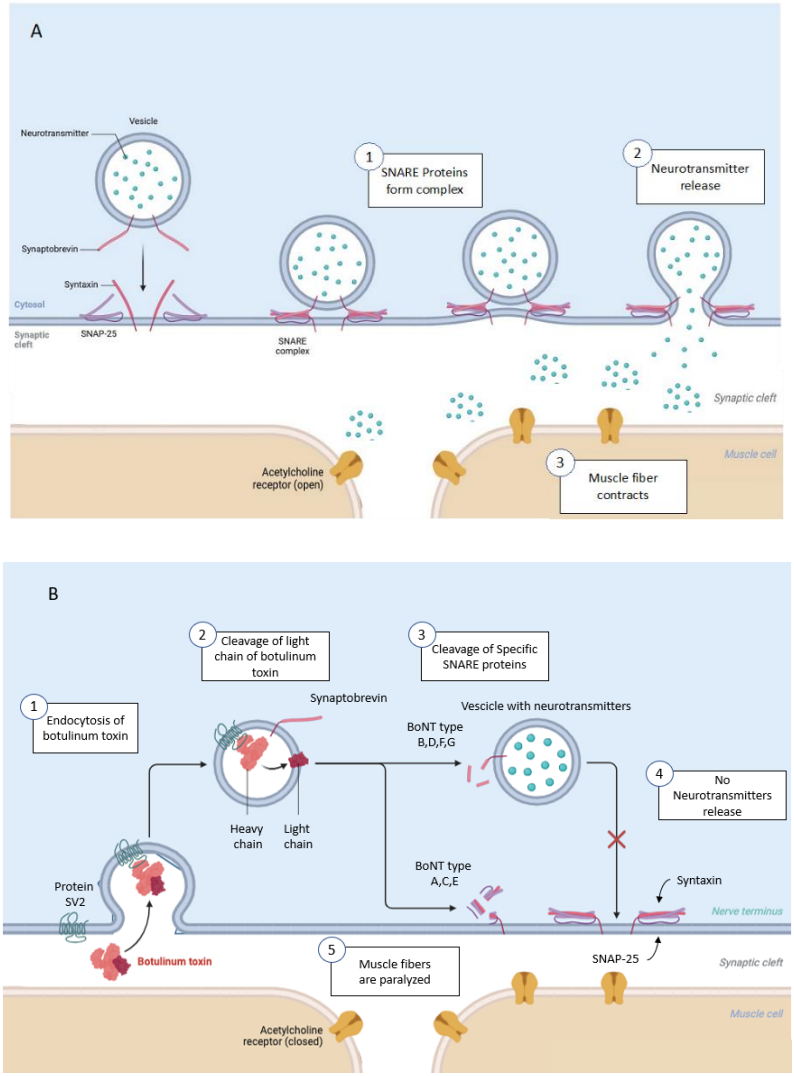


***Figure 2.*** *The mechanism of action of the botulinum neurotoxin. (A) Normal neurotransmitter release; (B) block of release of acetylcholine as a consequence of cleavage of protein complex SNARE by botulinum neurotoxin. Created in BioRender. Di Spirito M.*

The cleavage of these proteins disrupts SNARE complex formation and function, thereby preventing neurotransmitter release and causing a prolonged but reversible blockade of neuromuscular transmission. Importantly, BoNTs do not induce neuronal death but rather produce a reversible functional paralysis. Recovery depends on the toxin type, administered dose, and host species, and may require prolonged supportive care, including respiratory assistance [7].

Among the different serotypes, BoNT/A1 produces the longest-lasting paralysis (approximately 3–4 months in human skeletal muscle and up to 12–15 months in autonomic nerve terminals), whereas BoNT/E1 has a significantly shorter duration of action, typically lasting 2–4 weeks [9].

The remarkable potency and specificity of BoNTs underlie both their severe pathological effects and their therapeutic applications. Indeed, their ability to selectively inhibit neurotransmission has led to their use in the treatment of various spastic disorders, highlighting their dual nature as both highly toxic agents and valuable pharmacological tools.

## 1.3 CURRENT THERAPEUTIC APPROACHES AND LIMITATION

To understand the importance of our study regarding the chance to develop a new therapeutical approach, is essential to contextualize the preventive and therapeutic options for botulism. Currently, a limited number of strategies, mainly relying on immunological neutralization of the toxin and, more recently, on targeted pharmacological interventions are known. Despite advances in understanding the toxin's mechanism of action, these approaches remain only partially effective and highlight the need for alternative therapeutic solutions.

Regarding to vaccination, it has been primarily restricted to individuals at occupational risk, such as laboratory personnel and military staff. Earlier toxoid-based vaccines demonstrated the ability to induce protective immunity; however, their use was limited by reactogenicity, declining antibody titers over time, and the complexity of administration schedules. In addition, the presence of multiple toxin

serotypes poses a major challenge for the development of broadly protective vaccines. Although recombinant vaccine candidates based on the heavy-chain binding domain or inactive holoproteins have shown encouraging immunogenicity, they are not yet available for clinical use [19–25].

In clinical practice, treatment mainly relies on passive immunotherapy. Human-derived immunoglobulin (BIG-IV) is effective in infant botulism caused by serotypes A and B, while the heptavalent equine antitoxin (HBAT) is used in adults due to its broader serotype coverage. These antitoxins act by neutralizing circulating toxin and are most beneficial when administered early in the course of disease. However, they do not affect toxin molecules that have already entered neuronal cells, and their use—particularly for equine-derived preparations—may be associated with hypersensitivity reactions [2,26,27].

To address these limitations, newer immunotherapeutic strategies are under development. Monoclonal antibodies provide a more specific and potentially safer alternative, with preclinical evidence supporting their efficacy against selected serotypes. In parallel, camelid-derived single-domain antibodies (nanobodies) have attracted interest because of their small size and unique ability to access intracellular compartments. Engineered variants capable of entering neurons may directly interfere with the toxin's enzymatic activity, representing a significant step forward compared to conventional antibody-based therapies [28-31]. These approaches are shown ad summarized in the following Table 1.

| Approach | Mechanism | Strengths | Limitations |
|---|---|---|---|
| **Toxoid / Recombinant Vaccines** | Induce anti-BoNT immunity | Long-term protection (high-risk groups) | Reactogenicity, serotype diversity, not widely available |
| **BIG-IV** | Neutralizes circulating toxin (A, B) | Effective in infant botulism | No intracellular activity, limited spectrum |
| **HBAT** | Neutralizes circulating toxin (A–G) | Broad coverage, standard therapy | Hypersensitivity risk, early use required |
| **Monoclonal antibodies** | Target specific toxin epitopes | High specificity, scalable | Preclinical stage, limited coverage |
| **Nanobodies** | Intracellular toxin inhibition | Potential neuronal penetration | Experimental, delivery issues |

***Table 1.*** *Overview of current prophylactic and therapeutic strategies for botulism with mechanisms of action, main advantages, and key limitations.*

Our study has focused on small-molecule inhibitors targeting host-dependent steps required for toxin activation. In particular, the Trx-TrxR plays a crucial role in enabling the light chain of the toxin to exert its catalytic activity. Several compounds, as reported in the chapter 2, have been investigated for their ability to inhibit this pathway, including Auranofin, Curcumin, and Ebselen, with promising results in both in vitro and in vivo models that gave as the opportunity to proceed to clinical study.

## 1.4 BoNT: BIOLOGICAL THREAT

In light of the significant role of biological threats, it has been imperative to account for this aspect in the development of our study. Botulinum neurotoxins have historically been regarded as highly suitable agents for biological warfare [8,18,33]. This is largely due to several key features, including their extreme potency, the severity of the clinical effects they induce, and their lack of color, taste, and odor, which makes detection particularly challenging. Furthermore, BoNTs can be relatively easily produced, transported, and disseminated, and they remain highly effective through multiple routes of exposure, including ingestion, inhalation, and

injection. Their incubation period is generally short, ranging from a few hours to several days, depending on factors such as serotype, dose, and route of exposure [6,18].

In light of these characteristics and their potential public health impact, the Centers for Disease Control and Prevention (CDC) classified BoNTs in 1999 as Category A biological agents, representing the highest level of threat [33].

Historically, BoNTs have been investigated and used in biological weapons programs. In 1930s Manchuria, the leader of the Japanese Unit 731, a group dedicated to biological warfare research, reportedly conducted lethal experiments by administering *Clostridium botulinum* cultures to prisoners [18]. During the same period, other nations, including France, the United Kingdom, Canada, and the United States, were also actively engaged in biological weapons research [34].

During World War II, the United States produced over one million doses of botulinum toxoid vaccine to immunize Allied troops in preparation for the Normandy landings (D-Day) [34]. Despite the prohibition of offensive biological weapons development under the Biological and Toxin Weapons Convention, some signatory countries, including Iraq and the Soviet Union, continued to produce BoNTs for military purposes. BoNT was among the agents tested at the Soviet facility Aralsk-7, located on Vozrozhdeniye Island in the Aral Sea [18]. Reports from former scientists involved in the Soviet bioweapons program indicate attempts to genetically engineer other bacteria by inserting the *bont* gene [18].

Following the dissolution of the Soviet Union, concerns arose that expertise from former bioweapons scientists may have been transferred to countries identified by the United States as state sponsors of terrorism, such as Iran, Iraq, North Korea, and Syria, which are suspected of pursuing BoNT-based weapons [18].

With regard to Iraq, investigations conducted by the United Nations Special Commission after the First Gulf War revealed the production of approximately 19,000

liters of concentrated BoNT, of which 10,000 liters had already been weaponized. This quantity was theoretically sufficient to cause fatalities on a global scale if dispersed via inhalation [18].

Between 1990 and 1995, the Japanese cult Aum Shinrikyo attempted multiple times to release aerosolized BoNT in Tokyo and at U.S. military facilities in Japan, although these attempts were unsuccessful [6,18]. The failure was likely due to ineffective aerosol preparation [6], similar to their unsuccessful attempts to disseminate anthrax spores that were not adequately processed into particles small enough to reach the alveoli.

The inhalational route of BoNT exposure is relatively inefficient, as the toxin is unstable in environmental conditions and can be degraded by sunlight within 1–3 hours, with an estimated decay rate of 1–4% depending on environmental factors and dispersal conditions [35]. Other hypothetical dissemination methods include contamination of food, beverages, or pharmaceuticals; however, these approaches are considered impractical due to strict safety and quality control measures in production and distribution systems.

Regarding water supplies, standard chlorination processes are capable of inactivating BoNT, and environmental exposure to air and sunlight further contributes to toxin degradation [6]. A more plausible, although limited, scenario involves the illicit acquisition of BoNT/A intended for therapeutic use via online sources, where unregulated or counterfeit products with higher-than-declared toxin concentrations may be available [36]. However, such an approach would likely be limited to targeting individuals rather than large populations [6].

# 2. SCIENTIFIC BACKGROUND

Botulinum neurotoxins are protein toxins produced by anaerobic, spore-forming, rod-shaped bacteria of the genus *Clostridium* [4]. The term “botulinum” derives from the Latin *botulus* (sausage), historically linked to early outbreaks of botulism, while

*Clostridium* originates from the Greek *kloster*, meaning spindle. The main toxin-producing species include *Clostridium botulinum*, which is divided into Groups I–III, and Group IV, also known as *Clostridium argentinense*. Groups I and II are primarily responsible for human botulism, whereas Group III is mainly associated with botulism in animals. Group IV strains produce BoNT/G, a serotype that has not been definitively linked to disease in both humans or animals [7,37].

In addition to *C. botulinum*, certain strains of *Clostridium baratii* and *Clostridium butyricum* are able to produce BoNT/F7 and BoNT/E subtypes (E4 and E5), respectively, all of which have been associated with human botulism [37]. To date, seven immunologically distinct BoNT serotypes (A–G) have been identified, along with more than 40 subtypes [5] (Table 2). These serotypes were discovered over several decades: BoNT/A and BoNT/B were first identified in 1919 [38], whereas BoNT/G was characterized in 1970 [39].

