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ORIGINAL ARTICLE

Neutralizing Antibody and Botulinum Toxin Therapy: A Systematic Review and Meta-analysis

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Abstract The formation of neutralizing antibodies (NAbs) directed specifically against the active neurotoxin part of the botulinum neurotoxin (BoNT) complex is often cited as a major cause of secondary non-responsiveness (SnR) to treatment. This systematic and meta-analytic review evaluates the frequency of NAbs among patients treated with BoNT therapy for any clinical indication. A comprehensive database search strategy was designed to retrieve relevant clinical data from the published literature up to April 2013. All English-language publications that analyzed NAbs prevalence in more than ten patients were included, regardless of BoNT formulation, assay method, and study design. For the meta-analysis, patients were divided into three categories: secondary non-response (SnR) patients, clinically responding patients and all patients, independently of BoNT responsiveness. The meta-analysis included 61 studies reporting data for 8525 patients; 4972 dystonic patients, 1170 patients with spasticity, 294 patients with urologic indications, 396 patient with hyperhidrosis, 1659 patients with glabellar line, and 34 patients with hypersalivation. Among the "all patients" group NAbs frequency was 20 % for dystonia, 5.9 % for spasticity, and 2.7 % for urologic patients and 1.1 % for other conditions. The prevalence of NAbs was lower (3.5 %) among clinically responding patients and higher in 53.5 % SnR patients. About a half of patients with SnR do not have NAbs. NAbs was high among patients treated with RIMA but it was not associated with clinical non-responsiveness. Meta-analysis of the frequency of NAbs and SnR are limited by the heterogeneity of study design and reported outcomes. Indeed the analysis of several factors that can influence the development of NAbs, i.e., MHC of patients, frequency and site of injection, injection technique, cumulative dose, and toxin denaturation, was not specifically evaluated due to the paucity and heterogeneity of data. The identification of all these missing data should be taken into account in order to improve the methodology of future studies.

Keywords Botulinum toxin · Systematic review · Meta-analysis · Neutralizing antibodies

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Introduction

Treatment with Botulinum neurotoxin (BoNT) is considered effective even when treatment cycles are repeated over years (Albanese et al. 2011; Colosimo et al. 2012; Ramirez-

Castaneda and Jankovic 2013) However, a minority of patients develop secondary non-responsiveness (SnR) to BoNT, which has been broadly defined as a lack of clinical response to further BoNT treatment in a patient who previously showed adequate clinical improvement (Benecke 2012; Brin 2007). The formation of neutralizing antibodies (NAbs) directed against the active neurotoxin part of the BoNT complex is often cited as a major cause of SnR (Benecke 2012; Dressler 2004). Clinical immunogenicity has been reported to be dose dependent and tends to occur within the first 1–4 years of treatment. However, there is variation in the reported prevalence of immunoresistance/NAbs formation (Jankovic and Schwartz 1995; Mohammadi et al. 2009) and no controlled, long-term studies have compared the immunogenicity of different BoNT products (Dressler and Hallett 2006). Further, recent studies have also suggested that NAb formation and efficacy are not always strongly correlated (Lange et al. 2009; Brin et al. 2008), suggesting that other factors (e.g., worsening of disease baseline condition, changes in the pattern of muscle hyperactivity, and inappropriate dosing or target muscle selection) may be involved in the development of SnR.

We therefore conducted a systematic review and meta-analysis to clarify the clinical impact of NAbs in patients treated with BoNT, correlating the prevalence of NAbs among BoNT treated patients with the patients' response to therapy. Variability in the prevalence of NAb formation could be related to factors such as indication, administered dosages, whether or not patients had previously received BoNT, timing of serum sample testing, and duration of treatment and assay methods (Benecke 2012 ;Goschel et al. 1997).These factors were therefore systematically evaluated as part of the review.

Methods

Search Strategy and Selection Criteria

A comprehensive search strategy using the PubMed, Biosis, and EMBASE databases was designed to retrieve relevant clinical data from the published literature up to April 2013. The following search terms were pre-defined: "botulinum", "BoNT", "Botox", "Xeomin", "Dysport", "Lanzhou", "onabot* abobot* or incobot* or rimabot* or myobloc* or neurobloc*together with "neutralising antibodies" or "neutralizing antibodies" or "neutralizing antibody" or "neutralising antibody" or nonrespon* or fail* or immunores* or resis* or antibod* or immunogen* or NAb or NAbs, and "botulinum toxin database".

Two independent authors selected studies and extracted data. Disagreements were resolved by discussion between the two reviewers. This analysis used broad inclusion criteria to comprehensively capture all relevant data. Inclusion and exclusion criteria are specified in Table 1.

Data Extraction

The following information were extracted from each study: (1) study design; (2) number of patients studied for NAbs prevalence; (3) clinical indication; (4) BoNT formulation and mean dose per treatment; (5) follow-up (months); (6) methods of assay; (7) frequency of NAbs among all patients, independently of responsiveness condition; (8) frequency of NAbs among clinically responding patients; (9) frequency of NAbs among SnR patients.

Clinical non-responsiveness and SnR were defined according to the criteria adopted by each single study.

Statistical Analysis

Secondary non-response has been classically attributed to the presence of NAbs and many studies investigated NAbs only among SnR patients. In order to clarify the association of NAbs development to BoNT responsiveness, this analysis considered three principal categories of patients: all patients, independently from BoNT responsiveness, responder patients and SnR patients. We used DerSimonian–Laird random-effects meta-analysis to summarize the point prevalence of NAbs in these different populations (with 95 % confidence intervals—CIs). For all meta-analyses and meta-regressions, prevalence estimates were transformed to logits to improve their statistical properties, and later back transformed. We assessed heterogeneity of prevalence estimates between studies using the I^2 statistics. Studies with less than ten participants were excluded.

