PhD Course

Tecnologie Innovative nelle malattie dello scheletro, della cute e del distretto oro-cranio-facciale

PhD Thesis

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Modulation of trigeminal nociceptive input induced by Onabotulinum Toxin

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Index

1. INTRODUCTION

1.1 CHRONIC MIGRAINE: DEFINITION

Chronic migraine (CM) has only recently been recognized as a distinguished entity (distinct pathophysiology, epidemiology, and response to treatments) from episodic migraine (EM). [19,20, 21, 22] It was only in 2013, with the International Classification of Headache Disorders, third edition beta (ICHD-3b), that CM was formally defined. [23]

CM is actually coded at ICHD3b 1.3 where it is defined as "headache occurring on 15 or more days per month for more than 3 months, which has the features of migraine headache on at least 8 days per month". [23]

ICHD3b diagnostic criteria for CM are the following [23]:

A. Headache (tension-type-like and/or migraine-like) on ≥ 15 days per month for >3 months 2 and fulfilling criteria B and C

B. Occurring in a patient who has had at least five attacks fulfilling criteria B-D for 1.1 Migraine without aura and/or criteria B and C for 1.2 Migraine with aura

C. On ≥ 8 days per month for >3 months, fulfilling any of the following 3:

1. criteria C and D for 1.1 Migraine without aura
2. criteria B and C for 1.2 Migraine with aura
3. believed by the patient to be migraine at onset and relieved by a triptan or ergot derivative

D. Not better accounted for by another ICHD-3 diagnosis.

1.2 CHRONIC MIGRAINE: EPIDEMIOLOGIC CONSIDERATIONS

The estimated prevalence of CM throughout the world ranges from 0% to 5.1%. Most general population studies report 1.4-2.2%. Such high variability probably reflects differences among populations and the use of different definitions. Studies on US population show increasing prevalence throughout adolescence, peak-prevalence in midlife, and reduction after age 50 years. The highest prevalence is documented in women aged 18-49 years. [31]

CM accounts for about 8% of all migraine cases[32] and patients with daily or near-daily headache account for up to 45% of patients referring to headache specialist worldwide. However, according to a recent US study, only 20% of patients who meet the criteria for chronic migraine, are properly diagnosed. [22,32] Accordingly, CM should be regarded as a disabling, underdiagnosed and undertreated disorder.

CM causes substantially greater disability compared to EM. People with CM result to have lower income and are more likely to be occupationally disabled. A large population-based study conducted in 2008 revealed that 57% of people with CM but only 24% of those with EM missed at least 5 days of work or school over a three months period. Moreover, 58% of CM patients but only 18% of EM reported reduced productivity for at least 5 days over the three months.[33] Chronic migraineurs reported working at approximately half of their full effectiveness when experiencing headache symptoms.[32] About 25% of people with CM have very severe headache related disability and about 90% have at least moderate disability. [31]

Two questionnaires are commonly used to assess the burden of disease and functioning in daily activities for both episodic and chronic migraine patients: the Migraine Disability Assessment (MIDAS) and the Headache Impact Test-6 (HIT- 6). Both of these measures are available in numerous languages and may detect changes during a course of treatment. Patients with CM show significantly higher MIDAS score compared with EM patients. [32]

Compared with EM people, those with CM tend to have more intense and longer duration headaches, whether treated or not. [31] In addition, people with CM refer to healthcare facilities and

require diagnostic tests and migraine drugs more often. Annual total medical costs (direct and indirect) are 4.4 times greater in patients with CM compared with EM. [31]

The epidemiological distinction between CM and EM has been highlighted by three large observational studies: the International Burden of Migraine Study (IBMS), the American Migraine Prevalence and Prevention (AMPP) study, and the German Headache Consortium (GHC) study. [28]

The International Burden of Migraine Study (IBMS) provides the most robust epidemiological comparison between clinical features of CM and EM [26] demonstrating that CM patients experience longer duration of headache attacks and are more likely to experience severe pain intensity and to report comorbidities, notably non-headache pain, psychiatric disorders and vascular disease. Both IBMS and AMPP study [25] revealed differences in sociodemographic status between the two groups with higher prevalence of CM in a slightly older age and among Caucasians. A large observational study conducted in Germany [28] also found in these patients a significantly higher body mass index (BMI), lower levels of education, and more frequent smoking. The IBMS confirmed that CM patients in the USA visit primary care physicians two times more often than EM patients. [32]

Chronic migraineurs commonly suffer from neurological and medical comorbidities: obesity, ischemic stroke, cardiovascular disease, sleep disorders, chronic pain disorders, low back pain, asthma and allergic rhinitis. These comorbidities may impact disease prognosis, treatment and clinical outcomes. Psychiatric comorbidities, including depression, anxiety, and post-traumatic stress disorder (PTSD), have a higher incidence in patients with CM than in those with EM.

A linear relationship between the number of headache days and the degree of depression and anxiety measured by questionnaires is documented until the number of headache days reaches the chronic variant, when the linearity is lost and a high impact of psychiatric impairment is observed in all patients. [32]

Baseline results from a large internet survey of the US population designed to characterize the course of both EM and CM (CaMEO Study) confirmed previous findings that CM is associated with increased headache-related disability, psychiatric comorbidities, and greater financial and occupational burden compared with EM and that CM patients are more likely to be female, white, obese and more likely to have depression or anxiety than EM patients and have a lower socioeconomic status. [24]

1.3 PATHOPHYSIOLOGY OF MIGRAINE CHRONIFICATION

As highlighted by the authors of ICHD3b classification, it is impossible to distinguish the single headache episodes in patients with CM. Moreover, headache characteristics may change from day to day but also within the same day. [23] The use of a 15 days cut-off is somewhat arbitrary but allows clinicians to identify a group of migraineurs with a different epidemiological, clinical, functional and social profile. [20]

The relationship between EM and CM is complex. EM evolves to CM at the rate of 2.5% per year. On the other hand, CM remits to EM at 2-year rate of 26%. [27]

Response to acute medication is quite similar between EM and CM. However a less robust response to triptans has been observed in CM patients. CM is tipically poorly responsive to profilactic drugs commonly used in EM.

Several risk factors for progression to CM in EM patients have been identified and can be grouped into two main categories: modifiable and unmodifiable. [20] Unmodifiable risk factors include age, female sex, Caucasian race, low educational level or socioeconomic status, and head injury. Modifiable risk factors include obesity (defined as BMI>30), depression, major life changes (divorce, moving, employment changes, or problems with children), medication overuse (defined as use of more than 10 or 15 doses a month, depending on the class), intake of certain classes of drugs (regardless of overuse), high caffeine consumption.

AMPP study data revealed an about 1.28-fold increased risk of progression to CM in patients with severe depression and in patients using compounds containing barbiturates and opiates. Chronic

consumption of caffeine in high doses, whose pronociceptive effect is well documented in literature, is indicated in a population-based study as a modest risk factor. [29]

Clinical, neurological and functional studies of CM suggest a pathophysiological state characterized persistent and pervasive brain alterations, in contrast to the intermittent changes noted in EM during attacks. The neurological alterations observed in CM that are evident even in the absence of headache or as more extreme or severe changes. [28]

CM is characterized by three main physiological changes: altered brain metabolism, neuronal hyperexcitability, and central sensitization of nociceptive pathways. Although the precise mechanisms underlying headache chronification are not jet completely understood, central sensitization and dysfunctioning pain control systems appear to play a pivotal role. [48,49]

Population-based family studies showed higher levels of family aggregation in migraneurs with severe and disabling headache. An early age of onset was also associated with increased severity. Hence it could be hypothesized that probands with more severe and disabling pain may have a greater genetic load. [60,61] Such genetic vulnerability could be reflected phenotypically by increased responsiveness of the cerebral cortex to sensory information, dysfunctioning brainstem neurons, reduced mitochondrial energy reserve, and NO hypersensitivity. [48]

Many symptomatic treatments, such as triptans, analgesics, and opioids, can induce transformation from episodic headache to MOH. The neurobiological bases of the vicious circle of medication overconsumption are not completely understood. [48]

Neurophysiological methods are particularly suitable for study of functional brain abnormalities as they can be repeated noninvasively and at low cost.

Most evoked-potential studies in EM patients revealed a deficit of habituation during stimulus repetition for a number of different sensory modalities. This abnormality fluctuates over time being most pronounced during the days immediately preceding migraine attack and then normalizing during the attack. These methods have recently been applied also to CM patients, and in particular to those with associated medication overuse. [48]

Azyenberg and colleagues detected in a group of 29 CM patients and medication overuse increased pain-related evoked potentials (PREP) amplitudes both after cephalic and extracephalic stimulation normalizing after drug withdrawal. [50]

Contingent negative variation (CNV) is a slow cortical potential related to higher mental functions, consisting of a negative wave generated in a reaction-time paradigm and composed by two components: an early component (CNV1), related to both warning stimulus and level of expectation, and late one (CNV2), related to motor readiness. [48] Siniatchkin et al. found a reduction of both components and CNV1 habituation in patients with transformed migraine, which they attributed to difficulty to cope with environmental demands and susceptibility for depression in these patients.

Visual evoked potentials (VEPs) are the standard method to assess excitability in the visual cortex, which is supposed to be unrelated to pain processing. Lack of habituation in VEP is known to be present in EM interictally. Visual evoked magnetic fields (VEF) recorded using magnetoencephalography (MEG) showed normal habituation in CM. [55] This response pattern is similar to that found in EM patients in ictal phase.

