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Inter-ictal assay of peripheral circulating inflammatory mediators in migraine patients under adjunctive cervical non-invasive vagus nerve stimulation (nVNS): A proof-of-concept study☆

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ABSTRACT

Objective: To assay peripheral inter-ictal cytokine serum levels and possible relations with non-invasive vagus nerve stimulation (nVNS) responsiveness in migraineurs.
Methods: This double-blinded, sham-controlled study enrolled 48 subjects and measured headache severity, frequency [headache days/month, number of total and mild/moderate/severe classified attacks/month], functional state [sleep, mood, body weight, migraine-associated disability] and serum levels of inflammatory markers [inter-ictal] using enzyme-linked immunoassays at baseline and after 2 months of adjunctive nVNS compared to sham stimulation and suitably matched controls.
Results: No significant differences were observed at baseline and after 2 months for headache severity, total attacks/month, headache days/month and functional outcome [sleep, mood, disability] between verum and sham nVNS. However, the number of severe attacks/month significantly decreased in the verum nVNS group and circulating pro-inflammatory IL-1β was elevated significantly in the sham group compared to nVNS. Levels of anti-inflammatory IL-10 were significantly higher at baseline in both groups compared to healthy controls, but not at 2 months follow-up [$p < 0.05$]. Concentrations of high-mobility group box-1 (HMGB-1), IL-6, tumor-necrosis factor-α (TNF-α), leptin, adiponectin, ghrelin remained unchanged [$p > 0.05$]. No severe device-/stimulation-related adverse events occurred.
Conclusion: 2 months of adjunctive cervical nVNS significantly declined the number of severe attacks/month. Pro-inflammatory IL-1β plasma levels [inter-ictal] were higher in sham-treated migraine patients compared to verum nVNS. However, pro- [IL-6, HMGB-1, TNF-α, leptin] and anti-inflammatory [IL-10, adiponectin, ghrelin] mediators did not differ statistically. Profiling of neuroinflammatory circuits in migraine to predict nVNS responsiveness remains an experimental approach, which may be biased by pre-analytic variables warranting large-scale biobank-based systematic investigations [omics].

Abbreviations: BDI, Beck Depression Inventory; CAP, cholinergic anti-inflammatory pathway; CGRP, Calcitonin gene related peptide; CM, chronic migraine; CRP, C-reactive protein; DAMP, damage associated molecular patterns; ELISA, enzyme-linked immunoassay; EM, episodic migraine; HMGB, high mobility group box; IL, interleukins; TNF, tumor necrosis factor; IHS, International Headache Society; MIDAS, migraine disability scale; nVNS, cervical non-invasive vagal nerve stimulation; PSQI, Pittsburgh Sleep Quality Inventory.

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Introduction

Cervical non-invasive vagus nerve stimulation (nVNS) emerged as an adjunctive treatment option for the abortive and preventive use in episodic and chronic migraine. Both uncontrolled explorative studies and randomized-controlled trials have effectively underpinned the impact and safety of nVNS for the treatment of migraine and other primary headache disorders with a more favorable responsiveness for episodic migraine subtype [1–4]. In addition, several explorative human studies determined a variety of neuro-inflammatory markers in serum (jugular or peripheral vein) and found ictally increased interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, endocan and claudin-5, in migraine patients compared to healthy controls [5–7].

In humans, the effect of surgically implanted VNS devices on the neuro-immune host response was assessed in two small-scale uncontrolled clinical studies with refractory focal seizures and refractory depression. The inflammatory assessments demonstrated significant changes in plasma cytokine levels (IL-6, IL-8, TNF-α, TGF-β) after 3 months of VNS compared to baseline [8,9]. In healthy humans, Lerman et al., applied nVNS and assessed the peripheral neuro-immune axis. Significantly suppressed levels of pro-inflammatory IL-1β, TNF-α, IL-8 and macrophage attractant peptides were quantified in the nVNS group compared to sham stimulation indicating an anti-inflammatory potential of nVNS treatment [10].

An increasing body of experimental evidence suggests that vagal nerve stimulation modulates immune response and systemic inflammation by influencing cytokine release through the cholinergic anti-inflammatory reflex, thus re-balancing the neuro-immune axis [11,12]. The parasympathetic cholinergic response, which is in part regulated by reciprocal anatomic connectivity of the afferent vagal nerve fibers and the hypothalamic-pituitary axis (HPA), impacts the neuro-immune axis. The neuro-inflammatory pathway is modulated by the interaction of pro- and anti-inflammatory mediators (cytokines, chemokines), including the interleukins (IL)-1β, IL-6, IL-8, IL-10, IL-13, interferon-γ (IFN-γ), TNF-α and HMGB-1 protein. This modulation is likely promoted by the neuro-immune interaction between peripheral immune cells, such as mononuclear monocytes, and the autonomic nervous system [13,14].

However, clinical data from human migraine studies investigating the impact of VNS on the neuro-inflammatory axis are lacking [1,15].

In this double-blinded, sham-controlled study, we firstly attempted to test the feasibility of peripheral molecular profiling based on immune-assays in refractory migraine patients treated with adjunctive nVNS. This feasibility study assessed the clinical outcome of nVNS treated migraineurs based on score-evaluation (severity, frequency, functional capacity) and on the neuro-immune host response (pro- and anti-inflammatory cytokines, adipokines) under a double-blinded sham-controlled study design.

Materials and methods

Study design

This double-blinded, sham-controlled cohort study included patients with refractory episodic migraine (EM) and chronic migraine (CM) and compared nVNS to sham stimulation. This study protocol was performed according to the guidelines of the Helsinki declaration and was approved by the Ethics Committee of the Medical Faculty, University of Bonn (No.: 259/15) and indexed at the German Register for Clinical Trials (DRKS ID 00009944) on 08.02.2016. https://www.drks.de/drks_web/navigate.do?navigationId=trial.HTML&TRIAL_ID=DRKS00009944.

Study population and clinical assessment

Patients were referred by a headache specialist (neurologist/anesthesiologist) with their diagnosis and refractory condition (defined as having failed at least four classes of preventive medications) confirmed by a multidisciplinary pain board according to the International Classification of Headache Disorders criteria (3rd edition, beta version) [16–19]. The inclusion and exclusion criteria are outlined in Table 1.