Whenever data were available, we performed different subgroup analyses for the following a priori defined study-level covariates: clinical indications (dystonia, spasticity, urologic conditions, hyperhidrosis, glabellar line, and hypersalivation), laboratory screening tests/clinical functional tests or laboratory functional tests for NAbs (BoNT formulations (ABO, INCO, ONA, RIMA), exposure time (above or below 24 months), and BoNT dose (above or below mean dose of BoNT normally used for CD, i.e., 240 U for ONA). Laboratory screening tests include first screening tools that are not able to distinguish between neutralizing and non-NAbs. In this category, we have included the enzyme-linked immunosorbent assay, Western blot assay, fluorescence immunoassay, and immunoprecipitation assay (IPA). Clinical functional tests include functional assessment performed directly on the patients. In this category, we included the extensor digitorum brevis test, the sternocleidomastoid test, and the sudomotor sweat test laboratory functional tests are the only laboratory techniques able to detect NAbs and

Table 1 Inclusion and exclusion criteria

Inclusion criteria	Rationale
Population	
Human	The patient population included all type patients who could undergo BoNT therapy, without restrictions
Age: Any	
Gender: any	
Race: any	
Disease: all clinical indications for BoNT (dystonia, urologic diseases, spasticity, hemifacial spasm, hyperhidrosis, cosmetic, hypersalivation)	
Intervention	
BoNT (abobotulinumtoxinA [ABO, Dysport®], incobotulinumtoxinA [INCO, Xeomin®], onabotulinumtoxinA [ONA, Botox®], rimabotulinumtoxinB [RIMA, Neurobloc/Myobloc®], Neuronox®] and the Langzhou formulation)	We included all BoNT formulations
Comparator	
All patients (independently from responsiveness condition)	These comparators were selected to potentially enable the comparison between several responsiveness-based categories of patients, clinical indications and BoNT formulations
Responders patients	
SnR patients	
Several clinical indications	
BoNT formulation	
Outcomes	
NAbs prevalence among patients treated with BoNT, assessed by any assay method, across all clinical indications and BoNT formulations, divided for BoNT responsiveness	No restrictions regarding the assay method as several methods are routinely employed
Study design	
All type of manuscripts, including clinical trials and observational studies (retrospective, prospective, cross-sectional) that have described the frequency of NAbs to BoNT as a primary or secondary outcomes that have analyzed NAbs frequency in more than 10 patients	To enhance the completeness of the review observational and experimental studies were included. 10 patients has been considered a rational but arbitrary cut off to consider the number of examined patients
Language restrictions	
English paper	Due to the high number of publications on this topic we considered only English papers
Publication timeframe	
No date or publication status restrictions were imposed.	The review was designed to be as complete as possible
Exclusion criteria	**Rationale**
Population	
Animal model	We restricted the study to human participants
Patients already antibody positive for NAbs as inclusion criteria	Studies with already antibody positive patients could not be used to extrapolate the real incidence of NAbs frequency
Study design	
Case reports, abstracts from congresses; papers reporting the efficacy of using one type of BoNT formulation to treat patients resistant to another formulation; reviews, unless they report unpublished data	Full papers with new data have been considered for the review
No assay method specified	Assay method should be specified in order to analyze the changes in NAbs frequency due to the assay technique

SR systematic review, *BoNT* botulinum toxin, *NAbs* neutralizing antibodies

this category includes mouse diaphragm assay (MDA) or mouse protection assay (MPA). For the purpose of this study, clinical tests and laboratory screening tests have been considered a single category. Among the "laboratory functional tests'" category we evaluated the overall frequency of NAbs and we subsequently extrapolated the results obtained using only the MDA technique, as it is more sensitive compared to MPA. There is no universally accepted conversion ratio between BoNT formulations; the product labeling for each formulation clearly states that dosing units are not interchangeable, and reported ratios in the literature differ according to the indication under study and methodology used. For the purposes of this review, the following conversion factors were operationally adopted: 1:3 (ONA:ABO), 1:1 (ONA:INCO), and 1:50 (RIMA: ONA). Borderline titers of antibody were not considered; only clearly positive patients were considered as NAbs positive. If no titer threshold was mentioned, we assumed that positive patients had clear positive titers and not borderline ones. Among "all patients" we also performed random-effects meta-regression analysis including both clinical indications (dichotomized as dystonia versus other clinical indications) and BoNT formulations (BoNT-A versus BoNT-B). We performed the analyses using Open Meta-analyst (Tufts University, U.S., url: http://tuftscaes.org/open_meta/).

Results

Figure 1 shows the flow of studies through the systematic review process. The literature database search yielded 1482 references, published between 1991 and April 2013. Of these, 61 eligible studies were quantitatively evaluated, through meta-analysis. A total of 30 papers reported the frequency of NAbs among clinically responding patients and 12 papers among SnR patients; Table S1 (Supplementary material) summarizes the salient information from these papers (Jankovic and Schwartz 1995; Lange et al. 2009; Brin et al. 2008; Goschel et al. 1997; Hatheway and Dang 1994; Cordivari et al. 2006; Kessler et al. 1999; Greene et al. 1994; Hambleton et al. 1992; Jankovic and Schwartz 1991; Chinnapongse et al. 2012; Mezaki et al. 1994; Zuber et al. 1993; Schulte-Baukloh et al. 2008; Mejia et al. 2005; Jankovic et al. 2003, 2006; Naumann et al. 2013; Borodic et al. 1996; Jankovic and Schwartz 1991; Cruz et al. 2011).