Advances in genomic sequencing have enabled the identification of numerous subtypes [40,41], defined by sequence differences greater than 2.6% while still being neutralized by the same antiserum [42]. In 2016, a BoNT-like metalloprotease was identified for the first time in *Weissella oryzae*, a non-clostridial organism [43]; similar findings have also been reported in *Enterococcus faecium* and *Chryseobacterium piperi* [44].

Furthermore, mosaic toxins will be described. For example, a toxin initially designated as "BoNT/H" in 2013 was later reclassified as a hybrid toxin, in which the light chain shares approximately 80% identity with BoNT/F5 and the heavy chain about 84% identity with BoNT/A1. This toxin is also referred to as BoNT/FA or BoNT/HA [45]. In addition, genomic analysis of *C. botulinum* strain 111 identified a putative novel toxin, serotype named BoNT/X (GenBank no: BAQ12790.1), which cleaves SNARE proteins at a previously unrecognized site on VAMP2 (between Arg66 and Ala67) [46]. This strain produces two toxins, with bont/x located on the

chromosome and bont/b2 on a plasmid. However, BoNT/X appears to be inactive or non-toxic in vertebrates, including humans [47].

| ***C.botulinum* groups** | **BoNT types produced** | **Subtypes** | **Associated botulism types** |
|---|---|---|---|
| Group I *C.botulinum* (proteolytic) | A, Proteolytic B, F, H | A1, A2, A3, A4, A5, A6, A7, A8, B1, B2, B3, B4, B5(Ba), B6, B7, F1, F2, F3, F4, F5, Ab, Af, Bf, A(B), FA | Human |
| Group II *C.botulinum* (non-proteolytic) | E, Non-Proteolytic B, F | B4, E1, E2, E3, E6, E7, E8, E9, E10, E11, F6 | Human |
| Group III *C.botulinum* | C, D | C, D, CD, DC | Animals only |

***Table 2.*** *Neurotoxin serotypes, subtypes, and associated botulism (from Ref. [7]).*

BoNTs are considered the most potent natural toxins known. Botulinum neurotoxins, produced by different bacteria belonging to the genus Clostridium (botulinum, baratii and butyricum), cause botulism, a neuroparalytic syndrome of vertebrates of varying severity, which can cause death [17,48]. These toxins are the most potent currently known (minimum lethal dose in humans estimated between 0.1 and 1 nanogram/kg) [49]. Due to their extreme potency and potential for large-scale impact, botulinum neurotoxins are classified as Category A agents with potential application in bioterrorism [18,32,50].

The estimated median lethal dose (LD50) is extremely low and varies according to the route of exposure: approximately 0.09–0.15 μg following intravenous or intramuscular administration in a 70 kg adult, 0.70–0.90 μg via inhalation, and about 70 μg when ingested [18].

From a clinical standpoint, serotypes A, B, E, and, less commonly, F are responsible for human botulism, which can occur through various routes of exposure. However,

these serotypes differ in their virulence, and recovery time following intoxication depends on both the toxin type and the dose. The relative severity can be broadly ranked as follows: BoNT/A ≈ BoNT/C > BoNT/B ≈ BoNT/D ≈ BoNT/F ≈ BoNT/G > BoNT/E [51].

Serotypes are classified based on their recognition and neutralization by specific antibodies. Amino acid sequence variability can reach up to 70% between different serotypes [42] and up to approximately 50% among subtypes [37]. Additionally, strains belonging to different *Clostridium* groups may produce the same toxin serotype (for example, BoNT/F is produced by Groups I, II, and V), and subtypes within the same serotype may display differences in biological activity [42].

BoNTs are the causative agents of botulism, a potentially life-threatening disease that primarily affects wild and domesticated animals, but can also occur in humans, although less frequently [8].

## 2.1 PRECLINICAL RATIONALE FOR TARGETING BoNTs ACTIVATION

As part of the documentation preparatory phase for the clinical study, a comprehensive review of the literature-based research and preclinical studies on the mechanisms of action of BoNTs was conducted. In particular, host TrxR has been shown to play a key role in the reduction of the interchain disulfide bond, a highly conserved structural feature across all BoNT serotypes [14]. This enzymatic system is responsible for the activation of the light chain (L) once it reaches the cytosol. Because disulfide bond reduction represents a common step in the activity of all BoNTs, targeting the Trx–TrxR redox system with selective inhibitors may represent a promising antitoxin strategy.

## 2.2 IN VITRO STUDIES

This PhD project is based on the results published between 2013 and 2015 [14-16] relating to preclinical research activities conducted by the University of Padova in

collaboration with the Institute of Biomedical Sciences of Defence within research projects funded by the Military Health Research Plan (PNRM) of the Ministry of Defence. These research projects led to the identification and validation of the Trx–TrxR redox system as a pharmacological target involved in the intracellular activation process of botulinum neurotoxins (BoNTs) (Figure 3).

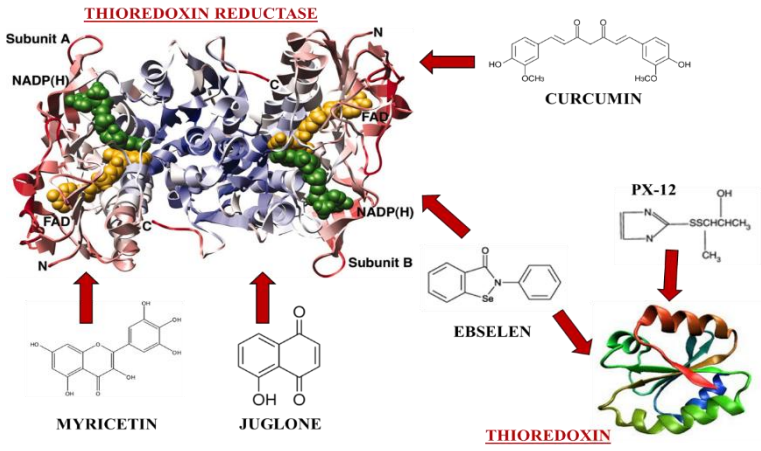


***Figure 3.*** *Structure of the Redox System: NADPH-Thioredoxin - Thioredoxin Reductase and some specific inhibitors.*

These findings have been further confirmed and expanded through subsequent preclinical investigations, with the aim of supporting the initiation of a clinical study designed to evaluate the efficacy of Ebselen in preventing BoNT/A-induced peripheral neuroparalysis in human skeletal muscle.

Several small molecules, including Auranofin, Curcumin, Myricetin, Juglone, Quercetin, PX-12, and Ebselen, have been investigated for their ability to inhibit this system, along with evaluations of their toxicity profiles in view of potential clinical applications [52–57].

These compounds were subsequently evaluated in vitro using primary cerebellar granule neuron (CGN) cultures to assess their intrinsic cytotoxicity as well as their efficacy in inhibiting BoNT activity [14,16].

Primary cerebellar granule neurons (CGNs) were preincubated with increasing concentrations of TrxR inhibitors and subsequently exposed to BoNT/A (1 nM).

SNAP-25 proteolysis was then assessed by immunoblotting, using VAMP2 as an internal control. Building on this finding, a series of in vitro experiments were performed to evaluate a dose-dependent reduction in toxin-induced SNAP-25 cleavage in the presence of the inhibitors [14,16] (Figures 4–9).

These initial results provide direct evidence that inhibition of the Trx–TrxR system interferes with the intracellular activation of BoNT/A, preventing the proteolytic cleavage of its primary substrate, SNAP-25 (Figures 4,5). Notably, the observed dose–response relationship strongly supports a specific and pharmacologically controllable mechanism of action.

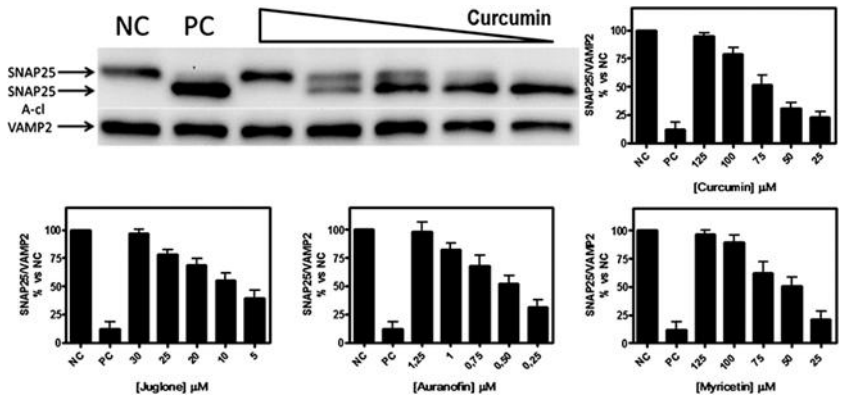


***Figure 4.*** *The proteolysis of SNAP-25 induced by BoNT/A is prevented in cerebellar granule neurons by thioredoxin reductase inhibitors. Neurons were preincubated with increasing concentrations of TrxR inhibitors and subsequently exposed to BoNT/A (1 nM). SNAP-25 cleavage was assessed by immunoblotting using VAMP2 as an internal control. Data, obtained from three independent experiments performed in triplicate, demonstrate a clear dose-dependent reduction of toxin-induced SNAP-25 cleavage. (from Ref. [14])*

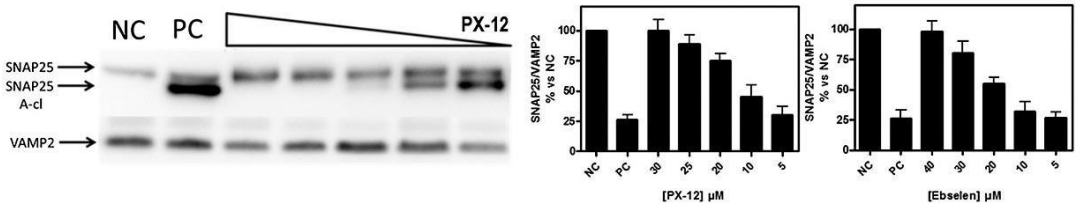


***Figure 5.*** *Thioredoxin inhibitors (PX-12 and Ebselen) prevent SNAP-25 proteolysis induced by BoNT/A in cerebellar granule neurons. Cells were pretreated with increasing concentrations of PX-12 or Ebselen prior to toxin exposure. SNAP-25 cleavage was analyzed by immunoblotting, using VAMP2 as a loading control.*

*Results from three independent experiments in triplicate show a marked dose-dependent inhibition of SNAP-25 cleavage at both 1 nM and 10 pM toxin concentrations. (from Ref. [14])*

Importantly, the efficacy of Ebselen and PX-12 across different toxin concentrations indicates that inhibition of the Trx–TrxR system is effective even at low toxin exposure, suggesting a robust protective mechanism that may be relevant under physiological or pathological conditions. This finding further strengthens the rationale for targeting host-dependent pathways rather than the toxin itself.

These findings provide direct experimental evidence that inhibition of the Trx–TrxR system effectively blocks the intracellular activation of BoNT/A, thereby preventing the proteolytic activity of the toxin on SNARE proteins [14]. Importantly, this effect is not limited to a single toxin serotype.

