Title: Serum alpha and beta-CGRP levels in chronic migraine patients before and after monoclonal antibodies against CGRP or its receptor.

Running head: Alpha and beta-CGRP levels after monoclonal antibodies.

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- If you and/or a co-author has a Twitter handle that you would like to be tagged, please enter it here.
 @juliopascualg @glezquintanilla @mpascualmato
- 2. What is the current knowledge on the topic? Migraine is a diagnosis just based in clinical symptoms and as it happens in many other neurological disorders, there is a need for a biomarker able to confirm/support its diagnosis or to objectively evaluate the efficacy of the new migraine therapies. A number of recent data indicate that CGRP is the most plausible migraine biomarker, mainly in chronic migraine, in several body fluids but especially in blood.
- 3. What question did this study address? This study analyses for the first time the behavior of CGRP alpha and beta levels in chronic migraine patients before and after treatment with CGRP antibodies. None of the previous studies have determined separately the two CGRP isoforms.
- 4. What does this study add to our knowledge? Our results confirm a basal increase in CGRP levels in chronic migraine patients as compared to healthy controls, but restricted to the CGRP alpha isoform. More importantly, we demonstrate the progressive normalization of CGRP alpha-levels after treatment with CGRP antibodies and its correlation with clinical response.
- 5. How might this potentially impact on the practice of neurology? These data support a role for serum CGRP levels as a biomarker for migraine and in particular for chronic migraine, which in the future could be of help both in its diagnosis and for an objective follow-up of treatment response not only in clinical trials, but also in clinical practice.

Objective: To analyse the evolution of alpha and beta-CGRP circulating levels throughout CGRP monoclonal antibodies (mAb) treatment in chronic migraine (CM) patients.

Methods: We recruited CM patients beginning mAb along with sex and age paired healthy controls (HC). Blood was extracted before, two-weeks (M0.5) and three months (M3) after first dose of mAb, always in free-migraine periods, and once for HCs. Alpha and beta-CGRP serum levels were measured using ELISAs specific for each isoform.

Results: Baseline alpha-CGRP levels were significantly elevated in 103 CM patients (median [95% CI]: 50.3 [40.5-57.0] pg/mL) compared to 78 HC (37.5 [33.9-45.0] pg/mL; 95% CI of differences: 2.85-17.08 pg/mL) and significantly decreased (n=96) over the course of mAb treatment (M0.5: 40.4 [35.6-48.2] pg/mL; M3: 40.9 [36.3-45.9] pg/mL). Absolute decrease of alpha-CGRP throughout the treatment positively correlated with the decrease in monthly migraine days. Negative modulation of alpha-CGRP significantly associated with positive scores at the patient global impression of change scale and with analgesic overuse reversal. Beta-CGRP did not differ at baseline between CM patients (4.2 [3.0-4.8] pg/mL) and HC (4.4 [3.4-5.6] pg/mL; -1.09 to 0.60) nor was modulated by mAb treatment (n=96) (M0.5: 4.5 [3.5-5.2] pg/mL; M3: 4.6 [3.7-5.2] pg/mL).

Interpretation: Treatment with mAb, regardless of its target, is able to progressively normalize basally increased alpha-CGRP levels in CM and this effect correlates with efficacy measures, which supports a role of this neuropeptide as the first CM biomarker.

Introduction

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Migraine is a highly prevalent and disabling neurological disorder. ¹ Despite many aspects about its pathophysiology remain unclear, the activation of the trigeminovascular system (TVS) is necessary for the migraine headache to occur.^{2,3} TVS connects the intracranial vasculature and meninges to the brainstem with its peripheral and central afferents. Its

activation leads to release of vasoactive neuropeptides, driving neurogenic inflammation, nociceptive modulation and central and peripheral sensitization.²

Among these neuropeptides, the calcitonin gene-related peptide (CGRP) has been linked to migraine pathophysiology³ by a series of studies which have shown increased CGRP levels during the migraine attack from jugular vein blood;⁴ peripheral blood⁵ and other biological fluids;⁶⁻⁸ a normalization of this elevation after abortive^{9,10} and prophylactic^{11,13} treatment; interictally elevated concentrations in patients with chronic migraine (CM) or episodic migraine (EM) compared to controls without headache;¹⁰⁻¹⁵ and its ability to induce migraine-like attacks when infused in humans.¹⁶ All these findings have converged into the development of migraine therapies that specifically target CGRP signalling. These are the gepants, specific antagonists of the CGRP receptor (CGRPr) used both as abortive and preventive treatments, and the monoclonal antibodies against CGRP or CGRPr (mAbs) for migraine prophylaxis.¹⁷⁻¹⁹