Magnetic suppression of perceptual accuracy (MSPA) is a test measuring suppression of accuracy in visual perception induced by transcranial magnetic stimulation (TMS) over the occipital area, which is believed to be induced by preferential activation of inhibitory neurons and is thus considered as an indirect index of cortical excitability. Aurora et al. applied the MSPA paradigm to study cortical inhibition in 25 CM patients and, for comparison, in EM patients and healthy controls. [51] EM patients showed a reduced suppression of visual accuracy compared to healthy controls. In CM patients suppression was further reduced or absent, which can be attributed to impaired intracortical inhibitory mechanisms and consequent persistent cortical hyperexcitability.[28,51] Of these 25 CM patients, 10 underwent an 18 F-fludeoxyglucose positron emission tomography (PET) scan, which showed increased metabolism in the pons and right

temporal cortex, contrasting with decreased metabolism in bilateral medial frontal, parietal, and somatosensory areas and caudate nuclei. [52] This suggests a reduced inhibitory capacity of the cortex in CM. [28]

In a pilot study Brighina and colleagues [53] found a long-lasting reduction in attack frequency, number of tablet intake, and headache index in chronic migraineurs treated with high-frequency rTMS over the left dorsolateral prefrontal cortex (DLPFC), which is thought to exert an inhibitory control on pain pathways in humans.

Chronic migraineurs exhibit a brainstem activation pattern similar to that observed during migraine attacks. Moreover electrophysiological abnormalities detected in CM patients are similar to those reported during attacks in EM and indicate both an increased cortical excitability and a decreased activity of inhibitory cortical interneurons. It can be argued that CM looks like a "never ending migraine attack". [48]

Increased amplitude of non-noxious and noxious evoked responses is probably a marker of central sensitization. It is supposed that central sensitization occurs in ictal phase of EM and manifests itself clinically as cutaneous allodynia and neurophysiologically as an increase in trigeminal reflexes. [56,57] Cutaneous allodynia, which is correlated with headache frequency severity and disability in over 60% of migraineurs and also with therapeutic response, can be regarded as a marker of central sensitization. CM subjects are supposed to undergo plastic changes in the pain matrix area. [46] Animal studies suggest a central sensitization in the trigeminocervical complex receiving convergent input from both the trigeminal and the occipital nerves. [42,43] Central sensitization in the pain network is associated with neuronal hyperexcitability, resulting in decreased nociceptive thresholds, increased responsiveness to both noxious and non-noxious stimuli, and an expansion of the receptive fields of nociceptors, which could explain extra-cranial disease at the back of the head or upper neck. [48,58,59] Sensitized central nociceptive neurons may also account for CM resistance to treatment. Alterations in central glutamate neurotransmission have been reported in the anterior cingulate cortex and insula using magnetic resonance spectroscopy. Studies by Coppola and colleagues [49] suggest a facilitation of thalamo-cortical activity, which could maybe take part in central sensitization.

A dysfunction of discendent noxious inhibitory control (DNIC) resulting from changes in PAG may also be an important factor in the pathophysiology of CM. Brainstem has been suggested as ''the generator'' of migraine attacks. Morphologic changes related to duration of migraine disorder include reduced cortical gray matter of the pain matrix areas and iron accumulation in the PAG, red nucleus, and basal ganglia structures [40]. These changes are more marked in CM compared to EM. [35] As reported by Raskin and Veloso, stimulation of the PAG region can produce migraine symptoms in otherwise non-headache people. [38,39] Moreover, CM symptoms can develop after bleeding of a cavernoma in the region of the PAG or lesion of the pons. [41] PET studies, as already seen, similarly demonstrated activation of the dorsal midbrain, including the PAG, and the dorsal pons, near the locus coeruleus, in spontaneous EM and CM. [42] Functional MRI studies showed significant decreases in the MRI values R2′ and R2* in the substantia nigra and red nucleus in CM, probably attributable to impaired iron homeostasis PAG, secondary to hyperoxia associated with head pain. [28]

Peripheral sensitization also plays a role in CM. CM patients were found to have a significant increase in TRPV1 (transient receptor potential vanilloid type-1 receptor) immune-reactive nerve fibers in the scalp arteries wall compared with healthy controls. [70] Expressed mainly in C fibres, TRPV1 is an ionotropic receptor inducing release of several neuropeptides involved in central sensitization: Calcitonin Gene Related Peptide (CGRP), Vasoactive Intestinal Peptide (VIP), Pituitary adenylate cyclase-activating polypeptide (PACAP), Substance P. These molecules are peripherally secreted from trigeminal afferents and induce intracellular increase in cAMP or cGMP with consequent vasodilation and inflammatory events within both the dura mater and trigeminal ganglion, which is important in triggering and amplification of pain. [71]

A crucial role in migraine triggering and chronification seems to be played by CGRP. [72,73] Increased interictal CGRP levels in peripheral blood have been found in CM patients compared with both EM

patients and non-headache controls. [73] CGRP receptors are also widely expressed in the CNS and may exert pain-modulation effects. Both CGRP and PACAP, when administered exogenously, can induce a migraine-like headache in otherwise non-migraine subjects. Recent studies reported that interictal PACAP plasma levels negatively correlated with attack duration in CM patients. [74] This results pair with animal studies showing decreased PACAP content in plasma and trigeminal ganglia and increased PACAP related receptor expression in the trigeminal ganglia in rats after repetitive dural inflammatory stimulation. These findings can be explained by headache-induced decrease of PACAP and subsequent upregulation of related receptors. [75] A selective PACAP effect on extra-cerebral arteries is hypothesized. [76] Recent human migraine models have also pointed to the PAC1 receptor and the PACAP molecule itself as target sites for drug testing. [77] Even though the pathophysiology and significance of subcortical white matter lesions and infarct like cerebellar lesions are not fully understood, their occurrence in frequent migraine is a further evidence of structural alterations in the brain in CM.

1.4 CHRONIC MIGRAINE: TREATMENT OPTIONS

The primary goals of migraine treatment have traditionally been relieving pain, restoring function and reducing headache frequency. Patients with EM should be counseled on avoiding risk factors associated with transformation to chronic migraine. Treatment strategies include educational interventions, lifestyle modifications, and trigger management, as well as acute and preventive pharmacotherapy. Optimal treatment should also include management of comorbidities. A headache diary is an important tool providing information about headache triggers, frequency and intensity of attacks, drug intake and possible patterns of medication overuse headache (MOH). [62]

Once CM is established, finding an appropriate and beneficial treatment is challenging.

The first step is the rigorous control of predisposing factors, such as MOH. Both the role of MOH in migraine chronification and the optimal treatment of MOH are subject to debate. European Federation of Neurological Sciences (EFNS) recommends early discontinuation or tapering down of the overused medication combined with a prophylactic migraine treatment. [66] By contrast, some authors advocate withdrawal alone, at least in case of uncomplicated MOH (short duration of MOH, lower doses of acute medications, minimal psychiatric symptoms, no history of relapse after withdrawal).[67] Moreover independent trials have shown that patients with and without MOH benefited from preventive medication without any explicit drug detoxification. Unfortunately, no randomized controlled trials specifically designed for comparison of different approaches exist at the moment, so that it is not possible to make definite, evidence-based recommendations. A systematic review of available studies of MOH published in 2016 showed more evidence for withdrawal or tapering in combination with early prophylaxis than for withdrawal alone.

Not all patients with EM need a preventive treatment. Conversely, the treatment of CM with only pain killers is ineffective and should be avoided because it predisposes to MOH. Topiramate is the only drug that has been investigated in this context in more than one double-blinded RCT although it is not approved for the prevention of CM. Both the TOPMAT-MIG- 201 (TOP-CHROME) Study Group and the Topiramate Chronic Migraine Study Group revealed that topiramate was relatively well tolerated (paraesthesia and fatigue were the most common adverse effects) and significantly reduced the mean number of monthly migraine days, even in the presence of medication overuse. Topiramate also showed to improve various measures of quality of life and migraine-accompanying photophobia, phonophobia and vomiting. It had been suggested topiramate to prevent progression from EM to CM. Howewer in the topiramate Intervention to Prevent Transformation of Episodic Migraine (INTREPID) trial, 100 mg topiramate per day for 26 weeks could not prevent progression from high-frequency EM to CM. An open-label study suggested that combining topiramate with betablockers could bring further benefit to patients with refractory migraine, including refractory chronic migraine, while an RCT investigating the use of propranolol with topiramate for chronic

migraine showed no benefit of the combination over topiramate alone. [63,64,65] Given the effects of topiramate on mood and the high comorbidity rates of chronic migraine and depression, it might not be considered the drug of choice for all patients.
Single RCTs showed efficacy of other preventive medications in CM treatment: candesartan, amitriptyline, sodium valproate, gabapentin and tizanidine. Smaller, mostly open-label, studies support the effectiveness of memantine, pregabalin, milnacipran, atenolol and zonisamide. In one small, open-label, study duloxetine improved the number of headache days per week and depressive symptoms in 30 patients with CM and comorbid depressive disorder. These drugs appear to have a common effect of suppression of cortical spreading depression. Chronic use of topiramate, valproate or propranolol has been demonstrated to reduce cortical spreading depression in rats. Suppression of cortical spreading depression by these agents is correlated with the dosages and the duration of treatment. Not all patients with CM improve with the above mentioned oral preventive medications and adverse effects can be observed.
Neuromodulatory methods can be useful in patients with otherwise intractable CM. Peripheral neuromodulation methods include pharmacological blockade of the greater occipital nerve (GON) and electrical stimulation of occipital nerves, supraorbital nerves or vagal nerve. Central neuromodulation methods include transcranial magnetic stimulation (TMS) and transcranial direct current stimulation (tDCS). [68]
Nonpharmacological therapies (biofeedback, exercise, cognitive therapies, stress management, manual therapy, electroceutics) have also been used to treat chronic migraine, but few well-controlled clinical trials have evaluated their efficacy. [68]
In the last few years we have seen the development of four monoclonal antibodies targeting CGRP or its receptor (CGRP-mAbs). Such antibodies have been tested successfully in animal models and are currently in phase 3 trials in the US. The major concern is that blocking CGRP may cause ubiquitous vasoconstriction with consequent cardiovascular effects, including medication-induced hypertension, interactions with the efficacy of anti-hypertensive drugs and coronary vasoconstriction. Biological effects within other organ systems typical of all mAbs can also be expected . Infusion and immunological reactions are further potential adverse events. Moreover, the long half-lives of mAbs could prevent immediate clearance in case of severe adverse effects. OnabotulinumtoxinA (BoNT-A) is, to date, the only treatment specifically approved for CM.