Patients and Selected Studies

This comprehensive meta-analysis is based on data from 61 studies reporting data for 8525 patients tested for NAbs. Studies reported data from several clinical indications including dystonia (37 studies reporting data for 4972 patients with CD [3661, 73.3 % of patients, distributing among 23 studies], blepharospasm, various forms of focal dystonia), spasticity (13 studies reporting data for 1170 patients), hyperhidrosis (3 studies reporting data for 396 patients), glabellar lines (two studies reporting data for 1659 patients), hyperactive detrusor or sphincter dysfunction (five studies reporting data for 294 patients), and hypersalivation (one study reporting data for 34 patients). The dystonia papers also included 23 patients with hemifacial spasm, 3 with spastic hemiplegia and 2 with segmental myoclonus, whose data were not separable from the dystonic group. Ten out of 61 of the selected studies had a follow-up >24 months (9 of those for dystonic patients) and 10 papers did not state the exposition time before NAbs assay. Table 2 summarizes the patients assessed for NAbs presence, stratified for BoNT formulations and clinical indications.

Overall NAbs Frequency

Across all clinical indications, NAbs frequency was 12.1 % (95 % CI: 0.092–0.158, with $I^2 = 92$ %, $P < 0.001$) in studies that used laboratory functional tests. Among dystonic patients, the overall frequency of NAbs was 20 % (95 % CI: 0.165–0.235) in studies that used laboratory functional tests and 27.6 % (95 % CI: 0.164–0.427) in studies with laboratory screening tests/clinical functional assays (Table 3). Significance between studies heterogeneity was high for both assay methods (respectively $I^2 = 98$ % and $I^2 = 85$ % with $P < 0.001$). Across all clinical indications, the 50 % of papers that presented a NAbs frequency >12 % reported data on non-responders patients (Dressler and Hallett 2006; Goschel et al. 1997; Mejia et al. 2005; Jankovic et al. 2003), SnR patients (Lange et al. 2009; Hatheway and Dang 1994) or used a BoNT-B formulation (Chinnapongse et al. 2012; Jankovic et al. 2006) (Fig. 2). Patients treated with BoNT-B were specifically investigated for antibodies against this serotype. Among the other clinical indications, we found the following NAbs prevalence: spasticity: 5.9 % (95 % CI: 0.035–0.082); urologic indications: 2.7 % (95 % CI: 0.009–0.064) with $I^2 = 56$ % and P = 0.058; c) hyperhidrosis, glabellar line, and hypersalivation: 1.1 % (95 % CI: 0.001–0.075) with $I^2 = 74$ % and $P = 0.009$ (Fig. 2; Table 2). Meta-regression analysis detects the "dystonic condition" as a significant effect modifier of the NAbs frequency [dystonic versus non-dystonic patients, $P = 0.005$ with OR 0.25 (95% CI: 0.09–0.66)] while borderline results were found for formulations [BoNT-A versus BoNT-B; $P = 0.059$, OR 3.39 (9 % CI: 0.95–12.06)]. Heterogeneity remains high for meta-regression analysis too ($I^2 = 96$ %). Data on booster injections were available only for four studies and were not systematically reviewed for subgroup analysis.

