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Plasma CGRP but not PACAP-38 concentrations are associated with response to anti-CGRP monoclonal antibodies in migraine

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ABSTRACT

Background: Monoclonal antibodies (mAbs) targeting the calcitonin gene-related peptide (CGRP) pathway have revolutionized migraine prophylaxis. However, a subset of patients does not respond to these therapies, highlighting an urgent need for predictive biomarkers. This study investigates whether baseline plasma levels of CGRP or pituitary adenylate cyclase activating peptide (PACAP)-38 may predict the clinical response to anti-CGRP mAbs in patients with high-frequency episodic migraine (HFEM) or chronic migraine (CM).

Methods: A prospective, longitudinal study was conducted on migraine patients treated with erenumab, fremanezumab, or galcanezumab. Clinical outcomes, including monthly migraine days (MMD), Migraine Disability Assessment (MIDAS), and monthly medication intake (MMI), were assessed at baseline (T0), 3 months (T1), and 6 months (T2). A group of healthy subjects was used as controls. Plasma CGRP and PACAP-38 levels were measured using an enzyme-linked immunosorbent assay (ELISA). Treatment responses were tested using bivariate and multivariate analyses.

Results: 30 females, 7 males (17 HFEM, 20 CM), and 16 healthy controls were included in the study. Baseline plasma CGRP and PACAP-38 concentrations were higher in migraine patients than in controls ($p<0.01$ and $p<0.001$, respectively). Baseline CGRP levels significantly correlated with worse clinical outcomes at 6 months: higher CGRP was associated with greater MMD ($r=0.470$, $p=0.003$), MIDAS scores ($r=0.601$, $p<0.001$), and MMI ($r=0.410$, $p=0.010$) at T2. Additionally, higher baseline CGRP levels were associated with a lower likelihood of achieving a ≥50% reduction in MIDAS scores. Multivariate regression confirmed that elevated CGRP independently predicted poorer response, particularly in CM patients. Conversely, PACAP-38 levels did not emerge as significant predictors of any clinical outcome measures.

Conclusions: Our study shows that higher baseline plasma CGRP levels predict a reduced clinical response to anti-CGRP mAbs in patients with HFEM and CM after 6 months, supporting CGRP as a potential biomarker for treatment stratification. PACAP-38 levels did not influence treatment outcomes, indicating a distinct role in migraine pathophysiology. These results encourage further research into personalized treatment approaches based on neuropeptide profiling.

Key words: migraine, chronic migraine, anti-CGRP monoclonal antibodies, CGRP, PACAP-38, MIDAS.

Introduction

Migraine is a chronic, neurovascular disease characterized by long-lasting headache attacks accompanied by nausea, vomiting, phono/photophobia, and focal neurological symptoms in some patients. (1,2) Migraine affects over one billion people worldwide and represents a disabling condition that affects the ability to work and carry out daily activities. (3,4) Furthermore, the financial burden that migraine imposes on the patient and society, encompassing both direct and indirect costs, is substantial. (5-7)

The trigeminovascular system plays a key role in migraine pathogenesis and calcitonin gene-related peptide (CGRP) is crucial in this process. (8,9) Drugs targeting the CGRP pathway, such as CGRP monoclonal antibodies (mAbs) and gepants, are increasingly used as preventive treatments for migraine. Monoclonal antibodies, such as erenumab, galcanezumab, fremanezumab, and eptinezumab, have been successful in reducing migraine frequency and severity, with up to 65% of patients seeing a 50% reduction in monthly migraine days. (10-14) Their effectiveness and safety are well established. (15) However, a percentage of patients still do not respond to anti-CGRP therapies, (16) highlighting the need for biomarkers to predict treatment response.

