



# Association Between Secondary Botulinum Toxin A Treatment Failure in Cosmetic Indication and Anti-Complexing Protein Antibody Production

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## ABSTRACT

**Introduction:** Botulinum toxin A (BoT/A) treatment failure (BTF) affects patients subjected to repeated BoT/A exposure for cosmetic indications. BoT/A's general formulation contains core BoT/A and complexing proteins. BTF may be caused by antibody-induced treatment failure. Antibodies against core BoT/A can occur; however, anti-complexing protein antibodies have never been demonstrated, and tools for anti-complexing protein antibody detection have not been developed. The aim of this study was to evaluate immune involvement in BoT/A-nonresponsive patients.

**Methods:** Patients suspected of nonresponsiveness to BoT/A for cosmetic indications were recruited. All volunteers were categorized as BoT/A-responsive or BoT/A-tolerant according to frontalis testing with onabotulinumtoxinA

(onaA). Twenty-two BoT/A-tolerant volunteers were recruited separately for frontalis testing with incobotulinumtoxinA (incoA). Anti-BoT/A and anti-complexing protein antibodies were quantified by special ELISA using sera from blood sampled before and after frontalis testing. **Results:** Significantly higher levels of IgG against complexing protein were detected in onaA-tolerant sera but not in onaA-responders, leading to proposals that anti-complexing protein antibodies could cause onaA unresponsiveness. Some onaA-tolerant patients according to frontalis test with incoA were responsive to incoA. Newly developed absorption ELISA confirmed that incoA-responsive sera predominantly contained IgG against complexing proteins, whereas incoA-tolerant sera contained significant levels of IgG against core BoT/A. The presence of anti-complexing protein antibodies higher than 90.75% in sera of onaA-tolerant patients could respond to incoA. The ELISA technique might be employed as a tool to predict incoA responsiveness. Our frontalis testing after incoA treatment showed that anti-incoA IgG levels were not increased by incoA.

**Conclusions:** BoT/A-exposed patients may develop antibodies against core botulinum toxin and complexing proteins. Our study is the first to demonstrate that anti-complexing protein antibodies cause BTF. High levels of antibodies against complexing proteins can cause onaA unresponsiveness, although some patients

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were still incoA-responsive. Our developed ELISA to detect anti-complexing protein antibodies can determine whether onaA-tolerant patients respond to incoA without incoA frontalis testing.

**Keywords:** Antibody; Botulinum toxin A; Complexing protein; ELISA; Secondary botulinum toxin A treatment failure

### Key Summary Points

#### Why carry out this study?

Botulinum toxin A (BoT/A) treatment failure (BTF) affects patients subjected to repeated BoT/A (core BoT/A and complexing proteins) exposure for cosmetic indications.

BTF may be caused by antibodies against core botulinum toxin or complexing proteins.

Anti-complexing protein antibodies involved in BTF and how to detect such antibodies have never been demonstrated.

#### What was learned from the study?

High levels of anti-complexing protein antibodies associated with BTF in onabotulinum toxin (onaB/T)-treated patients.

OnaB/T-tolerant patients with high levels of anti-complexing protein antibody could respond to incobotulinum toxin (incoB/T).

Our newly developed ELISA could be a promising tool to predict whether onaB/T-tolerant patients respond to incoB/T.

weaken hyperkinetic lines caused by muscular contractions [1]. BoTs are also used in facial sculpting and shaping, and to correct facial asymmetry [2–12]. US Food and Drug Administration (FDA) approvals have also been granted for botulinum toxin type A in axillary hyperhidrosis, limb spasticity, chronic migraine and idiopathic overactive bladder [13–21].

Commercially available BoTs in aesthetic medicines include type A and type B subtypes, each of which contains a unique complement of accessory proteins. Type A neurotoxins comprise light and heavy chains, which constitute the core 150-kDa neurotoxin. Type A BoTs (BoT/A) are frequently used and commercially available in preparations including onabotulinumtoxinA (onaA; Botox<sup>®</sup>, Allergan Inc, Irvine, CA, USA), abobotulinumtoxinA (aboA; Dysport<sup>®</sup>, Ipsen Ltd, Slough, Berkshire, UK), and incobotulinumtoxinA (incoA; Xeomin<sup>®</sup>, Merz Pharmaceuticals GmbH, Frankfurt am Main, Hessen, Germany). Of these toxins, incoA is the only product lacking accessory proteins known as complexing proteins [22]. In cosmetic indications, effective treatment with BoT/A requires periodic retreatment, which might trigger immune responses leading to immune resistance and failure to achieve desired outcomes. At least one study has linked the development of immunogenicity and neurotoxin-neutralizing antibodies to complexing proteins and in turn to producing partial or total toxin treatment failure [7, 22]. With complexing protein-containing BoT/A preparations, such failures can occur within 2–3 years of the first treatment and may be caused by BoT/A-neutralizing antibodies produced 1 year following treatment [8]. Shorter treatment intervals, lower toxin doses and using low-antigenicity preparations can minimise the risk of developing immune reactions and antibody production against BoT/A [23–25]. Treatments can be complicated by a patient's or the antigen's capacity to elicit immune responses [24].