CGRP actually comprises 2 different 37 amino acid-length peptides with potent vasodilator activity encoded in different genes of the 11th human chromosome. Alpha-CGRP is located at the central and peripheral nervous system, produced by tissue-specific alternative splicing of the transcript of the Calcitonin I gene. The beta isoform, differing in 3 amino acids from the alpha, is mainly expressed in the enteric nervous system (ENS) and is encoded in the Calcitonin II gene. These two peptides share structure, biological functions, and receptor affinity.²⁰ However, researchers have focused on alpha-CGRP or total CGRP rather than beta-CGRP, whose specific implications in the processes where CGRP plays a role remain unknown.

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All migraine mAbs have shown similar efficacy and safety independently of their target or design, though there is still a proportion of patients who do not respond to these medications.¹⁷⁻¹⁹ Because migraine is defined solely by clinical criteria and lacks specific biomarkers it has been impossible to consistently describe profiles with tendency to benefit from these or other preventive migraine therapies. Serum CGRP levels have been proposed as a diagnostic biomarker for CM^{12,13} and as predictor of its response to onabotulinumtoxin-A,^{11,13} but not every study has been able to find increased serum CGRP levels.^{21,22}

Nonetheless it is an undergoing topic, with studies analysing the source of such discrepancies^{23,24} and which keep evaluating its role as diagnostic and or response biomarker from multiple sample sources.²⁵

In the present study, we assessed serum alpha and beta-CGRP levels in patients with CM before and through their treatment with mAbs to evaluate its association with the clinical response and to compare its values with HC.

Methods

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Study Participants

Subjects who met CM criteria according to the International Classification of Headache Disorders-3 (ICHD-3)²⁶ were recruited from our Headache Unit at the University Hospital Marqués de Valdecilla. To be included in the study, patients with CM had to be older than 17 years, fulfil criteria for anti-CGRP/CGRPr mAb prescription in Spain (no response/intolerance to at least three preventives, including onabotulinumtoxinA) and begin the use of mAbs. Detailed clinical data of the participants with CM, including presence of comorbidities, date of migraine diagnosis and length of CM status, presence of aura, historical and current headache treatments, compliance with analgesic overuse (AO) criteria and monthly headache days, according to calendar, at the initiation of treatment was recorded. Preventive drugs for migraine were allowed during the study, but restricted as much as possible and remained stable in all patients during the three months of the study period and also for one month prior to mAB administration. For monitoring treatment outcome and efficacy of mAbs, we kept record of the evolution of the patients in terms of compliance with AO criteria, monthly headache days (MHD), monthly migraine days (MMD), response rates, patient global impression of change (PGIC) scale ²⁷ and appearance of adverse effects, all of them at the third month of mAb treatment. Patients underwent medical revision with the specialist clinician at 1-month and 3-months after the first dose of mAb and with the use of a detailed calendar they kept record of frequency and characteristics of their headaches throughout the study.

As healthy controls (HC), we recruited individuals with similar age and sex distribution as the group with CM (mostly medical students, residents, nurses, or physicians

from our region network of hospitals and primary care centres) with subjective absence of headache, without previous history of migraine and who were not taking any medication on the previous 24 hours to blood extraction. Exclusion criteria were being pregnant or breastfeeding women, excessive use of alcohol and serious, active somatic, or psychiatric diseases or taking any kind of medication on a daily basis. Recruiting process was performed simultaneously for the HC and CM group.

Based on a previous study measuring serum CGRP in CM and HC^{12,13} we calculated that with an expected increase of 25% between the CM group and the HC, an enrollment ratio of 1:1, alpha equal to 0.05 and a power of 80%, we had to include a minimum of 56 individuals per group. On the other side, based on the results of a different work analyzing the evolution of serum CGRP in a cohort of CM beginning treatment with OnabotulinumtoxinA^{11,13} we calculated that with a similar decrease of 30%, alpha equal to 0.05 and a power of 80% patients with CM.

Standard protocol approvals, registration, and patient consents.

The study was approved by the Ethics Committee of Investigations with Medications of Cantabria and its approval has been published in the record 28/2020 of the 11th December 2020. All participants gave written informed consent for their inclusion in the study.