To address the question of whether this mechanism is conserved across different BoNT variants, the analysis was extended to additional serotypes (BoNT/B, D, F, and G), thereby evaluating the potential of Trx–TrxR inhibition as a broad-spectrum therapeutic strategy [16]. (Figures 6,7,8,9)

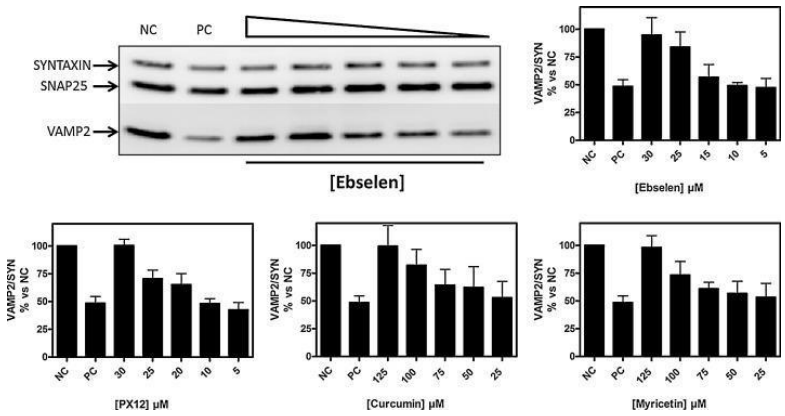


***Figure 6.*** *Inhibitors of the thioredoxin–thioredoxin reductase system prevent VAMP2 proteolysis induced by BoNT/B in cerebellar granule neuron cultures. Cells were preincubated with inhibitors and subsequently exposed to 2 nM toxin for 12 hours. Intact VAMP2 levels were assessed by immunoblotting, using Syntaxin and SNAP-25 as loading controls. Quantitative analysis (VAMP2/Syntaxin ratio, untreated cells = 100%) shows a significant preservation of VAMP2, based on at least three independent experiments. (from Ref. [16])*

These results demonstrate that the inhibitory effect is not restricted to SNAP-25-targeting toxins but also extends to toxins that cleave alternative SNARE substrates,

such as VAMP2, indicating a mechanism acting upstream of substrate specificity.

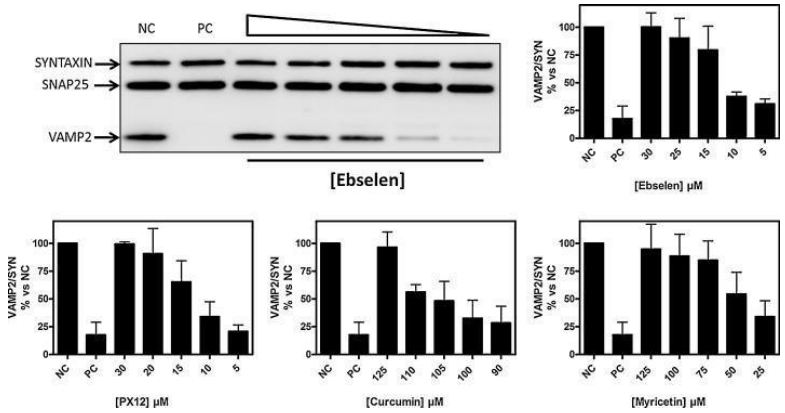


***Figure 7.*** *Thioredoxin–thioredoxin reductase inhibitors prevent VAMP2 proteolysis induced by BoNT/D in cerebellar granule neurons. Cells were pretreated with the inhibitor, briefly exposed to 0.025 nM toxin, and then reincubated for 12 hours. Immunoblot analysis of intact VAMP2, normalized to Syntaxin, demonstrates a significant protective effect across independent experiments. (from Ref. [16])*

The reproducibility of the protective effect across different experimental conditions and toxin concentrations further confirmed the robustness of Trx–TrxR inhibition as a pharmacological strategy [14,16].

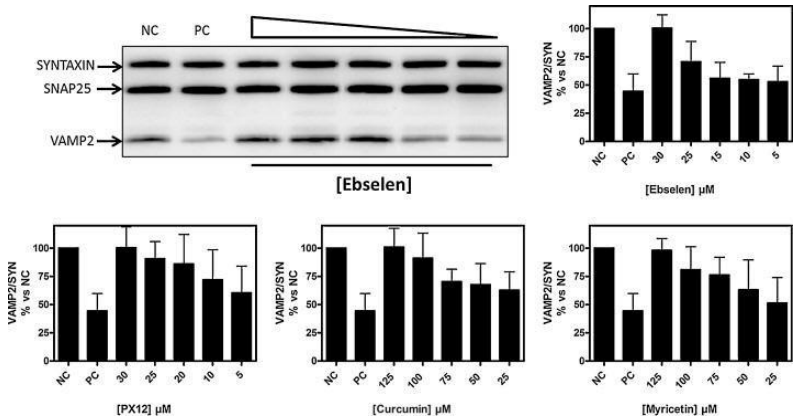


***Figure 8.*** *Inhibitors of the thioredoxin–thioredoxin reductase system prevent VAMP2 proteolysis induced by BoNT/F in cerebellar granule neurons. Following preincubation with inhibitors and exposure to 4 nM toxin for 12 hours, immunoblot analysis shows preservation of VAMP2 levels relative to controls, confirming a significant inhibitory effect. (from Ref. [16])*

Consistent results obtained with BoNT/F reinforced the hypothesis that the reduction of the interchain disulfide bond represents a shared and indispensable step in the activation of multiple BoNT serotypes.

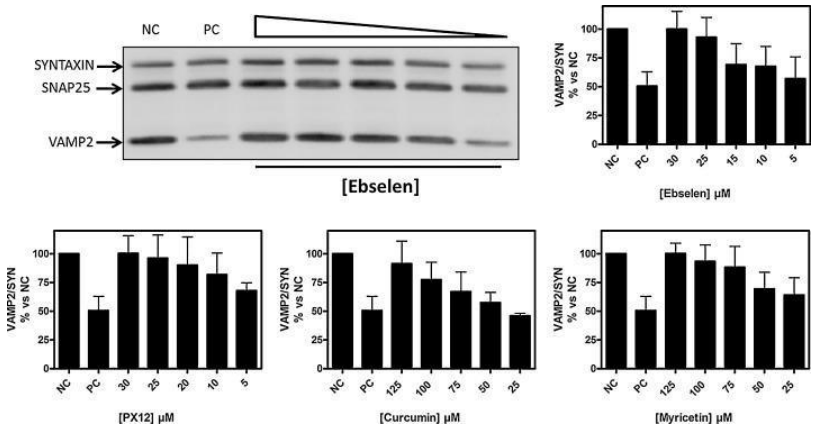


***Figure 9.*** *Thioredoxin–thioredoxin reductase inhibitors prevent VAMP2 proteolysis induced by BoNT/G in cerebellar granule neurons. Cells were pretreated with inhibitors and exposed to 4 nM toxin for 12 hours. Immunoblot quantification (VAMP2/Syntaxin ratio) demonstrates a robust and statistically significant protection from toxin-induced cleavage. (from Ref. [16])*

Taken together, the convergence of evidence across all tested serotypes highlights the central role of the Trx–TrxR system in BoNT activation. The ability of its inhibition to consistently prevent SNARE protein cleavage across structurally and functionally diverse toxins strongly supports its identification as a universal and highly promising pharmacological target.

These in vitro findings demonstrated that inhibition of the Trx–TrxR redox system effectively blocks BoNT activation at the intracellular level. These results provided the rationale for subsequent in vivo studies aimed at evaluating whether this protective effect could be translated into a reduction of neuroparalysis and improved clinical outcomes.

## 2.3 IN VIVO STUDIES

The in vivo relevance of Trx–TrxR system inhibition has been evaluated in murine models of botulism, providing critical evidence supporting the translational potential of this therapeutic strategy.

In particular, the effects of Trx–TrxR system has been assessed using the digit abduction score (DAS) assay, a well-established functional method for quantifying the degree and progression of botulinum toxin-induced neuromuscular paralysis. In

this model, intramuscular injection of BoNTs resulted in a progressive flaccid paralysis, characterized by an increased DAS score over time. Pre-treatment with TrxR inhibitors, including auranofin, myricetin, and curcumin, as well as Trx inhibitors such as PX-12 and Ebselen, significantly reduced both the severity and duration of paralysis, compared to untreated animals [14,16]. Treated mice exhibited a faster recovery of motor function, indicating that inhibition of the Trx–TrxR system effectively limits the functional consequences of BoNT intoxication. (Figure 10)

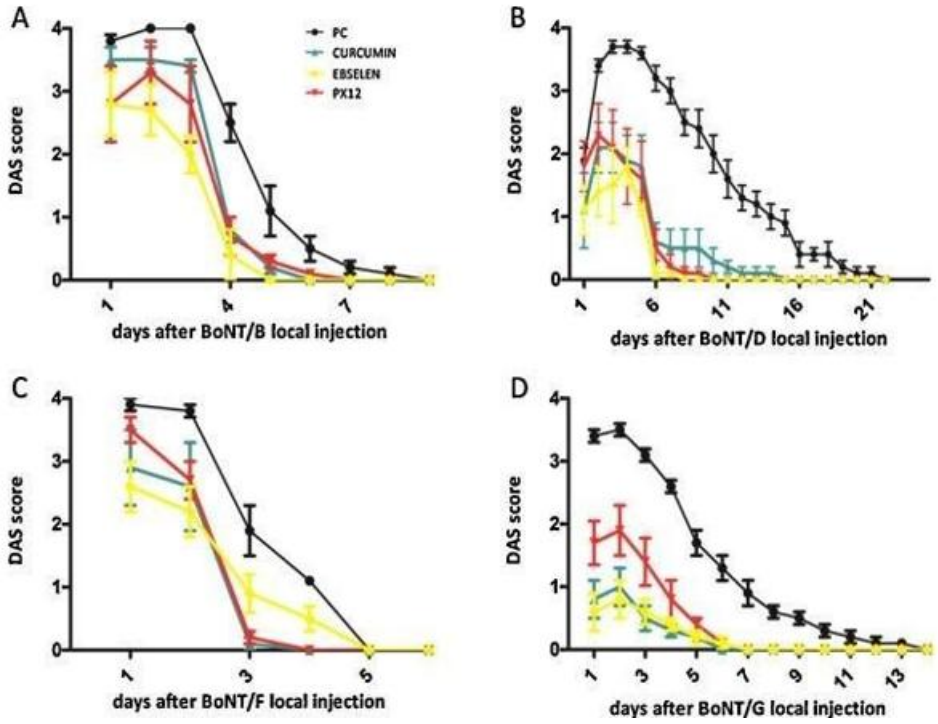


***Figure 10.*** *Inhibitors of the thioredoxin–thioredoxin reductase system (Curcumin, PX12, Ebselen), administered to the hind limb of CD1 mice 30 minutes before BoNT/B, D, F, or G injection, significantly reduce toxin-induced local paralysis, assessed by DAS score. The data (mean ± SEM of three experiments with at least eight animals per condition) show a clear attenuation of paralysis compared to the vehicle control. (from Ref. [16])*

These functional findings were further supported by electrophysiological analyses, which demonstrated a recovery of evoked end-plate potentials in treated animals, consistent with the restoration of neuromuscular transmission following inhibition of toxin activation [16].

Among the tested compounds, Ebselen emerged as the most promising candidate, due to its potent inhibitory activity and favorable pharmacological profile. (Figure 11)

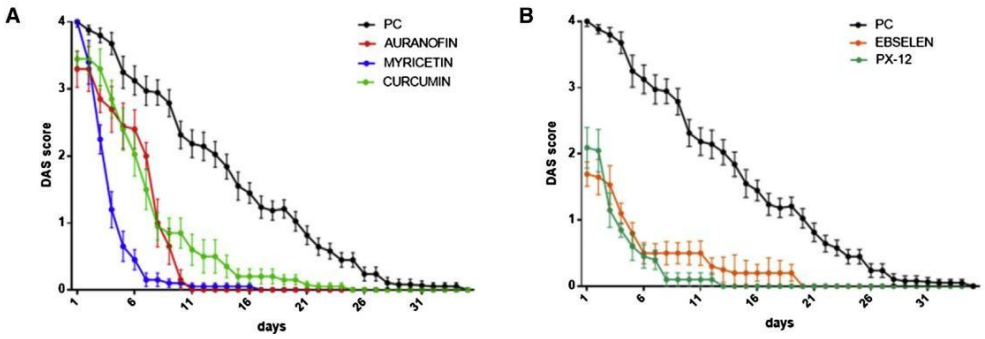


***Figure 11.*** *Effect of various inhibitors on the extent and duration of BoNT/A-induced neuromuscular paralysis. The digit abduction score (DAS) was measured over time after injection of 15 pg BoNT/A into the hind limb of mice, preceded by administration of TrxR inhibitors (A) or Trx (B) or vehicle alone (PC). Data, expressed as the mean ± SEM of four experiments with at least three animals per condition, exclude groups treated with inhibitors alone for clarity. (from Ref. [14])*

Notably, systemic administration of Ebselen (7.5 mg/kg) prior to toxin exposure significantly improved survival in mice challenged with a lethal dose of BoNT/A. Treated mice displayed reduced clinical signs of botulism and a marked increase in survival compared to controls, with many animals achieving full recovery [14] (Figure 12).

