



**Prospective analysis of neutralising antibody titres in secondary non-responders under continuous treatment with a botulinumtoxin type A preparation free of complexing proteins – a single cohort 4-year follow-up study**

Journal:	<i>BMJ Open</i>
Manuscript ID:	bmjopen-2011-000646.R3
Article Type:	Research
Date Submitted by the Author:	14-Jun-2012
Complete List of Authors:	Hefter, Harald Hartmann, Christian Kahlen, Ulrike Moll, Marek Bigalke, Hans
<b>Primary Subject Heading</b>:	Neurology
Secondary Subject Heading:	Medical management, Pharmacology and therapeutics
Keywords:	secondary non-responder , cervical dystonia , cessation of therapy , neutralising antibody titre, complexing proteins , botulinum neurotoxin

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3 **Prospective analysis of neutralising antibody titres in secondary non-responders**  
4 **under continuous treatment with a botulinumtoxin type A preparation free of**  
5 **complexing proteins – a single cohort 4-year follow-up study**  
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1  
2  
3 **Word count (text):** 1807  
4

5 **Word count (abstract):** 296  
6

7 **Tables/Figures:** 0/2  
8

9 **References:** 18  
10

11 **Short title:** Low antigenicity of incobotulinumtoxinA  
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**ABSTRACT**

**Objective.** In long-term botulinum neurotoxin treatment, loss of therapeutic efficacy may occur due to neutralising antibody formation. Preliminary results with incobotulinumtoxinA, a preparation free of complexing / accessory proteins, have indicated a low antigenicity. We hypothesised that continuous treatment with this botulinum neurotoxin preparation would not result in an increase in neutralising antibody titres (NABTs) in patients with preexisting NABTs.

**Design.** Prospective, blinded cohort study.

**Setting.** Single centre in Germany.

**Participants.** Thirty-seven cervical dystonia patients with NABTs and partial secondary non-responsiveness to their previous botulinum neurotoxin type A treatment.

**Intervention.** 3-monthly intramuscular injections of incobotulinumtoxinA with a constant dose of 200 MU per injection during the first year; thereafter up to 500 MU for the next 36 months.

**Outcome measures.** Primary outcome measure: number of patients in whom NABTs declined below the initial titre after 48 months of incobotulinumtoxinA treatment or in whom titres had become negative within the 48 months. Secondary outcome measure: steepness of changes in NABT. NABTs were determined by mouse hemidiaphragm assay. Findings were compared to long-term data from 24 cervical dystonia patients who had developed NABTs and in whom treatment had been discontinued.

**Results.** Following a transient increase in the first 24 months under incobotulinumtoxinA treatment in some patients, NABTs declined well below the initial titre in the majority of patients. Test assay results were negative in most of the patients followed for more than 36 months. NABTs seemed to decline into the negative detection range as rapidly under incobotulinumtoxinA treatment as after cessation of botulinum neurotoxin therapy.

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3 **Conclusions.** The reduction of neutralising antibody titres despite continuous treatment with  
4 incobotulinumtoxinA indicates low antigenicity of incobotulinumtoxinA. This might have  
5 implications on restrictions such as minimum injection intervals of  $\geq 10$  weeks currently in  
6 place for maintaining successful long-term application of botulinum neurotoxin.  
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14 **Key words:** secondary non-responder - cervical dystonia - cessation of therapy - neutralising  
15 antibody titre - complexing proteins – botulinum neurotoxin  
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## ARTICLE SUMMARY

### Article focus

- Evaluation of antigenicity of incobotulinumtoxinA, a botulinum neurotoxin type A preparation free of complexing proteins for the treatment of cervical dystonia.

### Key messages

- Secondary non-responders to conventional type A preparations showed a decline in neutralising antibody titres despite continuous treatment with incobotulinumtoxinA over a period of up to 50 months.
- Neutralising antibody titres seemed to decline into the negative detection range as rapidly under incobotulinumtoxinA treatment as after cessation of botulinumtoxin therapy.
- These results indicate low antigenicity of incobotulinumtoxinA.

### Strengths and limitations of this study

- Up to date this study is the largest investigation of secondary non-responders with neutralising antibodies against botulinumtoxinA.
- The continuous treatment with incobotulinumtoxinA in secondary non-responders according to current knowledge of immunogenicity of botulinumtoxins should have resulted in boosting of antibody titres. Instead an unexpected decline of antibody titres was observed.
- This is an interesting finding despite the small sample size (n=37). Monocentric data have to be confirmed in multicentre studies.

## INTRODUCTION

Intramuscular injections of botulinum neurotoxin (BoNT) have become the treatment of choice for the symptomatic treatment of focal dystonias;<sup>1</sup> a recent evidence-based assessment gave a Level A recommendation for the treatment of cervical dystonia (CD).<sup>2</sup> Therapeutic BoNT type A (BoNT/A) preparations usually consist of a high molecular weight complex containing the biologically active 150 kDa neurotoxin, non-toxic complexing / accessory proteins, and excipients.<sup>3</sup> Repeated BoNT injections can trigger an immune response and can result in the formation of neutralising antibodies against the botulinum neurotoxin which might lead to non-responsiveness to treatment.<sup>4</sup> To minimise this loss of therapeutic effect, it is recommended to avoid risk factors such as booster injections, the use of high doses, and short intervals of less than 10 to 12 weeks between injections.<sup>5</sup> Thus, optimal BoNT injection management leads to therapy restrictions in order to avoid secondary non-response.

Current treatment recommendations reduce the frequency of secondary non-response to approx. 2% over a treatment period of 2 years in patients with cervical dystonia.<sup>6</sup> However, a considerable percentage of patients would prefer shorter injection intervals and more individualised treatment.<sup>7</sup> There is thus still a need for a BoNT preparation with an extremely low antigenicity to avoid therapy restrictions and meet patients' needs. The new BoNT/A preparation free of complexing proteins seems to be such a candidate.