Blood extraction and serum processing

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Patients with CM had 3 blood extractions, one basal, before initiation of mAb treatment (M0), at two-weeks (M0.5) and at 3-months (M3). All samples were obtained in migraine-free days and having taken no symptomatic treatment in the previous 24 hours. Blood samples were drawn up to a maximum of 5 days later in a migraine-free day, in case a migraine attack occurred at two-weeks or 3-months visits. HC subjects had at least one blood extraction. All fasting blood samples were extracted in the morning (9-12am), let clot for 10 minutes, then centrifuged at 3500rpm and 4°C for 10 minutes, immediately transferred into sterile tubes and stored at -80°C until assayed. Full processing time in the samples of this study ranged from 23 to 29 minutes. Samples were always frozen within the first 30 minutes since blood extraction, and none remained cryopreserved for more than 6 months before being analysed.

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Laboratory procedures

Alpha and beta-CGRP serum levels were determined using commercial enzyme-linked immunosorbent assays (ELISAs) (Abbexa, UK; CUSABIO, China; respectively) and strictly following manufacturers' instructions. Regarding the last step of the ELISA processes, in which manufacturers give a window of time, specifying that the user must determine the optimum, we incubated the substrate for 15 minutes for alpha-CGRP and for 20 minutes for beta-CGRP. Both kits have been internally validated for the determination of the said molecules. All the samples were measured in duplicate, and all measurements fulfil the quality criteria set by manufacturers, having an intra-assay coefficient of variation below 8%. A standard curve was generated for every single batch, and they were calculated using a four-parameter logistic (4-PL) regression with r^2 >0.999. Besides the standards, to ensure the reproducibility of the results, we included in each ELISA plate samples from at least 10 HC and obtained an inter-assay variability within subjects of <6%.

Data analysis

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Categorical variables are reported as percentages while continuous variables are displayed as mean \pm SD for normally distributed data and as median with [95% confidence interval (CI) of median] for non-normally distributed data unless stated differently in text. Normality assumption of quantitative variables has been checked using D'Agostino & Pearson test (p<0.05 to refuse Ho). Statistical significance between groups of continuous variables following normal distribution (age) was assessed by student's *t* test. For non-normally distributed data (alpha and beta-CGRP), Mann-Whitney U test was performed. For group comparison of categorical variables Fisher's exact test was carried out. For multiple group comparisons among same individuals at different time-points, Friedman test was used followed by Dunn's test; for multiple group comparisons of sub-groups created upon posthoc division, we performed Kruskal-Wallis test followed by Dunn's test. Correlation relationships were evaluated by Pearson's correlation test.

Data Availability

Researchers may obtain access to data upon formal request to the corresponding author.

Results

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The initial sample included 103 patients with CM (mean age: 50.1 ± 9.9 years; range: 21-71; 85.4% women mAb treatment for CM prophylaxis, including erenumab (Aimovig[®], Novartis, Basile, Switzerland) and galcanezumab (Emgality[®], Eli Lilly, Indianapolis, U.S.) and matched 78 HC (mean age: 52.9 ± 17.6 ; range: 26-91; 73.1% women). Alpha-CGRP circulating levels were higher in patients with CM at baseline (median [95% CI]: 50.3 [40.5-57.0] pg/mL) compared to HC (37.5 [33.9-45.0] pg/mL; p=0.004; 95% CI of differences: 2.85 to 17.08). Basal beta-CGRP serum concentrations in patients with CM (4.2 [3.0-4.8] pg/mL) were not significantly different to those of HC (4.4 [3.4-5.6] pg/mL; p=053; 95% CI of differences: - 1.09 to 0.60).

We could not obtain the two CGRP follow-up samples from 7 cases (in 6 cases samples were not obtained as the patient was not migraine-free and the remaining one stopped mAB due to dizziness). Therefore, the final sample included 96 patients with CM (mean age: 50.0 ± 9.9 ; range: 21-71; 86.5% women) initiating mAb treatment and the 78 HC. Age differences were not significant nor sex distribution among groups. Baseline alpha-CGRP did not correlate with age (p=0.60; 95% CI "r": -0.15 to 0.25), while beta-CGRP did so with older ages associated to higher concentrations (p<0.05; 95% CI "r": 0.06 to 0.44). No statistical differences were found in pre-treatment measurements between women and men for any group nor molecule.

According to their medical history, patients with CM had been diagnosed migraine 33.0±12.7 years ago and had remained in a CM situation for an average of 12.1±8.6 years (range: 1-45), with 22.4±6.3 MHDs, 20.6±6.3 MMDs and a mean monthly intake of symptomatic treatments of 21.0±6.4 days, being triptans (n=83) and NSAIDs (n=81) the most commonly used acute medications. Patients had tried on average 7.1 different preventives and were currently on 1.8±0.2 per patient. Clinical characteristics of our final CM cohort are illustrated (Table 1). Sixty-three patients were treated with erenumab and thirty-three were with galcanezumab. After three months of treatment, patients experienced a mean decrease of 11.3 ± 8.7 in MHDs, of 10.1 ± 9.0 in MMDs, and reduced their monthly intake of symptomatic treatment to 11.3 ± 8.2 days. Patients scored 4.4 ± 2.1 at the PGIC scale, showing a general improvement throughout the mAbs therapies. Treatment outcome and efficacy parameters at the third month of treatment are displayed (Table 2).