1.5 BOTULINUM TOXIN: HISTORICAL ASPECTS

The first report on botulism could be dated 1820, when Kerner published a series of 76 cases of "sausage poisoning" and described a clinical syndrome which is now recognized as botulism. [1]
In 1897 van Ermengem first identified a toxin produced by an anaerobic bacillus as the responsible for this syndrome but the exact mechanism of action was clarified only after the 2nd World War. [2,3]
In 1980 the ophtalmologist Scott published the first report of therapeutic use of BoNT- A for strabismus.
BoNT-A is derived from the anaerobic spore-forming gram-positive bacteria Clostridium botulinum. Seven toxin serotypes (A–G) are known but only serotypes A and B are used medicinally.
Food and Drug Administration (FDA) approved its use in 1989 for two therapeutic indications: blepharospasm and strabismus. At present BoNT-A is approved by the FDA for eight therapeutic and two cosmetic indications and is globally approved in more than 85 countries for at least 27 indications [4] including cervical dystonia, severe primary axillary hyperhidrosis, upper limb spasticity, blepharospasm, strabismus, overactive bladder, urinary incontinence from neurogenic detrusor overactivity, and CM. Over 60 randomized placebo-controlled trials in the last 25 years proved the safety and efficacy of BoNT-A across these indications. [4]

BoNT- A is popularly known for cosmetic in temporary treatment of moderate to severe glabellar wrinkles associated with corrugator and/or procerus muscle activity and moderate to severe lateral canthal ("crow's feet") wrinkles associated with orbicularis oculi activity.
An effect on intensity and frequency of headache was first observed in patients treated for cosmetic purposes in 1990. Efficacy of BoNT-A in patients with frequent migraine was first reported in an open label study in 1998 [5,8] and the first peer-reviewed publication appeared in 2000 [9] but it was only in 2010 that FDA approved its use in CM.
Laboratory research conducted between 2005 and 2011 hypothesized an antinociceptive action of BoNT-A. [10,11] These findings encouraged exploratory studies for migraine and other headache subtypes. [12,13,14,15,16] Phase II results showed that the drug was well tolerated and the most consistent efficacy was in patients with high frequency headache and with migraine characteristics. Because of the severity and complexity of this kind of patients, they were previously explicitly excluded from clinical trials but, on the basis of these results, a selected population of patients with transformed migraine (later renamed CM) underwent phase III clinical trials (PREEMPT 1 and PREEMPT 2) between 2006 and 2010. [6,7]
In 2014 Blumenfeld and colleagues initiated a phase IV multicenter trial for evaluation of long term efficacy, safety and tolerability of BoNT-A as a prophylactic treatment for CM: the Chronic migraine BoNT-A Prolonged Efficacy open-Label (COMPEL) study. [17] In addition, a phase IV long term study using real-world medical records data (CLARITY) and a phase IV in adolescents with CM receiving BoNT-A (NCT01833130) were initiated between 2012 and 2014 and are still ongoing. [18]

1.6 RATIONALE FOR USE OF ONABOTULINUM TOXIN A IN CHRONIC MIGRAINE

The beneficial effect of BoNT-A in CM may be attributed to its antinociceptive effect. Changes in the glutamate and CGRP at the peripheral nerve endings reduce peripheral sensitization, which eventually leads to reduced central sensitization. [35]
Botulinum toxin acts as a protease. Its specific target is soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein. The SNARE proteins are essential for the fusion of synaptic vescicles and include synaptosomal-associated protein of 25 kDa (SNAP-25), target membrane proteins, syntaxin and vesicle-associated membrane protein (VAMP)/synaptobrevin. Botulinumtoxin serotypes A and E cleave SNAP-25 at two different sites. By blocking fusion of synaptic vescicles botulinum toxin prevents the release of acetylcholine at nerve endings and this mechanism is recognized as responsible for botulinum toxicity.
A similar mechanism has been suggested in nociceptive neurons, with consequent inhibition of both release of neuropeptides (substance P, CGRP and glutamate), and expression of TRPV1. Through this process, BoNT-A may inhibit neurogenic inflammation and peripheral sensitization. [78] Recent studies have suggested that BoNT-A is effective for the treatment of different clinical conditions that present with neuropathic pain. [120] According to the American Academy of Neurology BoNT-A is effective (Level A) in postherpetic neuralgia, trigeminal neuralgia, and spinal cord injury-induced neuropathic pain, while it is probably effective (Level B) in post-surgical neuralgia, diabetic neuropathy, and central post-stroke pain. A large and well-designed blinded and randomized controlled trial is recommended in occipital neuralgia, CRPS, and phantom limb pain. [120]
However, the entire process by which BoNT-A exerts pain relief is not yet clear.
BoNT-A inhibits the release of neurotransmitters involved in pain and inflammation in animal models of formaline-induced pain.[79,80] In cultured embryonic rat dorsal root ganglia neurons the release of substance P is inhibited by with different sensitivities for each serotype of BoNT and higher sensitivity to BoNT-A, suggesting differences in receptor affinity between the various serotypes. [82] Substance P secretion was inhibited after four hours and this effect lasted for up to 15

days. A similar effect was seen on the release of CGRP. [81] In a guinea pig formalin-induced pain model BoNT injection showed to reduce glutamate concentrations compared with an injection of saline. In a synaptosome preparation from the cerebral cortex of a guinea pig BoNT also inhibited glutamate secretion. [84,85]

An additional effect of BoNT is to inhibit the SNARE-mediated translocation of TRPV1 to neuron cell membranes. TRPV1 is known to intensify the excitability of nociceptors in response to noxious stimuli (heat, proalgesic substances) and also to play an important role in the processing of peripheral thermal and inflammatory nociceptive input. On the other hand, proalgesic agents can up-regulate TRPV1 expression and channel activity. [83,84,85]

Paterson and colleagues demonstrated that BoNT-A induces a selective increase in mechanical pain threshold without any effect on thermal detection or pain threshold.[106] Such effect was evaluated even at the cellular level on cultured mouse dorsal root ganglion neurons in which exposition to BoNT-A did not induce changes in rapidly and intermediately adapting, mechanically activated inward currents but significantly decreased slowly adapting, mechanically gated current, which is believed to be linked to noxious mechanosensation. The responsible channels for this current are not known yet. It is hypothesized that BoNT-A could block vescicular trafficking of mechanosensitive channels, whose expression could be upregulated by inflammatory mediators in sensory neurons. [106,107]

Xiao *et al.* discovered a further mechanism of action of BoNT-A on purinergic $P2X_3$ receptor, a transmembrane channels/receptor expressed on nociceptive neurons. They demonstrated nerve transaction- induced over-expression of $P2X_3$ receptors in the dorsal root ganglion of the rats associated with bilateral mechanical allodynia, both reverted by subcutaneous intraplantar injection of BoNT-A. [108]

Besides the peripheral actions on first order nociceptive neurons, a trans-synaptic transport within central nociceptive neurons.[112] Antonucci and colleagues provided biochemical evidence that BoNT-A can cleave SNAP-25 distant from an injection site [109] and there are studies reporting bilateral effects of BoNT-A after a unilateral injection. [110,111,112] Bach-Rojecky et al found bilateral improvement in mechanical and thermal hyperalgesia lasting for more than 15 days induced by monolateral BoNT-A injection in a rat model of streptozocin-induced diabetic neuropathy. [110] The same authors demonstrated an axonal transport of BoNT-A in a “mirror pain” model. [109] Unilateral injection of acidic saline. induced bilateral hyperalgesia of presumably central origin, which was bilaterally reduced by unilateral subcutaneous injection of BoNT-A on the ipsilateral side. The same effect was seen when it was injected into a proximal region of a distally transected sciatic nerve. In an animal study, monolateral injection of BoNT-A induced bilateral analgesic effect in rats with paclitaxel-induce peripheral neuropathy. [111] These data support a central effect of BoNT-A, that cannot be explained neither by actions on peripheral nerve endings nor by hematogenous diffusion, as the dose administered in experimental settings was not sufficiently high to induce systemic side effects and the protein is not sufficiently small to pass through the blood-brain barrier. [112]