IncobotulinumtoxinA (Xeomin®, NT 201, Merz Pharmaceuticals GmbH, Germany) treatment has not only been proven efficacious and well tolerated in patients with CD<sup>8-10</sup> but preliminary results also indicate a low antigenicity.<sup>3,11-13</sup> The present study was designed to support these hints of low antigenicity. We hypothesised that continuous treatment with incobotulinumtoxinA would not result in an increase in neutralising antibody titres (NABTs) in patients with preexisting NABTs. To test our hypothesis, we prospectively analysed NABTs in an immunologically critical subgroup of patients with NABTs and partial

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3 secondary non-responsiveness who were switched from long-term treatment with other  
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5 botulinumtoxin A preparations to incobotulinumtoxinA treatment.  
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## 8 9 **METHODS**

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11 Thirty-seven adult patients suffering from idiopathic cervical dystonia (CD) partly in  
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13 combination with other focal or generalised dystonia had been satisfactorily treated long-term  
14  
15 with the BoNT/A preparations abobotulinumtoxinA (Dysport®, Ipsen Ltd., Slough, UK) or  
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17 onabotulinumtoxinA (Botox®, Allergan Inc, Irvine, USA). They were included in this  
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19 prospective, blinded, monocentric study 1. when clinically secondary non-responsiveness  
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21 with systematic worsening of the severity of cervical dystonia occurred –severity was  
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23 measured by means of the objective TSUI score<sup>14</sup> (12 weeks after injection) at three  
24  
25 consecutive injection visits over a period of at least 6 months despite increased BoNT/A  
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27 doses– and 2. when patients had given their informed consent to be switched to  
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29 incobotulinumtoxinA.  
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33 IncobotulinumtoxinA was administered as intramuscular injections of 200 MU without  
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35 electromyography guidance every 3 months according to their previous BoNT/A injection  
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37 protocols. IncobotulinumtoxinA doses were kept constant during the first year; thereafter,  
38  
39 doses could be adjusted according to the patients' requirements, reduced or increased up to  
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41 500 MU per injection.  
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45 The study was carried out according to the Declaration of Helsinki and Good Clinical  
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47 Practice.  
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50 Blood samples for BoNT antibody testing were collected at the start of the study and in yearly  
51  
52 intervals. Antibody titres were determined by an independent blinded contractor (Toxogen  
53  
54 GmbH, Hannover, Germany) using the sensitive mouse hemidiaphragm assay for neutralising  
55  
56 antibodies.<sup>15</sup> Base10 logarithms are presented for all titres ( $\log_{10}$  (NABT) [mU/ml]); the value  
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58 of -1.3 was assigned to a negative test result. The upper limit of neutralising antibody  
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3 detection in the mouse hemidiaphragm assay was 10 mU/mL ( $\log_{10}(10) = 1$ ), the lower limit  
4 was 0.1 mU/ml ( $(\log_{10}(0.1) = -1)$ ). The use of the logarithmic scale allows the comparison of  
5 NABTs over the broad range of titres between 10 mU/ml and 0.1 mU/ml.  
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9 The primary outcome measure of the study was the number of patients in whom NABTs  
10 declined below the initial titre after 48 months of incobotulinumtoxinA treatment or who had  
11 negative titre results within the 48 months. The steepness of changes in NABT was chosen as  
12 secondary outcome measure. This parameter turned out to be difficult to calculate, because  
13 the titre had dropped below the detection limit in too many patients.  
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22 To test our hypothesis, the formation of neutralising antibodies in the present patient  
23 population was followed over a period of up to 50 months.  
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26 The temporal course of neutralising antibody titres (NABTs) was compared to the course of  
27 NABTs of 24 CD patients who had their BoNT treatment discontinued after development of  
28 antibody-induced treatment failure. NABTs of eight of these 24 patients have been described  
29 in the literature,<sup>16</sup> the remaining 16 are follow-up patients from our centre.  
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38 For quantification of the slope of NABT decline, patient data were divided into three groups.  
39 Group 1 included the titres of the 16 patients observed in our department with partial therapy  
40 failure who had their BoNT treatment discontinued. Group 2 were the NABTs of the 8  
41 patients with complete therapy failure reported in the literature<sup>16</sup> and group 3 included the  
42 titres of 37 patients with partial therapy failure who received continuous incobotulinumtoxinA  
43 treatment after positive NABTs were detected. The decline in NABTs was quantified by  
44 calculating a linear regression line through  $\log_{10}$ AB-titre values against time  
45 ( $\log_{10}(\text{mU/ml/year})$ ) for each patient. A negative titre was substituted by  $\log_{10}(0.01)$ , because  
46 0.01 is the next titre value below the detection limit of 0.01 mU/ml.  
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3 For group comparisons, an ANOVA was performed (Kruskal-Wallis test), for group mean  
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5 comparisons the non-parametric Mann-Whitney U test was used. These tests are part of the  
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7 commercially available statistics package SPSS.  
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## 10 11 **RESULTS**