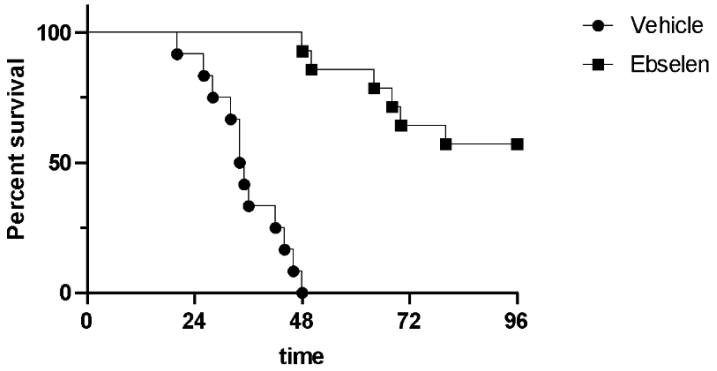


***Figure 12.*** *Ebselen protects mice from BoNT/A-induced death. Adult male CD1 mice pretreated with ebselen (7.5 mg/kg) or vehicle were injected intraperitoneally with 2×MLD50 BoNT/A and monitored for 96 hours. Survival curves were significantly different ($p < 0.0001$), indicating marked protection induced by ebselen treatment. (from Ref. [14])*

Kaplan–Meier survival curves showing the protective effect of Ebselen in mice challenged with a lethal dose of BoNT. Systemic administration of Ebselen (7.5

mg/kg) prior to toxin exposure significantly improved survival compared to untreated controls. Treated mice displayed reduced clinical signs of botulism and increased survival mice, with a substantial proportion of mice achieving full recovery. These results provide in vivo evidence supporting the therapeutic potential of Ebselen as an inhibitor of BoNT activation.

These findings provide a crucial proof-of-concept that targeting the Trx–TrxR system can effectively prevent BoNT-induced neuroparalysis in vivo. Moreover, the observation that Ebselen is effective when administered prior to toxin activation highlights the importance of the intracellular disulfide bond reduction step as a therapeutic target.

Collectively, these in vivo results strongly support the translational potential of Trx–TrxR inhibitors, and particularly Ebselen, as new therapeutic agents for the prevention and treatment of botulism.

Collectively, these in vivo findings provide strong evidence that inhibition of the Trx–TrxR system significantly mitigates BoNT-induced neuroparalysis and improves survival outcomes. The consistency of these effects across functional, electrophysiological, and survival endpoints highlights the robustness of this therapeutic strategy.

Importantly, the demonstrated efficacy of Ebselen in reducing both morbidity and mortality establishes a solid translational foundation, supporting its clinical evaluation as a novel pharmacological intervention for botulism.

## 3. CLINICAL BACKGROUND OF EBSELEN

In the preclinical models described above, Ebselen demonstrated significant efficacy in preventing peripheral neuroparalysis induced by multiple botulinum neurotoxin serotypes, including BoNT/A, B, D, F, and G [14,16]. More broadly, these findings indicate that Ebselen may represent a promising candidate for protection against botulism caused by all clinically relevant BoNTs, including those potentially

employed in bioterrorism scenarios [17].

In order to comply with the principles of reduction in animal experimentation, a key requirement for approval by the Organismo per il Benessere Animale (OPBA), subsequent studies were focused specifically on Ebselen as the most promising compound among the tested inhibitors. This strategic decision is further supported by the availability of pre-existing clinical data in humans demonstrating a favorable safety profile. In particular, two clinical trials conducted in healthy volunteers reported no evidence of toxicity at daily doses up to 7.5 mg/kg [58,59].

In addition to its protective effects on neuroparalysis, Ebselen has been shown to significantly delay or prevent mortality in mice exposed to botulinum toxin via systemic administration, further reinforcing its potential as a therapeutic agent in severe forms of botulism.

## 3.1 PHARMACOLOGICAL PROFILE OF EBSELEN

Ebselen, (2-fenil-1,2-benzoisosenelazol-3 (2H) -one), is a low-molecular-weight organoselenium compound characterized by antioxidant and cytoprotective properties. Its pharmacological activity is primarily related to its glutathione peroxidase-like function (GPx), allowing the reduction of peroxides through interaction with thiol-containing molecules, thereby limiting oxidative stress [60-62]. In addition, Ebselen can interact with the Trx–TrxR system, acting as both a substrate and modulator of redox-dependent pathways [63,64,65] (Figure 13).

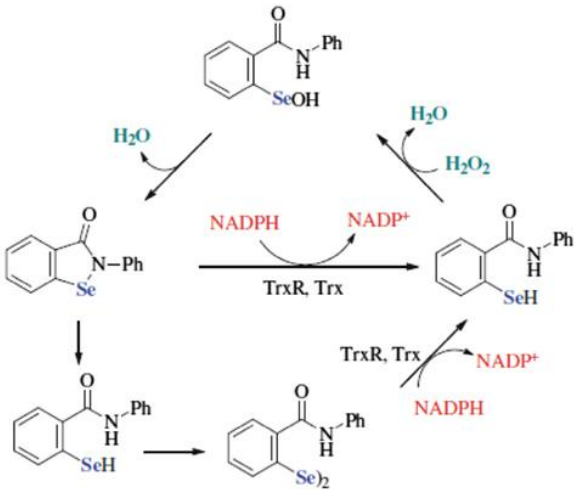

***Figure 13.*** *Redox cycle of Ebselen showing its glutathione peroxidase-like activity and interaction with the thioredoxin–thioredoxin reductase system. (from Ref. [62])*

From a pharmacokinetic perspective, Ebselen is orally bioavailable, with rapid absorption and measurable systemic exposure.

## 3.2 CLINICAL DATA IN HUMANS

Alongside the evaluation of preclinical data, it is essential to assess existing clinical studies conducted with the same molecule when preparing the documentation supporting our clinical study. This approach allows the acquisition of information on safety, tolerability, pharmacokinetics, and efficacy in humans, thereby contributing to a more accurate definition of the risk–benefit profile and guiding study design. Furthermore, the analysis of existing clinical data helps to avoid unnecessary duplication, optimize resources, and ensure adherence to ethical principles in research. In effect, Ebselen has been previously evaluated in humans across multiple clinical studies involving different therapeutic indications, particularly in disorders of the central nervous system and conditions associated with oxidative stress [66]. Clinical trials conducted in patients with ischemic stroke and reperfusion injury demonstrated a favorable safety profile at the tested doses [58,59]. Furthermore, a Phase II clinical trial conducted in the United States showed that orally administered Ebselen (SPI-1005) was effective in preventing temporary noise-induced hearing loss in healthy volunteers, with good tolerability [48]. (Table 3)

| Indication / Study Type | Population | Dose & Regimen | Exposure (Approx.) | Key Safety Findings |
|---|---|---|---|---|
| Acute ischemic stroke (Yamaguchi et al., 1998) | Patients with acute stroke | Up to 600 mg/day, oral, multiple days | >300 subjects | Well tolerated; no increase in serious adverse events vs placebo |
| Cerebrovascular disease (Ogawa et al., 1999) | Patients with cerebral infarction | 300–600 mg/day, oral | ~200 subjects | Mild GI and headache events; no organ toxicity |
| Bipolar disorder (Singh et al., 2013) | Adults with bipolar disorder | 600 mg/day, oral | ~60 subjects | Generally mild AEs; no significant laboratory abnormalities |
| Metabolic / vascular studies (Beckman et al., 2016) | Adults with diabetes | 600 mg/day, oral | ~40 subjects | No clinically relevant safety signals |

***Table 3.*** *Overview of clinical trials*

More recently, Ebselen has been investigated in advanced clinical studies for inner ear disorders, including Ménière's disease. A Phase III randomized, double-blind, placebo-controlled trial (ClinicalTrials.gov Identifier: NCT04677972), completed in 2024, evaluated its efficacy in adults with active symptoms such as hearing loss, tinnitus, and vertigo, further confirming its safety and tolerability. Additional clinical studies have explored the use of Ebselen in neuropsychiatric and metabolic conditions, including bipolar disorder and diabetes, without identifying significant safety concerns [67–71].

Across these studies, Ebselen was administered orally at doses up to 7.5 mg/kg/day or in repeated dosing regimens over several consecutive days, with no evidence of significant toxicity or serious treatment-related adverse events. The availability of an established clinical dossier in humans therefore represents a critical advantage for the present study in botulism, as it supports the safety of the compound at the proposed doses, reduces translational uncertainty, and facilitates a potentially accelerated clinical repurposing pathway.

From a mechanistic perspective, it is important to note that Ebselen exerts its inhibitory effect only prior to the release of the BoNT light chain into the cytosol; once the catalytic light chain is activated, the compound is no longer effective. However, because Ebselen targets a host-dependent mechanism that is conserved across all BoNT serotypes, its administration does not require prior identification of the specific toxin involved. This represents a significant advantage, particularly in light of the large number of known BoNT serotypes and subtypes (>40).

This therapeutic approach may be particularly relevant in clinical settings characterized by continuous toxin production, such as infant botulism, where BoNT is persistently released by vegetative bacteria colonizing the intestine [17], as well as in adult intestinal botulism, especially in immunocompromised patients or in individuals with altered gut microbiota. In addition, Ebselen may also have potential applications in a prophylactic context, particularly for individuals operating in high-risk environments where exposure to BoNTs cannot be excluded [14].

Taken together, the strong preclinical evidence and the well-established safety profile of Ebselen provide a solid rationale for its clinical evaluation. On this basis, further studies in humans, including clinical trials involving healthy volunteers, are warranted to assess its efficacy in the prevention and treatment of botulism [14,16,59].

## 4. TRANSLATIONAL DEVELOPMENT

Overall, this study represents a paradigmatic example of translational clinical research, integrating mechanistic insights, preclinical evidence, and innovative human challenge models [14,16].

By enabling the controlled evaluation of Ebselen's pharmacological activity in humans, this study provides a critical step toward the development of a new host-directed therapeutic strategy for botulism. Furthermore, it establishes a methodological framework that may be applicable to the development of medical countermeasures against other neurotoxins of high clinical and public health relevance. By enabling controlled evaluation of the pharmacological activity of Ebselen in humans, our study

constitutes an important intermediate step between preclinical proof-of-mechanism and later-stage therapeutic development. More broadly, it provides a framework for the clinical investigation of host-directed strategies targeting toxin activation rather than toxin neutralization alone.

In addition to its potential relevance for botulism, this methodological framework may also be informative for the development of medical countermeasures against other neurotoxins of clinical and public health importance.

## 4.1 FROM PRECLINICAL EVIDENCE TO CLINICAL TRANSLATION

In the preclinical models described above, Ebselen demonstrated significant efficacy in preventing peripheral neuroparalysis induced by multiple botulinum neurotoxin serotypes, including BoNT/A, B, D, F, and G [14,16]. More broadly, these findings indicate that Ebselen may represent a promising candidate for protection against botulism caused by all clinically relevant BoNTs, including those potentially employed in bioterrorism scenarios [17].