The following baseline characteristics of patients were assessed: headache severity with a visual analogue scale (VAS), headache frequency (number of headache days per month; number of total attacks/month and number of mild − moderate − severe categorized attacks/month). Depending on head pain intensity (quantified by VAS; visual analogue scale), migraine attacks were categorized as severe (severe = VAS 7-10/10), moderate (moderate = VAS 4-6/10) or mild (mild = VAS 1-3/10). Pain relief was defined as a ≥50% reduction in severity and/or frequency. In addition, relevant migraine co-morbidities such as impaired sleep quality assessed by the Pittsburgh Sleep Quality Index (PSQI), severity of depressive symptoms assessed by the Beck Depression Inventory (BDI), impact of headache on life by the Migraine Disability Assessment (MIDAS), and the metabolic state (Body Mass Index (BMI)) were recorded [20–22]. Patients were instructed to record severity and frequency on a daily basis (headache diary) within the study period. Data for all study parameters including head pain intensity/frequency were recorded from the patients' headache diaries and through interviews during the outpatient visits after two months during the inter-ictal period (48 h apart from an attack). Data of all reported and treated attacks within the two months of nVNS therapy were pooled and analyzed. Peripheral blood samples were collected from the cubital vein during the inter-ictal period (defined as 48 h from the last attack) at a defined time (08:00–09:00 a.m.).

All patient-reported migraine scores, peripheral blood collection and analysis were performed again after two months of nVNS treatment by an independent third party to assure a double-blinded design. Adjunctive medication remained unchanged four weeks prior to baseline and within the study period.

Baseline characteristics of the study cohort

The study population included 48 subjects consisting of 30 migraine patients and 18 age-/gender-matched healthy controls. Of the 30 migraineurs, 29 were female and one was male, with an average age of 46.96 years (range, 27–66 years). Episodic migraine (EM) was diagnosed in 19 patients (14 without aura; five with aura) and chronic migraine (CM) was diagnosed in the other 11 patients (five with aura; six without aura). An impaired functional state was present in the migraineurs affecting sleep architecture (non-sleep onset of attacks) in 21/30 patients (average PSQI global: 7; average MIDAS Grade: IV/MIDAS score: 54; BDI score: 15; BMI: 23.2).

Eighteen healthy controls (HC) were recruited from the local population by means of online advertisement, public postings and contacts to assisted living facilities. Subjects were free of any current physical or psychiatric illness as assessed by medical history and were assessed at baseline and follow-up (score evaluation and serum sampling for cytokine assay). HC participants did not receive nVNS treatment. Baseline assessment of the healthy control group (HC) demonstrated similar characteristics compared to the migraine group (HC group: 15 female/3 male; mean age: 43.16 years, ranging from 22 to 59 years; BMI: 24.2). Adjunctive prophylactic and abortive medication was unchanged one month prior

Table 1
Inclusion and exclusion criteria according to the study protocol.

Inclusion criteria	Exclusion criteria
➢ chronic refractory headache disorder according to the International Classification of Headache Disorders criteria (3rd edition, beta version) age equal/greater than 18	➢ no informed consent
➢ informed consent (Study, nVNS)	➢ other concomitant neuropsychiatric comorbidity not adequately classified and/or requiring specific diagnosis/treatment
➢ refractory to medical and/or behavioural therapy	➢ pregnancy
➢ eligible for vagus nerve stimulation	➢ Previously performed invasive, noninvasive and ablative procedures
➢ willingness to a defined follow-up interval	➢ not willing to complete pain diary regarding severity and frequency
➢ stable pain medication four weeks prior to nVNS	➢ intracranial and cervical pathologies confirmed by magnetic resonance scan
	➢ medication overuse headache

to baseline/nVNS initiation and remained unchanged within the entire study.

Table 2 summarizes baseline characteristics of 26 migraine patients, who accomplished the two months visit (four patients excluded due to study protocol deviation).

Determination of peripheral cytokines

Different cytokines such as IL-1β, TNF-α, IL-6 and IL-10 were quantified in serum of the control and migraine patients by enzyme-linked immunoassays. Serum IL-1β, TNF-α and IL-6 high sensitivity ELISA kits were employed to quantify the levels of these cytokines by following the manufacturer's instructions (Catalog # HSLB00D, HSTA00E and HS600B respectively; R&D Systems, MN, USA). The high sensitive ranges were 0.1–8 pg/mL, 0.2–10 pg/mL and 0.2–10 pg/mL, respectively.

Serum IL-10 was quantified by BD OptEIA™ ELISA kit from BD Biosciences (Catalog # 550613, San Jose, CA, USA) within an assay range of 2–500 pg/mL. HMGB-1 ELISA kit was supplied by IBL International (Catalog # ST51011, Hamburg, Germany) and was performed in the high sensitive range 0.313–10 ng/mL. Human total adiponectin and leptin ELISA kits were obtained from R&D Systems (Catalog # DRP300 and DLP00, Minneapolis, MN, USA), while Ghrelin serum levels were determined by ELISA kit obtained from eBioscience (Catalog # BMS2192, Bender MedSystems GmbH, Vienna, Austria). The serum levels of these adipokines were determined by following the manufacturers' instructions and were performed within assay ranges of 3.9–250 ng/mL, 15.6–1000 pg/mL and 15.6–1000 pg/mL, respectively. The lower values of the assay ranges represent the lowest standard dilutions used and the sensitivities of these kits were following: IL-1β - 0.063 pg/mL, TNF-α - 0.049 pg/mL, IL-6 - 0.11 pg/mL, IL-10 - 2 pg/mL, HMGB-1 - 0.2 ng/mL, adiponectin - 0.891 ng/mL, leptin - 7.8 pg/mL and ghrelin - 11.8 pg/mL.

nVNS stimulation pattern and randomization/blinding

The nVNS therapy was self-administered by patients twice daily (in the morning and late afternoon) bilaterally (one application on each side). Each application lasted 120 s. Patients were also instructed to administer one additional bilateral application at the onset of each headache attack in conjunction to already-prescribed acute rescue medication (one additional bilateral application was permitted after 15–30 min). The nVNS device (CE-approved;

Table 2
Baseline characteristics according to migraine subtype, gender, age, relevant co-morbidities, abortive and preventive medications. f = female; m = male; CM = chronic migraine; EM = episodic migraine; +/− = with/without aura; TRIP = triptans; TCA = tricyclic antidepressants; SS(N)RI = selective serotonin (noradrenaline) reuptake inhibitor; ACD = anticonvulsant drug (i.e. topiramate); NSAID = nonsteroidal anti-inflammatory drugs; VA = valproic acid; BB = β-blocker; DA = dopamine-antagonist (i.e. domperidone); CORT = cortisone; THC = tetrahydrocannabinol, BDI = Beck depression inventory, BMI = Body mass index, MIDAS = Migraine disability assessment, PSQI = Pittsburgh sleep quality index.