Interictal alpha-CGRP circulating levels were higher in the final sample of 96 patients with CM at baseline (47.7 [38.9-54.1] pg/mL) compared to HC (37.5 [33.9-45.0] pg/mL; p=0.019; 95% CI of differences: 1.22 to 14.45). Significant differences against HC disappeared at 2-weeks (40.4 [35.6-48.1] pg/mL) and at 3-months of mAb treatment (40.9 [36.3-45.9] pg/mL). Intra-individual differences between baseline measurements and the 2-weeks and 3-months determinations after treatment initiation were significant. No significant differences were found between 2-weeks and 3-months alpha-CGRP circulating concentrations (Figure 1). Differences in baseline concentrations between erenumab and galcanezumab treated subgroups were not significant and their alpha-CGRP reduction at 3-months were significant for both treatment options (erenumab mean reduction: 9.2 pg/mL, median reduction: 7.2 pg/mL; galcanezumab mean reduction: 7.6 pg/mL, median reduction: 6.2 pg/mL).

Regarding response, when patients with CM were divided into those patients with a decrease of monthly pain days over 50% (responders) and those who did not (nonresponders), only the responder group had significantly higher alpha-CGRP circulating levels at baseline (52.6 [38.6-59.4] pg/mL) compared to HC, being the non-responder group nonsignificant (40.4 [37.3-54.1] pg/mL). However, decrease in alpha-CGRP content at 3months was significant for both sub-groups (Figure 2). Similarly, when response was determined by PGIC, with a score over 4 considered responders and equal or below it, nonresponder, only the responder group had higher alpha-CGRP at baseline (53.4 [38.6-61.0] pg/mL) compared to HC (p=0.004), being the non-responder group non-significant (40.3 [37.3-53.9] pg/mL). Attending to this criterion, only the sub-group with PGIC>4 had a significant decrease of alpha-CGRP circulating levels at 3-months (45.9 [36.3-56.3] pg/mL) (Figure 3). Patients who stop fulfilling AO criteria after mAb treatment presented higher pretreatment alpha-CGRP content (53.0 [43.9-59.4] pg/mL) against HC (p<0.01), while those who kept fulfilling the criteria did not (39.9 [36.4-53.9] pg/mL) (p=0.84). On this first subgroup alpha-CGRP decreased significantly over the time (M0.5: 44.3 [39.1-57.0] pg/mL; M3: 45.9 [35.0-53.1] pg/mL) while on the other sub-group the concentration remained unaltered (M0.5: 38.0 [31.3-48.0] pg/mL; M3: 37.5 [30.9-44.1] pg/mL) (Figure 4).

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The decrease in MMDs at the 3^{rd} month correlated with the absolute decrease in alpha-CGRP content at the same time-point (p=0.02; 95% Cl "r": 0.03 to 0.41) (Figure 5).

When analysing the presence of aura, comorbidities listed in Table 1, baseline levels of alpha-CGRP, baseline MHDs, baseline MMDs or time since first diagnosis of CM condition none of these baseline parameters could acted as statistically significant predictors of response at the third month.

Basal interictal beta-CGRP serum concentrations in the final sample of 96 patients with CM (4.3 [3.3-5.0] pg/mL) were not significantly different to those of HC (4.4 [3.4-5.6] pg/mL; p=073; 95% CI of differences: -1.04 to 0.67). The evolution of circulating levels of beta-CGRP did not show any trend or difference at two-weeks (4.5 [3.5-5.2] pg/mL) nor three-months (4.6 [3.7-5.2] pg/mL) and no differences were found against HC for these two time-points (p=0.82; p=0.74, respectively) (Figure 1). Average peptide levels did not differ significantly from erenumab and galcanezumab treated patients at any time point. When classified into responders, both attending on monthly pain days decrease and PGIC, none of the resulting sub-groups showed differences against HC nor between them. Beta-CGRP content did not correlate with alpha-CGRP content at any time point nor group.