An axonal transport of BoNT-A from the periphery to the CNS has been hypothesized. This theory, however, is still matter of debate. [112] Wiegand *et al.* injected [125] I-BoTN/A unilaterally into the gastrocnemius muscle of a cat to trace the toxin and could observe radioactivity was first in the sciatic nerve and then in the ipsilateral spinal ventral roots and the ipsilateral spinal cord segment until 48 h after injection suggesting the possibility of axonal transport of the toxin. However, this finding does not prove that the enzymatically active toxin reached the CNS. [113] The studies of Marinelli and colleagues on chronically injured sciatic nerves of mice revealed immunostained cl-SNAP-25 from the peripheral nerve endings to the spinal cord in response to peripheral injection of BoNT- A. [115] *In vitro* experiments on Schwann cells in proliferative state showed that BoNT-A is able to modulate the proliferation these cells, inhibiting the acetylcholine release. These results support the retrograde transport of the BoNT- A along the nerve. [122] Restani *et al.* detected BoNT-A induced cleavage of SNAP-25 in retinal neurons of adult rat at optic tectum, which is distanced

from the injection site. This is consistent with a possible effect of the retrograde trafficking of BoNT-A. [121]

BoNT- A induced reduction of neurogenic inflammation in peripheral neuropathies has also been suggested [117] and common peripheral mechanisms for antinociceptive and anti-inflammatory activities of BoNT have been proposed. [118] The anti-inflammatory effects were thought to be due to reducing the release of peripheral neurotransmitters and inflammatory mediators. [117] However, injection of BoNT-A was injected into an animal model of carrageenan-induced hyperalgesia [111,118] did not show any effect on local tissue inflammatory edema or plasma protein extravasation suggesting a dissociation between the antinociceptive and anti-inflammatory effects. A study by Cui *et al.* suggested that the anti-inflammatory effects of BoNT could be dose-dependent, unlike its antinociceptive effects and experimental studies in humans showed similar results. [119] While capsaicine-induced pain was be reduced only into a BoNT-A-pretreated area, capsaicine-induced neurogenic inflammation was reduced even when capsaicin was injected adjacently into a toxin-pretreated region where pain was not reduced. Therefore, the relationship between the anti-inflammatory and antinociceptive effects of BoNT-A is still controversial.

Based on such premises and given that chronic migraine pathogenesis is complex and still poorly understood, it is quite hard to define the exact mechanism of action of BoNT-A in chronic migraine. As for neuropathic pain, an antinociceptive action via peripheral mechanisms with direct inhibition of peripheral sensitization by attenuating neuropeptide and neurotransmitter exocytosis from peripheral sensory neurons and indirect reduction of central sensitization can be postulated. [86] The above mentioned effect of BoNT-A on mechanical nociception to suprathreshold stimuli mediated by reduced exocytosis of TRPV1, TRPA1, and ATP-gated P2X3 receptors has been demonstrated even in peripheral trigeminal neurons [123], as well as its effect on capsaicine-induced inflammation and pain in humans. [124,125]

However, even in chronic migraine a pure peripheral mechanism of action has been questioned. [86] In an animal model of formalin-induced pain, the analgesic effects of peripheral BoNT-A injection were completely reverted by colchicine injection into the trigeminal ganglion. [114] Furthermore, immuno-histochemical studies revealed the presence of truncated SNAP-25 in trigeminal nucleus caudalis. [114] These findings support the hypothesis that BoNT-A undergoes axonal transport via trigeminal sensory neurons and can affect second order sensory nociceptive nuclei into trigeminal spinal nucleus. [114, 86]

1.7 LASER-EVOKED POTENTIALS

Laser Evoked Potentials (LEPs) is the currently accepted neurophysiological method for assessing nociceptive pathways according to EFNS guidelines on neuropathic pain assessment. [89]

The sensation perceived after laser skin stimulation is made up of a first components, similar to a pricking sensation (related to Aδ -fibre activation), and a second one, more diffuse and burning (related to C-fibre activation). The resulting afferent input is conducted through the spinothalamic pathway. [90,91,92]

Although laser pulses concomitantly activate Aδ and C-fibres, the corresponding brain evoked potentials remain strictly limited to the Aδ component, without any response consistent with C-fibre activation. [99] Even small C-fibres activation generates a corresponding cortical component which is however difficult to reproduce because of central inhibitory interaction with Aδ-fibre mediated component.

Aδ LEPs consists of two components: a lateralised component N1 and a vertex potential consisting of a N2-P2 complex. The N1- LEP component is a small negative potential recorded at temporal sites, generated in the in the opercular cortex (SII area and insula) bilaterally. N1 is followed, about 50 ms later, by the larger biphasic potential N2-P2. The N2-LEP component is believed to reflect neuronal activity in insular networks and possibly the anterior cingulate cortex while the P2-LEP originates from the anterior cingulated cortex alone. [93,94,99]

N2-P2 complex is mainly used in clinical practice [99] while the N1 component is less easy to reproduce requiring more trials and is mainly used in experimental settings.
LEP recordings after trigeminal stimulation are easier and quicker to obtain than those after stimulation of the limb extremities because of a higher receptor density in the facial skin and a shorter conduction distance. Lower signal dispersion along a shorter distance yields a highly synchronised volley that exerts a strong spatial and temporal summation at central synapses and thus provides higher amplitude responses. Lower-threshold and higher-amplitude LEPs after facial stimulations are reported in all studies dealing with trigeminal LEPs. [96,97,98,105]
Use of neurophysiological methods exploring nociceptive pathways may improve knowledge of the functional changes subtending pain processing in the different forms of headache and facial pain as well as in chronic pain syndromes. [98,104,105]
In migraine patients a normal amplitude of basal LEPs with reduced inter-critical habituation to repetitive multimodal stimuli and altered attentive modulation has been observed. This seems to express a general dysfunction of cortical pain processing, which may contribute to migraine chronification. [100,101,102,103]
Trigeminal LEPs have been used measure the effects of symptomatic and preventive treatments on pain pathways in EM patients. [101,102] These patients show increased N2-P2 amplitude during migraine attack reverted by both almotriptan and lysine-acetil salicylate. [101] Moreover preventive treatment with topiramate seems to normalize the N1 habituation pattern suggesting modulating action on cortical processing of the sensory-discriminative component of pain stimuli. [102]
In medication overuse headache recovery of reduced habituation has been observed after detoxification. [103]
Di Tommaso and colleagues found a trend for an excessive baseline increase in laser evoked potentials (LEP) N2-P2 amplitudes in a group of 25 CM patients. As it is postulated that N2-P2 complex is originated in insula and anterior cingulate cortex the authors interpreted these results as evidence for a functional reorganization of the cortical pain matrix due to the attack repetition, a phenomenon similar to that described in other long-lasting pain conditions. Moreover, in a brain-mapping analysis of the cortical source of P2 peak after supraorbital laser stimulation, the same authors found greater activation of the rostral portion of the anterior cingulate cortex in CM patients compared to EM and normal controls, which resulted to be significantly correlated with headache frequency, but not with other clinical features or with greater self-evaluated depression and anxiety. [46,47]

2. METHODS

2.1 STUDY DESIGN

Our prospective clinical and neurophysiological study aims to evaluate pain processing modifications in CM patients after three BoNT-A administration in pericranial muscles at 12 weeks intervals, according to PREEMPT paradigm, by means of CO2 LEPs obtained by the stimulation of the skin over both the supraorbital and the perioral region, and also to correlate main LEPs findings with clinical outcome after 6 months of BoNT-A treatment.

The skin of periorbital area shares a common peripheral innervation with injected muscle areas of anterior face, that is the ophthalmic division of fifth cranial nerve. Therefore, a peripheral action of Bont-A on first order nociceptive neurons could account for any modification of LEP components after stimulation of this territory.

On the other hand, perioral region is innervated by a different trigeminal division and in is distant enough from injected areas to rule out a peripheral effect of Bont-A.

We enrolled 16 patients (11 F, 5 M, aged 28-56) with CM undergoing treatment with BoNT-A, at the outpatient service of the Department of Human Neurosciences, "Sapienza" University of Rome.

Inclusion criteria were a diagnosis of CM according to ICHD3b diagnostic criteria and consent and ability to participate to the study.

Exclusion criteria were other neurological diseases and cognitive disturbances, as assessed with clinical history and examination.

Patients enrolled received three BoNT-A injection session at 12 weeks intervals, according to PREEMPT schedule.[6,7]

At baseline, detailed information on life-style, behavioral, socio-demographic and clinical migraine features were assessed in each participant via face-to-face interviews using a semi-structured questionnaire. [86]

Patients were seen at follow up visit every 3 months up to the end of the trial.

All patients were asked to exhibit their headache diary with recording of headache days, intensity and duration of pain, use of analgesics at baseline and at follow-up visits after 3 and 6 months. MIDAS and HIT-6 questionnaires were used to assess migraine-induced disability.

LEPs after supraorbital and perioral stimulation were performed in each patient before the first injection and after the second and third injections (after 3 and 6 months).

During the study period patients were asked to maintain stable all the concomitant treatments and to avoid analgesics at least 48 hours before LEP recording.

The study was approved by the local Institutional Review Board.

The number of headache days per month and MIDAS and HIT-6 score were assessed as clinical outcome variables before the first BoNT-A injection and after 3 and 6 months.

The amplitude of the main LEP components after supraorbital and perioral stimulation were assessed at the same time as neurophysiological outcome variables

Sample design was based on the number of headache days/month reported on headache diary.

Patients reporting < 15 headache days/month at 6 moths follow-up time were considered as "Responders" (R). The other patients were considered as "Non Responders" (NR).

2.2 INJECTION PROTOCOL

Our BoNT-A injection protocol was strictly adherent to PREEMPT Paradigm, defined in the PREEMPT 1 and PREEMPT 2 trials. [6,7]

Therefore, muscle selection, dose, and treatment interval were fixed for all patients. According to PREEMPT Paradigm, a trial of two treatments, 12 weeks apart, was performed with a further re-treatment after 12 weeks.