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14 The two figures show the temporal course of neutralising antibody titres over a period of up to  
15  
16 50 months in secondary non-responders who were either switched to incobotulinumtoxinA  
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18 (Figure 1), or did not receive any further BoNT treatment (Figure 2).  
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21 Antibody titre values of one of the CD patients with cessation of BoNT treatment (Figure 2)  
22  
23 were included for a visual marker in Figure 1 (symbol: black square, bold line). This patient  
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25 had high titres (upper limit of detection); after a slow decline over the first 30 months, the  
26  
27 neutralising antibody test was negative at the end of the 50-month observation period.  
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30 Despite a transient increase in 10 patients in the first 24 months under incobotulinumtoxinA  
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32 treatment (Figure 1), neutralising antibody titres declined well below the initial titre in the  
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34 majority of patients (31 patients, 84%,  $p < 0.001$ , chi-square test). Test assay results were even  
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36 negative or below the lower detection limit in 23 (62%) of the patients in the follow-up, i.e.  
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38 antibody titres were  $\leq 0.1$  mU/ml. In Figure 2 decline of titres is demonstrated for the patients  
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40 in whom therapy was stopped (light grey squares = published patients, coloured circles own  
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42 patients).  
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45 There was considerable inter-individual variability in the steepness of the titre decline in all  
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47 three patient populations (Figure 1 and 2). In most patients receiving incobotulinumtoxinA  
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49 treatment (Figure 1), baseline titres were lower than in the patients with discontinued  
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51 treatment (Figure 2). Under incobotulinumtoxinA treatment, antibody titres seemed to decline  
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53 into the negative detection range as rapidly as after cessation of therapy and in some cases  
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55 became negative even earlier than in those patients who had discontinued their BoNT/A  
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57 treatment, probably because of lower initial titres.  
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3 Mean slope of NABT decline ( $-0.0516 \log_{10}(\text{mU/ml})/\text{year}$ ) was lowest in the patients with  
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5 partial therapy failure in whom BoNT treatment was discontinued after detection of positive  
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7 NABTs (group 1). Variability of slopes was also highest in this group (min:  $-0.2168$ ; max:  
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9  $0.0048$ ; SD:  $0.6463$ ). The NABTs reported in the literature for patients with complete  
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11 treatment failure (group 2) declined rather homogeneously ( $-0.0664$ ; min:  $-1.1620$ ; max:  $-$   
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13  $0.0348$ ; SD:  $0.2897$ ). However, the mean slope for group 2 did not differ significantly from  
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15 the mean slope for group 1 ( $p=0.12$ ). The steepest mean slope of NABT decline ( $-0.0750$ ) was  
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17 observed in group 3 (min:  $-0.9484$ ; max:  $0.1505$ ; SD:  $0.1725$ ).

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20 The Kruskal-Wallis test did not show any significant differences of slopes of NABT decline  
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22 between the groups ( $p=0.269$ ). Even when groups 1 and 2 were combined and slopes of  
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24 patients with and without incobotulinumtoxinA treatment (group 3 vs. groups 1+2) were  
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26 compared, no significant difference could be detected ( $p=0.816$ ).

## 27 28 29 30 31 32 33 34 **DISCUSSION**

35  
36 IncobotulinumtoxinA is a preparation free of complexing proteins with a high specific  
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38 biological activity.<sup>3,17</sup>

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40 The patients included in our investigation had already developed secondary treatment failure  
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42 and neutralising antibodies under the former treatment with botulinum toxin type A  
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44 formulations containing complexing proteins. Despite their sensitivity to react to BoNT/A  
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46 treatment with antibody formation and despite a possible increase of doses up to 500 MU  
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48 incobotulinumtoxinA, more than 80% of these patients showed a decrease in antibody titres  
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50 and in more than 60% a reduction down to the detection limit was observed. The antigen load  
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52 of repetitive injections with 200 to 500 MU incobotulinumtoxinA every 3 months was  
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54 apparently so low that in this cohort of immunologically critical patients no permanent  
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56 boosting of antibody titres was observed. This confirms our hypothesis. We do not know  
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3 whether this occurs because the protein load of 0.88 ng to 2.2 ng (200 to 500 MU) of active  
4 neurotoxin is not detected by the human immune system after intramuscular injections or  
5 because of the lack of complexing proteins. The role of complexing proteins regarding the  
6 efficacy of treatment in secondary non-responders with BoNT/A is still unclear but deserves  
7 further interest.  
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11 Since NABTs under incobotulinumtoxinA treatment declined at least as rapidly as after  
12 cessation of therapy, we recommend to detect antibody-induced therapy failure as early as  
13 possible to avoid any further increase in NABTs and development of complete treatment  
14 failure. Comparison of Figures 1 and 2 indicates the tendency that in patients with partial  
15 therapy failure (group 1 and 3) titres return to negative values earlier than in patients with  
16 complete therapy failure and higher antibody titres (group 2).  
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27 This is important for further continuation of BoNT/A treatment. It has been observed that in a  
28 patient with antibody-induced therapy failure and negative antibody titres after cessation of  
29 therapy after several years, no new formation of neutralising antibodies and clinical response  
30 occurred when continuously high doses of incobotulinumtoxinA were injected in the  
31 following years.<sup>18</sup> Clinical response was also observed in the majority of our patients after  
32 switching to incobotulinumtoxinA.<sup>13</sup>  
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40 The present paper does not only show that NABTs did not increase by incobotulinumtoxinA  
41 treatment but we also present evidence that the decline in NABTs was not significantly  
42 different in patients who were continuously treated with incobotulinumtoxinA following  
43 partial therapy failure with other BoNT preparations compared to patients who no longer  
44 received BoNT treatment after neutralising antibody titres were detected.  
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53 The present study has a potential impact on patient management. If it is confirmed in  
54 additional studies that the antigenicity of incobotulinumtoxinA is as extremely low as  
55 suggested here – it has to be kept in mind that the presented data were produced in an  
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3 uncontrolled, non-randomised study from a single centre— injection intervals and dosages can  
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5 possibly be modified to meet patients' need for an optimised individual treatment.  
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10 **Acknowledgments** Neutralising antibody assays were carried out by Toxogen GmbH,  
11  
12 Hannover, Germany. Editorial assistance was provided by Elke Grosselindemann (Brett  
13  
14 Medical Writing Australia).  
15