No.	Sex	Age (year)	BMI kg/m^2	Type	Attacks per month	Headache days per month	Preventive medication	Abortive medication	MIDAS score/grade	BDI score/grade	PSQI score
1	f	53	23,0	EM+	10	14	BB, VA	TRIP, NSAID	42/IV	16/II	7
2	f	49	20,8	CM+	12	20	0	0	70/IV	23/III	7
3	f	42	24,3	EM-	9	14	0	0	22/IV	8/0	9
4	f	37	27,4	EM-	10	10	0	TRIP	84/IV	2/0	3
5	f	63	19,5	EM-	3	7	0	TRIP	27/IV	4/0	9
6	f	30	19,0	EM+	12	12	0	0	174/IV	12/0	10
7	f	66	24,3	EM-	11	13	0	TRIP, NSAID	30/III	18/II	5
8	f	36	23,4	CM-	30	30	SSRI, ACD	TRIP	85/IV	15/II	6
9	f	40	20,9	CM-	10	30	SNRI	TRIP, NSAID	90/IV	19/II	6
10	f	45	19,6	EM-	3	8	THC	TRIP, NSAID	85/IV	13/0	13
11	f	52	22,3	EM+	7	7	0	TRIP, NSAID	20/III	6/0	3
12	m	27	26,2	CM-	25	17	ACD	TRIP	65/IV	17/II	4
13	f	50	18,8	CM+	16	19	0	TRIP	30/IV	8/0	9
14	f	54	23,4	EM-	12	15	0	TRIP	87/IV	15/II	9
15	f	48	22,5	EM-	12	12	0	TRIP	23/IV	19/II	8
16	f	55	23,3	EM-	15	15	BB	TRIP	60/IV	20/III	11
17	f	58	19,7	EM-	2	8	0	TRIP, NSAID	43/IV	11/0	4
18	f	38	26,5	CM-	30	30	BB	TRIP	90/IV	41/IV	15
19	f	50	21,7	EM-	12	30	0	NSAID, CORT	90/IV	36/IV	10
20	f	45	19,3	EM-	10	10	0	TRIP	37/IV	18/II	5
21	f	51	23,4	EM+	5	13	0	NSAID	25/IV	8/0	5
22	f	27	22,1	EM-	15	15	0	NSAID	30/IV	16/II	13
23	f	45	33,9	EM+	15	15	0	TRIP	25/II	14/I	9
24	f	58	22,7	EM-	12	12	BB	TRIP, NSAID	54/IV	7/0	12
25	f	41	21,3	EM-	5	13	0	TRIP	14/II	13/0	5
26	f	61	33,5	CM+	30	30	0	TRIP	13/I	6/0	5

provided by electroCore, LLC, Basking Ridge, NJ, USA) was positioned medial to the sternocleidomastoid muscle and lateral to the larynx. The constant voltage-driven device employed the following stimulation specifications: 1-ms bursts of 5 kHz sine waves, repeated every 40 ms (25 Hz) with an adjustable stimulation intensity (from 0 to 24 V). A conducting gel was applied in order to ensure transdermal signal conductivity. Prior to nVNS initiation, all participants were trained by the same independent and unblinded instructor for appropriate and standardized use of the nVNS device. nVNS sham stimulation was achieved by producing a 0.1 KHz biphasic signal that could be perceived physically without stimulating the vagus nerve or neck muscles.

The randomization schedule was designed by an independent statistician to assign study participants 1:1 to verum group with nVNS or sham stimulation. The end of the two months randomized phase was defined as month 2. Participants, principal investigators and study coordinators were blinded to treatment assignment within the randomized phase. Four out of 30 migraine patients were excluded from the final analysis (verum group: 1 device dysfunction; sham group: 1 device malfunction, 1 cold, 1 worsening of head pain requiring change of medication), hence, 26 migraine (14 verum nVNS versus 12 sham nVNS) and 18 healthy participants fulfilled the 2 months follow-up visit.

Statistical analysis

Study parameters including migraine severity (VAS), migraine frequency (headache days, total attacks, mild/moderate/severe attacks per month), functional state (PSQI, BDI, MIDAS, BMI) and peripheral levels of inflammatory mediators (IL-1β, IL-6, HMGB-1, TNF-α, leptin, IL-10, adiponectin, ghrelin) at baseline and at follow-up visit were represented as mean ± SEM. Different groups such as healthy controls, sham and verum were compared to each other by using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test unless otherwise stated. A p-value of less than 0.05 was considered significant. Pearson's correlation coefficients were used to assess linear associations between different parameters, while Spearman's correlation coefficients were determined to assess non-linear associations. The results were analyzed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA).

Results

Migraine severity

No significant difference was found for mild [$F(3,48) = 1.45$, $p = 0.241$], moderate [$F(3,48) = 0.820$, $p = 0.489$] and severe [$F(3,48) = 1.87$, $p = 0.148$] rated head pain between sham and verum nVNS at baseline and follow-up. Although not statistically significant, we found an increase of the severity in the sham nVNS group for mild rated head pain. No significant differences were found, when comparing baseline and follow-up of verum nVNS group [mild attacks (VAS): 1.68 ± 0.49 versus 2.5 ± 0.33; moderate attacks (VAS): 4.68 ± 0.58 versus 5.43 ± 0.22; severe attacks (VAS): 8.56 ± 0.27 versus 7.75 ± 0.64; $p > 0.05$] and sham nVNS treated subjects [mild attack (VAS): 1.38 ± 0.43 versus 5.95 ± 3.6; moderate attacks (VAS): 5.17 ± 0.53 versus 5.54 ± 0.26; severe attacks (VAS): 9.13 ± 0.25 versus 8.63 ± 0.31; $p > 0.05$](Fig. 1).