In order to comply with the principles of reduction in animal experimentation, a key requirement for approval by the *Organismo per il Benessere Animale* (OPBA), subsequent studies were focused specifically on Ebselen as the most promising compound among the tested inhibitors. This strategic decision was further supported by the availability of pre-existing clinical data in humans demonstrating a favorable safety profile. In particular, two clinical trials conducted in healthy volunteers reported no evidence of toxicity at daily doses up to 7.5 mg/kg [58,59].

In addition to its protective effects on neuroparalysis, Ebselen has been shown to significantly delay or prevent mortality in mice exposed to botulinum toxin via systemic administration, further reinforcing its potential as a therapeutic agent in severe forms of botulism.

## 4.2 RATIONALE FOR CLINICAL DEVELOPMENT OF EBSELEN

According to the stakeholders of our clinical trial, in light of the evidence generated in the preceding preclinical investigations, Ebselen was selected as a candidate for a phase I/II clinical study. As previously described, the Trx–TrxR redox system plays a critical role in the intracellular activation of BoNTs, mediating the reduction of the interchain disulfide bond required for the release of the catalytic light chain [14,15]. The identification and validation of this pathway as a pharmacological target have provided a solid mechanistic framework for therapeutic intervention.

Within this context, Ebselen has consistently demonstrated a marked ability to inhibit this redox system and to prevent SNARE protein cleavage in neuronal models, resulting in effective blockade of toxin activity [14,15]. Notably, as highlighted in the in vitro and in vivo studies described above, Ebselen showed superior efficacy compared to other tested inhibitors, significantly reducing both the severity of neuroparalysis and mortality in animal models [15].

Importantly, this effect has been observed across multiple BoNT serotypes, including BoNT/A, B, D, F, and G, confirming that inhibition of the Trx–TrxR system targets a conserved and essential step in toxin activation [15]. This broad-spectrum activity represents a key advantage over existing serotype-specific therapies, particularly in clinical scenarios where rapid toxin identification may not be feasible.

In addition to its demonstrated efficacy, Ebselen benefits from an extensive clinical background, as previously discussed. Clinical trials conducted in humans have consistently shown that the compound is well tolerated and non-toxic at the proposed doses [48,58,59,66], thereby significantly reducing the uncertainty associated with translational development. Furthermore, the availability of prior clinical data enables a realistic and accelerated drug repurposing strategy.

Taken together, the mechanistic validation of the target, the robust preclinical efficacy across multiple BoNT serotypes, and the well-established safety profile in humans converge to position Ebselen as the candidate with the highest degree of translational maturity. These considerations support its selection as an ideal compound for the

initiation of our clinical study aimed at evaluating its efficacy in preventing BoNT/A-induced peripheral neuroparalysis.

# 5. CLINICAL TRIAL DEVELOPMENT

## 5.1 DEFINITION AND PHASES OF CLINICAL TRIALS

A clinical trial is defined as any investigation conducted in human subjects with the aim of evaluating the safety, efficacy, pharmacokinetics, and pharmacodynamics of a medicinal product [72]. Clinical trials represent a fundamental step in the development of new therapeutic strategies, enabling the translation of preclinical findings into clinical practice [73].

Clinical development is conventionally divided into four sequential phases, each characterized by specific objectives and methodological approaches [74] (Figure 14).

Phase I trials are typically the first studies conducted in humans and are primarily designed to assess safety, tolerability, and pharmacokinetics.

Phase II trials aim to evaluate preliminary efficacy while further characterizing safety in a target population.

Phase III trials are large-scale randomized studies aimed at confirming efficacy and safety compared to standard treatments or placebo, forming the basis for regulatory approval. Should the results of the present study be favorable, they would provide the foundation for future Phase III investigations.

Finally, Phase IV trials are conducted post-marketing to evaluate long-term safety and effectiveness in real-world settings [74].

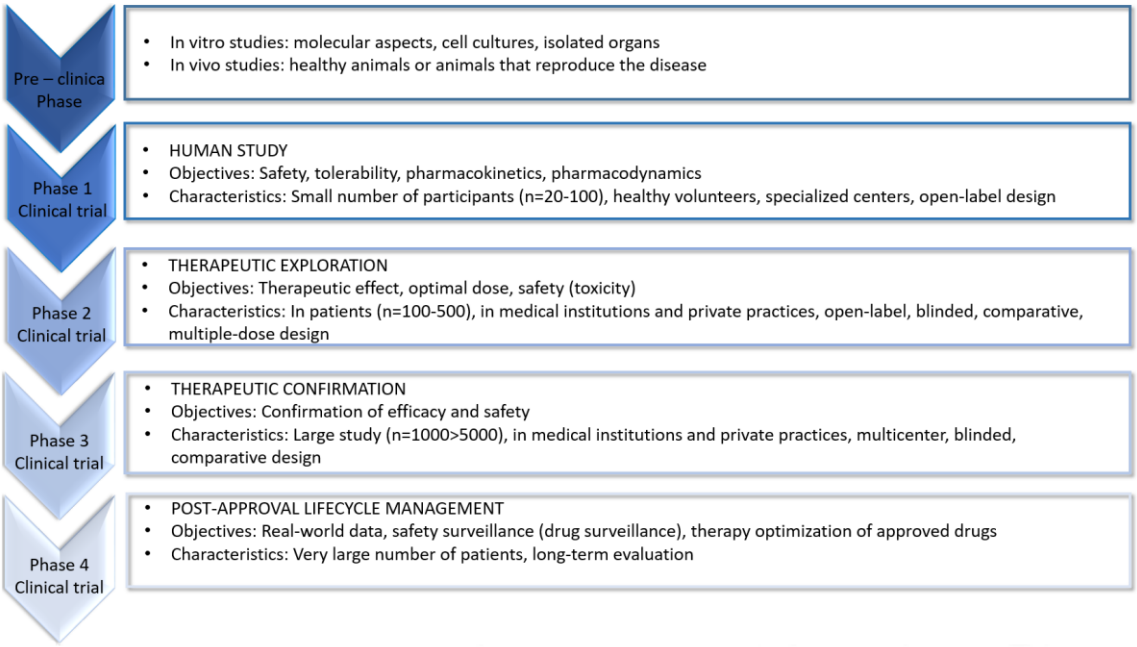


***Figure 14.*** *Schematic representation of the drug development pathway, including preclinical studies and clinical trial phases (I–IV), highlighting their objectives and key characteristics.*

On the basis of data derived from in vitro and in vivo preclinical studies, together with previous clinical trials, in accord with our stakeholders it has been determined that a Phase I/II would be developed.

## 5.2 GOOD CLINICAL PRACTICE (GCP) AND ICH GUIDELINES

The conduct of clinical trials is regulated by internationally recognized standards aimed at ensuring the protection of participants and the reliability of generated data. Among these, GCP guidelines represent the cornerstone of clinical research [75].

GCP is an international ethical and scientific quality standard for designing, conducting, recording, and reporting clinical trials involving human subjects. Compliance with GCP ensures that the rights, safety, and well-being of participants are protected, and that the data generated are credible and accurate, in accordance with the principles of the Declaration of Helsinki [76].

The International Council for Harmonisation (ICH) has developed a comprehensive set of guidelines to harmonize regulatory requirements across regions. In particular, the ICH E6 guideline on GCP provides a unified framework that is applicable to the clinical

study proposed in this work, ensuring that its design and conduct meet international standards [75].

In the present study on Ebselen, adherence to GCP principles is essential to guarantee the scientific validity of the results and the protection of participants, particularly considering the innovative mechanism of action of the compound and its potential application in a severe condition such as botulism.

In compliance with regulatory requirements, the stakeholders draft the necessary documentation such as: protocol design, investigator brochure, data integrity document, and monitoring procedures. In the European and Italian context, clinical trials are regulated by Regulation (EU) No 536/2014 and by national implementing provisions overseen by *Agenzia Italiana del Farmaco* - AIFA, which ensure a high level of protection for participants and the scientific quality of clinical research [77,78].

## 5.3 ETHICAL ASPECTS: INFORMED CONSENT AND ETHICS COMMITTEE

Ethical considerations are central to the conduct of clinical trials and must be rigorously addressed at every stage of the study. The primary ethical requirement is the protection of the rights, dignity, and well-being of participants [76].

A fundamental component of ethical clinical research is the process of informed consent.

The essential documents required by the Ethics Committee include the informed consent form and the privacy information notice, ensuring compliance with applicable data protection regulations General Data Protection Regulation (GDPR, Regulation (EU) 2016/679. The drafts of the aforementioned documents have been approved by the stakeholders in order to submit them to the Ethic Committee of the Clinical Structure where the study will be conducted.

Before enrollment, participants must will receive comprehensive information regarding the purpose of the study, the procedures involved, potential risks and benefits, and their right to withdraw at any time without consequences. This aspect is particularly relevant

in the proposed clinical study, where participants will be exposed to an investigational use of Ebselen in the context of botulinum toxin exposure [75,76].

Another key element is the role of the Ethics Committee (EC), also known as the Institutional Review Board (IRB), which is responsible for the independent evaluation and approval of the study protocol prior to its initiation [76]. For the clinical study described in this thesis, Ethics Committee approval will be required to ensure that the risk–benefit balance is acceptable and that all procedures comply with ethical and regulatory standards.

The Ethics Committee assessed multiple aspects of the study, including the scientific rationale, the adequacy of the informed consent process, and the safety monitoring plan. Continuous oversight will be ensured throughout the study duration.

In this framework, the integration of scientific rigor with ethical responsibility is essential to guarantee that the clinical investigation of Ebselen is conducted in a manner that is both methodologically sound and ethically acceptable. (Figure 15)

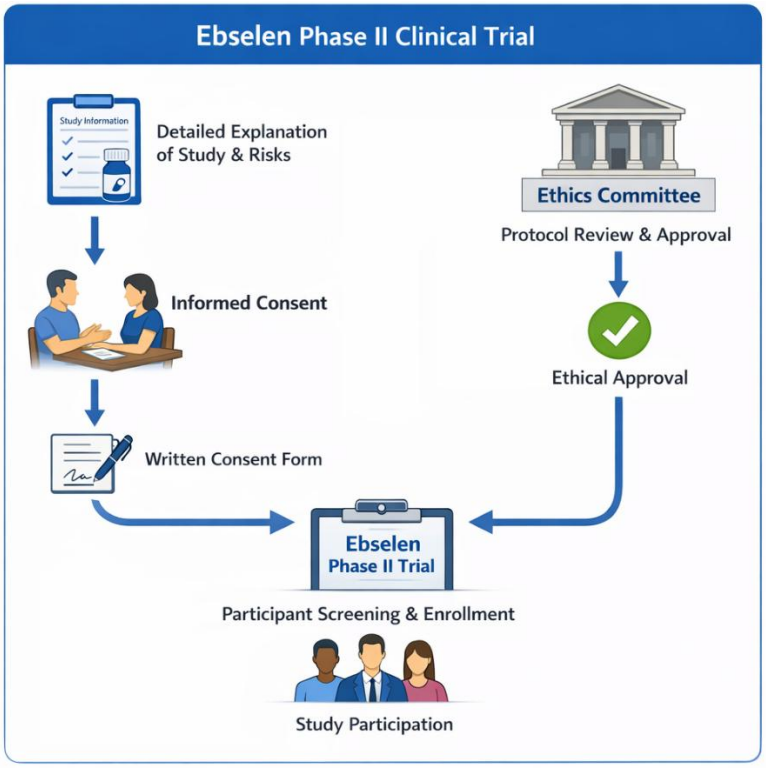


***Figure 15.*** *Ethical approval and informed consent process in the Ebselen Phase II clinical trial, from protocol review to participant enrollment.*

## 5.4 INVESTIGATIONAL MEDICINAL PRODUCT (IMP) AND IMP DOSSIER (IMPD)

In the context of clinical research, the investigational medicinal product (IMP) is defined as any pharmaceutical form of an active substance or placebo being tested or used as a reference in a clinical trial. This definition includes products already authorized but used outside their approved indication, or modified in formulation or packaging for research purposes [79]. The quality, safety, and consistency of the IMP are critical elements to ensure both the scientific validity of the study and the protection of trial participants.