16  
17  
18 **Funding** All costs associated with the publishing of the present manuscript were met by Merz  
19  
20 Pharmaceuticals GmbH, Frankfurt, Germany. Merz Pharmaceuticals also paid for the  
21  
22 neutralising antibody assays. Merz Pharmaceuticals was not involved in study design, data  
23  
24 collection, and data analysis and interpretation. The authors were independent from the  
25  
26 funder; they had full access to all presented data and take responsibility for all data and  
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28 analyses.  
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34 **Competing interest** HH: participated in research studies for which unrestricted grants were  
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36 provided by Allergan, Merz and Ipsen. He has been the recipient of honoraria and fees for  
37  
38 presentations at meetings and conferences and for participating in Advisory Boards from  
39  
40 Allergan, Ipsen and Merz; MM and UK: received honoraria and fees for presentations at  
41  
42 meetings and conferences; CH: nothing to declare; HB: is shareholder and CEO of Toxogen  
43  
44 GmbH, a spin-off of Medical School Hannover.  
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49 **Patient consent** Obtained.  
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51  
52 **Ethics approval** The local ethics committee was asked and agreed on the continuation of  
53  
54 botulinumtoxin treatment with the new preparation, incobotulinumtoxinA, as a possible  
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56 treatment option. Usually the recommendation for secondary non-responders is to stop  
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58 botulinumtoxin treatment.  
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3 **Data sharing** There are no additional study data.  
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5 **Contributors** Study design, data collection, analysis and interpretation were carried out by all  
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7 authors. HH prepared the first draft of the manuscript; all authors participated in the final  
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9 preparation of the manuscript and approved the final version.  
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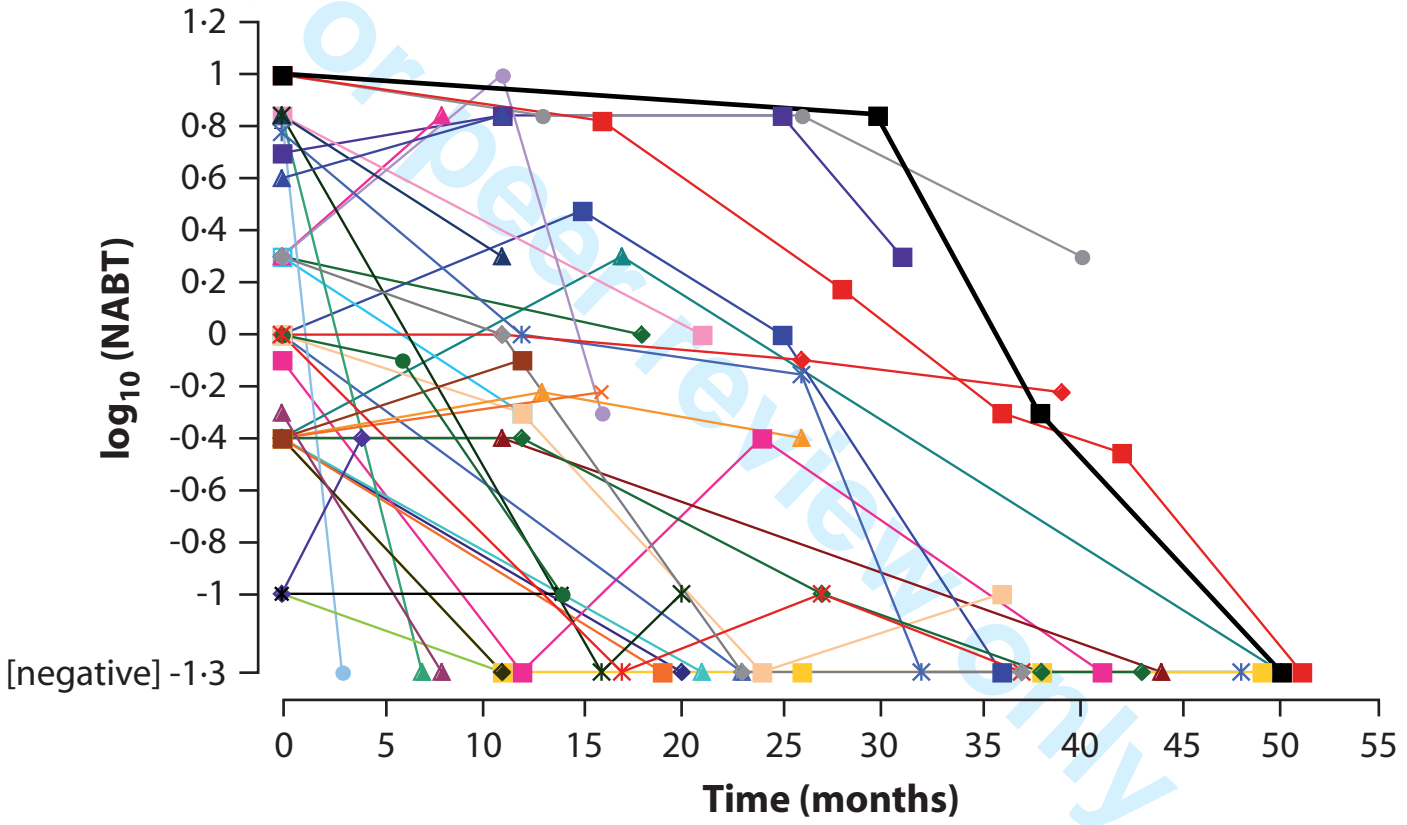