Migraine frequency (headache days per month - attacks per month)

No significant difference was found for the number of headache days/month [$F(3,48) = 0.724$, $p = 0.542$] and for the total number of attacks/month [$F(3,48) = 0.675$, $p = 0.572$] between sham and verum nVNS at baseline and follow-up (Fig. 2A and B).

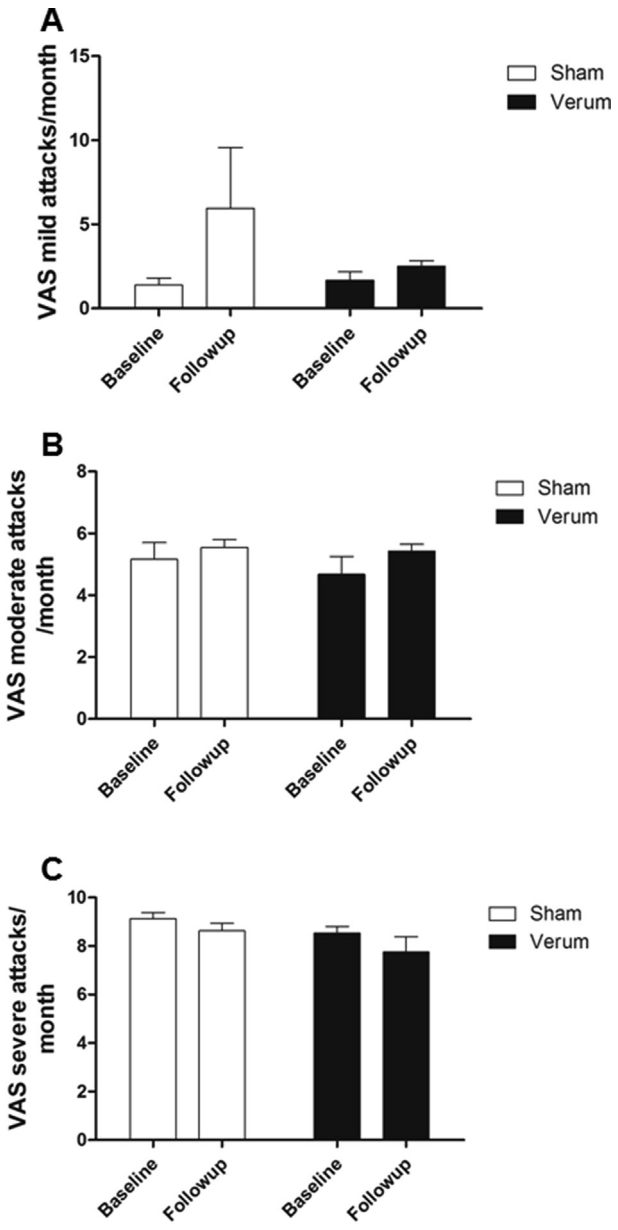

Fig. 1. Comparison of migraine severity assessed separately for mild [VAS 1-3/10] – moderate [VAS 4-6/10] – severe [VAS 7-10/10] rated head pain using the visual analogue scale [VAS] given at the vertical axis. Mild, moderate and severe attacks between sham nVNS [n = 12] and verum nVNS [n = 14] group at baseline and follow-up after 2 months [horizontal axis]. Values represent mean ± SEM and One-way ANOVA followed by Tukey's multiple comparisons test was used. P value < 0.05 was considered significant. Although no significant differences were present, mild rated attacks increased in severity in the sham nVNS group.

However, categorization of the number of total attacks into mild – moderate – severe rated attacks demonstrated no significant difference for the number of mild attacks/month [$F(3,48) = 0.950$, $p = 0.424$] and moderate attacks [$F(3,48) = 0.0429$, $p = 0.988$], but a significant difference was found for the number of severe rated attacks/months [$F(3,48) = 2.81$, $p = 0.049$] between sham and verum nVNS at baseline and follow-up. Post hoc Tukey's test showed a significantly lower number of severe rated attacks/months after 2 months of nVNS in verum group [verum nVNS (VAS): 7.64 ± 1.44 versus 2.93 ± 1.03; sham nVNS (VAS): 6.75 ± 1.54 versus 4.79 ± 1.05; $p < 0.05$](Fig. 2C).