Frevort and Dressler postulated that incoA's lack of complexing proteins may reduce both immunogenicity and the risk of antibody production [22]. So far, no tool exists to determine the causes of secondary nonresponse or to confirm the presence of BoT/A-neutralizing

## INTRODUCTION

Botulinum toxins (BoTs) have been a principal tool for both clinical and aesthetic physicians. Cosmetic medicine leverages its ability to

antibodies. Currently available methods rely on animal models to establish antibody presence, including mouse protection assays using affected patient sera to neutralize toxin and prevent death [26, 27]. However, these tests are impractical for daily, clinical use and are unethical because of their high and often lethal consumption of animals.

We previously investigated the existence of antibodies against BoT/A active sites in sera of secondary BoT/A treatment failure patients [25]. Some serum samples of treatment failure subjects contained low levels of anti-BoT/A active site antibodies, suggesting that BoT/A treatment failure was due to other factors in the sera. One possibility was that such sera contained antibodies against complexing proteins. Therefore, the aim of the present study was to investigate the existence of anti-complexing protein antibodies in the sera of BoT/A treatment failure patients. We thus developed a technique to detect anti-complexing protein antibodies.

## METHODS

### Volunteers

This was a prospective cohort study performed at a single centre in Thailand. Seventy-four healthy subjects aged 18 years or older, who were suspected of BoT/A tolerance, were enrolled. Frontalis testing with onA (Botox<sup>®</sup>) was performed on all subjects to confirm the diagnosis of onA-tolerant (onA-T). Thirty-five (47.3%) subjects responded to onA (onA-responsive, onA-R) while 39 (52.7%) subjects did not respond (onA-T). All 39 onA-T subjects were asked to repeat frontalis testing with inA (Xeomin<sup>®</sup>). Twenty-two onA-T subjects agreed to frontalis testing with inA as illustrated in Fig. 1. The average interval between frontalis testing with onA or inA was 7.52 months. These 22 subjects were divided into two groups based on the results of inA frontalis testing: onA-tolerant with inA-responsive (onA-T with inA-R) and onA-tolerant with inA-tolerant (onA-T with inA-T). Blood samples were collected immediately before and 2 weeks after frontalis tests were performed with either

onA or inA (Fig. 1). Absorption ELISA to detect anti-non-BoT/A antibodies and inhibition ELISA to detect the anti-active sites of BoT/A antibodies were conducted on the second and third collections of blood samples. This prospective analytic study was approved by the ethical committee, Faculty of Medicine, Siriraj Hospital, Mahidol University (COA No. Si 087/2020). All procedures performed in studies involving human participants were in accordance with the institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

### Frontalis Test

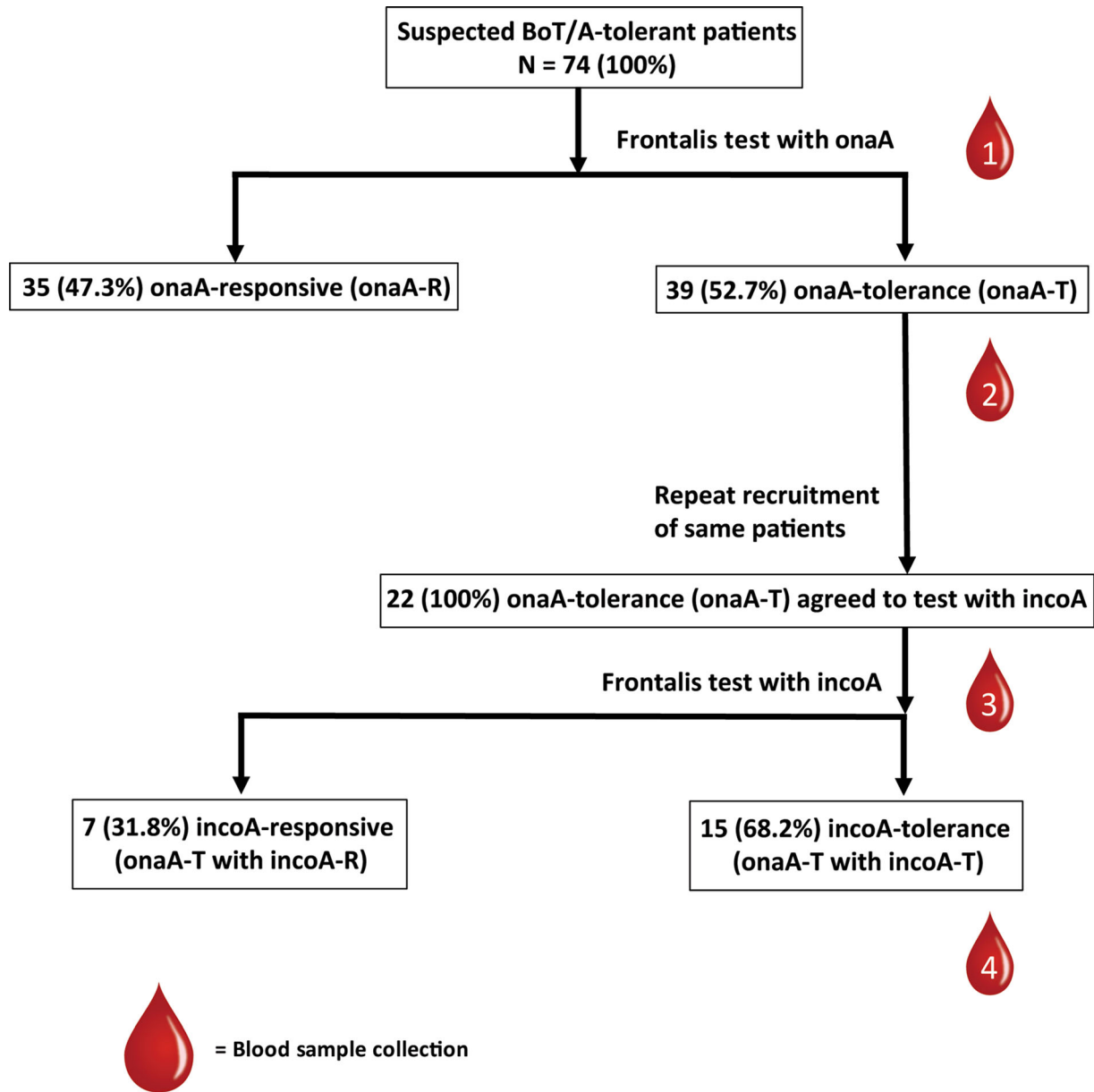
The frontalis muscle was injected unilaterally with onA in all patients and onA tolerance was assessed as described previously [28]. Briefly, 20 units of either onA or inA was injected at a location 3 cm above the lateral and medial canthus of one eye but not into the contralateral eye area. The patient's ability to raise both eyebrows was analysed 2 weeks post-injection. Asymmetric muscular activity proved BoT/A efficacy and patients were included or excluded on the basis of these outcomes.