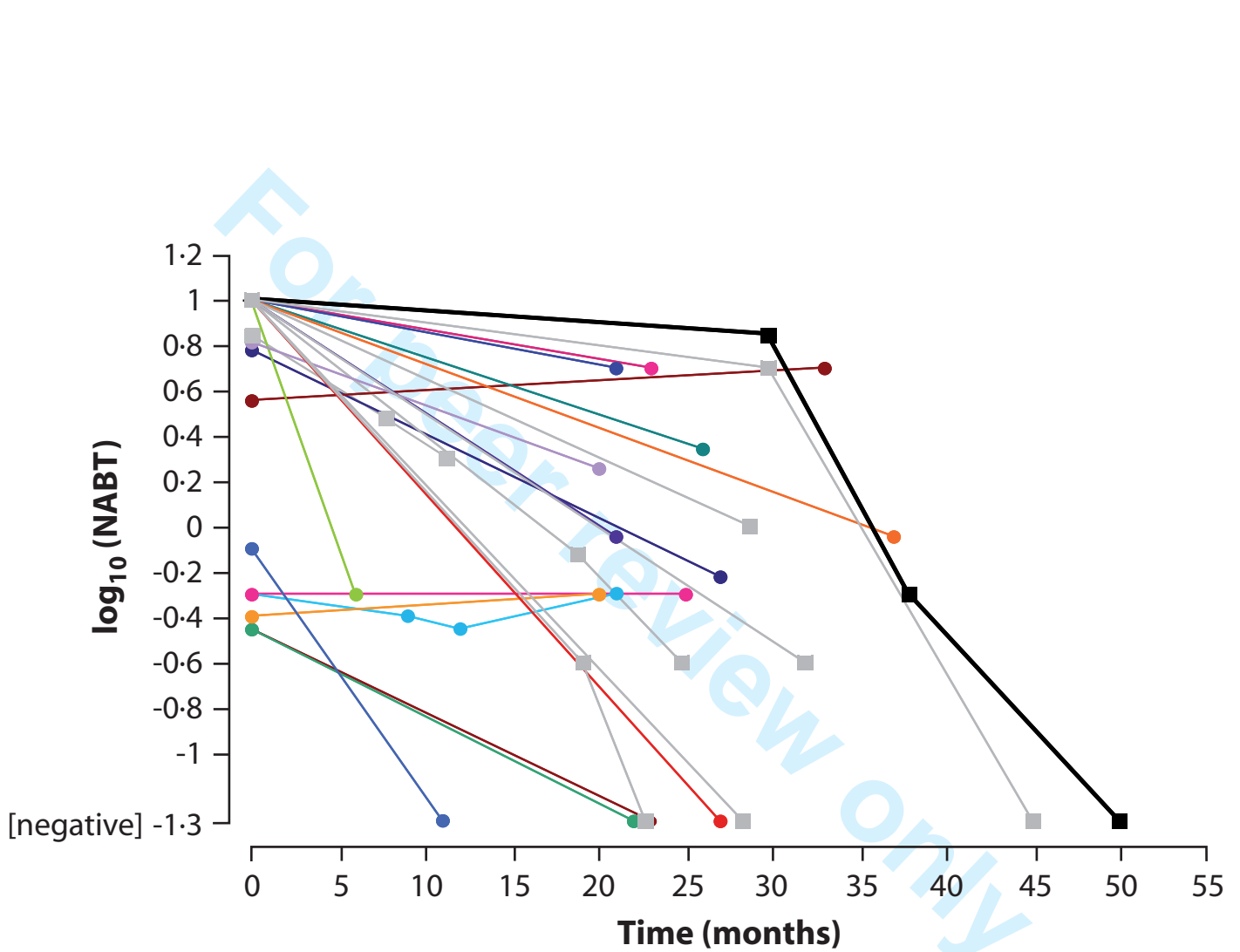
### Figure legends

**Figure 1** Decline in neutralising antibody titres in 37 partial secondary non-responders (group 3) with cervical dystonia who were switched from their previous botulinum neurotoxin type A therapy to incobotulinumtoxinA treatment following the detection of neutralising antibodies. (coloured symbols ●, ▲, ×, ■, ◆) patients receiving incobotulinumtoxinA; (■, bold line) data from one patient with cessation of botulinum toxin therapy were included as visual marker for comparison with Figure 2. NABT, neutralising antibody titre

**Figure 2** Decline in neutralising antibody titres in 24 patients with cervical dystonia after cessation of botulinum neurotoxin type A therapy following the detection of neutralising antibodies. (■) patients described in Dressler *et al.*<sup>16</sup>, group 2 with complete therapy failure; (coloured ●) follow-up patients from our centre, group 1 with partial therapy failure; data of one patient (■, bold line) were highlighted for comparison with Figure 1. Please note that titre changes can only be presented for the time span when titres were within the detection limits of the mouse hemidiaphragm assay. NABT, neutralising antibody titre

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3 **Prospective analysis of neutralising antibody titres in secondary non-responders**  
4 **under continuous treatment with a botulinumtoxin type A preparation free of**  
5 **complexing proteins – a single cohort 4-year follow-up study**  
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11 Harald Hefter<sup>1</sup>, Christian Hartmann<sup>1</sup>, Ulrike Kahlen<sup>1</sup>, Marek Moll<sup>1</sup>, Hans Bigalke<sup>2</sup>

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3 **Word count (text):** 1807  
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5 **Word count (abstract):** 296  
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7 **Tables/Figures:** 0/2  
8

9 **References:** 18  
10

11 **Short title:** Low antigenicity of incobotulinumtoxinA  
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**ABSTRACT**

**Objective.** In long-term botulinum neurotoxin treatment, loss of therapeutic efficacy may occur due to neutralising antibody formation. Preliminary results with incobotulinumtoxinA, a preparation free of complexing / accessory proteins, have indicated a low antigenicity. We hypothesised that continuous treatment with this botulinum neurotoxin preparation would not result in an increase in neutralising antibody titres (NABTs) in patients with preexisting NABTs.

**Design.** Prospective, blinded cohort study.

**Setting.** Single centre in Germany.

**Participants.** Thirty-seven cervical dystonia patients with NABTs and partial secondary non-responsiveness to their previous botulinum neurotoxin type A treatment.

**Intervention.** 3-monthly intramuscular injections of incobotulinumtoxinA with a constant dose of 200 MU per injection during the first year; thereafter up to 500 MU for the next 36 months.