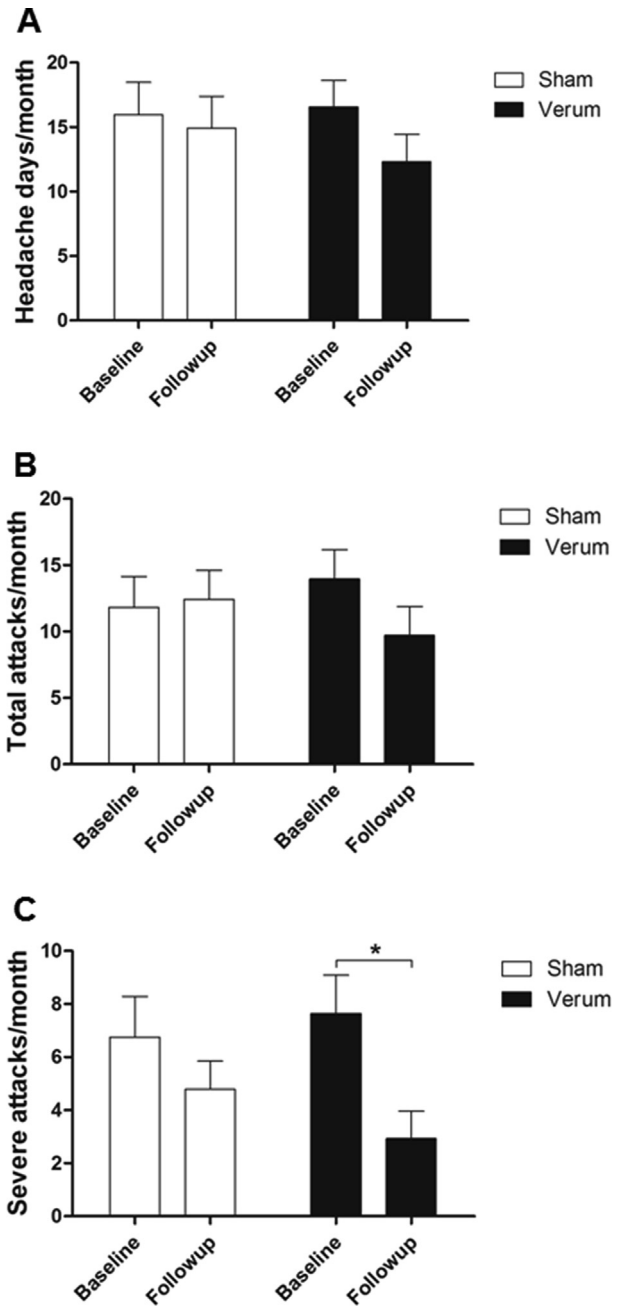

Fig. 2. Comparison of (A) number of headache days [y-axis] and (B) the number of total attacks per month [y-axis] between sham [n = 12] and verum [n = 14] groups at baseline and follow-up after 2 months [x-axis]. In addition, (C) the number of severe rated attacks per month were assessed. Values represent mean ± SEM and One-way ANOVA followed by Tukey's multiple comparisons test was used. P value < 0.05 was considered significant. A significant difference was found for the number of severe attacks/month between sham and verum group at baseline and follow-up [$F(3,48) = 2.81$, $p = 0.049$].

Migraine-associated impaired functional state assessed by subjective self-rated scores

No significant difference was found for different functional scores such as BDI grade [$F(3,48) = 0.827$, $p = 0.486$], BDI score [$F(3,48) = 2.20$, $p = 0.0996$], MIDAS [$F(3,48) = 1.45$, $p = 0.241$], PSQI [$F(3,48) = 1.08$, $p = 0.368$] and BMI [$F(2,41) = 0.578$, $p = 0.566$] between sham and verum nVNS group at baseline and after 2 months. So, the functional impairment remained unchanged at baseline and after 2 months among the verum nVNS group [BDI grade (1.71 ± 0.34 versus 1.43 ± 0.25); BDI score (16.57 ± 2.77 versus 12.64 ± 1.85); MIDAS (3.86 ± 0.1 versus 3.57 ± 0.25); global PSQI (8.57 ± 0.92 versus 7.5 ± 1.17); $p > 0.05$] and the sham treated nVNS cohort [BDI grade (1.58 ± 0.19 versus 1.17 ± 0.17); BDI score (12.75 ± 1.88 versus 8.67 ± 1.84); MIDAS grade (3.92 ± 0.08 versus 3.83 ± 0.11); global PSQI (6.83 ± 0.87 versus 6.0 ± 1.17); $p > 0.05$]. BMI was similar at baseline and after 2 months nVNS [HC: 24.2 ± 1.08 vs verum nVNS: 23.7 ± 1.25 vs sham nVNS: 22.6 ± 0.779; $p > 0.05$] (data not shown).

Levels of circulating pro-inflammatory HMGB-1, TNF-α, IL-1β, IL-6 and leptin

A significant difference of pro-inflammatory IL-1β was found between healthy control, sham and verum nVNS at baseline and follow-up [$F(5,82) = 3.51$, $p = 0.006$] with a significant increase of IL-1β in the sham stimulation group compared to the verum nVNS and healthy controls [HC: 0.11 ± 0.02 pg/mL versus sham nVNS: 0.16 ± 0.06 pg/mL versus verum nVNS: 0.05 ± 0.01 pg/mL; $p < 0.05$] after 2 months. No significant difference was found for pro-inflammatory HMGB-1 [$F(5,82) = 1.43$, $p = 0.221$], IL-6 [$F(5,82) = 0.774$, $p = 0.571$], TNF-a [$F(5,82) = 1.31$, $p = 0.266$] and leptin [$F(5,82) = 0.639$, $p = 0.671$] between healthy control, sham and verum nVNS treated group at baseline and follow-up (Figs. 3 and 4). Further subgroup analysis comparing pooled HMGB-1 values between migraine patients with and without aura demonstrated similar levels for both groups [aura: 1.93 ± 0.32 ng/mL versus non-aura: 2.2 ± 0.68 ng/mL; $p > 0.05$] (Fig. 4). Metabolic-associated leptin remained similar at baseline and follow-up [HC: 23666.67 ± 4202.21 versus 20244.45 ± 3225.41 pg/mL; sham nVNS: 24708.33 ± 5071.47 versus 27991.67 ± 6587.08 pg/mL; verum nVNS: 31850 ± 10523.13 versus 34064.29 ± 9486.9 pg/mL; $p > 0.05$].

Table 3 provides an overview of the concentrations of these cytokines and adipokines determined in this study. Assessment of pre- and post-nVNS IL-1β [baseline: $r = 0.189$; $p = 0.519$ versus follow-up: $r = -0.022$; $p = 0.94$] and IL-10 [baseline: $r = 0.11$; $p = 0.708$ versus follow-up: $r = -0.006$; $p = 0.985$)] levels showed no significant correlation or trend with the number of severe attacks per months (Fig. 5A–D).

Levels of circulating anti-inflammatory IL-10, adiponectin and ghrelin

Anti-inflammatory IL-10 significantly differed between the healthy control, sham nVNS and verum nVNS comparing baseline and follow-up ($F(5,82) = 7.41$, $p = 0.0001$). After post-hoc testing, no significant difference was found at baseline and follow-up in HC, the sham and nVNS groups. However, a significant difference existed among baseline IL-10 values in HC and baseline IL-10 levels in sham as well as verum nVNS group and also follow-up levels of IL-10 in verum nVNS group (Fig. 3D, Table 3). Follow-up IL-10 levels in HC also significantly differed from baseline IL-10 levels of both sham and verum groups (HC: 13.78 ± 4.5 pg/mL versus sham nVNS: 59.56 ± 12.44 pg/mL versus verum nVNS: 64.48 ± 9.68 pg/mL; $p < 0.05$), but not at follow-up after two months of nVNS (HC: 17.04 ± 3.78 pg/mL versus sham nVNS: 43.56 ± 9.3 pg/mL versus verum nVNS: 43.68 ± 10.66 pg/ml; $p > 0.05$) (Fig. 3). No significant difference was found for metabolic-related anti-inflammatory concentrations of adiponectin ($F(5,82) = 1.27$, $p = 0.285$) and ghrelin ($F(5,82) = 0.744$, $p = 0.592$) between healthy control, sham and verum nVNS groups at baseline and follow-up (Table 3).