### Blood Sample Collection

Ten millilitres of blood was collected from each subject and centrifuged at 1000 rpm for 10 min at room temperature to separate sera from solid components. Sera was collected and stored at – 20 °C until use.

### Absorption ELISA to Detect Anti-Non-BoT/A Antibodies

Ninety-six-well plates were labelled either as “absorption” or “working” plates. An inA solution (0.028 unit/ml) was used to coat selected wells in absorption plates at 50 µl/well, while selected working plate wells were coated with 0.051 unit/ml of onA solution. Absorption was performed in inA-coated plates by washing wells with 0.05% Tween-20 in



**Fig. 1** Schematic plan. A patient suspected of BoT/A nonresponsiveness was recruited for frontalis testing and blood collection thereafter. Blood was sampled four times and subjected to ELISA. The first and third collections

were performed prior to frontalis testing, while the second and fourth samples were collected 2 weeks after frontalis testing. Blood droplet illustrations represent blood sample collection events

phosphate buffered saline (PBST) and blocking with 1% bovine serum albumin (BSA) in PBS for 1 h at 37 °C in a humidified chamber. After washing, 100 µl/well of 1:50 diluted patient serum was added and plates were placed in a humidified shaking incubator at 37 °C for 1 h. Approximately 100 µl of absorbed serum was

transferred to other incoA-coated wells and the absorption step was repeated twice. Following absorption, approximately 100 µl of absorbed sample was transferred to the working plate (onaA-coated and blocked with 1% BSA) and incubated at 4 °C for 2 h in a humidified chamber. In parallel, 100 µl of freshly diluted

(1:50) un-absorbed serum samples was added to adjacent, matched wells. After washing, freshly prepared, HRP-conjugated rabbit anti-hIgG antibody solution was added and incubated at 37 °C for 1 h. After the wells were washed five times, TMB was added to each well and colour developed for 10 min in the dark at room temperature. Colour development was stopped with 1 N HCl and the optical density of each well was measured at 450 nm.

### Inhibition ELISA to Detect Anti-BoT/A Active Site Antibodies

The ELISA plate was coated with incoA and blocked with a mixture of three mouse monoclonal antibodies prior to incubating with 1:50 diluted sera. We developed and previously published this method of detecting human IgG against the active sites of BoT/A [25].

### Statistical Analysis

Results were analysed by either parametric or non-parametric paired tests, where appropriate. The differences between the ELISA values for the non-absorbed and absorbed samples were analysed and used to calculate the percentage of anti-complexing protein antibody using the formula:

$$\left[ 1 - \left( \frac{\text{the numerical difference between the values for non-absorbed and absorbed samples}}{\text{divided by the numerical values for non-absorbed sample}} \right) \right] \times 100\%.$$

Threshold values were obtained by applying receiver operating characteristic (ROC) data analysis. Statistical significance was reached when  $p < 0.05$ .

## RESULTS

### OnaA-T Patient Demographics and Clinical Data

The demographic data of 22 onaA-T is shown in Table 1. Seven (31.8%) onaA-T subjects, six women and one man with a median age of 35 years, were incoA-responsive during frontalis testing and were categorized as onaA-T with incoA-R. Fifteen (68.2%) onaA-T subjects, 14 women and one man with a median age of 33 years, were incoA-unresponsive and were categorized as onaA-T with incoA-T. When comparing between incoA-T and incoA-R groups, the number of BoT/A injections and total doses for cosmetic indications were significantly different. Tolerant patients had more injections and higher doses per injection and total cumulative dose prior to the study.