**Outcome measures.** Primary outcome measure: number of patients in whom NABTs declined below the initial titre after 48 months of incobotulinumtoxinA treatment or in whom titres had become negative within the 48 months. Secondary outcome measure: steepness of changes in NABT. NABTs were determined by mouse hemidiaphragm assay. Findings were compared to long-term data from 24 cervical dystonia patients who had developed NABTs and in whom treatment had been discontinued.

**Results.** Following a transient increase in the first 24 months under incobotulinumtoxinA treatment in some patients, NABTs declined well below the initial titre in the majority of patients. Test assay results were negative in most of the patients followed for more than 36 months. NABTs seemed to decline into the negative detection range as rapidly under incobotulinumtoxinA treatment as after cessation of botulinum neurotoxin therapy.

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3 **Conclusions.** The reduction of neutralising antibody titres despite continuous treatment with  
4 incobotulinumtoxinA indicates low antigenicity of incobotulinumtoxinA. This might have  
5 implications on restrictions such as minimum injection intervals of  $\geq 10$  weeks currently in  
6 place for maintaining successful long-term application of botulinum neurotoxin.  
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14 **Key words:** secondary non-responder - cervical dystonia - cessation of therapy - neutralising  
15 antibody titre - complexing proteins – botulinum neurotoxin  
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## ARTICLE SUMMARY

### Article focus

- Evaluation of antigenicity of incobotulinumtoxinA, a botulinum neurotoxin type A preparation free of complexing proteins for the treatment of cervical dystonia.

### Key messages

- Secondary non-responders to conventional type A preparations showed a decline in neutralising antibody titres despite continuous treatment with incobotulinumtoxinA over a period of up to 50 months.
- Neutralising antibody titres seemed to decline into the negative detection range as rapidly under incobotulinumtoxinA treatment as after cessation of botulinumtoxin therapy.
- These results indicate low antigenicity of incobotulinumtoxinA.

### Strengths and limitations of this study

- Up to date this study is the largest investigation of secondary non-responders with neutralising antibodies against botulinumtoxinA.
- The continuous treatment with incobotulinumtoxinA in secondary non-responders according to current knowledge of immunogenicity of botulinumtoxins should have resulted in boosting of antibody titres. Instead an unexpected decline of antibody titres was observed.
- This is an interesting finding despite the small sample size (n=37). Monocentric data have to be confirmed in multicentre studies.



## INTRODUCTION

Intramuscular injections of botulinum neurotoxin (BoNT) have become the treatment of choice for the symptomatic treatment of focal dystonias;<sup>1</sup> a recent evidence-based assessment gave a Level A recommendation for the treatment of cervical dystonia (CD).<sup>2</sup> Therapeutic BoNT type A (BoNT/A) preparations usually consist of a high molecular weight complex containing the biologically active 150 kDa neurotoxin, non-toxic complexing / accessory proteins, and excipients.<sup>3</sup> Repeated BoNT injections can trigger an immune response and can result in the formation of neutralising antibodies against the botulinum neurotoxin which might lead to non-responsiveness to treatment.<sup>4</sup> To minimise this loss of therapeutic effect, it is recommended to avoid risk factors such as booster injections, the use of high doses, and short intervals of less than 10 to 12 weeks between injections.<sup>5</sup> Thus, optimal BoNT injection management leads to therapy restrictions in order to avoid secondary non-response.

Current treatment recommendations reduce the frequency of secondary non-response to approx. 2% over a treatment period of 2 years in patients with cervical dystonia.<sup>6</sup> However, a considerable percentage of patients would prefer shorter injection intervals and more individualised treatment.<sup>7</sup> There is thus still a need for a BoNT preparation with an extremely low antigenicity to avoid therapy restrictions and meet patients' needs. The new BoNT/A preparation free of complexing proteins seems to be such a candidate.

IncobotulinumtoxinA (Xeomin®, NT 201, Merz Pharmaceuticals GmbH, Germany) treatment has not only been proven efficacious and well tolerated in patients with CD<sup>8-10</sup> but preliminary results also indicate a low antigenicity.<sup>3,11-13</sup> The present study was designed to support these hints of low antigenicity. We hypothesised that continuous treatment with incobotulinumtoxinA would not result in an increase in neutralising antibody titres (NABTs) in patients with preexisting NABTs. To [provetest](#) our hypothesis, we prospectively analysed NABTs in an immunologically critical subgroup of patients with NABTs and partial

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3 secondary non-responsiveness who were switched from long-term treatment with other  
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5 botulinumtoxin A preparations to incobotulinumtoxinA treatment.  
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## 8 9 **METHODS**

10  
11 Thirty-seven adult patients suffering from idiopathic cervical dystonia (CD) partly in  
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13 combination with other focal or generalised dystonia had been satisfactorily treated long-term  
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15 with the BoNT/A preparations abobotulinumtoxinA (Dysport®, Ipsen Ltd., Slough, UK) or  
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17 onabotulinumtoxinA (Botox®, Allergan Inc, Irvine, USA). They were included in this  
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19 prospective, blinded, monocentric study 1. when clinically secondary non-responsiveness  
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21 with systematic worsening of the severity of cervical dystonia occurred –severity was  
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23 measured by means of the objective TSUI score<sup>14</sup> (12 weeks after injection) at three  
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25 consecutive injection visits over a period of at least 6 months despite increased BoNT/A  
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27 doses– and 2. when patients had given their informed consent to be switched to  
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29 incobotulinumtoxinA.  
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34 IncobotulinumtoxinA was administered as intramuscular injections of 200 MU without  
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36 electromyography guidance every 3 months according to their previous BoNT/A injection  
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38 protocols. IncobotulinumtoxinA doses were kept constant during the first year; thereafter,  
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40 doses could be adjusted according to the patients' requirements, reduced or increased up to  
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42 500 MU per injection.  
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46 The study was carried out according to the Declaration of Helsinki and Good Clinical  
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48 Practice.