When classifying patients with CM by constipation as an adverse effect of mAb treatment, average baseline interictal beta-CGRP levels in patients who suffered from it (4.3 [2.9-6.7] pg/mL) were not higher than in the non-constipated patients (4.3 [2.6-5.7] pg/mL) (p=0.57), neither showed significant differences at 3-months (constipated: 4.2 [2.2-5.5] pg/ml; non-constipated: 4.6 [4.0-5.5] pg/mL) (p=0.36). However, the tendency in the constipated group along the treatment was a decrease on average beta-CGRP (-22%) while in the non-constipated group it increased (+7%).

Discussion

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We have used, for the first time in the same work, different methodologies to discriminate between alpha and beta-CGRP using ELISA kits specifically designed for the detection of exclusively one of these forms of CGRP. Here we show a significant and specific increase in basal interictal serum alpha-CGRP levels in a big group of CM patients as compared to HC without headache, possibly as a reflection of the permanent or almost permanent activation and secondary sensitization known to occur in CM.^{2,28} As stated above, a few works could not find such increase in total serum CGRP levels,^{21,22} but our results concur with those

coming from most studies showing significantly increased serum CGRP levels in CM patients and reinforce the value of serum alpha-CGRP levels as a potential CM biomarker.^{11-15,25,29-31} It is important to emphasize that this increase in serum alpha-CGRP levels has been found despite our patients were on average on 1.8 other preventive treatments and took abortive medications an average of 21 days per month, two clinical situations which have been shown to reduce CGRP levels.⁹⁻¹¹

Our novel objective was to describe how the serum levels of CGRP evolve throughout the course of anti-CGRP/CGRPr mAb treatments. A very recently published study has found erenumab numerically decreases the baseline serum CGRP levels at 2-4 weeks since first dose, though the differences were not significant.³² Here, our results show that these therapies have a lowering effect on the circulating concentrations of alpha-CGRP not only at 2-weeks since its initiation but also at 3-months since first dose, identical for both antibodies against the ligand and its receptor. On top of that, there was a correlation between the amount of circulating alpha-CGRP decreased by the therapies within the first three months and the reduction of MMD at the third month of treatment. Furthermore, we have seen that positive outcome parameters are related to higher baseline alpha-CGRP concentrations and negative modulation of the circulating levels. Patients with ≥50% reduction in MMD (responders) showed higher levels of circulating peptide in their baseline than those who did not respond to the therapies, although the lowering effect on alpha-CGRP levels was found in both sub-groups. When the criteria applied to classify between responders and non-responders was the PGIC scores, we found again higher alpha-CGRP levels in the responder group and, moreover, that only this group had a significant decrease of their circulating levels of the peptide after 2 weeks and 3 months. Patients who stopped fulfilling AO criteria due to the mAbs, classified as responders, had higher pre-treatment alpha-CGRP concentrations than those who kept fulfilling AO criteria after 3-months and only in this first group a significant negative evolution of alpha-CGRP levels was found. Our results concur with those of Alpuente et al in a group of episodic and chronic migraine patients showing that a higher headache frequency is associated with higher CGRP levels, but in this case in saliva, and that this basal increase converge to controls after 12 weeks on erenumab.³³

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Because, in contrast to previous studies, we included both erenumab and galcanezumab we have demonstrated the lowering effect happens independently of blocking the receptor or its ligand, which suggests a common final mechanism of action regardless the target and which also seems to be linked to the signalling blocking of CGRP. The mechanism of how these two different targeting converge or how the signalling blockade results in decreasing alpha-CGRP concentrations is still obscure and goes beyond the scope of this study, though, as occurs in experimental models, it is tempting to propose that the reduction seen in serum alpha-CGRP levels reflects a successful deactivation of the TVS induced by the two mAbs regardless of their individual target.³⁴

Notwithstanding the results obtained with alpha-CGRP, we had different findings with beta-CGRP. For this form of CGRP, the baseline circulating levels of the peptide were not elevated in comparison with our HC group. Additionally, we could not measure a modulation on its concentration throughout the course of the mAb therapies nor find differences when patients were grouped by positive outcome parameters. Because beta-CGRP is mainly expressed within the ENS, it has been linked with the gastrointestinal effects of CGRP in humans.³⁵ We hypothesized before the study that constipation caused by anti-CGRP/CGRPr mAbs, the most common adverse effect described for these therapies,³⁶ could be caused by a negative modulation of this form of CGRP. However, despite the finding that the group who suffered constipation showed an average negative modulation of their beta-CGRP circulating levels while the non-constipated did not, these changes were nonsignificant, and we cannot conclude it is a possible cause for the constipation, though the number of cases with constipation in our study was low. This lack of differences between CM and HC and between constipated and non-constipated CM, together with the low measured concentrations of beta-CGRP compared with alpha-CGRP could also indicate that our sample source, serum, is not the ideal source for beta-CGRP determinations. One recent study using the same ELISA kit we have assayed, but different sample source (saliva), has found far higher peptide concentrations and have been able to describe differences between circulating levels in EM and HC.⁶