In the past decade, several efforts have been made to identify biomarkers able to predict and monitor treatment response in migraine. Studies showed that plasma concentrations of CGRP are elevated mainly in patients with chronic migraine. (17) Higher baseline plasma CGRP levels have been linked to better responses to onabotulinumtoxinA therapy. (18) Similarly, a recent study showed a significant association between higher pre-treatment salivary CGRP levels and a higher probability of achieving a 50% or greater reduction in headache frequency. (19) Finally, a recent study showed that baseline alpha-CGRP levels were significantly elevated in patients with chronic migraine (CM) and significantly decreased over the course of mAb treatment. (20) Nevertheless, further studies are needed to confirm these findings.

Additional neuropeptides that may be related to migraine pathogenesis are currently identified as potential therapeutic targets for migraine. Similar to CGRP, the pituitary adenylate cyclase activating polypeptide (PACAP), a peptide belonging to the vasoactive intestinal peptide, secretin, and glucagon superfamily, is found in sensory nerve fibers. It dilates cranial arteries and induces migraine when infused in humans. (21) Interestingly, a clinical trial with monoclonal antibodies targeting PACAP-38 has been demonstrated to be effective as a prophylactic therapy for migraine. (22)

The aim of this study was to evaluate whether pretreatment plasma levels of CGRP or PACAP-38 could predict the effective-

ness of subcutaneous anti-CGRP monoclonal antibody response at 3 and 6 months in patients with high-frequency episodic migraine (HFEM) and CM.

Results

Clinical characteristics of the study cohort. A group of 37 patients with HFEM or CM (median age: 54.3 years; interquartile range [IQR]: 47.0-61.0) were included in the analysis. Of these, 17 patients presented with HFEM, while 20 were diagnosed with CM. Among the CM patients, 18 also presented with medication overuse headache (MOH) at baseline. Sixteen healthy participants were also included. **Table 1** presents the demographic and clinical characteristics of the patients included in the study.

The median number of previously failed prophylactic treatments was 4.00 (IQR=3.0-4.75). The prescribed mAbs included: 7 erenumab (140 mg subcutaneously every 4 weeks), 16 fremanezumab (225 mg subcutaneously monthly), 14 galcanezumab (120 mg subcutaneously monthly following a 240 mg loading dose). The prescription of mAbs was based on patient-physician shared decision at baseline (T0) visit, considering factors such as injection device characteristics and dosing schedules. In this cohort, none of the patients received other concurrent preventive treatments during the study period to avoid potential confounders.

The mean scores obtained from the questionnaires State-Trait Anxiety Inventory (STAI)-Y1 (state anxiety), STAI-Y2 (trait anxiety), and the Beck Depression Inventory-II (BDI-II) did not reveal statistically significant differences between patients with HFEM and CM (p>0.05).

Treatment response at 3- and 6-month. As expected, the treatment efficacy of mAbs confirmed previously published data, with monthly migraine days (MMD), Migraine Disability Assessment (MIDAS), monthly medication intake (MMI), and Numerical Rating Scale (NRS) pain levels significantly reduced at 3 and 6 months (p<0.001, repeated-measures analysis of variance [ANOVA] with Tukey's honestly significant difference [HSD] *post hoc* comparisons).

Baseline CGRP levels and clinical characteristics. At baseline, CGRP levels were significantly higher in overall patients with migraine (HFEM and CM) compared to healthy controls (median CGRP in patients: 93.3 pg/mL (IQR 88.1-146) *vs.* 9.3 pg/mL in controls (IQR 4.3-58.9), with the difference being statistically significant (p=0.003) (**Table 2**). Pairwise comparisons confirmed significantly higher CGRP concentrations in both HFEM and CM relative to controls (p<0.05 and p<0.01, respectively). Plasmatic CGRP levels did not significantly differ when comparing HFEM and CM patients (**Table 1**).

We found no correlations between CGRP concentrations and baseline migraine parameters (MMD, MIDAS, MMI, and NRS). No significant differences were found in the migraine characteristics according to sex (p=0.522).