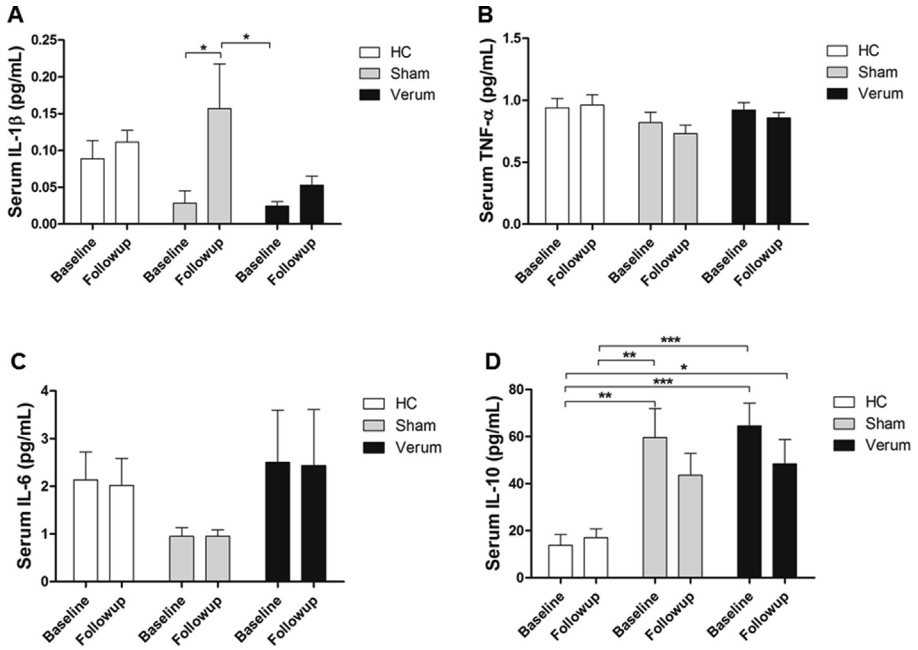

Fig. 3. Comparison of peripheral levels [y-axis] of IL-1β [pg/mL], IL-6 [pg/mL], TNF-α [pg/mL] and IL-10 [pg/mL] between healthy controls [n = 18], sham nVNS [n = 12] and verum nVNS [n = 14] treated subjects at baseline and follow-up after 2 months. Values represent mean ± SEM and One-way ANOVA followed by Tukey's multiple comparisons test was used. P value < 0.05 was considered significant. Anti-inflammatory IL-10 was significantly lower in healthy controls compared to migraineurs [sham and verum nVNS] at baseline and follow-up [$F(5,82) = 7.41$, $p = 0.0001$] and pro-inflammatory IL-1β significantly increased in the sham nVNS group at follow-up compared to verum nVNS. Abbreviations: Interleukin-1, 6, 10 [IL-1β, IL-10, IL-6] and tumor necrosis factor [TNF], *HC* [healthy controls], *ns* [not significant].

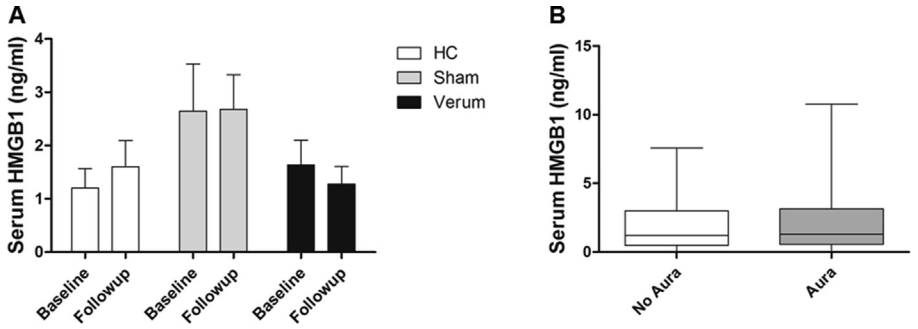

Fig. 4. A. Comparison of **s**erum levels of pro-inflammatory HMGB-1 [y-axis; ng/mL] between healthy controls [n = 18], sham nVNS [n = 12] and verum nVNS [n = 14] groups at baseline and follow-up after 2 months [x-axis] demonstrating no significant differences ($F(5,82) = 1.43$, $p = 0.221$). **B.** Pooled HMGB-1 [y-axis; ng/mL] analysis comparing migraine with aura (n = 16) versus migraine subjects without aura (n = 36) demonstrated similar values for migraineurs with and without aura [x-axis]. Values represent median with range and Mann Whitney *U* test was used. P value < 0.05 was considered significant. Abbreviations: HMGB-1 [high-mobility group box-1], *HC* [healthy controls], *ns* [not significant].

nVNS associated adverse events

One device-related adverse event (DAE) was noted for both groups (dysfunction), while in the sham group two non-device-related adverse events occurred (1 cold, 1 worsening of headache requiring changed medication).

Discussion

Summary of the main findings (primary outcomes first followed by secondary outcomes)

This double-blinded, sham-controlled study demonstrated cervical nVNS to be safe and to effectively reduce the number of severe attacks per month, while the overall frequency (headache days/month − total attacks/month) and severity did not differ statistically between verum and sham nVNS treatment (combined with pharmacotherapy) in refractory EM and CM patients (with/without aura). In our trial, nVNS headache responsiveness was low compared to the findings of previously published data related to the utility of nVNS in the acute and preventive treatment of migraine and cluster headaches [1–4].