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This study has several limitations. Reflecting clinical practice, our participants with CM were not homogenous, with a variable use of preventives, abortive medications,

different presence of comorbidities and history of failure to preventives. To obtain more definite results, patients would have ideally stopped taking preventives and acute phase medications but this is not easy in clinical practice in CM, not only because some patients notice some improvement on preventives -though they still meet CM criteria-, but because drugs with potential antimigraine efficacy are prescribed due to comorbidities, such as betablockers or candesartan for concomitant hypertension in one-quarter of patients or amitriptyline for anxiety-depression in almost 2 out of 3 patients. Our policy here was to try to reduce their use as much as possible, allowing only those necessary potential preventives, and keep them stable during the study period, but we are fully aware of this limitation, which has to be addressed in future studies. Additionally, due to the high headache frequency in our CM patients, it was only possible to obtain blood samples at migraine free days, but not always on headache free days. Our study has highlighted the importance of alpha-CGRP reduction over the course of treatment, but could not prove the existence of two different profiles, prone and not prone to respond to mAb depending on their CGRP levels, as there was no placebo arm and some patients showing a >50% reduction in MHD could not be true responders. The two limitations of this and all the previous studies, the use of preventatives and the lack of blinding and a placebo arm, should be tested in future controlled, multicentre clinical trials in which patients have to stop preventatives to participate. Last, contrary to alpha-CGRP, we could not find significant differences in the levels of beta-CGRP and one of the explanations could be that our sample source may have not been ideal for the determination of beta-CGRP, but with the lack of studies focusing only on this subtype of peptide it will need a confirmation of not being so. Our sample size was not big enough to analyse the effect of beta-CGRP on constipation and futures studies will have to try to unveil the mechanism behind this adverse effect and its relative high incidence.

In conclusion, in this observational study, serum interictal alpha-CGRP levels, but not beta-CGRP levels, were shown to be increased in CM patients as compared to HC. Treatment with mAb was able to progressively restore alpha-CGRP levels by 3 months and there was a correlation between the alpha-CGRP levels normalization and several efficacy measures. Our data support a role of this neuropeptide as the first dynamic CM biomarker, though these results should be confirmed in future controlled studies which should also 5318249, ja, Downloaded from https://onlineLibrary.wiley.com/doi/10.1002/ana.26658 by Universidad De Cantabria, Wiley Online Library on [20/04/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

explore further the preliminary negative modulation of beta-CGRP as a potential explanation for the constipation seen with anti-CGRP therapies.

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Authors contribution

J.P. and V.G-Q. contributed to the conception and design of the study; G.G., A.G., M.P., S.P-P., V.G-Q. and J.P. contributed to the acquisition and analysis of the data; G.G. and J.P. contributed to drafting the text or preparing the figures.

Conflict of interest

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. J.P. and V.G-Q. have received honoraria as an advisory/speaker from Novartis and Lilly, the companies manufacturing erenumab and galcanezumab, respectively. The remaining authors have nothing to disclose.

Data availability

The dataset from the current study is available from the corresponding author on reasonable request.

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Figure 1.- Circulating levels of A) alpha-CGRP and B) beta-CGRP in healthy controls (HC) and patients with CM at baseline (CM M0), at two-weeks (CM M0.5) and at three-months (CM M3) after first dose of mAb treatment. All data is presented as median with IQR range. Mann-Whitney U test for HC vs. CM M0 comparison; Friedman test followed by Dunn's test for CM M0 vs. M0.5 vs. M3 comparisons; ns: non-significant * p<0.05 *** p<0.001.

Figure 2.- Circulating levels of alpha-CGRP in: A) healthy controls (HC) and patients with CM with a reduction in their monthly headache days \geq 50% (responders) and <50% (non-responders) at baseline. Comparisons are established using Kruskal-Wallis test followed by Dunn's test. B) patients with CM responders to mAb at baseline (CM M0), at two-weeks (CM M0.5) and at three-months (CM M3) after first dose. Comparisons are established using Friedman test followed by Dunn's test. C) patients with CM non-responders to mAb at baseline (CM M0), at two-weeks (CM M0.5) and at three-months (CM M3) after first dose. Comparisons are established using Friedman test followed by Dunn's test. C) patients with CM non-responders to mAb at baseline (CM M0), at two-weeks (CM M0.5) and at three-months (CM M3) after first dose. Comparisons are established using Friedman test followed by Dunn's test. All data is presented as median with IQR range ns: non-significant. * p<0.05 ** p<0.01.