### Different Quantities of Anti-Complexing Proteins Between OnaA-Responsive and OnaA-Tolerant Patients

Sera from all patients tested with onaA (both onaA-R and onaA-T; blood sample 2) were subjected to absorption ELISA. Total sera (un-absorbed) were suspected of containing antibodies against core botulinum toxin and complexing

proteins whereas absorbed sera, having been depleted of antibodies specific to core botulinum toxin, were suspected of containing only antibodies against complexing proteins. After absorption, differences in hIgG were observed between sera from onaA-responsive and onaA-tolerant patients. OnaA-responsive sera hIgG

**Table 1** Demographic data of onaA-tolerant patients

Group	Median age (years)	Gender		Median duration (years)	Median dose/visit (units)	Median number of injections	Median interval (months)	Median total dose (units)
		Female, <i>n</i> (%)	Male, <i>n</i> (%)					
All	34	25 (73.5)	9 (26.5)	–	–	–	–	–
IncoA-responsive patients ( <i>n</i> = 7)	35	6 (85.70)	1 (14.30)	7	70	12	5	800
IncoA-tolerant patients ( <i>n</i> = 15)	33	14 (93.30)	1 (6.70)	7	100	16	5	1600
<i>p</i> value				0.731	0.07	< 0.05	0.267	< 0.01

levels were significantly decreased ( $p < 0.05$ ) after absorption (Fig. 2a), whereas onaA-tolerant sera hIgG levels were not significantly changed ( $p > 0.05$ ) (Fig. 2b). Therefore, antibodies persisting after absorption in onaA-tolerant sera may be hIgG that are reactive to complexing proteins and suggested that onaA-tolerant patients are affected by antibodies against complexing proteins.

### OnaA-T with IncoA-R Group

On the basis of the present results, we questioned whether onaA-tolerant patients would respond to pure BoT/A. Therefore, the frontalis test was repeated with incoA on 22 of the original 39 onaA-tolerant patients as illustrated in Fig. 1. Approximately 31% (7 of 22 patients) of patients responded to incoA, supporting our hypothesis and suggesting that incoA tolerance might arise as a result of factors unrelated to incoA.

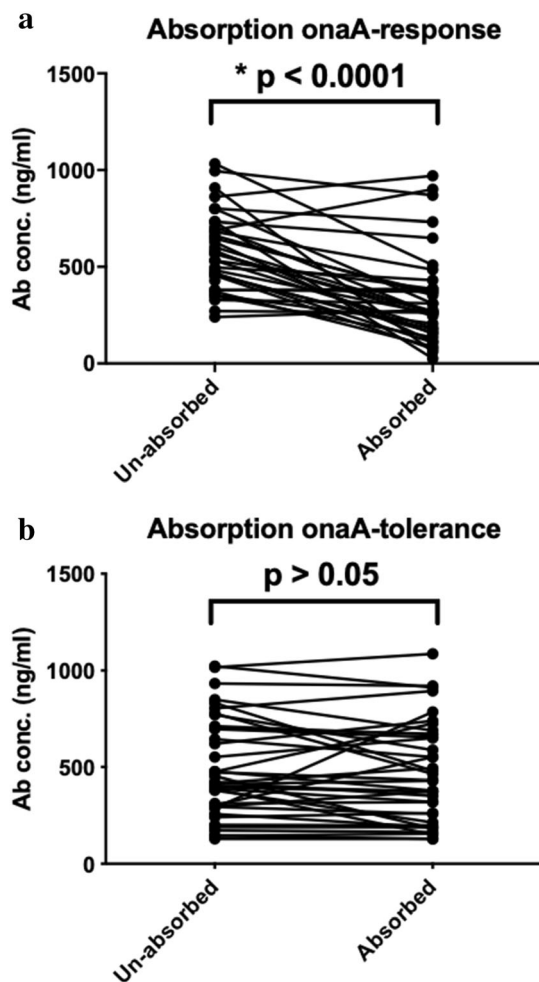
### Sera of OnaA-T with IncoA-R Patients Contained Significant Levels of Anti-Complexing Protein Antibodies

As proof of concept that onaA-tolerant patients could still respond to incoA because the observed interference was only due to anti-

complexing protein antibodies reacting against onaA, absorption ELISA was repeated on serum samples from the 22 onaA-T patients. Following absorption, differences were observed in the detected hIgG levels between incoA-responsive (Fig. 3b) and incoA-tolerant patients (Fig. 3a). These findings were reversely different from the detected hIgG levels from onaA-responsive and onaA-tolerant patients as shown in Fig. 2. After absorption, all sera from incoA-responsive patients contained no significant change in levels of hIgG ( $p > 0.05$ ), whereas hIgG levels in incoA-tolerant sera were significantly decreased ( $p < 0.05$ ). These findings suggest that incoA-responsive sera could contain impactful levels of hIgG that target complexing proteins while incoA-tolerant sera contains fewer anti-complexing protein antibodies.