In the present study, Ebselen is an IMP, as it is being evaluated for a new therapeutic indication, namely the prevention of botulinum neurotoxin (BoNT)-induced neuroparalysis. Although, Ebselen has been previously administered in humans for other clinical conditions and its use in this specific context requires full compliance with the regulatory framework governing investigational medicinal products.

The manufacturing, formulation, packaging, and labeling of the IMP must comply with Good Manufacturing Practice (GMP) guidelines, which ensure that medicinal products are consistently produced and controlled according to predefined quality standards. These requirements are essential to guarantee the identity, purity, potency, and stability of the investigational product throughout the clinical trial.

According to European regulatory guidance, IMPs must be manufactured in compliance with GMP principles, regardless of the development phase, to ensure that risks related to product quality are adequately controlled [80]. In addition, the compliance with GMP must be formally documented within the clinical trial application, including evidence of manufacturing authorization and certification by a qualified person (QP), where applicable [79].

For the clinical study described in this thesis, Ebselen has been produced and handled in accordance with GMP requirements, ensuring full traceability of manufacturing batches and proper documentation of production processes. Specific procedures for storage, transport, and accountability of the IMP will be implemented to maintain

product integrity and to comply with regulatory requirements, including those established by AIFA.

As the coordinator of this study, I set up the drafting of the Investigational Medicinal Product Dossier (IMPD), which provides comprehensive information on the quality, manufacture, and control of the IMP, as well as relevant non-clinical and clinical data supporting its use in humans [79].

The IMPD is structured according to a risk-based approach, whereby the extent of required information depends on the stage of clinical development, the characteristics of the product, and the anticipated exposure of participants. Unlike the dossier required for marketing authorization, the IMPD focuses primarily on aspects relevant to the safe use of the product within the clinical trial setting [80].

From a structural perspective, the IMPD includes three main domains (Figure 16):

- **Quality documentation**, covering the drug substance and drug product, including composition, manufacturing process, control of materials, specifications, analytical methods, impurity profile, and stability data.
- **Non-clinical data**, summarizing pharmacological and toxicological evidence supporting the safe administration of the IMP.
- **Clinical data**, including previous human exposure and available clinical experience with the investigational product.

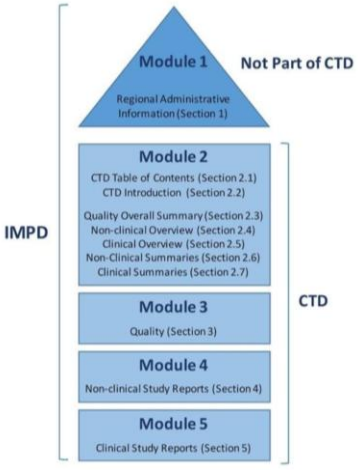


***Figure 16.*** *Structure of IMPD*

Particular emphasis is placed on the quality section, which must follow a clearly structured format and provide sufficient detail to ensure that the IMP is reproducible and suitable for clinical use. This includes detailed information on manufacturing processes, control strategies for impurities, validation of analytical methods, and stability testing under defined conditions [80].

In the context of the present study, the IMPD for Ebselen has been included detailed information on its pharmaceutical characteristics, manufacturing process, and prior clinical use, thereby supporting its safe administration in healthy volunteers. Given that, Ebselen has been previously investigated in humans, certain elements of the dossier benefit from a simplified approach through cross-reference to existing data in accordance with current regulatory provisions [79].

The preparation of a complete and scientifically robust IMPD represents a fundamental prerequisite for obtaining clinical trial authorization. Together with the clinical protocol and other regulatory documents, the IMPD will allow competent authorities and Ethics Committees to assess the overall risk–benefit balance of the study and to ensure that the investigational product meets appropriate quality and safety standards.

In this framework, adherence to GMP principles and the accurate preparation of the IMPD are essential to guarantee that the clinical evaluation of Ebselen is conducted using a product of defined, controlled, and reproducible quality [76,78]. This is particularly relevant in early-phase translational studies, where variability in product quality could directly impact both safety outcomes and the interpretation of pharmacodynamic effects.

## 5.5 STAKEHOLDERS IN CLINICAL TRIALS

The conduct of a clinical trial is a complex and highly regulated process that involves the coordinated interaction of multiple stakeholders, each with clearly defined roles and responsibilities. This collaborative framework is essential to ensure the scientific validity of the study, compliance with regulatory requirements, and the protection of participants' rights, safety, and well-being [75,81,82]. In our specific case, the main

stakeholders include the Defence Institute of Biomedical Sciences as Sponsor, University of Padova as a Nonclinical Study Director and both the CRO and the Clinical Research Associate (CRA). Within this frame, I am nominated as Monitor of this study.

At the core of the clinical trial organization is the sponsor, defined as the individual, institution, or organization responsible for initiating, managing, and financing the study. The sponsor has played a pivotal role in the design of the clinical protocol, the preparation and submission of the Clinical Trial Application (CTA), and the overall oversight of trial conduct [81,82]. In the context of the present study, the sponsor has been also responsible for ensuring that the clinical development of Ebselen is carried out in accordance with applicable regulatory standards and GCP guidelines [75,83].

To support the operational aspects of the trial, the sponsor had delegated specific tasks to a CRO. CROs had provided specialized expertise in clinical trial management and has been involved in activities such as site selection, monitoring, data management, regulatory submissions, and coordination between stakeholders [75]. Despite this delegation, the sponsor retains ultimate responsibility for the integrity and quality of the study [72].

The principal investigator (PI) is responsible for the conduct of the trial at the clinical site and ensures adherence to the approved protocol, GCP principles, and local regulatory requirements. Generally, the PI oversees participant recruitment, informed consent procedures, data collection, and the reporting of adverse events, thereby playing a central role in safeguarding participant safety and ensuring data reliability.

Clinical trial participants represent the core of the research process. Their voluntary participation, based on a comprehensive informed consent process, is fundamental to the ethical conduct of the study. Particular attention must be paid to ensuring that participants are adequately informed and that their rights, dignity, and well-being are protected throughout the duration of the trial [76].

Regulatory authorities, such as the AIFA, are responsible for the evaluation and authorization of clinical trials, as well as for ongoing supervision to ensure compliance

with applicable legislation [75,78]. In parallel, the EC provides independent oversight of the study, assessing the scientific rationale, the risk–benefit balance, and the adequacy of the informed consent process before granting approval [76].

Additional key roles are fulfilled by CRA and monitor, who ensure that the trial is conducted in accordance with the protocol and GCP standards. These professionals perform regular site visits, verify source data, and ensure proper documentation. Data managers and statisticians further contribute to the integrity and scientific validity of the study by overseeing data handling, quality control, and statistical analysis [82].

As illustrated in Figure 17, the effective coordination among these stakeholders is essential for the successful execution of the clinical trial. In the specific context of the Ebselen Phase I/II study, this integrated framework ensures that the transition from preclinical research to clinical evaluation is conducted in a scientifically robust, ethically sound, and regulatory-compliant manner.

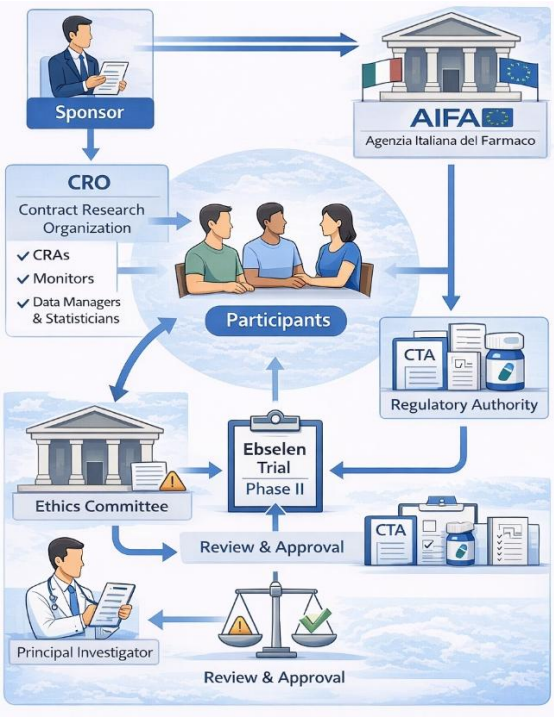


***Figure 17**. Overview of the stakeholders involved in the Ebselen Phase II clinical trial, including sponsor, CRO, principal investigator, regulatory authorities (AIFA), Ethics Committee, and supporting roles (CRAs, data managers, statisticians), highlighting their coordinated contribution to trial conduct and participant protection.*

## 5.6 CLINICAL TRIAL APPLICATION (CTA) AND REGULATORY SUBMISSION

The initiation of a clinical trial in the European Union requires formal authorization through a structured regulatory process, aimed at ensuring the scientific validity of the study and the protection of participants. This process is based on the submission of a Clinical Trial Application (CTA), which must be approved by both the competent national authority and the relevant Ethics Committee prior to the start of the study (Figure 18).


***Figure 18.*** *Regulatory submission pathway of the Ebselen Phase II clinical trial in the European Union. The CTA dossier is submitted via CTIS/EudraCT and evaluated by AIFA and the Ethics Committee, with assessment of GMP, safety monitoring, and risk–benefit balance prior to trial authorization.*

In Italy, the competent authority responsible for the evaluation of clinical trial applications is AIFA, which operates within the framework defined by Regulation (EU) No 536/2014 [77,78]. This regulation was introduced a harmonized system for the submission, assessment, and supervision of clinical trials across European Member States, promoting transparency and coordination.

A central component of this process is the European clinical trials database, commonly known as EudraCT (European Union Drug Regulating Authorities Clinical Trials Database) assigned a unique identification number to clinical trials referring to those

ones until 31 January 2023. This identifier ensured traceability and facilitated regulatory oversight throughout the trial's lifecycle. Under the current regulatory framework, the database has been replaced, only for new CTA submission, by the Clinical Trials Information System (CTIS), which supports the submission and evaluation process at the European level [84]. However, the EudraCT database continues to be accessible for specific purposes, including the submission of amendments, updates on trial status, and results for studies that were originally registered in EudraCT. In addition, it remains available for the submission of third-country trial data conducted exclusively outside the EU/EEA when such studies are part of a Pediatric Investigation Plan (PIP) or fall within the scope of Article 46 of the Pediatric Regulation (EC) No 1901/2006.

The CTA dossier includes several key elements, such as the clinical trial protocol, the Investigator's Brochure (IB), the IMPD, and documentation related to quality, safety, and manufacturing in compliance with GMP guidelines. In the context of the present study, this dossier will provide detailed information on the use of Ebselen as an investigational medicinal product, including its pharmaceutical characteristics, preclinical data, and prior clinical experience.

In addition, the CTA submission must include comprehensive documentation on risk assessment, safety monitoring procedures, and the informed consent process, ensuring that all ethical and regulatory requirements are met. Particular attention will be given to the justification of the risk–benefit balance, especially considering the use of Ebselen in the prevention of botulinum neurotoxin-induced neuroparalysis.

The approval of the CTA on the platform CTIS and the favorable opinion of the Ethics Committee represent mandatory prerequisites for the initiation of the clinical study. Once authorized, the trial will be conducted in full compliance with GCP principles and it will be subject of an ongoing monitoring and reporting obligations, including the notification of adverse events and periodic safety updates.

In this regulatory framework, the proper preparation and submission of the CTA dossier are essential to ensure that the clinical evaluation of Ebselen is conducted in accordance

with European and national regulations, thereby guaranteeing both scientific rigor and the protection of study participants.