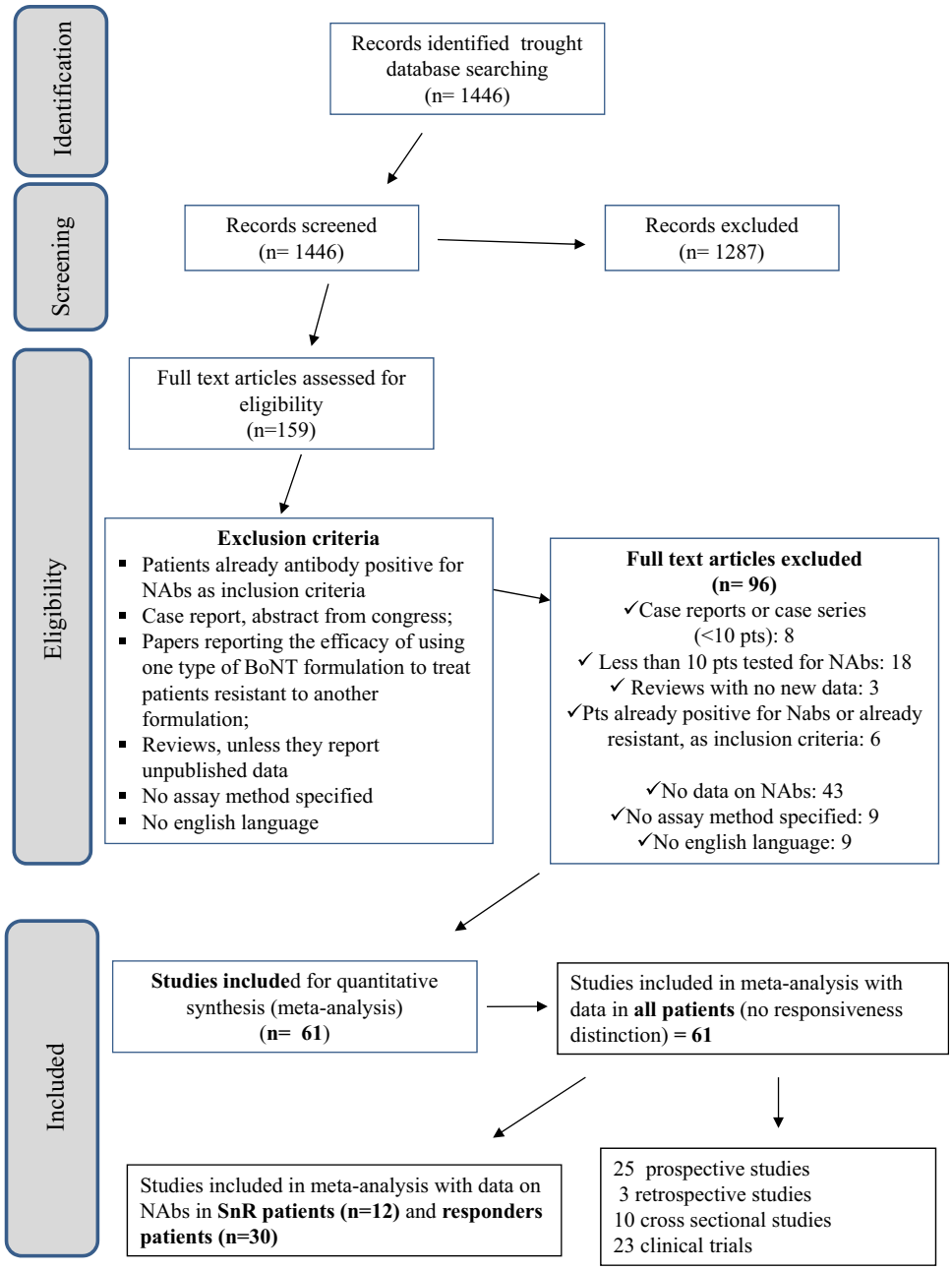

Fig. 1 Flow of studies through the systematic review process

Among all clinical indications 23 studies analyzed the NAbs frequency by means of the MDA and we found a frequency of 15.5 % (95 % CI: 0.094–0.243 with $I^2 = 92.47$ %). Among those studies, the detection limit for NAbs ranges from 0.3 to 1 mU/mL, being 0.3 mU/mL for half of them (four studies). Considering only dystonic patients (10 studies), the NAbs frequency was 16.5 % (95 % CI: 0.074–0.328), $I^2 = 95.4$ %.

NAbs in Clinically Responding Patients

Data from 4282 patients were analyzed to address the frequency of NAbs among responders. Across all clinical indications NAbs frequency was 3.5 % (95 % CI: 0.02–0.063, with $I^2 = 93$ %, $P < 0.001$). The level of heterogeneity did not reduce using the cut off dose of 240 U of ONA (93 % for high and low BoNT doses), but significantly decreased when analyzing across some clinical indications and BoNT formulations (Fig. 3; Table 3). Indeed across several clinical indications, the NAbs frequency was as follows: dystonia (6.3 %, 95 % CI: 0.36–0.107, $I^2 = 92$ %), spasticity (0.7 %, 95 % CI: 0.002–0.03, $I^2 = 0$ %), hyperhidrosis (1.4 %, 95 % CI: 0.001–0.127), urology (3.8 %, 95 % CI: 0.011–0.123), and glabellar line (0.4 %, 95 % CI 0.001–0.007, $I^2 = 20$ %). Two papers reported data from blepharospasm patients

Table 2 Summary of the number of patients examined for NAbs by means of laboratory functional test (I) or laboratory screening tests/clinical functional tests (II), stratifies for BoNT formulations and clinical indications, independently of BoNT responsiveness

BoNT formulation	ABO Dysport®		ONA Botox®		INCO Xeomin®		BoNT-A		BoNT-B NeuroBloc®		BoNT-A & BoNT-B		Crystallized BoNT-A	
Assay method	I	II	I	II	I	II	I	II	I	II	I	II	I	II
Dystonia	243	183	876	337	102	/	2133	345	1234	/	94	/	18	/
Spasticity	32	/	599	/	145	/	381	42	13	/	34	/	/	/
Hyperactive detrusor or sphincter dysfunction	20	/	238	/	/	/	25	/	/	/	/	/	/	/
Glabellar line	5	1554	/	/	105	/	/	/	/	/	/	/	/	/
Hypersalivation	/	/	/	/	/	/	/	/	/	/	34	/	/	/
Hyperhidrosis	/	/	179	/	/	/	207	/	10	10	128	/	/	/
Total number of patients	300	1737	1892	337	352		2746	387	1257	10	290	/	18	/

/: no data

showing absence of NAbs among responders treated with INCO and BoNT-A.

Analyzing several BoNT formulations we had the following frequencies across all clinical indications: BoNT-A (2.5 %, 95 % CI: 0.013–0.047), ONA (1.5 %, 95 % CI: 0.003–0.071), ABO (1.7 %, 95 % CI: 0.004–0.074), INCO (0.5 %, 95 % CI: 0.001–0.025), and RIMA (42.4 %, 95 % CI: 0.368–0.483).

NAbs Among Secondary Non-Responders Patients

To address the frequency of NAbs among SnR patients, we analyzed data from 793 patients with dystonia or spasticity (Fig. 4). The overall frequency was 53.5 % (95 % CI: 0.441–0.627, $I^2 = 71$ %, $P < 0.001$). Heterogeneity decreased when analyzing BoNT formulations, while no significant changes were evidenced examining clinical indications, the BoNT dose and the exposition time (cut off of 24 months) (Table 3). No studies that examined NAbs in SnR patients for urologic indications, glabellar line, hypersalivation, and hyperhidrosis were identified.

Discussion

In this comprehensive meta-analysis of 61 studies, we analyzed the frequency of NAbs among 8525 patients receiving any type of BoNT across several clinical indications. The analysis found that 20 % of patients treated for dystonia, 5.9 % of patients treated for spasticity and 2.7 % of patients treated for urologic indications, and 1.1 % for other clinical conditions (hyperhidrosis, glabellar line, and hypersalivation) developed NAbs, independent of clinical responsiveness to BoNT. The frequency of NAbs was much lower (overall 3.5 %) among responders (6.3 % in dystonia, 0.7 % in spasticity, 3.8 % for urologic conditions, 0.4 for glabellar line, and 1.4 for hyperhidrosis) and much higher (overall 53.6 %) among SnR patients. Such a difference between responders and SnR patients was evident analyzing data across all clinical indications and BoNT formulations, as no overlap of 95 % CI values occurred between these two categories. No data on migraine are yet available.