Figure 3.- Circulating levels of alpha-CGRP in: A) healthy controls (HC), patients with CM with a PGIC score >4 (PGIC>4) and those with a score \leq 4 (PGIC \leq 4) at baseline. Comparisons are established using Kruskal-Wallis test followed by Dunn's test. B) patients with CM with PGIC score >4 at baseline (CM M0), at two-weeks (CM M0.5) and at three-months (CM M3) after first dose. Comparisons are established using Friedman test followed by Dunn's test. C) patients with CM with PGIC score \leq 4 at baseline (CM M0), at two-weeks (CM M0), at two-weeks (CM M0.5) and at three-months (CM M0.5) and at three-months (CM M3) after first dose. Comparisons are established using Friedman test followed by Dunn's test. C) patients with CM with PGIC score \leq 4 at baseline (CM M0), at two-weeks (CM M0.5) and at three-months (CM M3) after first dose. Comparisons are established using Friedman test followed by Dunn's test. All data is presented as median with IQR range. ** p<0.01.

Figure 4.- Circulating levels of alpha-CGRP in: A) healthy controls (HC), patients with CM who stopped fulfilling AO criteria (Stopped AO) and those who kept fulfilling it (Kept AO) at baseline. Comparisons are established using Kruskal-Wallis test followed by Dunn's test. B) patients with CM who stopped fulfilling AO criteria at baseline (CM MO), at two-weeks (CM M0.5) and at three-months (CM M3) after first dose. Comparisons are established using Friedman test followed by Dunn's test. C) patients with CM who kept fulfilling AO criteria at baseline (CM M0), at two-weeks (CM M0.5) and at three-months (CM M3) after first dose. Comparisons are established using Friedman test followed by Dunn's test. C) patients with CM who kept fulfilling AO criteria at baseline (CM M0), at two-weeks (CM M0.5) and at three-months (CM M3) after first dose. Comparisons are established using Friedman test followed by Dunn's test. All data is presented as median with IQR range. ns: non-significant *p<0.05 ** p<0.01.

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Figure 5.- XY plot showing the correlation between Δ monthly migraine days (x axis) and Δ alpha-CGRP in pg/mL (y axis).

Table 1.- Summary of clinical characteristics of our patients with CM at baseline.

	Clinical features
	At least 1 como
	Anxiety and/
+	Fibromyalgia
	Arterial hype
	Hypercholest
	Obesity
	Analgesic overu
	Historical Preve
λ	Amitriptyline
	Topiramate
	Botulinum to
(1)	ß-Blockers
	Candesartan
	Valproic Acio
	Preventives at
	Amitriptyline
	Topiramate
	Botulinum to
()	ß-Blockers
	Candesartan
()	Valproic Acid
	Symptomatic tr
	Triptans
	NSAIDs
	Opioids

	Initial Sample ("N"=103)		Final Sample ("N"=96)	
Clinical features	"N"	%	"N"	%
Aura	22	21.4%	20	20.8%
At least 1 comorbidity	81	78.6%	76	79.2%
Anxiety and/or depression	65	63.1%	60	62.5%
Fibromyalgia	23	22.3%	22	22.9%
Arterial hypertension	22	21.4%	22	22.9%
Hypercholesterolemia	19	18.4%	17	17.7%
Obesity	10	9.7%	8	8.3%
Analgesic overuse	96	93.2%	89	92.7%
Historical Preventives				
Amitriptyline	99	96.1%	92	95.8%
Topiramate	97	94.2%	90	93.8%
Botulinum toxin A	96	93.2%	89	92.7%
ß-Blockers	93	90.3%	87	90.6%
Candesartan	64	62.1%	59	61.5%
Valproic Acid	39	37.9%	35	36.5%
Preventives at baseline				
Amitriptyline	28	27.2%	27	28.1%
Topiramate	13	12.6%	12	12.5%
Botulinum toxin A	55	53.4%	51	53.1%
ß-Blockers	20	19.4%	18	18.8%
Candesartan	26	25.2%	25	26.0%
Valproic Acid	4	3.9%	2	2.1%
Symptomatic treatment				
Triptans	88	85.4%	83	86.5%
NSAIDs	87	84.5%	81	84.4%
Opioids	12	11.7%	12	12.5%

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Table 1.- Summary of clinical characteristics of our patients with CM at baseline.