Baseline CGRP levels did not differ significantly between patients with and without acute medication intake within the preceding 24 hours (median 92.1 pg/mL *vs.* 112.4 pg/mL; Mann-Whitney U test, p=0.330).

Baseline CGRP levels and response to anti-CGRP mAbs at 3- and 6-month. No significant correlations were observed between baseline CGRP levels and clinical variables (MMD, MIDAS, MMI) at 3 months (T1). Interestingly, baseline CGRP levels were significantly correlated at six months (T2) with MMD (r=0.470; p=0.003), MIDAS (r=0.601; p<0.001), and MMI (r=0.410; p=0.010).

In the overall cohort, treatment response, assessed as a reduction in MMD at dichotomous thresholds of 30%, 50%, and 75%, showed no significant differences in baseline CGRP concentrations between responders and non-responders at either 3 or 6 months.

In subgroup analyses, female patients with chronic migraine who did not achieve a ≥50% reduction in MMD at 6 months showed significantly higher baseline CGRP concentrations compared with responders (p=0.038). Furthermore, in this subgroup

Table 1. Patients' characteristics at baseline (T0). Data are expressed as median and IQR (median, Q1-Q3) or as count and percentage of the total. Quantitative variables are compared through a two-tailed independent-samples Mann-Whitney U test. Categorical variables are compared through a two-sided Fisher's exact test.

	Patients (n=37)	CM (n=20)	HFEM (n=17)	p-value
Age at sampling	54.3, 47.0-61.0	52.2, 46.5-60.1	54.0, 48.9-62.0	0.737
BMI	22.5, 20.1-25.0	24.5, 21.8-26.0	22.0, 20.0-23.0	0.119
Females	30 (81.1%)	15 (75%)	15 (88.24%)	0.417
Age at onset	18.0, 13.0-30.0	18.0, 10.0-26.3	25.0, 14.0-22.0	0.232
Duration of disease	31.0, 18.9-41.0	31.9, 23.1-43.5	25.0, 14.0-32.0	0.404
Number of failed prophylaxis	4.0, 3.0-4.75	4.0, 3.0-4.0	3.0, 3.0-4.0	0.341
Migraine with aura	5 (13.51%)	2 (10%)	3 (17.65%)	0.644
CGRP (pg/mL)	93.3 (88.1-146)	91.1 (88.1-108)	112 (86.6-192)	0.411
PACAP-38 (pg/mL)	218 (184-271)	234 (184-277)	215 (189-259)	0.662

CM, chronic migraine; HFEM, high-frequency episodic migraine; BMI, body mass index; CGRP, calcitonin gene-related peptide; PACAP, pituitary adenylate cyclase activating polypeptide.

Table 2. Baseline levels of CGRP and PACAP-38 in patients and controls. Values are reported as median and IQR (median, Q1 -Q3); p-values from the Mann-Whitney U test.

	Cases (n=37)	Controls (n=16)	p-value
CGRP (pg/mL)	93.3 (88.1-146)	9.3 (4.3-58.9)	0.003
PACAP-38 (pg/mL)	218 (184-271)	109.4 (105.4-118.6)	p<0.001

CGRP, calcitonin gene-related peptide; PACAP, pituitary adenylate cyclase activating polypeptide.

each 20 pg/mL increase in baseline CGRP was associated with an approximately 10% lower odds of achieving a clinical response (odds ratio [OR]=0.90; 95% confidence interval [CI]: 0.82-0.99; p=0.035), while each 50 pg/mL increase was associated to a 22% reduction in the odds of improvement (OR=0.78; 95% CI: 0.66-0.94; p=0.035).

Conversely, when clinical response was assessed using MIDAS, baseline CGRP distinguished overall responders from non-responders at 6 months. Patients who failed to achieve a ≥50% reduction in MIDAS had higher baseline levels than responders (median 642.8, IQR 615.4-670.1 pg/mL *vs.* median 93.3, IQR 87.9-134.5 pg/mL, p=0.024). Logistic regression analyses supported this finding, indicating that higher baseline CGRP was associated with an increased likelihood of MIDAS non-response.