A NAb frequency of 20 % for dystonia, is in line with data on NAbs prevalence among CD patients treated with ONA (Jankovic et al. 2003, 2006) but it is considerably higher than what has been reported in other CD studies: 1.2 % or 1–3 % among CD patients (Brin et al. 2008; Naumann et al. 2013). However, it should be noted that studies reporting higher rates typically evaluated the frequency of NAbs among non-responsive patients (Mejia et al. 2005), while the studies reporting lower frequencies were conducted in populations comprising of largely clinically responsive patients (Brin et al. 2008). The inclusion of the pre-1997 ONA formulation is also likely to have skewed the results as it was well known to contain significant levels of inactive BoNT (Borodic et al. 1996). Indeed, 9 out of 32 studies on dystonic patients were published within the 1998 (Jankovic and Schwartz 1995; Goschel et al. 1997; Hatheway and Dang 1994; Greene et al. 1994; Hambleton et al. 1992; Jankovic and Schwartz 1991; Mezaki et al. 1994; Zuber et al. 1993; Hanna and Jankovic 1998). In line with this observation, the present meta-analysis found that the frequency of NAbs in the "all patients" group was strongly influenced by responsiveness condition, i.e., if patients were responders or SnR to BoNT and BoNT formulation (Fig. 2). It is also pertinent to note the lack of any data on NAbs prevalence among patients with oro-mandibular dystonia, task-specific, or limb dystonia. More work in other dystonias (i.e., not CD) is warranted.

Table 3 Meta-analysis results

Categories		Sub-categories	All patients	Responders	SnR patients
Clinical indications	All indications	//	12.1 % (95 % CI: 0.092–0.158), $I^2 = 92$ %	3.5 % (95 % CI: 0.020–0.063), $I^2 = 93$ %	53.5 % (95 % CI: 0.441–0.627), $I^2 = 71$ %
	Dystonia	Laboratory functional assay	20.0 % (95 % CI: 0.165–0.235), $I^2 = 98$ %	7.3 % (95 % CI: 0.04–0.13), $I^2 = 92$ %	47.6 % (95 % CI: 0.387–0.567), $I^2 = 61$ %
	Dystonia	Laboratory screening tests/clinical assay	27.6 % (95 % CI: 0.164–0.427), $I^2 = 85$ %	NA	NA
	Dystonia	MDA	16.5 % (95 % CI: 0.074–0.328), $I^2 = 95.4$ %	NA	NA
	Spasticity	Laboratory functional assay	5.9 % (95 % CI: 0.035–0.082), $I^2 = 92$ %	0.7 % (95 % CI: 0.002–0.03), $I^2 = 0$ %	75.9 % (95 % CI: 0.408–0.935), $I^2 = 82$ %
	Urology	Laboratory functional assay	2.7 % (95 % CI: 0.009–0.064) $I^2 = 56$ %	3.8 % (95 % CI: 0.011–0.123), $I^2 = 0$ %	NA
	Hyperhidrosis	Laboratory functional assay	1.1 % (95 % CI: 0.001–0.075) $I^2 = 74$ %	1.4 % (95 % CI: 0.001–0.127), $I^2 = 26$ %	NA
	Glabellar line			0.4 % (95 % CI 0.001–0.007), $I^2 = 20$ %	NA
	Hypersalivation			NA	NA
BoNT formulation	ABO Dysport®	All indications	NA	1.7 % (95 % CI: 0.004–0.074), $I^2 = 80$ %	56.7% (95 % CI 0.452–0.675), $I^2 = 0$%
	ONA Botox®	All indications	NA	1.5 % (95 % CI: 0.003–0.071), $I^2 = 84$ %	32.5 % (95 % CI: 0.228–0.439), $I^2 = 0$ %
	INCO Xeomin®	All indications	NA	0.5 % (95 % CI: 0.001–0.025), $I^2 = 0$ %	NA
	BoNT-A	All indications	NA	2.5 % (95 % CI: 0.013–0.047), $I^2 = 0$ %	59.3 % (95 % CI 0.443–0.728), $I = 78$ %
	BoNT-B NeuroBloc®	All indications	NA	42.4 % (95 % CI: 0.368–0.483), $I^2 = 56$ %	NA
BoNT dose	≤240 U ONA	All indications	NA	3.6 % (95 % CI: 0.019–0.066), $I^2 = 93$ %	60.4 % (95 % CI 0.371–0.797), $I^2 = 74$ %
	>240 U ONA	All indications	NA	3.5 % (95 % CI: 0.02–0.063), $I^2 = 93$ %	50.9 % (95 % CI 0.377–0.604), $I^2 = 76$ %
Follow-up	≤24 months	All indications	NA	NA	46.4 % (95 % CI 0.354–0.578), $I^2 = 47$ %
	>24 months	All indications	NA	NA	54.7 % (95 % CI 0.355–0.726), $I^2 = 80$ %

NA not available

Despite the high BoNT doses usually used to treat spasticity, the incidence of immunogenicity in patients with spasticity appeared to be very low among responders (0.7 %) although significantly increased in SnR patients (75.9 %). The low immunogenicity among spastic patients was already reported by Naumann and colleagues