# 6. STUDY DESIGN AND METODOLOGY

## 6.1 TRANSLATIONAL FRAMEWORK AND STUDY RATIONALE

The transition from preclinical research to clinical investigation represents a critical step in the development of new therapeutic strategies against BoNTs. As discussed, inhibition of the Trx–TrxR redox system has been identified as a key mechanism capable of preventing intracellular activation of BoNTs [14,16]. Among the tested compounds, Ebselen emerged as the most promising candidate due to its superior efficacy across multiple serotypes and its well-established safety profile in humans [48,58,59,66].

Within this context, the clinical study, described in this thesis, was designed to translate these mechanistic and preclinical findings into a controlled clinical trial. The study aims to evaluate whether the pharmacological inhibition of the Trx–TrxR system by Ebselen can modulate the neuromuscular effects induced by BoNT/A in vivo, thereby providing a proof-of-concept for its potential therapeutic application in botulism [14,16].

More specifically, the study is intended to bridge three levels of evidence: mechanistic validation of the target, demonstration of efficacy in preclinical models, and exploratory clinical evaluation in humans. In this sense, the aim of this trial is to establish whether the biological effects observed in vitro and in vivo can be detected in a controlled human model.

## 6.2 STUDY DESIGN

This clinical study is an early-phase, randomized, double-blind, placebo-controlled clinical trial conducted in healthy volunteers, in line with current methodological standards for exploratory clinical pharmacology studies [73]. The study integrates Phase I and Phase II elements, allowing the simultaneous evaluation of safety, pharmacokinetics (PK), and exploratory pharmacodynamic (PD) effects.

Participants are randomized in a 2:1 ratio to receive either Ebselen or placebo, ensuring adequate characterization of the safety profile while enabling preliminary assessment of treatment-related effects. A key methodological feature of the study is the adoption of a within-subject control design, in which each participant receives a BoNT/A injection in one extensor digitorum brevis (EDB) muscle and a saline injection in the contralateral muscle, in line with current methodological standards for exploratory clinical pharmacology studies [74]. Recruitment activities will be conducted in compliance with GCP ICH E6(R3) and in accordance with the inclusion and exclusion criteria defined in the study protocol. This approach significantly reduces inter-individual variability and increases the sensitivity of pharmacodynamic measurements [85,86].

Blinding will be maintained for participants and outcome assessors, while the investigator performing the intramuscular injections may remain unblinded, where necessary, to ensure procedural accuracy and correct administration of the challenge intervention. This partial unblinding is operationally justified and does not compromise the blinded evaluation of study endpoints. The randomized parallel-group design, combined with a within-subject challenge model, provides a robust methodological framework for detecting early biological signals while preserving an appropriate balance between scientific rigor and operational feasibility.

## 6.3 HUMAN CHALLENGE MODEL

A central component of the study is the use of the extensor digitorum brevis (EDB) challenge model, which represents a well-established and reproducible experimental paradigm for the assessment of localized BoNT-induced neuroparalysis [85,86].

Following intramuscular injection of BoNT/A, a controlled and reversible reduction in neuromuscular function is induced, which can be quantitatively measured through electrophysiological techniques. In particular, the compound muscle action potential (CMAP) amplitude is used as a sensitive biomarker of neuromuscular transmission [87]. The expected reduction in CMAP typically reaches 60–80% at peak effect and gradually recovers over a period of 60–90 days [85,88].

The EDB muscle is particularly suitable for this model due to its accessibility and limited functional impact, ensuring that the induced weakness does not significantly interfere with daily activities [89-91]. This makes the model ethically acceptable for use in healthy volunteers while maintaining high scientific value [85,86,91,92]. Within the present study, the challenge model serves as an experimental platform to detect whether Ebselen can attenuate the functional effects of BoNT/A at the peripheral neuromuscular level under controlled conditions. In this regard, the model is not intended to reproduce the full clinical complexity of naturally occurring botulism, but rather to provide an ethically manageable and scientifically informative translational system for proof-of-mechanism testing.

## 6.4 INVESTIGATIONAL MEDICINAL PRODUCT (IMP) AND DOSING RATIONALE

According to our protocol, Ebselen is administered as IMP for a new therapeutic indication. The selected dosing regimen consists of oral administration of 400 mg three times daily for three consecutive days, corresponding to a total daily dose of 1200 mg.

This regimen is based on previously available pharmacokinetic data demonstrating adequate systemic exposure, favorable absorption, and tolerability at comparable doses in humans [58,59,66,67]. The multiple-dose schedule is specifically designed to achieve sufficient systemic concentrations prior to the BoNT/A challenge, thereby maximizing the likelihood of interfering with toxin activation during its early intracellular phase [14]. The timing of toxin administration is aligned with the expected peak or near steady-state concentrations of Ebselen, ensuring optimal pharmacological coverage during the critical window of BoNT activity.

From a translational perspective, this regimen was selected to balance pharmacological plausibility, prior human safety experience, and the practical constraints of an early-phase experimental study in healthy volunteers.

## 6.5 STUDY OBJECTIVES AND ENDPOINTS

The primary objective of this integrated Phase I/II clinical study is to evaluate the safety and tolerability of a short multiple-dose regimen of Ebselen in healthy adult volunteers, both when administered alone and in combination with a localized BoNT/A challenge to the EDB muscle.

Safety will be comprehensively assessed throughout the study duration, up to Day 90, through the evaluation of the incidence, nature, severity, and causality of treatment-emergent adverse events (TEAEs) and serious adverse events (SAEs). Additional safety assessments include changes from baseline in vital signs, clinical laboratory parameters (including hematology, biochemistry, and urinalysis), physical and neurological examinations, as well as the evaluation of neuromuscular function to exclude any signs of generalized or clinically relevant weakness beyond the targeted EDB muscle [73].

Secondary objectives include the characterization of the pharmacokinetic (PK) profile of Ebselen following repeated oral administration. PK endpoints will be assessed exclusively in subjects receiving active treatment and will include standard non-compartmental parameters such as maximum plasma concentration (C_max), time to maximum concentration (T_max), area under the concentration–time curve (AUC), trough concentrations (C_trough), and apparent terminal half-life. These analyses will allow the assessment of systemic exposure and will be interpreted in the context of previously reported clinical data on Ebselen [48,58,59,66].

In addition, secondary and exploratory objectives include the evaluation of the pharmacodynamic (PD) effects of Ebselen in attenuating BoNT/A-induced peripheral neuroparalysis using the established within-subject EDB challenge model. The principal PD endpoint is the difference in percentage reduction of compound muscle action potential (CMAP) amplitude (Δ% CMAP) between the BoNT/A-injected EDB muscle and the contralateral saline-injected control within the same subject [85,86,89,91-93]. This parameter provides a sensitive, quantitative, and internally controlled measure of neuromuscular impairment and allows the detection of potential attenuation of toxin-induced effects [74,75].

Additional PD endpoints include the longitudinal assessment of Δ% CMAP over time, enabling the characterization of the onset, peak effect, and recovery of BoNT/A-induced neuroparalysis. Furthermore, neurophysiological parameters such as distal latency and motor conduction velocity of the peroneal nerve will be evaluated, together with the time to peak effect and time to recovery toward baseline CMAP values. These assessments are aligned with the well-characterized time course of BoNT/A activity in the EDB model, which typically involves onset within 24–48 hours, peak effect within 1–3 weeks, and recovery over 2–3 months [75,76].

Overall, this comprehensive set of endpoints enables an integrated evaluation of safety, systemic exposure, and biological activity of Ebselen, providing a robust framework for the interpretation of its potential therapeutic effects in the context of botulinum neurotoxicity (Table 4).

A further key objective of the study is the exploratory assessment of the pharmacodynamic effects of Ebselen on BoNT/A-induced peripheral neuroparalysis using the within-subject EDB challenge model.

Overall, this set of endpoints enables an integrated evaluation of safety, systemic exposure, and biological activity, thereby providing a structured framework for interpretation of the potential effects of Ebselen in the context of botulinum neurotoxicity (Table 4).

| Category | Endpoint | Description | Population |
|---|---|---|---|
| **Primary (Safety)** | TEAEs and SAEs | Incidence, nature, severity, seriousness, and causality of adverse events from first dose to Day 90 | All subjects |
| | Vital signs | Changes from baseline in blood pressure, heart rate, temperature | All subjects |
| | Laboratory parameters | Hematology, biochemistry (including liver and renal function), urinalysis | All subjects |
| | Physical and neurological exam | Detection of clinically relevant abnormalities | All subjects |
| | Neuromuscular function | Assessment of generalized or off-target muscle weakness | All subjects |
| **Secondary (PK)** | Plasma concentration–time profile | Measurement of Ebselen plasma levels over time | Ebselen-treated subjects |
| | Cmax | Maximum plasma concentration | Ebselen-treated subjects |
| | Tmax | Time to reach Cmax | Ebselen-treated subjects |
| | AUC | Area under the concentration–time curve | Ebselen-treated subjects |
| | Ctrough | Trough plasma concentrations | Ebselen-treated subjects |
| | Half-life (t1/2) | Apparent terminal elimination half-life | Ebselen-treated subjects |
| **Secondary / Exploratory (PD)** | Δ% CMAP (primary PD endpoint) | Difference in percentage reduction of CMAP between BoNT/A-injected and contralateral saline-injected EDB muscle | All subjects |
| | Time course of Δ% CMAP | Longitudinal evaluation at scheduled visits (Days 1–90) | All subjects |
| | Distal latency | Changes in distal motor latency of peroneal nerve | All subjects |
| | Motor conduction velocity | Nerve conduction velocity measurements | All subjects |
| | Time to peak effect | Time to maximum CMAP reduction | All subjects |
| | Time to recovery | Time to return toward baseline CMAP values | All subjects |

***Table 4.*** *Summary of the study endpoints*

## 6.6 ADAPTIVE DESIGN AND DECISION STRATEGY

The study incorporates an adaptive design, allowing for progressive evaluation of safety and pharmacodynamic signals and optimization of resource allocation, consistent with modern early-phase trial methodologies [92]. An initial cohort of participants is followed by potential expansion phases, depending on interim results.

Predefined decision points are established at key time intervals corresponding to the expected pharmacodynamic profile of BoNT/A [86], including early (Day 21), intermediate (Day 30), and recovery (Day 60) phases. This staged evaluation enables the identification of early efficacy signals, confirmation of delayed effects, and assessment of recovery dynamics.

This adaptive approach is particularly suited for exploratory studies based on mechanistic endpoints and is consistent with modern clinical trial design strategies.

The study incorporates adaptive elements intended to support progressive evaluation of safety and pharmacodynamic signals in a manner consistent with modern early-phase trial methodology [81]. In practical terms, the design allows staged review of emerging data from an initial cohort, with the possibility of subsequent cohort expansion depending on the overall pattern of safety, tolerability, and exploratory PD findings.

The adaptive component of the study therefore does not replace the core methodological structure of the trial, but rather provides a framework for data-driven progression through the planned study stages. This approach is particularly appropriate in an exploratory, mechanism-oriented setting in which the principal objective is to identify informative safety and PD signals to guide further clinical development.

## 6.7 STUDY PROCEDURES

After screening and confirmation of eligibility, participants receive the assigned treatment (Ebselen or placebo) over a three-day period. Pharmacokinetic sampling is performed to characterize systemic exposure.

On Day 4, participants undergo baseline neurophysiological assessment, followed by intramuscular injection of BoNT/A in one EDB muscle and saline in the contralateral muscle [86].

Subsequent follow-up includes serial electrophysiological assessments using electromyography and nerve conduction studies, allowing the monitoring of both the development and resolution of neuromuscular impairment over time (Figure 19). During this phase, pharmacokinetic sampling is performed to characterize systemic drug exposure.