Baseline PACAP-38 levels and baseline clinical characteristics. PACAP-38 concentrations were elevated in overall patients (median 218 pg/mL, IQR 184-271) compared to controls (109.4 pg/mL, IQR 105.4-118.6, p<0.001). In the subgroup analysis, median concentrations were 234 pg/mL (IQR 184-277.1) in HFEM and 215 pg/mL (IQR 189-259) in CM, both markedly higher than controls (109.4 pg/mL, IQR 105.4-118.6, p<0.001 in both subgroups). In our cohort, no difference was found in PACAP-38 levels between HFEM and CM.

Moreover, we compared PACAP-38 plasmatic concentrations across groups of patients based on medication intake during the 24 hours prior to sampling, and no significant differences were found.

We also tested the association between baseline PACAP-38 and CGRP levels and found no significant correlation, either in the overall cohort or when analyses were stratified by migraine subtype (high-frequency episodic migraine and chronic migraine) (p>0.05).

Baseline PACAP-38 levels and response to anti-CGRP mAbs at 3- and 6-month. Interestingly, baseline PACAP-38 concentrations showed no significant association with treatment response, regardless of whether outcomes were defined by MMD or MIDAS. Mann-Whitney U tests confirmed that baseline PACAP-38 levels were similar between responders and non-responders across all thresholds and time points (p>0.05). Logistic regression models, adjusted for migraine subtype, also confirmed the absence of predictive value.

Multivariate analyses. Multivariate analyses reinforced these findings. After adjusting for diagnosis, age, BMI, and sex, baseline CGRP levels remained significantly associated with worse outcomes at 6 months. Higher baseline CGRP predicted, at T2, increased MMD (β=0.018, p=0.007; R^2=0.349), higher MIDAS scores (β=0.088, p<0.001; R^2=0.469), and greater MMI (β=0.018, p=0.017; R^2=0.348). Stratified analyses indicated that these associations were stronger among CM patients compared with HFEM (**Figures 1-3**). In contrast, no significant associations were found for NRS scores at either time point.

Discussion

The main purpose of our study was to investigate whether baseline plasma concentrations of CGRP and PACAP-38 could serve as biomarkers for predicting the clinical response to anti-CGRP monoclonal antibodies. We found that patients with high-frequency migraine and chronic migraine who had elevated baseline CGRP levels demonstrated a lower clinical response to mAb therapy at six months. Intriguingly, PACAP-38 plasma concentrations showed no significant differences between responders and non-responders, suggesting that this peptide is not a predictive biomarker for anti-CGRP therapy.

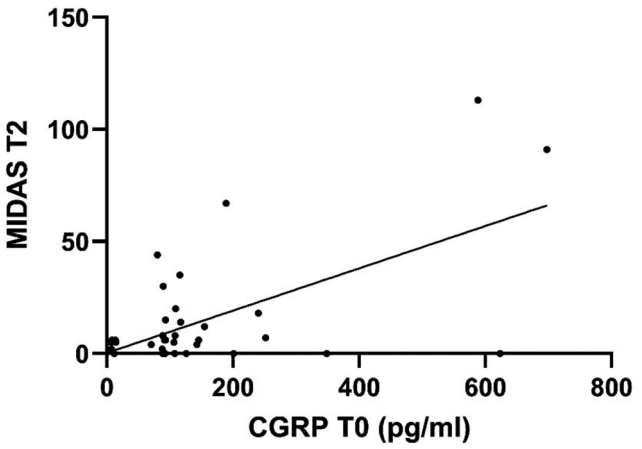

Figure 1. Correlation between plasma CGRP concentration at T0 and MIDAS at T2. The solid line represents the linear regression fit (R^2=0.469, p<0.001).