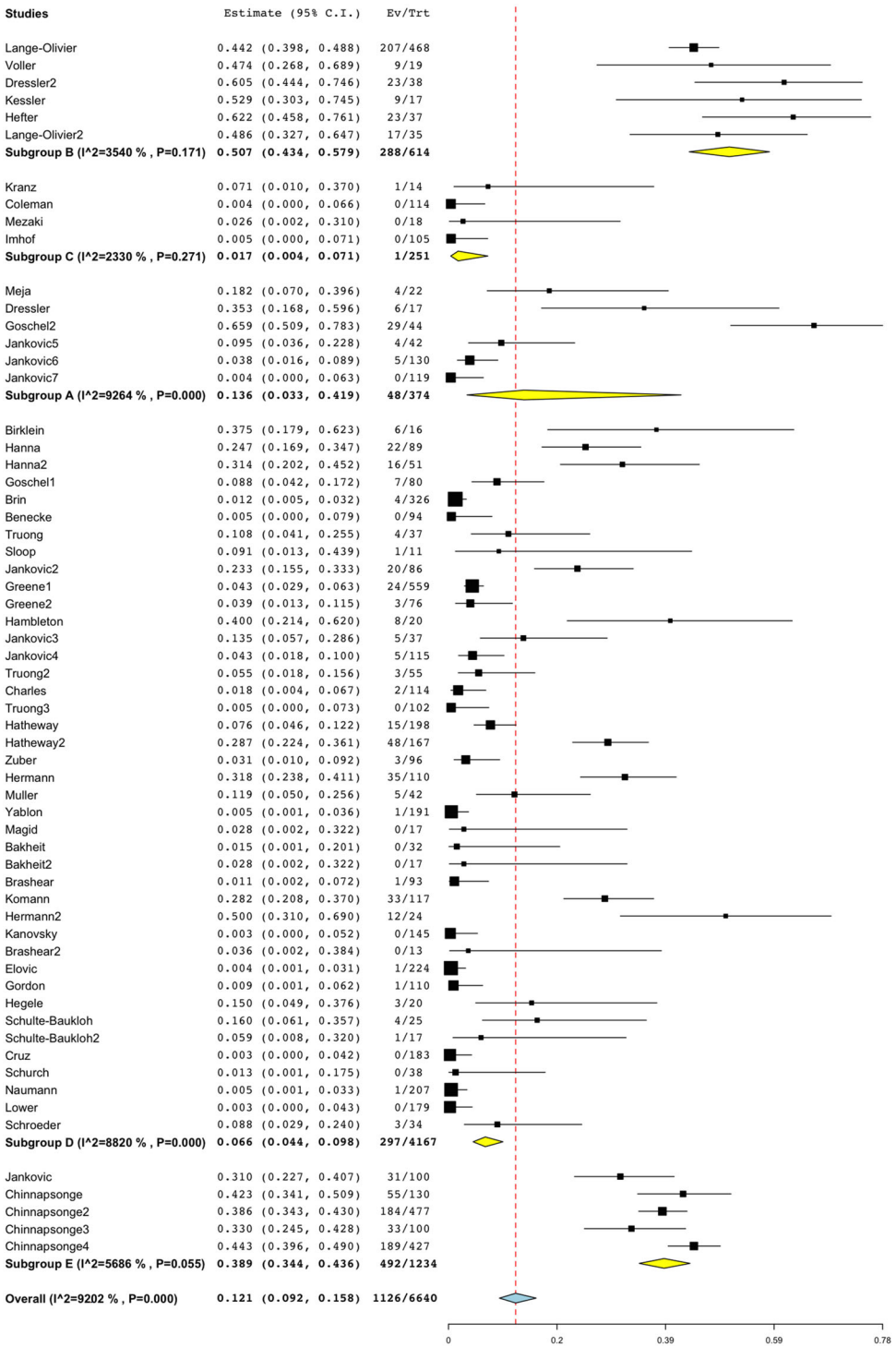

Fig. 2 Forest plot of NAbs frequency, across several responsiveness conditions. BoNT-A and BoNT-B results are shown separately. Subgroup A = non-respondent patients only (not specified if SnR or primary); Subgroup B = SnR patients only; Subgroup C = responders only; Subgroup D = mixed responsiveness condition or NA; Subgroup E = BoNT-B-treated patients; $I^{\wedge} = I^2$

(Naumann et al. 2013). However concerning our review, the number of selected studies for spasticity that included responders and SnR patients was low (respectively, four studies including 456 patients and three studies including 80 patients) and we did not perform a sub-analysis for pediatric spasticity patients who have been reported to develop a higher percentage of antibodies (a frequency of 30 % has been reported although the clinical responsiveness of the patients has not been reported) (Koman et al. 2001). By contrast, the incidence of NAbs amongst patients treated for urologic indications shows a paradoxical slight increase among the responding patients. This finding contradicts recent studies using ONA, which have reported an absence of NAbs (Cruz et al. 2011). It has been proposed