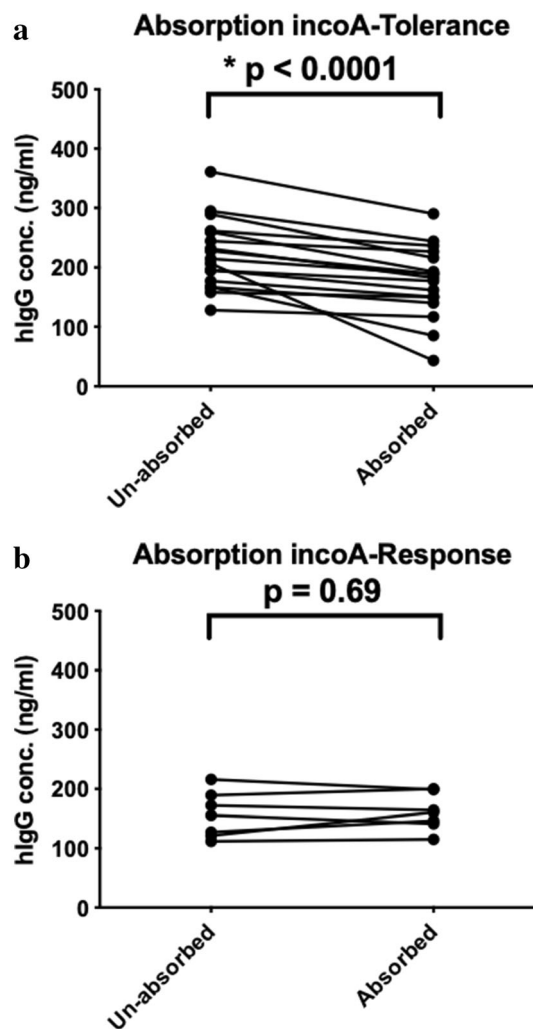
### Predictive Cut-Off Threshold for OnaA-T with IncoA-R Patients

According to our absorption ELISA results, if hIgG levels in absorbed sera were comparable to those in un-absorbed sera, such sera may contain predominantly complexing protein-specific hIgG. Conversely, if hIgG levels in absorbed sera were lower than those in un-absorbed sera, such sera may contain predominantly hIgGs against the core botulinum toxin and complexing



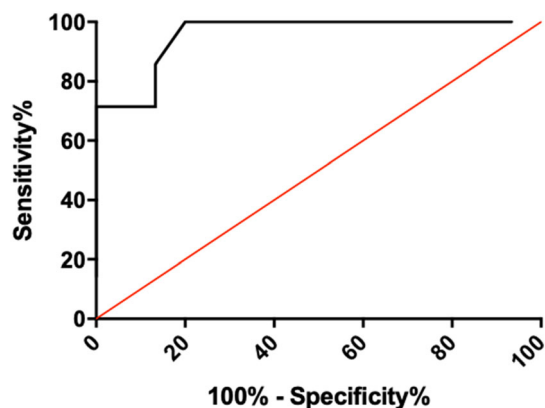
**Fig. 2** Absorption ELISA with onaA-responsive and onaA-tolerant sera. Sera from the second blood collection were subjected to absorption ELISA. ‘Un-absorbed’ denotes whole sera that were not depleted of antibodies, while ‘absorbed’ denotes sera depleted of antibodies against incoA prior to anti-complexing protein hIgG detection by ELISA. Match-paired line graphs represent levels of hIgG against BoT/A and complexing proteins (un-absorbed) or only complexing proteins (absorbed) from onaA-responsive volunteers (**a**) and onaA-tolerant (**b**) patients. \*Statistical significance at  $p$  value  $< 0.05$

proteins. Therefore, we interpreted the subtractive values of hIgG in the un-absorbed and absorbed sera as the quantity of hIgG against complexing proteins in the sera. To normalize the differences in basal levels, decreasing values were converted into percentages of reduction and analysed by ROC analysis (Fig. 4). The highest value of Youden’s index at 180 was



**Fig. 3** Absorption ELISA with incoA-tolerant and incoA-responsive sera. Sera from the third blood collection were subjected to absorption ELISA. ‘Un-absorbed’ denotes whole sera that were not depleted of antibodies, while ‘absorbed’ denotes patient sera that were depleted of antibodies against incoA before detecting anti-complexing protein hIgG by ELISA. Match-paired line graphs represent levels of hIgG against BoT/A and complexing proteins (un-absorbed) or only complexing proteins (absorbed) from incoA-tolerant patients (**a**) and incoA-responsive volunteers (**b**). \*Statistical significance at  $p$  value  $< 0.05$

chosen to achieve an optimal cut-off value at 90.75% (Table 2). Consequently, if the percentage of hIgG specific to complexing proteins (in absorbed serum) was higher than 90% of the percentage of hIgG against whole BoT/A (in un-



**Fig. 4** ROC curve demonstrates percentage of reduction in hIgG levels and incoA outcomes. Cut-off points for percentage of reduction in hIgG corresponded to sensitivity, specificity and Youden's index and are shown in Table 2

absorbed serum), the affected patient may respond to incoA with a toxin sensitivity of 100% and specificity of 80%.

#### OnaA-T with IncoA-R Patients Were Not Affected by Anti-Active Sites of Botulinum Toxin

We also hypothesized that patients who were both onaA-tolerant and incoA-responsive arose as a consequence of having antibodies against complexing proteins, but not antibodies against BoT/A active sites. Using an inhibition ELISA developed by our group [25], we found no significant difference in hIgG levels against core botulinum toxin ( $p > 0.05$ ) in either incoA-responsive or incoA-tolerant sera (Fig. 5) even when all three BoT/A active sites were specifically blocked with mouse monoclonal antibodies beforehand. These results indicated that hIgG against all three BoT/A active sites did not affect incoA function, since changes in hIgG levels were insignificant and not associated with the outcomes of incoA frontalis testing.