A fixed dose of 155 U was delivered in 31 fixed sites across 7 specific head and neck muscles areas adjacent to the peripheral nerve distribution of the trigeminal, occipital, and cervical sensory nerves.[87,88]The injection volume for each site was 0.1 mL (equivalent to 5 Units).
Patients were placed in a sitting position. Before injection each muscle area was carefully evaluated for individual anatomical variations function and potential effects of treatment (eg, weakening). Each muscle was visually inspected and palpated and patients were asked to activate the muscle.[88]

Injected areas at anterior face were corrugator, procerus, frontalis and temporalis muscles. (Fig.1)

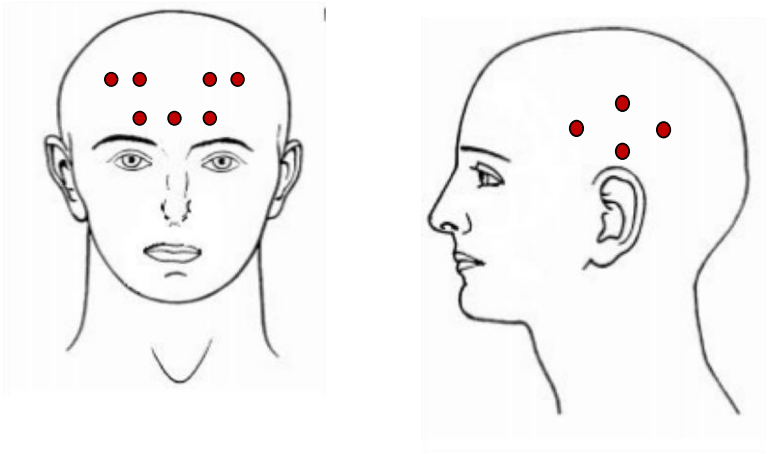


Fig.1

Corrugator muscle, crossed by supraorbital and supratrochlear nerves, attaches to the nasal-frontal bone medially and the skin of the eyebrow laterally. It is a brow depressor so its activation pulls the brow downward and creating vertical lines between the brow while its weakening may elevate the brow. According to standard PREEMPT protocol a total of 10 Units divided between 2 sites were injected about 1.5 cm (1 fingerbreadth) above the medial inferior edge of the superior orbital rim with possible variations based on individual anatomy. Patients were asked to furrow the brow in order to activate muscle, which was palpate, pinched and held between the thumb and index finger before injection, which was performed at a 90° angle into the belly of the muscle, remaining above the periosteum, to avoid medication spreading into a nearby muscle. (Fig.2)

(Fig.2)

Procerus muscle, crossed by supra- trochlear nerve, originates from the aponeurotic fascia of the nose and inserts into the glabellar skin. It draws down the medial aspect of the brow so its activation creates a transverse ridge over the nose. According to standard PREEMPT protocol a total of 5 Units in 1 site were injected at the base of the procerus, approximately midway between the 2 corrugator injections. Patient were asked to furrow the brow in order to use the vertical and horizontal lines as orientation sites. Injection was performed into the belly of the muscle at 90o to avoid medication spreading into a nearby muscle.

Frontalis muscle originates from the epicranial aponeurosis, and attaches distally to the skin of the forehead and eyebrow. It is a brow elevator, pulling the brow upward so its activation creates transverse lines on the forehead while its weakening may result in brow ptosis or worsen a preexisting ptosis. The supratrochlear and supraorbital nerves cross this muscle at their exit points through the supratrochlear and supraorbital foramen and then branche superficially providing sensation to the forehead and anterior border of the ramus of the mandible. According to standard PREEMPT protocol a total of 20 Units divided between 4 sites were injected. Two injection sites were identified on each side of the head: medial and lateral. Medial injection site is generally within the upper one-third of the forehead, and at least 1.5 cm (1 fingerbreadth) above the corrugator injection site with possible variations according to individual anatomy. It can be visually pinpointed drawing a vertical line up from the medial inferior edge of the superior orbital rim. Lateral injection site is parallel, lining up with the lateral limbus of the cornea, at least 1.5 cm (1 fingerbreadth) lateral to the medial injection site with possible variations based on individual anatomy. Injection was performed at 45° in the most superficial aspect of the muscle to avoid the periosteum.

Temporalis muscle originates from the temporal fossa and deep layer of the temporal fascia, and inserts into the top and medial surface of the coronoid process of the mandible. It is a masticatory muscle so it is activated by clenching the teeth. The auriculotemporal and zygomatico-temporal cutaneous nerve branches of the trigeminal nerve pass through this muscle. According to standard PREEMPT protocol a total of 40 Units divided between 8 sites were injected. On each side of head 4 injection sites were pinpointed. The first injection site was found 3 cm (2 fingerbreadths) above the tragus of the ear vertically up the side of the head. For the second injection site one moved about 1.5 cm to 3 cm 1-2 fingerbreadths) up from the first one, still in line with the tragus of the ear. The

third injection site was found about 1.5 cm to 3 cm (≈ 1-2 fingerbreadths) forward, toward the face, from the first and second injections, halfway vertically between the two. For the fourth injection, one moved about 1.5 cm (1 fingerbreadth) back from the second one, in line with the helix of the ear. Injections were made in the most superficial aspect of the muscle at 45°, after aspiration to ensure no blood return, strictly within the hairline and with the needle angled posteriorly. (Fig.3)

Fig.3

Injected areas at posterior head and neck were muscles occipitalis, cervical paraspinal and trapezius muscle. (Fig. 4)

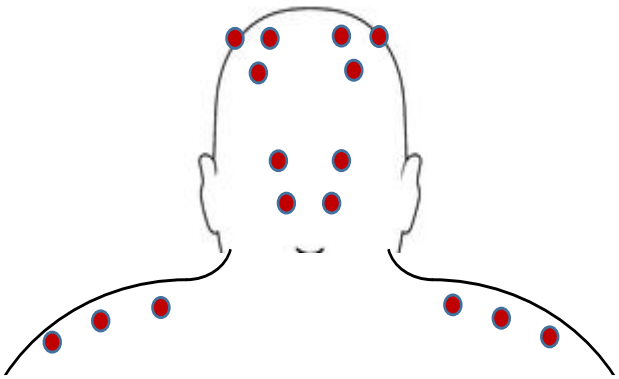


Fig. 4

Occipitalis muscle originates at the highest nuchal line and inserts into the epicranial aponeurosis, which is attached to the frontalis muscle. One function of the occipitalis is as an anchor for the frontalis. The greater occipital nerve lies medial to occipitalis, and the lesser occipital nerve lies on its lateral aspect. According to standard PREEMPT protocol a total of 30 units divided between 6 sites (3 on each side) were injected. On each side the occipital protuberance was palpated to find the most posterior point (inion) in the midline and the tip of the mastoid process was located behind the

ear. The first injection was placed just above the nuchal ridge at the midpoint of inion and mastoid process. For the second and third injection a diagonal fingerbreadth was measured up and lateral and up and medial respectively. Inject the most superficial aspect of the muscle, which will be just upon penetration of the dermis. Injections were performed in the most superficial aspect of the muscle, at 45°, angling the needle upward and away from the neck and above the nuchal ridge in order to avoid neck pain and weakness. (Fig.5)

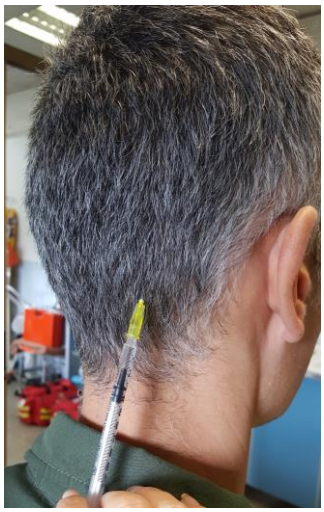


Fig.5

Cervical paraspinal muscles should be considered a group (including the splenius capitis and semispinalis capitis) running deep alongside the cervical spine, stabilizing and allowing for movement of the head and cervical spine. This group of muscles is crossed by both the third occipital nerve, near the mid- line, the greater and lesser occipital nerves, laterally.According to standard PREEMPT protocol a total of 20 Units divided into 4 sites (2 on each side) were injected. Patients were positioned upright, with the head in a neutral position. On each side the first injection site was pinpointed about 1 cm left of the midline of the cervical spine and about 3 cm (2 fingerbreadths) inferior to the lower border of the occipital protuberance. The second injection site was located about 1.5 cm (1 fingerbreadth) diagonally up at a 45o angle laterally. Injections were performed in the most superficial aspect of the muscle, angling 45°, in the hairline, above the line across the neck about 2 fingerbreadths down from the occipital protuberance in order to minimize the risk of neck weakness.

Trapezius is a flat, triangular muscle situated over the back of the neck and upper thorax that contributes to stabilize and bend the head and neck backward and laterally. The sensory rami of C2, C3 and C4 run across this muscle. According to standard PREEMPT protocol a total of 30 Units divided between 6 sites (3 on each side) are injected. On each side the upper portion of the muscle was divided in half, from the inflection point of the neck (necklace line) to the acromioclavicular joint and the first injection was located at this midpoint. The second and third injection points were located splitting the difference between this point and the acromioclavicular joint and the necklace line respectively. Injections were performed horizontal to the muscle to avoid injecting too deep.

2.3 LEP RECORDING PROCEDURE

The staff members recording LEPs were different from those carrying out BoNT-A treatment.