Impaired sleep architecture represents a frequently occurring co-morbidity in migraine. Engstrom et al. provided evidence for a significant relation between dysfunctional sleep and primary headache disorders [23,24]. Impaired functional capability in terms of migraine-associated disturbed sleep architecture, life quality/disability and clinical depressive symptoms remained unchanged after two months of nVNS in our cohort. Whether, and to what extent, decreased headache or improved sleep quality contribute to the functional outcome in nVNS headache studies remains unclear and needs further clarification via quantitative measures (polysomnography) [23,24]. Due to the possible impact of changes in medication on headache, sleep patterns and circulating cytokines, these factors were maintained for the duration of the study.

Table 3
Numeric presentation of baseline and follow-up serum concentrations for healthy controls, sham nVNS and verum nVNS groups of pro- [IL-1β (pg/mL), IL-6 (pg/mL), TNF-α (pg/mL), HMGB-1 (ng/mL) and leptin (pg/mL)] and anti-inflammatory [IL-10 (pg/mL), adiponectin (pg/mL), ghrelin (pg/mL)] cytokines demonstrating significant differences for pro-inflammatory IL-1β and anti-inflammatory IL-10. Abbreviations: Interleukin-1β, 6 and 10 [*IL-1ß, IL-6, IL-10*], tumor necrosis factor [*TNF*], high mobility group box-1 [*HMGB-1*], *HC* [healthy controls].

	healthy control	nVNS	sham nVNS	p-values
	baseline/follow-up	baseline/follow-up	baseline/follow-up	
IL-1β (pg/ml)	0.08 ± 0.02/0.11 ± 0.02	0.02 ± 0.01/0.05 ± 0.01	0.02 ± 0.01/0.16 ± 0.06	$p < 0.05$
IL-6 (pg/ml)	2.14 ± 0.58/2.01 ± 0.57	2.5 ± 1.1/2.44 ± 1.18	0.95 ± 0.19/0.95 ± 0.14	$p > 0.05$
IL-10 (pg/ml)	13.78 ± 4.5/17.04 ± 3.78	64.48 ± 9.68/43.68 ± 10.66	59.56 ± 12.44/43.56 ± 9.3	$p < 0.05$
TNF-α (pg/ml)	0.94 ± 0.08/0.96 ± 0.08	0.92 ± 0.06/0.86 ± 0.04	0.82 ± 0.08/0.73 ± 0.07	$p > 0.05$
HMGB-1 (ng/ml)	1.2 ± 0.37/1.6 ± 0.5	1.64 ± 0.46/1.27 ± 0.33	2.64 ± 0.89/2.68 ± 0.65	$p > 0.05$
Leptin (pg/ml)	23666.67 ± 4202.21/20244.45 ± 3225.41	31850 ± 10523.13/34064.29 ± 9486.9	24708.33 ± 5071.47/27991.67 ± 6587.08	$p > 0.05$
Adiponectin (pg/ml)	7391.68 ± 976.93/7021.68 ± 921.27	9896.43 ± 1209.41/9685 ± 1208.25	8304.17 ± 1330.01/7210 ± 1319.94	$p > 0.05$
Ghrelin (pg/ml)	3538.49 ± 251.25/3827.747 ± 472.63	3693.12 ± 366.2/4350.55 ± 822.8	4509.76 ± 661.67/3336.94 ± 356.74	$p > 0.05$

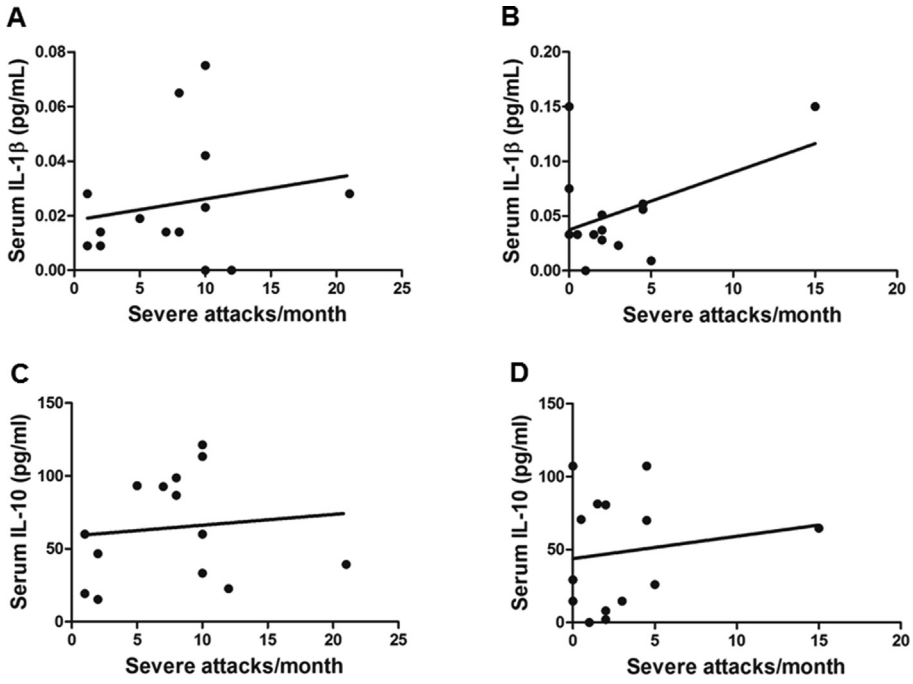

Fig. 5. A-B. Correlation analysis between pre- [A] and post-nVNS [B] levels of pro-inflammatory IL-1β [y-axis; pg/mL] and headache frequency [numbers of severe attacks/month; x-axis] showing no association with number of severe attacks [baseline: $r = 0.189$; $p = 0.519$ versus follow-up: $r = -0.022$; $p = 0.94$]. **C-D.** Assessment of pre- [C] and post-nVNS [D] concentrations of anti-inflammatory IL-10 [y-axis; pg/mL] levels showed no association with number of severe attacks [baseline: $r = 0.11$; $p = 0.708$ versus follow-up: $r = -0.006$; $p = 0.985$]. Abbreviations: *IL-1ß* [Interleukin-1β], *IL-10* [Interleukin 10], *HC* [healthy controls], *ns* [not significant].