#### IncoA Did Not Provoke Anti-Core Botulinum Toxin Antibody

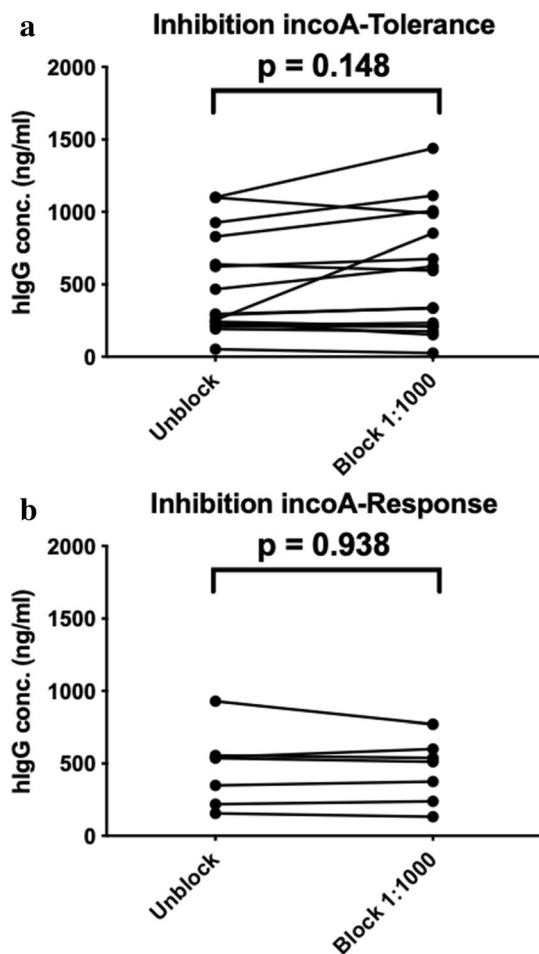
Using our ELISA test [25], we analysed sera from patients before and after incoA injection for

**Table 2** Cut-off threshold for percentage of reduction, sensitivity, specificity and Youden's index to predict incoA responsiveness

Cut-off	Sensitivity	Specificity	Youden's index
> 35.95	100	6.667	106.67
> 62.70	100	13.33	113.33
> 74.65	100	20	120.00
> 76.85	100	26.67	126.67
> 79.60	100	33.33	133.33
> 81.55	100	40	140.00
> 82.80	100	46.67	146.67
> 83.50	100	53.33	153.33
> 84.60	100	60	160.00
> 86.55	100	66.67	166.67
> 89.30	100	73.33	173.33
> 90.75	100	80	180.00
> 91.45	85.71	86.67	172.38
> 92.35	71.43	86.67	158.10
> 93.80	71.43	93.33	164.76
> 95.15	71.43	100	171.43
> 99.05	57.14	100	157.14
> 104.1	42.86	100	142.86
> 110.3	28.57	100	128.57
> 123.8	14.29	100	114.29

frontalis testing (blood samples 3 and 4) to quantify hIgG levels against botulinum toxin and determine if incoA provoked antibody responses. Levels of hIgG against core botulinum toxin were not significantly different between sera of patients before and after incoA frontalis testing ( $p > 0.05$ ; Fig. 6a). Match-pairing graphs also confirmed that hIgG levels were not significantly changed between both sera ( $p > 0.05$ ; Fig. 6b), suggesting that incoA does not cause or increase immunogenicity in patients previously exposed to BoT/A.

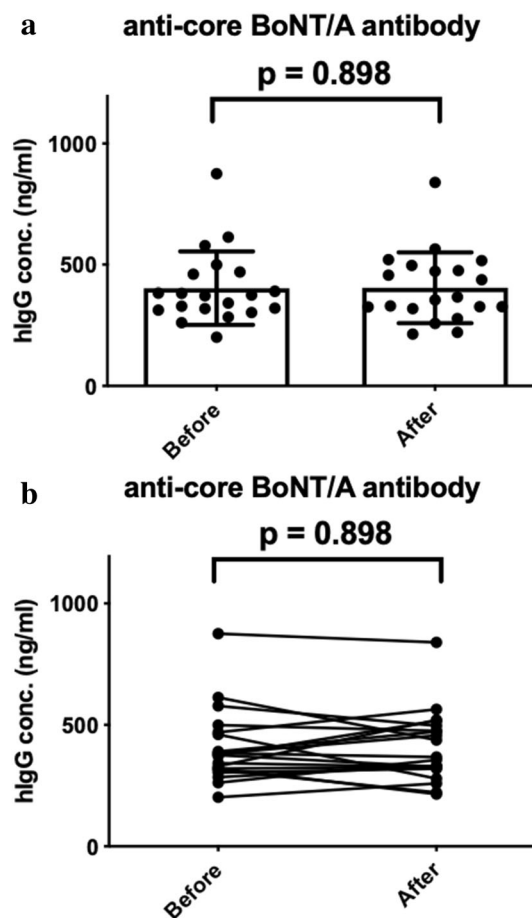




**Fig. 5** Inhibition ELISA to detect antibodies against BoT/A active sites. Sera from the third blood sample collection were subjected to inhibition ELISA. Match-paired line graphs represent levels of hIgG against active sites of BoT/A in sera from incoA-tolerant patients (a) and incoA-responsive patients (b) before (“Unblock”) and after (“Block 1:1000”) active site blockage by mouse monoclonal antibodies. Statistical significance at  $p$  value < 0.05