On Day 4, participants undergo baseline neurophysiological assessment, followed by intramuscular injection of BoNT/A into one EDB muscle and saline into the contralateral

muscle [86]. This within-subject procedure establishes the internal control necessary for subsequent pharmacodynamic comparisons.

Follow-up includes serial electrophysiological assessments using electromyography and nerve conduction studies, allowing monitoring of both the development and the resolution of neuromuscular impairment over time. These repeated measurements are scheduled to capture onset, peak effect, and recovery of the localized BoNT/A response (Figure 19).

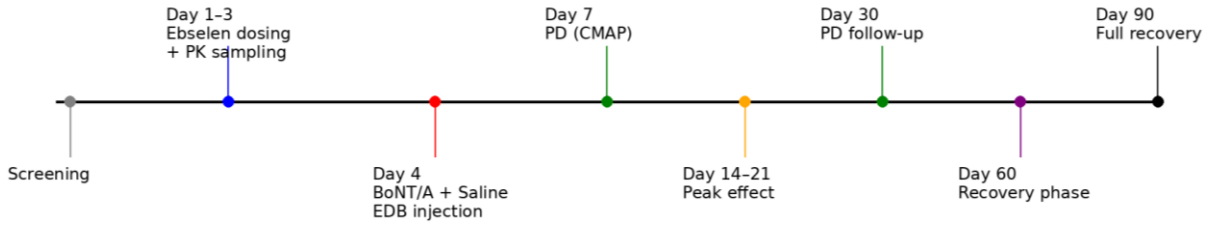


***Figure 19.*** *Graphical representation of the study timeline. Following screening, participants receive Ebselen or placebo over three consecutive days (Days 1–3), with pharmacokinetic (PK) sampling performed during this phase. On Day 4, a localized BoNT/A challenge is administered to one extensor digitorum brevis (EDB) muscle, with saline injected in the contralateral muscle. Pharmacodynamic (PD) assessments based on compound muscle action potential (CMAP) measurements are conducted longitudinally at predefined time points (Days 7, 14–21, 30, 60, and 90), allowing evaluation of onset, peak effect, and recovery of BoNT/A-induced neuromuscular impairment.*

## 6.8 SAFETY MONITORING

Safety is a primary focus of this early-phase clinical study and is ensured through continuous monitoring of adverse events, clinical parameters, and laboratory data, in accordance with GCP guidelines [75]. Predefined stopping rules and safety review procedures are implemented to promptly identify and manage potential risks. All adverse events are recorded and evaluated according to standardized criteria, and appropriate actions are taken in case of clinically relevant findings.

Safety oversight includes predefined stopping rules and review procedures intended to permit timely identification and management of potential risks.

All adverse events will be recorded, graded, and evaluated according to standardized criteria, with assessment of seriousness, severity, and causal relationship to study

treatment or study procedures. Particular attention will be paid to the detection of unexpected or clinically relevant neuromuscular findings, as well as to any evidence of effects extending beyond the intended local challenge site. Where clinically indicated, participants will undergo additional medical evaluation and appropriate follow-up measures.

This safety framework is particularly important in light of the controlled BoNT/A challenge model and is intended to ensure that risk remains proportionate, closely monitored, and ethically acceptable throughout the study.

## 6.9 ETHICAL CONSIDERATIONS

The study is conducted in accordance with the principles of the Declaration of Helsinki, GCP, and European regulatory requirements [75,76].

The use of a controlled BoNT/A challenge in healthy volunteers is justified by the localized, reversible, and low-risk nature of the model [86], as well as by the significant scientific value of the data generated.

All participants provide written informed consent before enrollment. The informed consent process is intended to ensure that participation is fully voluntary and based on an adequate understanding of the study objectives, procedures, foreseeable risks, and follow-up requirements. In this context, particular emphasis is placed on transparent communication regarding the challenge procedure and the expected course of localized and reversible neuromuscular effects. The study is conducted in accordance with the principles of the Declaration of Helsinki, GCP and applicable European regulatory requirements [75,76].

In this setting, the ethical acceptability of the model rests on careful participant selection, rigorous monitoring, minimization of procedural risk, and the use of objective endpoints capable of providing meaningful translational data.

# 7. STATISTICAL CONSIDERATIONS AND SAMPLE SIZE JUSTIFICATION

The present study is designed as an early-phase, exploratory clinical investigation aimed at evaluating safety, pharmacokinetics (PK), and pharmacodynamic (PD) signals associated with Ebselen administration in a controlled human model of botulinum neurotoxin exposure. As such, the study is not powered for confirmatory efficacy but rather to detect biologically meaningful trends that may support further clinical development [81].

The use of a within-subject control design, in which each participant serves as their own control through the comparison between BoNT/A-injected and saline-injected muscles, represents a key methodological strength. This approach significantly reduces inter-individual variability and increases statistical sensitivity, allowing meaningful interpretation of PD outcomes even in relatively small sample sizes [86].

The primary pharmacodynamic variable, represented by the change in CMAP amplitude, is expected to show a consistent and reproducible time course in response to BoNT/A administration. Previous studies using the EDB model have demonstrated relatively low intra-subject variability, supporting the feasibility of detecting treatment-related differences in small cohorts [86].

Statistical analyses will be primarily descriptive, with exploratory inferential approaches applied where appropriate. Safety data will be summarized using descriptive statistics, while PK parameters will be derived using standard non-compartmental analysis. PD data will be analyzed longitudinally, with particular attention to differences in CMAP reduction between treatment groups over time.

Accordingly, the study is not powered for confirmatory efficacy, but rather to detect biologically meaningful trends that may justify and inform subsequent stages of clinical development [81].

One of the main methodological strengths of the study is the within-subject control design, in which each participant serves as his or her own internal control through comparison between the BoNT/A-injected muscle and the contralateral saline-injected muscle. This design substantially reduces inter-individual variability and increases sensitivity for detection of PD signals, thereby improving interpretability even in relatively small exploratory cohorts [86].

The primary PD variable, represented by the change in CMAP amplitude, is expected to show a reproducible time course after BoNT/A administration. Previous studies using the EDB model have reported relatively limited intra-subject variability, supporting the feasibility of identifying treatment-related differences in small cohorts under controlled conditions [81,86].

Given the exploratory nature of the study, statistical analyses will be primarily descriptive, with inferential analyses used to support interpretation where appropriate. In particular:

- Safety data will be summarized using descriptive statistics;
- PK parameters will be derived using standard non-compartmental methods;
- PD data will be analyzed longitudinally, with particular attention to time-dependent differences in CMAP reduction between treatment groups and to the within-subject contrast between the BoNT/A-injected and saline-injected sides.

In this context, sample size justification is based primarily on the exploratory objectives of the study, the expected sensitivity of the within-subject EDB model, and the aim of generating sufficiently informative safety, PK, and PD data to support future confirmatory development, rather than on formal demonstration of efficacy.

# 8. EXPECTED RESULTS AND DATA INTERPRETATION

## 8.1 EXPECTED PHARMACODYNAMIC EFFECTS

Based on preclinical evidence, it is expected that Ebselen administration may attenuate the neuromuscular impairment induced by BoNT/A. Specifically, a reduction in the

magnitude of CMAP decrease in the BoNT/A-injected muscle, compared to placebo-treated participants, would represent a key indicator of pharmacodynamic activity [14,16].

Such an effect may manifest as a shift in the time course of CMAP reduction, a decrease in peak paralysis, or an acceleration of recovery. Even partial modulation of toxin-induced effects would provide important proof-of-concept evidence supporting the mechanism of action of Ebselen in vivo.

### 8.2 PHARMACOKINETIC AND PHARMACODYNAMIC RELATIONSHIP

The integration of PK and PD data is expected to provide insight into the relationship between systemic exposure to Ebselen and its potential biological effects. A correlation between plasma concentrations and attenuation of CMAP reduction would further strengthen the mechanistic rationale and support dose optimization strategies for future studies [48,58].

### 8.3 SAFETY EXPECTATIONS

Given the extensive clinical experience with Ebselen, no significant safety concerns are anticipated at the doses used in this study. Previous trials have consistently demonstrated a favorable tolerability profile, with no evidence of serious adverse events at comparable or higher dosing regimens [58,59,66].

## 9. STRENGTHS AND LIMITATIONS OF CLINICAL TRIAL

### 9.1 STRENGTHS

This study presents several methodological and scientific strengths. First, the use of the EDB challenge model provides a highly controlled and reproducible system for evaluating localized BoNT-induced neuroparalysis. This allows for precise and quantitative assessment of pharmacodynamic effects using objective electrophysiological endpoints [81,86].

Second, the within-subject design minimizes inter-individual variability and enhances statistical sensitivity, making it particularly suitable for early-phase exploratory studies. Third, the study is grounded in a strong mechanistic rationale, supported by extensive preclinical evidence demonstrating the role of the Trx–TrxR system in BoNT activation and the inhibitory effects of Ebselen across multiple serotypes [7,14,15].

Finally, the availability of an established clinical safety profile for Ebselen significantly reduces translational uncertainty and supports the feasibility of rapid clinical development [48,58,59,66].

### 9.2 LIMITATIONS

Despite its strengths, the study also presents several limitations. The use of healthy volunteers limits the generalizability of the findings to clinical populations affected by botulism, which may present different pathophysiological conditions.

Moreover, the EDB model reflects a localized and controlled exposure to BoNT, which may not fully replicate the systemic and often more severe manifestations of natural botulism. While the model offers high internal validity, its external applicability remains limited.

Additionally, the relatively small sample size and exploratory nature of the study preclude definitive conclusions regarding clinical efficacy.

Finally, the effectiveness of Ebselen is expected to be limited to the early phase of toxin action, prior to light chain activation, which may restrict its therapeutic window in real-world scenarios [14].

## 10. CLINICAL AND TRANSLATIONAL IMPLICATIONS

The findings of this study may have important implications for both clinical practice and biodefense preparedness. If Ebselen demonstrates the ability to attenuate BoNT-induced neuroparalysis in humans, it would represent a new host-targeted therapeutic strategy, distinct from currently available antitoxin-based approaches.

Unlike antitoxins, which neutralize circulating toxins, Ebselen acts at the intracellular level by targeting a host-dependent mechanism required for toxin activation. This

approach may provide advantages in terms of broader spectrum of activity and potential efficacy across multiple BoNT serotypes [14,16].

Clinically, this strategy may be particularly relevant in conditions characterized by ongoing toxin production, such as infant botulism or intestinal colonization in immunocompromised patients [94].

Furthermore, the potential use of Ebselen as a prophylactic or early intervention agent may be of particular interest in high-risk scenarios, including accidental exposure or deliberate release of BoNTs [95].

## 11. FUTURE PERSPECTIVES

Future studies will be required to further evaluate the clinical potential of Ebselen in botulism. These may include larger Phase II trials aimed at confirming pharmacodynamic effects and optimizing dosing strategies, as well as Phase III studies designed to assess clinical efficacy in affected patient populations.

Additional research should also explore the therapeutic window of Ebselen, particularly its effectiveness when administered after toxin exposure, which represents a critical aspect for real-world applicability.

The combination of Ebselen with existing antitoxin therapies may represent another promising avenue, potentially providing both extracellular and intracellular protection against BoNTs.

Moreover, the identification of additional inhibitors targeting the Trx–TrxR system or related pathways may further expand the therapeutic landscape and contribute to the development of next-generation countermeasures against neurotoxins.

## 12. CONCLUSIONS

In conclusion, this study represents a significant step in the translational development of a new therapeutic strategy for botulism. By integrating mechanistic insights, preclinical evidence, and a controlled human experimental model, this clinical study

provides a robust framework for the evaluation of Ebselen as a host-targeted inhibitor of BoNT activity.

The results of this investigation are expected to contribute to the advancement of innovative treatment approaches for botulism and to support the development of medical countermeasures against neurotoxins of high clinical and public health relevance.

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