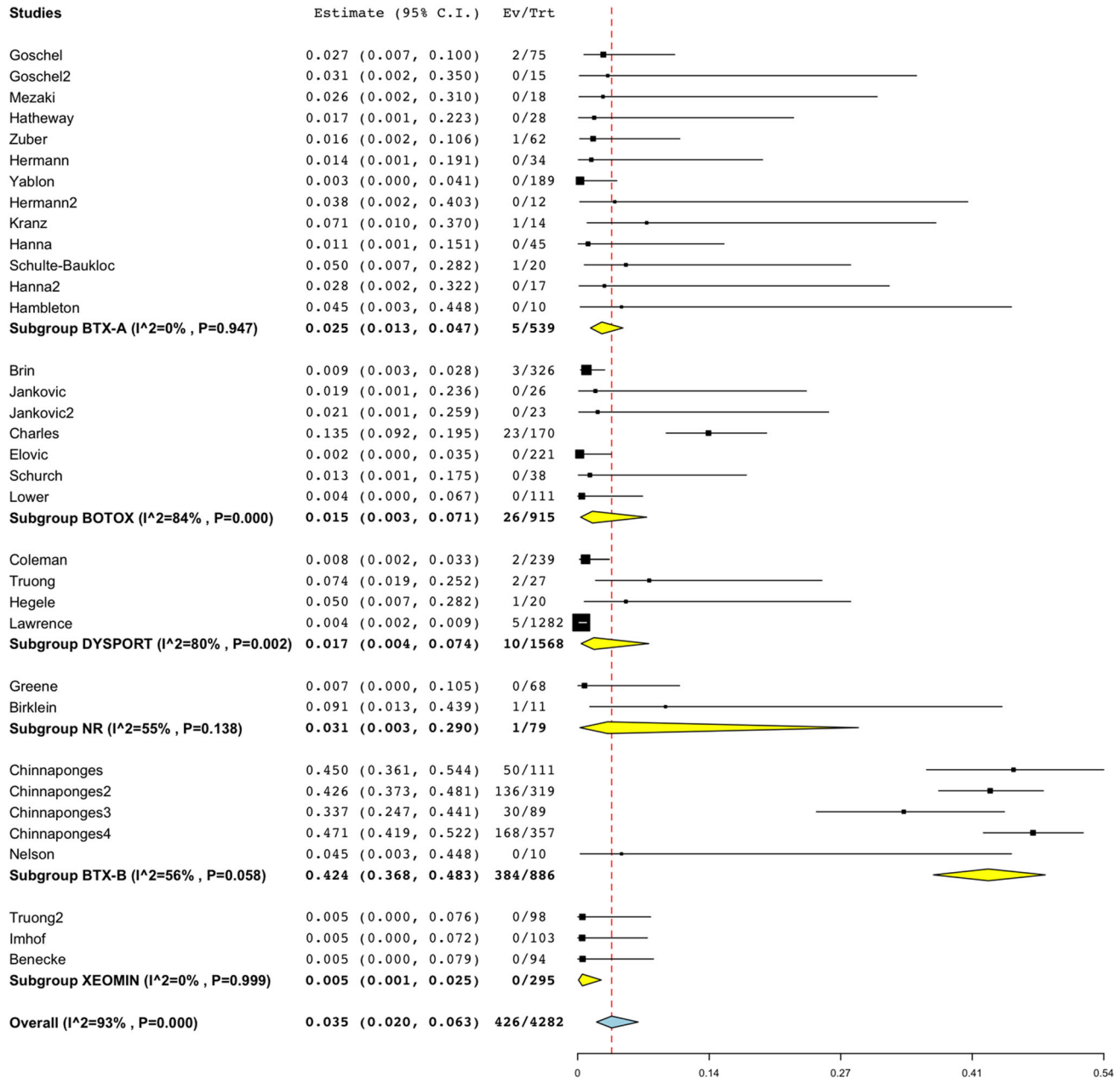

Fig. 3 Forest plot of NAbs frequency among responders, divided on BoNT formulations. $I^{\wedge} = I^2$

that the urinary bladder acts as an immunologic organ, and that other factors such as latex allergies and urinary infections may also influence the NAb development (Goschel et al. 1997; Hatheway and Dang 1994; Schulte-Baukloh et al. 2008). The number of papers reporting treatment of urologic conditions was too small and heterogeneous to tease out any risk factors linked to therapeutic methodology.

The current meta-analysis confirms the presence of NAbs in about the half of SnR patients, which is line with the recent estimate by Lange and colleagues who used the sensitive mouse phrenic nerve-hemidiaphragm assay to evaluate NAbs in a cohort of 503 SnR patients (Lange et al. 2009). The development of NAbs was not always correlated to clinical unresponsiveness, as NAbs were also present in a small group of clinically responsive patients as well as they can be absent in SnR patients. Other studies of SnR patients have reported a frequency of NAbs ranging from 33 to 100 % (Jankovic and Schwartz 1995; Goschel et al. 1997; Cordivari et al. 2006; Kessler et al. 1999; Jankovic et al. 2003). This variation is likely due to the small populations of CD patients studied and the different

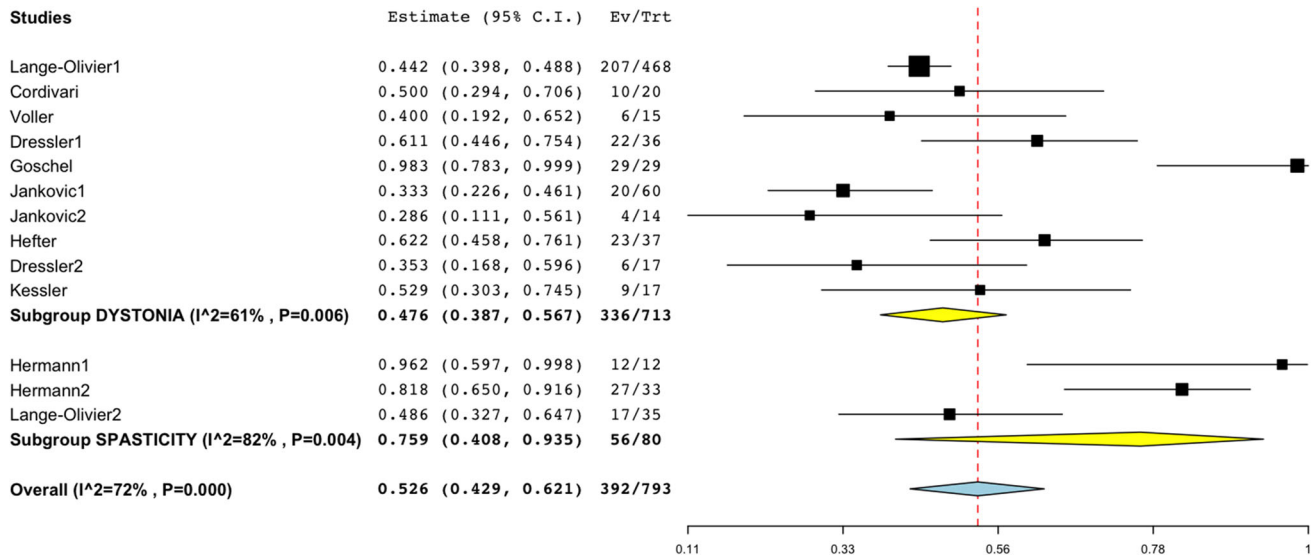

Fig. 4 Forest plot of NAbs frequency among SnR patients. *Panel A* dystonic patients; *Panel B* Spastic patients. $I^{\wedge} = I^2$

assay methods used. The present meta-analysis did not find substantial differences in the development of NAbs across the several indications studied. The clinical implication of this finding is that NAbs are responsible for SnR in only half of cases and other causes of treatment failure should be always suspected, such as changes in patterns hyperactivity, inappropriate muscles injections, expiration of placebo therapy effect or disease progression (Dressler 2004; Mohammadi et al. 2009). No data for urologic indications, glabellar line, hypersalivation and hyperhidrosis were found for SnR patients and considering the quite high incidence of NAbs among urologic patients, specific studies addressing their frequency among urologic SnR patients should be encouraged.