## DISCUSSION

Secondary treatment failures after botulinum toxin treatments are caused by clinical factors, patients’ requests for shorter injection intervals and more customized treatments, and the use of higher doses and increased injection frequencies [29]. Analysis of clinical data revealed that high toxin doses per visit and cumulative doses are associated with secondary BoT/A treatment



**Fig. 6** Levels of anti-core BoT/A antibodies in sera before and after incoA frontal testing. Antibodies against core BoT/A in sera from the third and fourth blood sample collections were quantified by ELISA using plates pre-coated with incoA. Match-paired linear plot (a) and bar graphs with overlapping data points (b) were generated from the same dataset. The match-paired linear plot suggested no significant change in levels of hIgG against incoA before and after incoA induction, as shown in the bar graphs. Statistical significance at  $p$  value < 0.05

failure even when the highly pure incoA preparation was used. This finding was similar to our previous report that botulinum toxin tolerance could be induced by, and correlated with, an increasing quantity of injected BoT/A [25]. Interestingly, more frequent BoT/A exposure correlated with BoT/A tolerance. We also noted that some patients could still respond to non-toxin-associated proteins in BoT/A formulations. As such, the demand for BoT/A with

minimal immunogenicity or antigenicity endures and may be suitably filled by commercial preparations such as incoA, which lacks complexing proteins [30]. To date, secondary treatment failure due to incoA has not been detected in toxin-naïve patients.

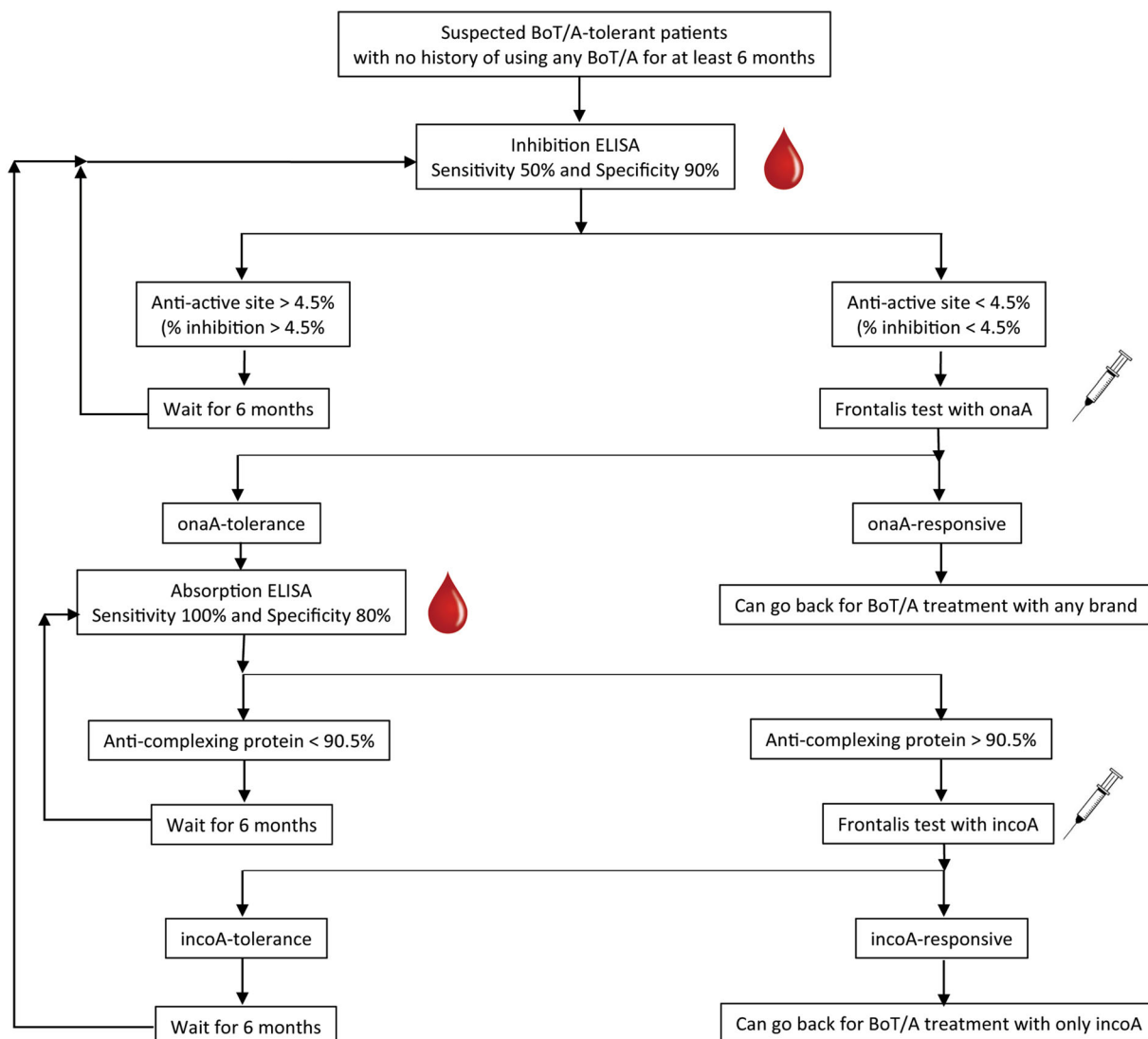
OnaA-responsive sera also contained hIgG against both core BoT/A and complexing proteins (Fig. 2a). Following depletion of anti-core BoT/A antibody, levels of hIgG against complexing proteins in some patients were lower than levels of hIgG against both BoT/A and complexing proteins (in un-absorbed sera). This is the first documented observation of the existence of anti-complexing protein antibodies determined by our newly developed absorption ELISA technique. These results suggested that there may be fewer anti-complexing protein hIgG than anti-core BoT/A hIgG in the sera of onaA-responsive patients. Conversely, levels of hIgG in some onaA-tolerant sera did not decrease significantly after absorption, suggesting that some of the hIgG in these sera were targeting anti-complexing proteins. This finding raised a possibility that onaA treatment failure might be caused by antibodies induced against onaA. This possibility was raised during an evaluation of neutralizing antibodies in patients with secondary treatment failure following use of complexing protein-free BoT/A preparations [30]. Over 80% of patients had decreased neutralizing antibody titres and over 60% of these patients experienced a decrease in titres to the limit of detection.