We used a stimulator Neodimium:Yap laser (Neurolas, Electronic Engineering, Florence, Italy), which is a solid-state laser. Such a kind of laser allows the use of shorter pulses than those delivered by CO2 lasers, enhancing the amplitude and shortening the latency of cortical responses.
To determine laser perceptive threshold (PTh) we delivered series of stimuli at increasing and decreasing intensity. PTh was defined as the lowest intensity at which the subjects perceived at least 50% of the stimuli. The stimulus intensity we used to record brain LEPs was 1.5—2 times the mean pain threshold.
Brief radiant heat pulses (intensity 76-127 J/cm2, duration 5 ms, beam diameter 5 mm) were delivered to the skin of the ophthalmic division (supraorbital region) and maxillary/mandibular divisions (perioral region) of the right side. This caused a clear pinprick sensation, mediated by Aδ afferents, and produced a subjective rating of at least 4 on a 0–10 numeric rating scale (NRS) (0 = no sensation, 10 = worst possible pain). To avoid skin burns, nociceptor fatigue and central habituation the laser beam was shifted slightly after each stimulus and the inter-stimulus interval (10–15 s) was varied pseudo-randomly.
Participants laid on a couch and wore protective goggles. They were instructed to keep their eyes open to minimize alpha contamination and gaze slightly downwards. We used tasks such as counting the number of stimuli to keep subject's attention level constant. Laser stimuli can produce some skin reddening, which disappears in a few days.
According to the recent ''Recommendations for the clinical use of somatosensory-evoked potentials'' [95], five recording disc electrodes were placed on subjects'scalp: two midline sites (Fz - Cz) referred to the nose to record the N2 and P2 components and two temporal electrodes (contralateral to each side of stimulation) referred to the midline (Fz) to record the N1 component. The early, lateralized component, N1, and the main complex, N2–P2, were recorded through disc electrodes from the temporal areas (Tc) referenced to frontal area (Fz) and vertex (Cz) referenced to the nose. Electroculographic (EOG) signals were simultaneously recorded using surface electrodes.
Ten trials devoid of artefacts were collected and averaged. We measured peak latency and amplitude (peak-to-peak) of the temporal N1 component and the N2–P2 vertex complex.

2.4 STATISTICAL ANALYSIS

Given that the amplitude of LEP components elicited after supraorbital and perioral stimulation has a normal parametric distribution, as assessed with the D'Agostino & Pearson normality test, we used parametric tests. The differences across the three LEP recordings corresponding to baseline (t0) and after the second injection at 3 months (t1) and after the third injection at 6 months (t2) were analyzed with the Repeated measures ANOVA. Since the analysis focuses on the differences between the baseline (t0) and the end of the recording session (t2), a paired t-test can be used.
P values <0.05 were considered to indicate statistical significance.

3 RESULTS

All patients completed the study. No patient reported adverse events due to BoNT-A treatment or LEP recording. In particular no systemic side effects or muscle weakness were identified. Of 16 patients, 11(70%) were considered as "Responders" (R), 5 (30%) as "Non Responders" (NR) according to the aforementioned sample design criteria.

3.1 CLINICAL EFFECTIVENESS

The measurements of number of headache days/month, MIDAS score and HIT-6 score decreased significantly ($P < 0.05$) across the examination period in all patients. These measurements at t2 showed lower mean values compared to t0. This difference resulted to be particularly prominent for number of headache days/month ($P = 0.00022$), MIDAS score ($P = 0.00035$) and HIT-6 score ($P = 0.00137$). (Fig. 6) The difference between the mean of these variables at t2 and t0 was less than 0.

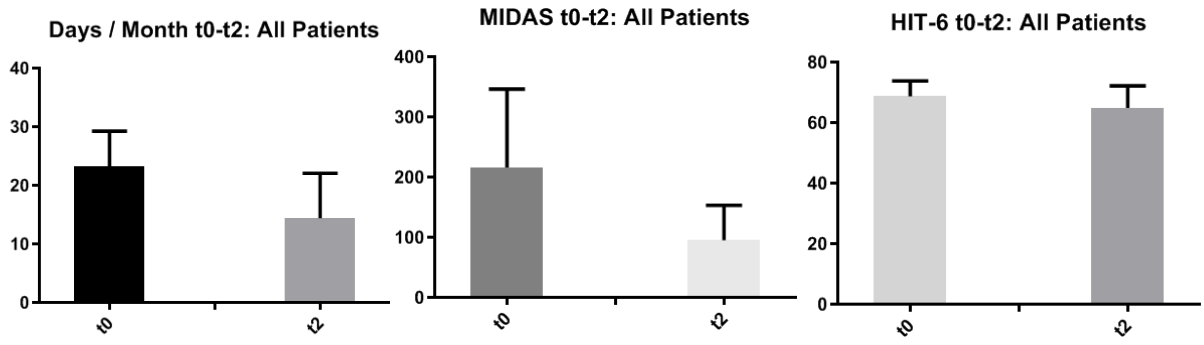


Fig. 6

Analyzing the two groups of patients separately (R/NR), we found that the number of headache days/month was significantly reduced only in the R group ($P = 0.0003$). (Fig. 6) In the NR group this variable was reduced, although not in a statistically significant fashion ($P = 0.14$). Reduction of MIDAS score from t0 to t2 was statistically significant in both groups with a more significant difference in the R group ($P = 0.00029$) compared to NR group ($P = 0.0427$). (Fig.7) A significant difference in HIT-6 score was statistically confirmed only in the R group ($P = 0,03$). (Fig. 8)

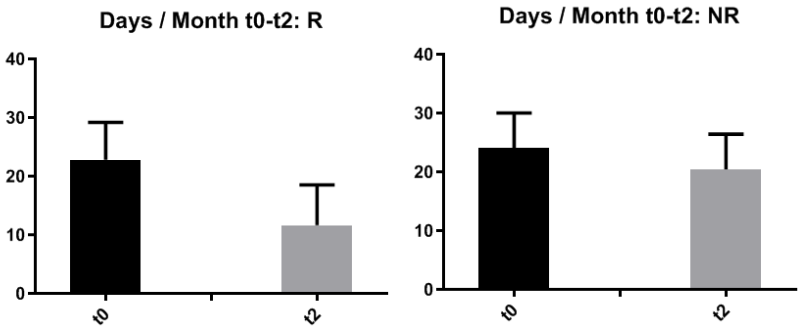


Fig.6

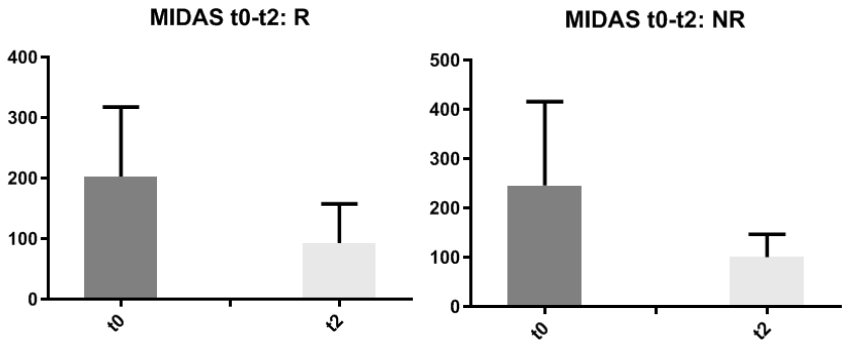


Fig.7

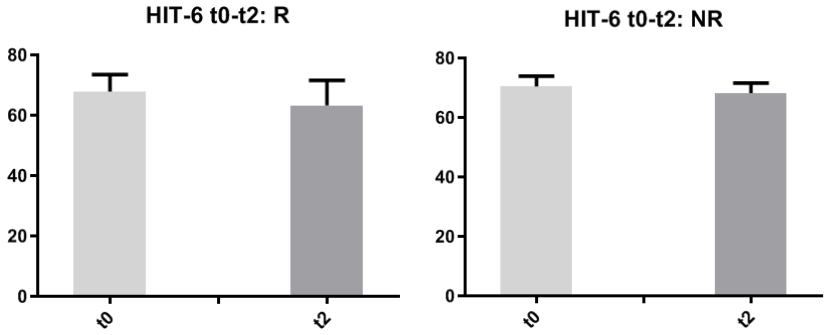


Fig.8

3.2 SUPRAORBITAL STIMULATION

In all patients, the amplitude of the N1 and N2-P2 LEP components after supraorbital stimulation significantly decreased across the three LEP recording sessions ($P < 0.05$). Paired t-test showed lower N1 amplitude at t2 compared to t0 ($P = 0.00005$). The N2-P2 amplitude showed the same behavior with a statistically significant decrease across the period of examination. At t2 N2-P2 amplitude was markedly reduced compared to t0 ($P = 0.00017$). (Fig.9)
Separate analysis of the two groups of patients (R/NR) confirmed this trend in both groups with higher significance in the R group. The NR group showed a significant reduction of N1 ($P = 0.042$) and N2-P2 ($P = 0.00853$) amplitude at t2 compared to and t0, Fig In R group this reduction was more prominent for both N1 ($P = 0.0001$) and N2-P2 ($P = 0.0077$). (Fig.9)