	Initial Sam	ple ("N"=103)	Final Samp	ole ("N"=96)
Clinical features	"N"	%	"N"	%
Aura	22	21.4%	20	20.8%
At least 1 comorbidity	81	78.6%	76	79.2%
Anxiety and/or depression	65	63.1%	60	62.5%
Fibromyalgia	23	22.3%	22	22.9%
Arterial hypertension	22	21.4%	22	22.9%
Hypercholesterolemia	19	18.4%	17	17.7%
Obesity	10	9.7%	8	8.3%
Analgesic overuse	96	93.2%	89	92.7%
Historical Preventives				
Amitriptyline	99	96.1%	92	95.8%
Topiramate	97	94.2%	90	93.8%
Botulinum toxin A	96	93.2%	89	92.7%
ß-Blockers	93	90.3%	87	90.6%
Candesartan	64	62.1%	59	61.5%
Valproic Acid	39	37.9%	35	36.5%
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Amitriptyline	28	27.2%	27	28.1%
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Candesartan	26	25.2%	25	26.0%
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Symptomatic treatment				
Triptans	88	85.4%	83	86.5%
NSAIDs	87	84.5%	81	84.4%
Opioids	12	11.7%	12	12.5%

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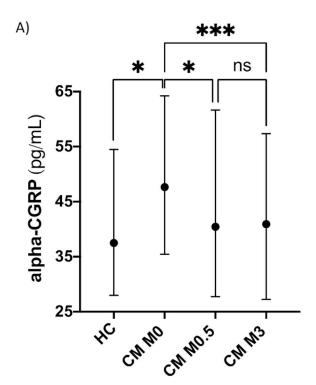
	"N"	%
Culcome parameters		-
At least 50% response	49	51.0%
<25% Response	23	24.0%
25-50% Response	24	25.0%
50-75% Response	35	36.5%
>75% Response	14	14.6%
PGIC scores		
scored ≤4	41	42.7%
scored >4	55	57.3%
An Igesic overuse	31	32.3%
eventives		
Amitriptyline	30	31.3%
Topiramate	9	9.4%
Fotulinum toxin A	30	31.3%
R-Blockers	14	14.6%
Landesartan	24	25.0%
Valproic Acid	3	3.1%
Sonptomatic treatment		
Triptans	83	86.5%
JSAIDs	72	75.0%
pioids	6	6.3%
At least 1 adverse effect	29	30.2%
onstipation	28	29.2%

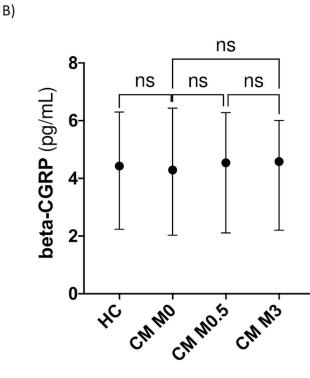
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Table 2.- Summary of clinical characteristics and treatment outcome parameters of our patients with CM at 3-months since first mAb dose.

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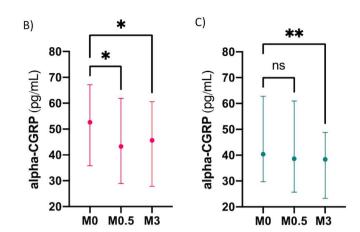


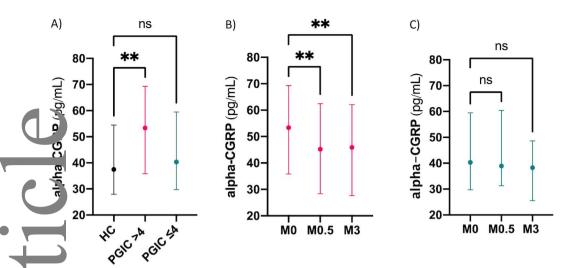




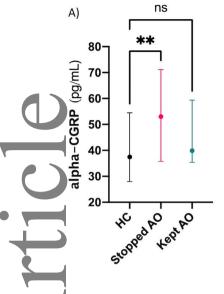
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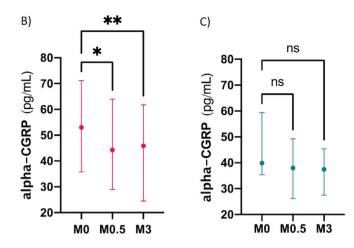




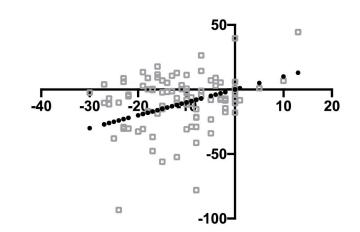


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- Δ Monthly headache Days at M3
- Δ alpha–CGRP en M3