Compared to the 150-kDa core neurotoxin, the complex-containing 900-kDa unit is a substantial foreign protein load and highly possible immune target. Thus, complexing proteins theoretically increase the risk of antibody production against themselves and their associated neurotoxin. Although the application of BoT/A at lower doses or with reduced protein content in aesthetic medicine may reduce the risk of neutralizing antibody development, recent reports suggest that they did not completely prevent the development of immunogenicity [31, 32]. As such, the presence of anti-complexing protein hIgG could induce BoT/A treatment failure and the absence of complexing proteins in incoA may be clinically

beneficial for physicians wishing to avoid antigenicity, as has been demonstrated in animal studies [33–35].

As proof of concept that anti-complexing protein antibodies interfere with onaA function, we challenged onaA-T patients with incoA. Approximately 30% of onaA-T patients could respond to incoA and their sera contained the most hIgG against complexing proteins (Fig. 3b). Even though high levels of anti-complexing protein antibody in incoA-responsive patients were detected, these antibodies did not affect incoA function. This study is the first to present scientific evidence indicating that secondary BoT/A treatment failure could be caused by anti-complexing protein antibodies. We also investigated why incoA-tolerant patients were nonresponsive to complexing protein-free BoT/A. Previously, we reported that onaA tolerance was associated with the existence of antibodies to the BoT/A active sites. We performed inhibition ELISA and showed that both incoA-tolerant and incoA-responsive sera did not contain significant levels of hIgG against all three BoT/A active sites. Therefore, incoA-tolerant patients may fail incoA treatment owing to the existence of antibodies against core BoT/A apart from active sites and other unknown factors unrelated to incoA. With this finding, we hypothesized that the low immunogenicity of incoA may provoke immune responses and production of significant levels of hIgG against the core BoT/A. However, we observed an insignificant change in levels of hIgG against the core BoT/A between sera obtained before and after incoA administration (Fig. 6a), suggesting that incoA did not provoke immune responses or significant antibody production against the core BoT/A. This was consistent with previous reports that repeated injections of incoA did not result in the development of any neutralizing antibodies to incoA [36, 37].

On the basis of our absorption ELISA results (Fig. 3), we analysed the percentage of anti-complexing protein antibodies in incoA-tolerant sera and incoA-responsive sera with ROC to determine cut-off thresholds for this evaluation. ROC analysis revealed an excellent curve and suggested that a percentage of anti-complexing protein antibodies of 90.75% was predictive for



**Fig. 7** Proposed treatment protocol for patients suspected of BoT/A tolerance. In patients suspected of BoT/A tolerance, we recommend completely ceasing injections of any brand of BoT/A for at least 6 months and performing diagnostic evaluations using both inhibition and

absorption ELISA. Blood droplet illustrations represent blood sample collection events for ELISA investigations and syringe illustrations represent frontalis testing events with various BoT/A formulations

patients who respond to incoA, with an excellent sensitivity of 100% and very good specificity (80%). This level of sensitivity and specificity suggests that our absorption ELISA technique may be useful for predicting the responsiveness of onaA-tolerant patients to incoA. It may also be indispensable for aesthetic physicians and patients when discussing and choosing the type of BoT/A required for further

treatment in the setting of onaA treatment failures.

Therefore, we propose a protocol and treatment approach for patients suspected of BoT/A tolerance: instead of switching BoT/A brands or injecting the same type or brand of BoT/A repeatedly, we recommend that all doctors stop injecting BoT/A for at least 6 months and instead use both inhibition and absorption

ELISA, as shown in Fig. 7, to prevent further antibody production or immune responses against BoT/A.

Lastly, there are a few limitations to our study. As a result of the prevalence of BTF being low, collection of prospective BTF cases in the future is needed to support these results. In addition, validation of our newly developed absorption ELISA is still required with new cohorts of BTF and botulinum toxin responsive groups.

## CONCLUSION

The results of this study demonstrated that BoT/A-exposed patients could produce antibodies against core botulinum toxin and complexing proteins. Levels of anti-complexing protein antibodies were quantified by our newly developed ELISA and their levels associated with BTF were firstly demonstrated. Our study suggested that onA-unresponsive patients produced high levels of antibodies against complexing proteins that could respond to incoA. Our ELISA technique might be employed as a tool to predict whether onA-tolerant patients respond to incoA without incoA frontalis testing.

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**Data Availability.** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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