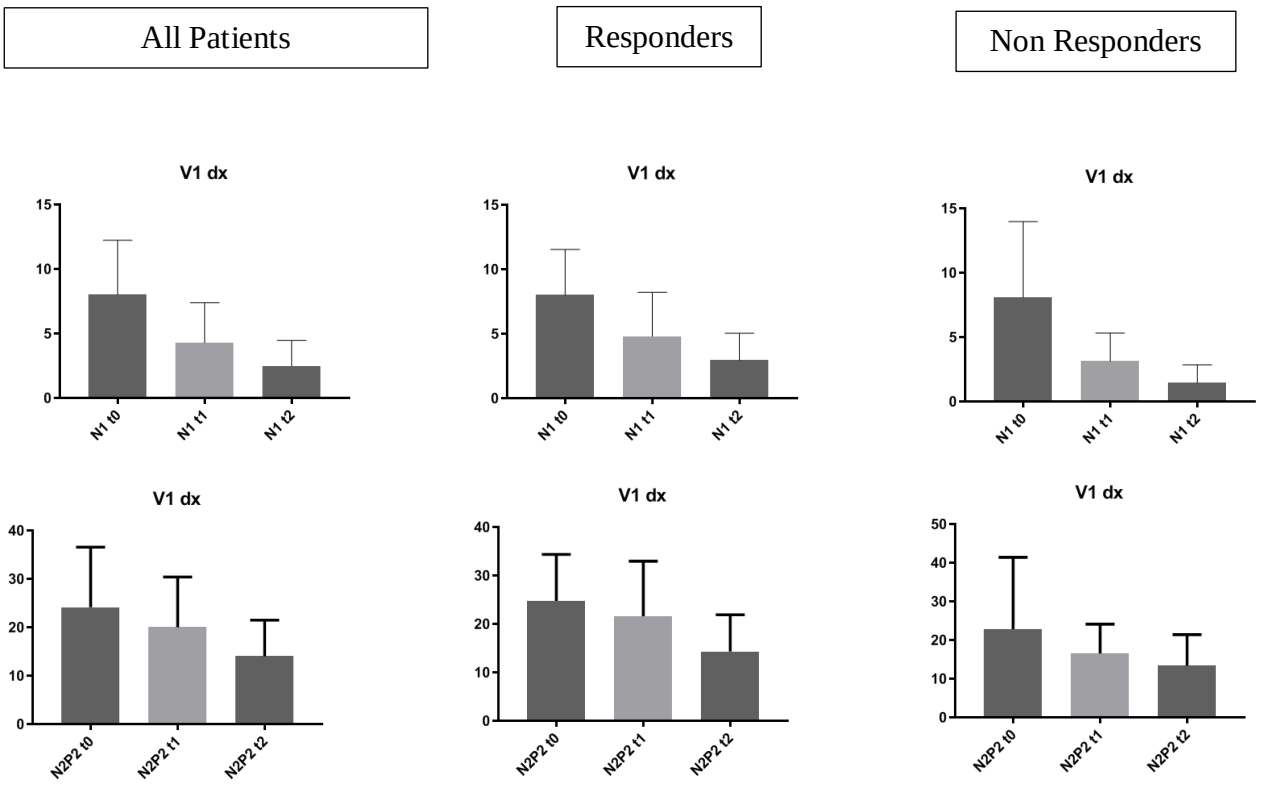
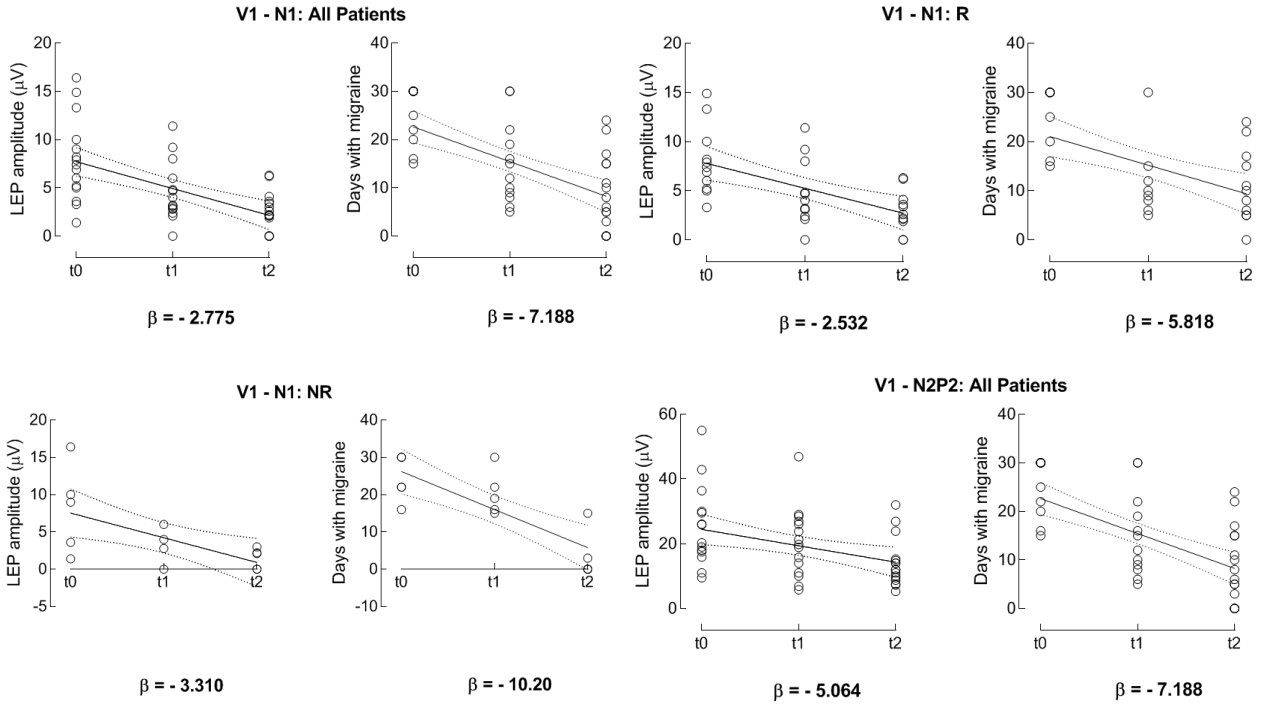


Fig.9

By performing the regression analysis of LEP amplitude and the days with migraine it is possible to evaluate difference of these two measurements across the recording sessions. All the plots are characterized by a decreasing behavior of the measurements at the different time of recording session. For each regression the slope is negative ($\beta < 0$) and statistically significant. The difference between the slopes in each pair of plots is not significant thus it can be argued that reduction of LEP amplitude and clinical improvement are concordant. (Fig.10)



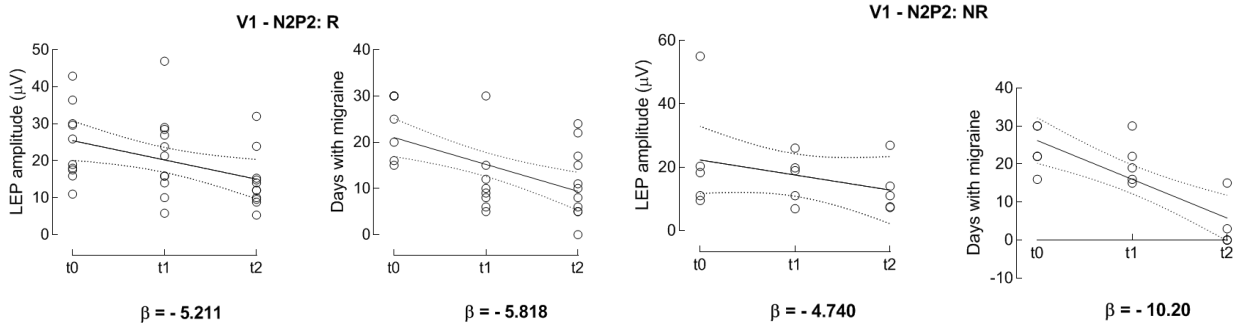


Fig.10

3.3 PERIORAL STIMULATION

Even after perioral stimulation amplitude of the N1 and N2-P2 LEP components significantly decreased across the three LEP recording sessions in all patients ($P < 0.05$). Paired t-test showed reduction of both N1 ($P = 0.0007$) and N2-P2 ($P = 0.0009$) amplitude at t2 compared to t0. fig
As for periorbital stimulation separate analysis of the two groups (R/NR) confirmed statistically significant reduction in the R group for both N1 ($P= 0.0038$) and N2-P2 amplitude ($P= 0.0021$) at t2 compared to t0. (Fig 11)
In the NR group a reduction of these variable was observed, although not statistically significant ($P= 0.07$ for N1 amplitude; $P= 0,13$ for N2-P2 amplitude) and can be graphically evinced by the barplots below. (Fig.11)