Levels of pro-inflammatory IL-1β were significantly increased in the sham group at follow-up, hence nVNS may have prevented such a significant upsurge of IL-1β in the verum group at follow-up. Furthermore, anti-inflammatory IL-10 was significantly elevated pre-nVNS in migraineurs (verum and sham) compared to healthy controls, but not post-nVNS treatment. Pro-inflammatory IL-6 and TNF-α levels demonstrated no significant differences between all groups at baseline and after 2 months nVNS. Prior to nVNS treatment and within the treatment period, no clinical systemic disease was observed (C-reactive protein below 0.4 mg/dL). IL-1β has been suspected to evoke activation of cyclo-oxygenase 2 (COX-2) associated pathways involving glial cells and neurons of the trigeminal ganglion (TG). These COX-2 dependent pathways lead to prostaglandins release from glial and neuronal trigeminal ganglion (TG) cells, which exclusively promotes neurons of the TG to immediately synthesize calcitonin-gene related peptide (CGRP), contrary to IL-1β, which demonstrated a delayed CGRP release pattern indicative for a glia-neuron interaction in the TG. Methylprednisolone antagonized the IL-1β effects, but was found to have no impact on prostaglandin induced CGRP release [25]. Preclinical evidence indicates that IL-1β and IL-6 interact with intracranial meningeal nociceptors promoting head pain and disrupted trigemino-nociceptive signaling [26].

The pro-inflammatory HMGB-1 (DNA-binding peptide), a member of the damage-associated molecular patterns (DAMPs) and early recognition marker of inflammation, is overly expressed extracellularly in the cerebrospinal fluid (CSF) and plasma after neuronal injury/damage. Elevated circulating HMGB-1 marker has been associated with cortical spreading depression (CSD) in preclinical models of migraine with aura [27,28]. Interestingly, a correlation has been observed between the HMGB-1 expression and the number of experimentally induced CSDs with a time-dependent pattern. HMGB-1 may reflect the bridging link between migraine aura, CSD and headache pain, by activation of the trigemino-vascular system leading to attack onset [27–30]. Invasive and non-invasive VNS equally suppressed CSD susceptibility

and increased electrical thresholds either ipsilateral or in the contralateral hemisphere lasting more than three hours after nVNS application. These observations indicate that nVNS may interact with the propagation of CSD as the electrophysiological correlate of migraine aura [31,32]. To our knowledge, this is the first study assessing peripheral HMGB-1 levels in human migraineurs, although we observed no changes between all groups. In addition, pooled assessment of migraine patients with aura compared to those without aura demonstrated similar levels of peripheral HMGB-1 levels for both groups.

A pre-obese state (BMI 20-25 kg/m^2) was present in 16 participants and overweight (BMI 25-30 kg/m^2) was present in four patients. However, we found no statistically significant changes for leptin (pro-inflammatory), adiponectin (anti-inflammatory) and ghrelin (anti-inflammatory) as BMI did not change. White adipose tissue (WAT), previously associated with a metabolic storage function, is now known to act as an inflammatory endocrine active organ with the potential to induce or inhibit systemic inflammation via crosstalk between adipocytes (e.g synthesis of leptin - adiponectin) and the innate and adaptive immune system, linking obesity to the pathogenesis of migraine [33–37]. For instance, leptin (pro-inflammatory), a metabolic marker produced by WAT cells, has been shown to interact with the COX-2 dependent pathways via crosstalks with IL-1β in glial cells and neurons of the hypothalamic-pituitary axis and ghrelin reduced photophobia and induced behavioural changes in an experimental trigeminal pain model [34,35]. Hence, immunometabolism may have a considerable impact on the molecular inflammatory profiling in migraine patients. Rising evidence highlights the contribution of adipose tissue in the development of systemic inflammatory associated neurological disorders, of which some have been linked to obesity. Circulating mediators of inflammation participate in the mechanisms of migraine pathology, and many of these inflammatory peptides are secreted from adipocytes and adipose tissue–derived immune cells (monocytes). The targeted inhibition of various pro-inflammatory pathways in adipocytes may represent a novel therapeutic approach for migraine [36,37].

Stimulation of the vagal nerve activates the cholinergic anti-inflammatory reflex of neuro-immunity via its afferent properties and reciprocal interaction with brainstem circuits leading to an efferent response and changes in cytokine/chemokine levels. Activation of this anti-inflammatory reflex is assumed to induce peripheral inhibition of IL-1β, IL-6, TNF-α, HMGB-1 and other pro-inflammatory mediators via α7 subunit of the nicotinic acetylcholine (Ach) receptor (α7nAchR) on immune cells (monocytes and macrophages) [11–13,36,37].

Conclusion

Non-invasive vagus nerve stimulation significantly decreased the number of severe attacks in our study, while there was no statistical difference between sham and verum nVNS with respect to migraine severity and migraine-associated co-morbidities. Systemic circulating pro-inflammatory IL-1β was significantly increased in sham-stimulated participants compared to verum nVNS and anti-inflammatory IL-10 (anti-inflammatory) remained significantly high before and after adjunctive verum nVNS treatment, which may be indicative for head pain susceptibility. Pro- and anti-inflammatory mediators such as IL-6, TNF-α, HMGB-1, leptin, ghrelin and adiponectin remained unchanged.

The strengths of this proof-of-concept study is that it is the first to investigate peripheral circulating cytokines suspected to be involved in migraine development and chronification in human migraineurs treated with nVNS. Main limitations include small sample size, short-term observation period and the heterogenous study cohort as episodic and chronic migraine differs in pathophysiology, prevalence, symptom phenotype, socio-demographics, individual/economic burden and co-morbidities [16–19].

Furthermore, pre-analytical confounders may bias our findings. In the future, blood-based phenotyping may help to identify distinct pain phenotypes more likely to respond to neurostimulation and facilitate the development of symptom-tailored personalized neurostimulation treatment. Hence, our results, though preliminary, certainly deserve to be further investigated in large-scale and homogenized population studies. Ultimately, biobank-based system biological approaches are on the way and represent an appropriate roadmap to substantially provide insights in such complex molecular circuits and the possible implications for head pain therapy [38,39].

Conflicts of interest

TMK works as a consultant for Abbott, Inc.

Author contributions

S.R. Chaudhry and I.S. Lendvai contributed equally.

All authors were involved in the study design and participated in data collection and data analyses. All authors contributed to the development of this manuscript and provided their critique and their approval of the final draft for submission to Brain Stimulation.

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