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50 Blood samples for BoNT antibody testing were collected at the start of the study and in yearly  
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52 intervals. Antibody titres were determined by an independent blinded contractor (Toxogen  
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54 GmbH, Hannover, Germany) using the sensitive mouse hemidiaphragm assay for neutralising  
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56 antibodies.<sup>15</sup> Base10 logarithms are presented for all titres ( $\log_{10}$  (NABT) [mU/ml]); the value  
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58 of -1.3 was assigned to a negative test result. The upper limit of neutralising antibody  
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3 detection in the mouse hemidiaphragm assay was 10 mU/mL ( $\log_{10}(10) = 1$ ), the lower limit  
4 was 0.1 mU/ml ( $(\log_{10}(0.1) = -1)$ ). The use of the logarithmic scale allows the comparison of  
5 NABTs over the broad range of titres between 10 mU/ml and 0.1 mU/ml.  
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9 The primary outcome measure of the study was the number of patients in whom NABTs  
10 declined below the initial titre after 48 months of incobotulinumtoxinA treatment or who had  
11 negative titre results within the 48 months. The steepness of changes in NABT was chosen as  
12 secondary outcome measure. This parameter turned out to be difficult to calculate, because  
13 the titre had dropped below the detection limit in too many patients.  
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22 To test our hypothesis, the formation of neutralising antibodies in the present patient  
23 population was followed over a period of up to 50 months.  
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27 The temporal course of neutralising antibody titres (NABTs) was compared to the course of  
28 NABTs of 24 CD patients who had their BoNT treatment discontinued after development of  
29 antibody-induced treatment failure. NABTs of eight of these 24 patients have been described  
30 in the literature,<sup>16</sup> the remaining 16 are follow-up patients from our centre.  
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38 For quantification of the slope of NABT decline, patient data were divided into three groups.  
39 Group 1 included the titres of the 16 patients observed in our department with partial therapy  
40 failure who had their BoNT treatment discontinued. Group 2 were the NABTs of the 8  
41 patients with complete therapy failure reported in the literature<sup>16</sup> and group 3 included the  
42 titres of 37 patients with partial therapy failure who received continuous incobotulinumtoxinA  
43 treatment after positive NABTs were detected. The decline in NABTs was quantified by  
44 calculating a linear regression line through  $\log_{10}$ AB-titre values against time  
45 ( $\log_{10}(\text{mU/ml/year})$ ) for each patient. A negative titre was substituted by  $\log_{10}(0.01)$ , because  
46 0.01 is the next titre value below the detection limit of 0.01 mU/ml.  
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3 For group comparisons, an ANOVA was performed (Kruskal-Wallis test), for group mean  
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5 comparisons the non-parametric Mann-Whitney U test was used. These tests are part of the  
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7 commercially available statistics package SPSS.  
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## 10 11 **RESULTS**

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14 The two figures show the temporal course of neutralising antibody titres over a period of up to  
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16 50 months in secondary non-responders who were either switched to incobotulinumtoxinA  
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18 (Figure 1), or did not receive any further BoNT treatment (Figure 2).  
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21 Antibody titre values of one of the CD patients with cessation of BoNT treatment (Figure 2)  
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23 were included for a visual marker in Figure 1 (symbol: black square, bold line). This patient  
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25 had high titres (upper limit of detection); after a slow decline over the first 30 months, the  
26  
27 neutralising antibody test was negative at the end of the 50-month observation period.  
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30 Despite a transient increase in 10 patients in the first 24 months under incobotulinumtoxinA  
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32 treatment (Figure 1), neutralising antibody titres declined well below the initial titre in the  
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34 majority of patients (31 patients, 84%,  $p < 0.001$ , chi-square test). Test assay results were even  
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36 negative or below the lower detection limit in 23 (62%) of the patients in the follow-up, i.e.  
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38 antibody titres were  $\leq 0.1$  mU/ml. In Figure 2 decline of titres is demonstrated for the patients  
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40 in whom therapy was stopped (light grey squares = published patients, coloured circles own  
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42 patients).  
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45 There was considerable inter-individual variability in the steepness of the titre decline in all  
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47 three patient populations (Figure 1 and 2). In most patients receiving incobotulinumtoxinA  
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49 treatment (Figure 1), baseline titres were lower than in the patients with discontinued  
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51 treatment (Figure 2). Under incobotulinumtoxinA treatment, antibody titres seemed to decline  
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53 into the negative detection range as rapidly as after cessation of therapy and in some cases  
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55 became negative even earlier than in those patients who had discontinued their BoNT/A  
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57 treatment, probably because of lower initial titres.  
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3 Mean slope of NABT decline ( $-0.0516 \log_{10}(\text{mU/ml})/\text{year}$ ) was lowest in the patients with  
4 partial therapy failure in whom BoNT treatment was discontinued after detection of positive  
5 NABTs (group 1). Variability of slopes was also highest in this group (min:  $-0.2168$ ; max:  
6  $0.0048$ ; SD:  $0.6463$ ). The NABTs reported in the literature for patients with complete  
7 treatment failure (group 2) declined rather homogeneously ( $-0.0664$ ; min:  $-1.1620$ ; max:  $-$   
8  $0.0348$ ; SD:  $0.2897$ ). However, the mean slope for group 2 did not differ significantly from  
9 the mean slope for group 1 ( $p=0.12$ ). The steepest mean slope of NABT decline ( $-0.0750$ ) was  
10 observed in group 3 (min:  $-0.9484$ ; max:  $0.1505$ ; SD:  $0.1725$ ).

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12 The Kruskal-Wallis test did not show any significant differences of slopes of NABT decline  
13 between the groups ( $p=0.269$ ). Even when groups 1 and 2 were combined and slopes of  
14 patients with and without incobotulinumtoxinA treatment (group 3 vs. groups 1+2) were  
15 compared, no significant difference could be detected ( $p=0.816$ ).