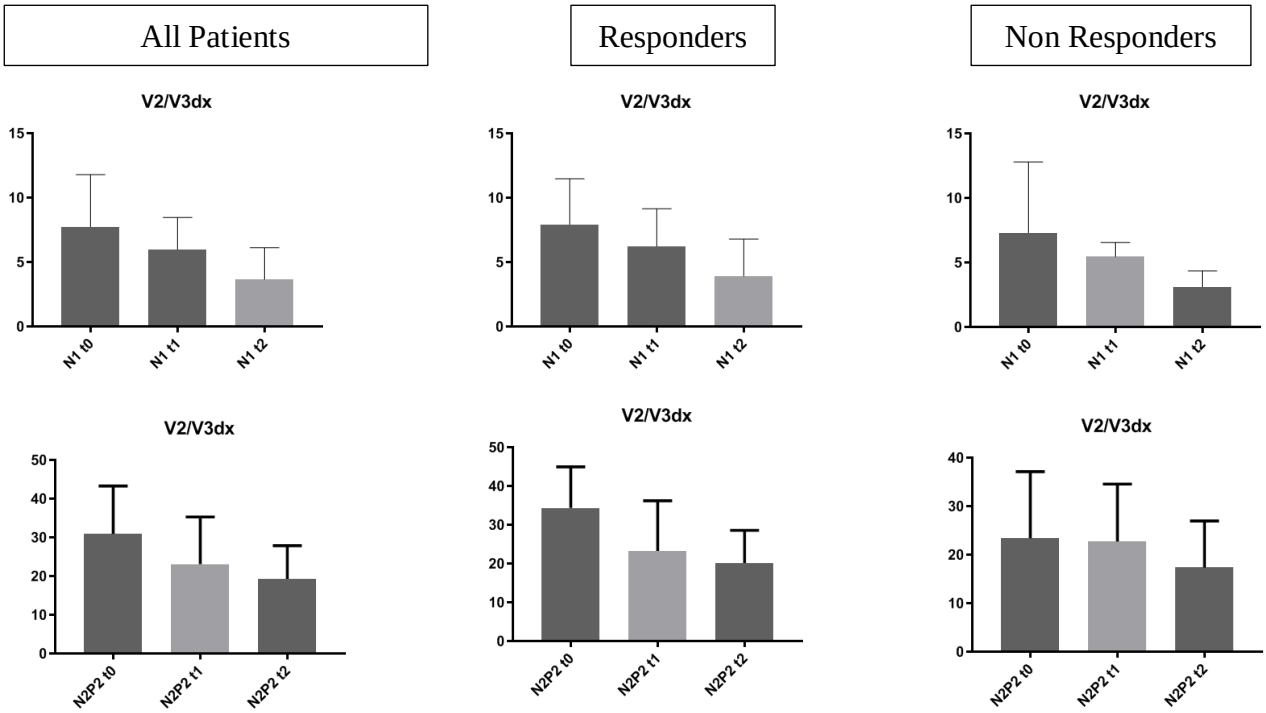


Fig.11

A joint analysis of the results above can be derived from the comparison of the both regression analysis of LEP amplitude and the days with migrain. All the plots below are characterized by a decreasing performance of the measurements at the different time of recording session. For each regression the slope is negative ($\beta < 0$) and statistically significant. The difference between the slopes in each pair of plots is not significant. (Fig.12)

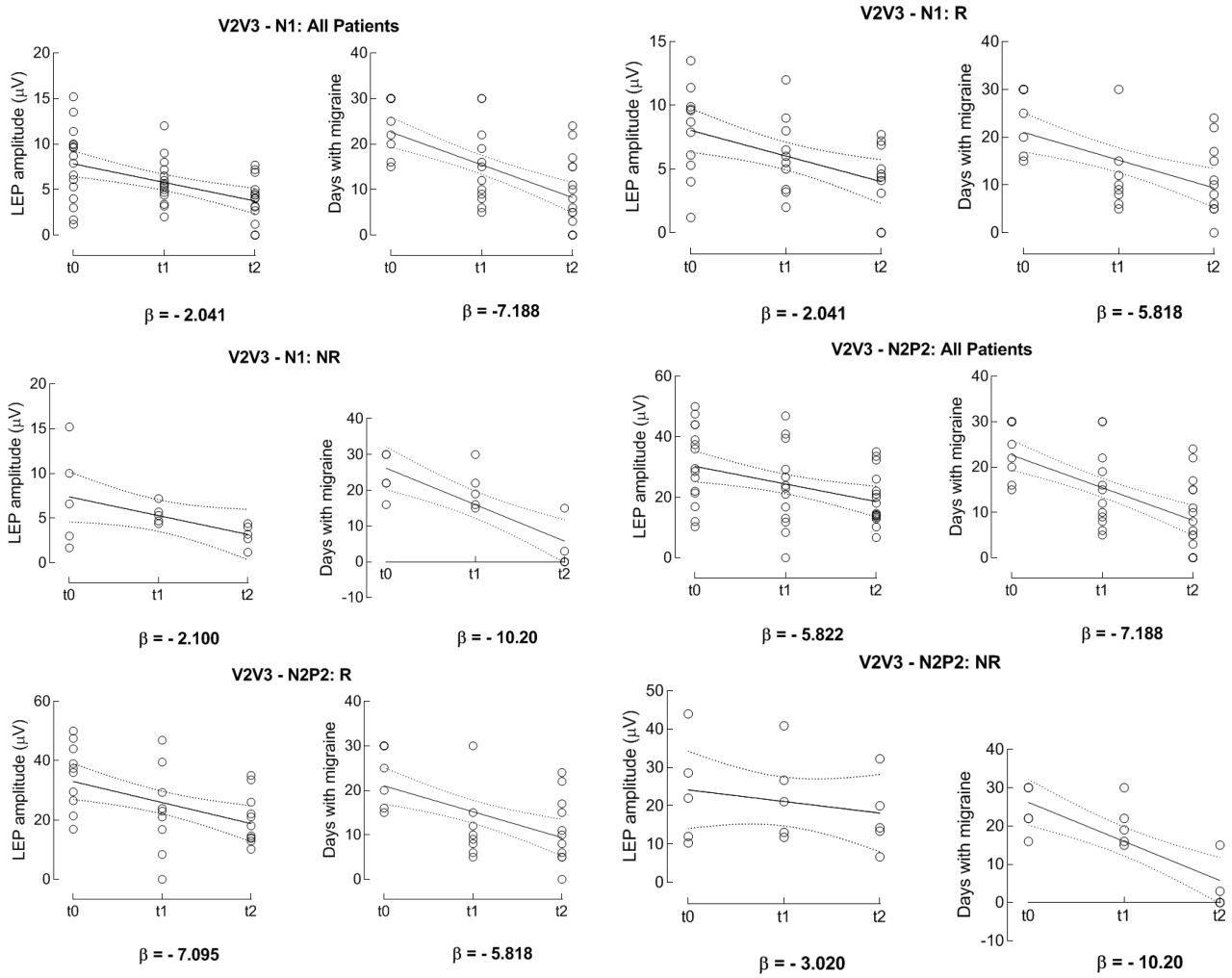


Fig.12

4. DISCUSSION

Peripheral and central sensitization into the trigeminocervical complex seems to play a crucial role in migraine chronification, as suggested by neurophysiological studies and clinical data (e.g. higher frequency of cutaneous allodynia an CM patients). A vicious circle of TRPV1 over-expression and neuropeptides over-release could, at least in part, account for this phenomenon. BoNT-A is the only FDA approved drug for CM treatment but if its exact mechanism of action is not completely understood. A direct inhibition of peripheral sensitization by attenuating neuropeptide and neurotransmitter exocytosis from first order nociceptive neurons has been strongly suggested by *in vitro,* animal and healthy human studies. However, increasing evidence exist of a possible trans-synaptic axonal transport into secondary nociceptive neurons at trigeminal spinal nucleus with consequent direct effect on central sensitization.

In this clinical and neurophysiological study in patients with CM we showed that LEP responses are reduced by BoNT-A treatment, thus objectively reflecting the antinociceptive effect of this drug. We found a LEP reduction also after stimulation of a trigeminal territory different from that injected with BoNT-A, thus suggesting that the effect of this drug is not restricted to the peripheral nervous system.

In our study we selected patients with CM to seek information on the antinociceptive activity of BoNT-A because many studies showed that this treatment is effective in reducing the frequency of migraine attacks. We used LEPs for assessing objectively the antinociceptive activity of BoNT-A because LEP recording is widely agreed as the best neurophysiological tool for investigating trigeminal nociceptive pathways. Previous studies have already used LEP recordings for clinical and research purposes in patients with migraine.

Laser stimulation activates Aδ- and C-mechanothermal nociceptors in the skin and evokes scalp potentials related to Aδ-fibres. Given that the afferent volley is conducted through the spinothalamic pathways, LEP recoding provides the opportunity to have an insight into the trigeminal nociceptive primary afferents. We found that BoNT-A injected in the territory of ophthalmic division reduced the amplitude of LEPs elicited after stimulation of the same trigeminal area. This finding is therefore compatible with the effect of the drug on trigeminal primary afferents, and agrees with previous human experimental studies suggesting that BoNT-A targets nociceptors, blocking neurotransmitter release and thus reducing pain. The comparison between the linear regression of LEP amplitude reduction and the linear regression of the reduction of days with migraine shows that LEP changes and clinical improvement were concordant, thus indicating that LEP amplitude reduction reflects the antinociceptive effect of BoNT-A.

In our study, however, we recorded LEPs also after stimulation of the perioral area, a trigeminal territory distant from the territory injected with BoNT-A. Laser stimulation of the perioral and supraorbital skin though activating distinct primary nociceptive neurones triggers an afferent volley relayed through shared nociceptive second order neurons lying in the dorsal horn of the spinal nucleus. Hence we deem our LEP findings reliably reflecting trigeminal dorsal horn function.

Our study now shows that BoNT-A modulates nociceptive-related responses evoked by stimulation of untreated region. As mentioned above, human studies reported contrasting hypothesis concerning the antinociceptive activity of BoNT-A. Whereas some experimental human studies indicated that BoNT-A reduces pain through a predominantly peripheral activity on TRPV1 other human experimental studies however reported that although BoNT-A reduces neurogenic flare evoked by capsaicin and cutaneous electrical stimulation it has poor analgesic effects. Animal studies showed

that BoNT-A has mild or no effect on acute nociceptive pain thresholds, conversely it affects predominantly pain associated with central sensitization phenomenon.

Our study provides the first neurophysiological evidence of both peripheral and central modulation of nociceptive pathway induced by BoNT-A in CM patients thus supporting the hypothesis of a pleiotropic effect of this molecule with a combined peripheral and central action.

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