A higher immunogenicity of RIMA has been already reported among CD patients (Chinnapongse et al. 2012; Jankovic et al. 2006) but, again, the studies demonstrated that the development of NAbs does not correlate with loss of effect, as patients responsiveness persists regardless of the presence of BoNT-B antibodies as detected by the mouse neutralizing assay (Chinnapongse et al. 2012). We also found a high frequency of NAbs among responders patients treated with RIMA. Due to the low number of patients in the SnR group, it was not possible to compare NAbs rates for the different formulations. However, no significant differences in terms of NAbs rates between BoNT-A formulations were found in the responder groups.

With regard to assay methods, the analysis confirms the higher NAbs frequency detected by laboratory screening tests/clinical assays than laboratory functional tests (27.6 versus 20 %). Moreover, extrapolating data obtained considering only the MDA technique we confirmed the higher sensitivity of this assay method as NAbs frequency slightly decreased if compared to results obtained using both MPA and MDA (passing from 20 to 16.5 %, among dystonic patients). Unfortunately, the adopted threshold titer of antibodies was specified only in a small group of studies (25 %) and we are aware that differences in the reported NAbs frequency could be accounted on those threshold disparity. NAbs have been reported to form primarily against the heavy chain of the core BoNT. However, NAbs that bind to epitopes on the light chain of the core BoNT have more recently been observed (Atassi et al. 2011). Laboratory screening tests cannot detect between the antigens produced against non-toxic accessory proteins (NAPs) or bind to the core BoNT, thus they are recommended as screening assays.

In spite of the non-significant results obtained analyzing BoNT dose (> or < than 240 U ONA, see Table 2), we can confirm that NAbs frequency is generally higher among those conditions that usually are treated with higher BoNT doses, such as urologic, spasticity and, especially, dystonia patients. Among the later, we have to highlight that dystonic patients primarily suffer from CD, the dystonic form that usually need higher BoNT dose.

Limitations of this meta-analysis follow the limitations of the studies included and the difficulties in performing a meta-analysis that could gather all these heterogeneous data. Indeed, only 16 % of the selected studies had a follow-up >24 months and 16 % of studies did not specify the exposition time before NAbs assay. This "short-term" follow-up could certainly influence NAbs detection. Indeed, there was a high level of heterogeneity in the meta-analysis, mainly due to the large number of included papers and the different methods (assay methods, BoNT formulations, clinical indications, and population responsiveness

to BoNT) adopted in these studies. While this could compromise the reliability of the results, further analysis of the sources of heterogeneity showed that I^2 significantly reduces when analyzing separate clinical indications (urology indication: $I^2 = 56$ %), responsiveness to BoNT (responders patients: $I^2 = 0$ % among spasticity, glabellar line, and urologic indications) and BoNT formulations ($I^2 = 0$ % for Xeomin and BoNT-A categories)—thereby achieving a more homogenous dataset. However, heterogeneity did not reduce even when performing meta-regression analysis. Another limitation of our review concerns the varying definitions of clinical non-responsiveness and SnR used in the studies and the tools used to define it, that could vary according to study methodology and clinical indications. However a consistent part of the selected studies, independently from clinical indication, used a 0–4 point "peak effect" scale in order to define clinical non-responsiveness, considering patients who scored 0 or 1 as non-responders. Equally SnR patients were usually defined as patients who had at least 2 unsuccessful treatments subsequent to treatments with satisfactory results.

Genetic predisposition or major histocompatibility (MHC) susceptibility of the patients may play a role in those patients who develop immunoresistance as the immune responses to a protein antigen and to the various epitopes on the antigen are each under separate genetic control (Atassi et al. 2011; Atassi 2004; Dolimbek et al. 2007). Considering the substantial role of those genetic factors in the development of immunoresistance during BoNT, we are aware that a meta-analysis on NAbs frequency should consider also the MHC of patients. However, no data on MHC of the patients were available among the 61 included studies.

In addition, the analysis of several factors that increase the likelihood of the development of NAbs or non-responsiveness to BoNT, i.e., protein load, cumulative dose, high dose injections, frequent injections/booster injections, and technique of injection (EMG or ultrasound use), have not been specifically evaluated due to the paucity and heterogeneity of studies reporting these type of data. Likewise, no significant results were obtained analyzing the influence of dose cut off (240 U) on NAbs frequency among responders and SnR patients, but the paucity of studies belonging to these sub-groups limited our ability to detect any differences.

To the best of our knowledge, this is the first meta-analysis to review the incidence of NAbs across all clinical indications, assay methods and BoNT formulations stratifying results on the responsiveness to BoNT. Even if our findings should be cautiously interpreted due to the implicit and above mentioned limitations, a meta-analysis on this topic has, we can individuate some major results along with relevant clinical and research implications. Indeed, we can affirm that even if the frequency of NAbs was clearly higher in SnR patients compared with clinically responsive patients, the development of NAbs did not always predict responsiveness to BoNT therapy and roughly half of SnR patients did not have NAbs, suggesting that NAbs are not the main cause in at least half of the patients and other factors should be investigated. NAbs frequency was high among patients treated with RIMA but it was not associated with clinical non-responsiveness. There is a need for a consensual and operational definition of SnR. Moreover clinicians and researches should be aware that the risk of NAbs and SnR could also vary depending on factors like treatment indication.

Analysis of the literature showed that there is more data from patients with dystonia (especially CD), while there is a relative lack of studies for other clinical indications. Further studies in these indications, particularly urologic conditions, are clearly warranted along with prospective studies with long-term follow-up.

Systematic reviews with meta-analysis on NAbs and SnR are limited by the heterogeneity of study design and reported outcomes. We have identified several missing data that may be key factors for the development of NAbs during BoNT and that should be taken into account in order to improve the methodology of further studies.

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