## 33 DISCUSSION

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35 IncobotulinumtoxinA is a preparation free of complexing proteins with a high specific  
36 biological activity.<sup>3,17</sup>

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38 The patients included in our investigation had already developed secondary treatment failure  
39 and neutralising antibodies under the former treatment with botulinum toxin type A  
40 formulations containing complexing proteins. Despite their sensitivity to react to BoNT/A  
41 treatment with antibody formation and despite a possible increase of doses up to 500 MU  
42 incobotulinumtoxinA, more than 80% of these patients showed a decrease in antibody titres  
43 and in more than 60% a reduction down to the detection limit was observed. The antigen load  
44 of repetitive injections with 200 to 500 MU incobotulinumtoxinA every 3 months was  
45 apparently so low that in this cohort of immunologically critical patients no permanent  
46 boosting of antibody titres was observed. This confirms our hypothesis. We do not know  
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3 whether this occurs because the protein load of 0.88 ng to 2.2 ng (200 to 500 MU) of active  
4 neurotoxin is not detected by the human immune system after intramuscular injections or  
5 because of the lack of complexing proteins. The role of complexing proteins regarding the  
6 efficacy of treatment in secondary non-responders with BoNT/A is still unclear but deserves  
7 further interest.  
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11 Since NABTs under incobotulinumtoxinA treatment declined at least as rapidly as after  
12 cessation of therapy, we recommend to detect antibody-induced therapy failure as early as  
13 possible to avoid any further increase in NABTs and development of complete treatment  
14 failure. Comparison of Figures 1 and 2 indicates the tendency that in patients with partial  
15 therapy failure (group 1 and 3) titres return to negative values earlier than in patients with  
16 complete therapy failure and higher antibody titres (group 2).  
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21 This is important for further continuation of BoNT/A treatment. It has been observed that in a  
22 patient with antibody-induced therapy failure and negative antibody titres after cessation of  
23 therapy after several years, no new formation of neutralising antibodies and clinical response  
24 occurred when continuously high doses of incobotulinumtoxinA were injected in the  
25 following years.<sup>18</sup> Clinical response was also observed in the majority of our patients after  
26 switching to incobotulinumtoxinA.<sup>13</sup>  
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31 The present paper does not only show that NABTs did not increase by incobotulinumtoxinA  
32 treatment but we also present evidence that the decline in NABTs was not significantly  
33 different in patients who were continuously treated with incobotulinumtoxinA following  
34 partial therapy failure with other BoNT preparations compared to patients who no longer  
35 received BoNT treatment after neutralising antibody titres were detected.  
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The present study has an [potential](#) impact on patient management. If it is confirmed in additional studies that the antigenicity of incobotulinumtoxinA is as extremely low as suggested here – it has to be kept in mind that the presented data were produced in an

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2  
3 uncontrolled, non-randomised study from a single centre— injection intervals and dosages can  
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5 possibly be modified to meet patients’ need for an optimised individual treatment.  
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8  
9 **Acknowledgments** Neutralising antibody assays were carried out by Toxogen GmbH,  
10  
11 Hannover, Germany. Editorial assistance was provided by Elke Grosselindemann (Brett  
12  
13 Medical Writing Australia).  
14

15  
16  
17 **Funding** All costs associated with the publishing of the present manuscript were met by Merz  
18  
19 Pharmaceuticals GmbH, Frankfurt, Germany. Merz Pharmaceuticals also paid for the  
20  
21 neutralising antibody assays. Merz Pharmaceuticals was not involved in study design, data  
22  
23 collection, and data analysis and interpretation. [The authors were independent from the](#)  
24  
25 [funder; they had full access to all presented data and take responsibility for all data and](#)  
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27 [analyses.](#)  
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32  
33 **Competing interest** HH: participated in research studies for which unrestricted grants were  
34  
35 provided by Allergan, Merz and Ipsen. He has been the recipient of honoraria and fees for  
36  
37 presentations at meetings and conferences and for participating in Advisory Boards from  
38  
39 Allergan, Ipsen and Merz; MM and UK: received honoraria and fees for presentations at  
40  
41 meetings and conferences; CH: nothing to declare; HB: is shareholder and CEO of Toxogen  
42  
43 GmbH, a spin-off of Medical School Hannover.  
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49 **Patient consent** Obtained.

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51 **Ethics approval** The local ethics committee was asked and agreed on the continuation of  
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53 botulinumtoxin treatment with the new preparation, incobotulinumtoxinA, as a possible  
54  
55 treatment option. Usually the recommendation for secondary non-responders is to stop  
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57 botulinumtoxin treatment.  
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3 **Data sharing** There are no additional study data.  
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5 **Contributors** Study design, data collection, analysis and interpretation were carried out by all  
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7 authors. HH prepared the first draft of the manuscript; all authors participated in the final  
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9 preparation of the manuscript and approved the final version.  
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### Figure legends

**Figure 1** Decline in neutralising antibody titres in 37 partial secondary non-responders (group 3) with cervical dystonia who were switched from their previous botulinum neurotoxin type A therapy to incobotulinumtoxinA treatment following the detection of neutralising antibodies. (coloured symbols ●, ▲, ×, ■, ◆) patients receiving incobotulinumtoxinA; (■, bold line) data from one patient with cessation of botulinum toxin therapy were included as visual marker for comparison with Figure 2. NABT, neutralising antibody titre

**Figure 2** Decline in neutralising antibody titres in 24 patients with cervical dystonia after cessation of botulinum neurotoxin type A therapy following the detection of neutralising antibodies. (■) patients described in Dressler *et al.*<sup>16</sup>, group 2 with complete therapy failure; (coloured ●) follow-up patients from our centre, group 1 with partial therapy failure; data of one patient (■, bold line) were highlighted for comparison with Figure 1. Please note that titre changes can only be presented for the time span when titres were within the detection limits of the mouse hemidiaphragm assay. NABT, neutralising antibody titre