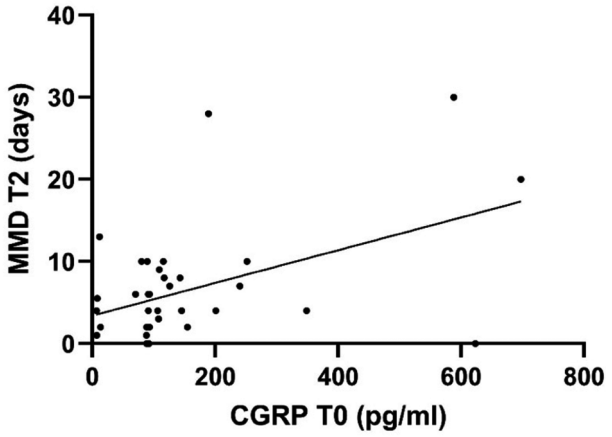

Figure 2. Correlation between plasma CGRP concentration at T0 and MMD at T2. The solid line represents the linear regression fit (R^2=0.349, p=0.007).

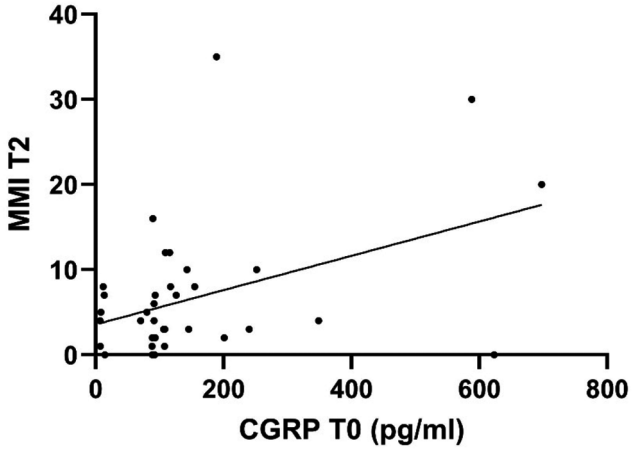

Figure 3. Correlation between plasma CGRP concentration at T0 and MMD at T2. The solid line represents the linear regression fit (R^2=0.349, p=0.007).

Our findings also showed that baseline plasma CGRP and PACAP-38 levels were significantly higher in migraine patients compared to healthy controls. These differences confirm that both neuropeptides are upregulated in patients with migraine and support their role in migraine pathophysiology. However, only CGRP, not PACAP-38, was associated with treatment outcomes, highlighting that the two peptides contribute differently to disease pathophysiology.

To the best of our knowledge, this is the first study to assess both CGRP and PACAP-38 levels as potential biomarkers of anti-CGRP mAbs efficacy. Previous studies have primarily focused on CGRP levels. A study investigated salivary CGRP levels and found that higher pretreatment levels were significantly associated with a higher probability of having a reduction of 50% or more in headache frequency in patients treated with erenumab. (19) This effect was observed exclusively in patients with HFEM but not in CM. Another recent study examining circulating levels of alpha and beta-CGRP in patients with CM treated with mAbs revealed that a decrease in alpha-CGRP levels during treatment was positively correlated with a reduction in MMDs. (20) Furthermore, the authors reported that patients who experienced a reduction of ≥50% in MMDs exhibited higher baseline alpha-CGRP levels compared to non-responders. (20) Finally, a recent study showed higher CGRP interictal levels in tears of migraine patients in comparison to controls, and markedly greater concentrations than plasma, which can normalize after 6 months of anti-CGRP mAb therapy, suggesting its potential as a sensitive, non-invasive biomarker of treatment response. (23)

Our findings suggest that higher baseline CGRP levels may be associated with a reduced response to anti-CGRP mAbs in patients with HFEM and CM. Patients with elevated baseline CGRP levels exhibited a reduced improvement in MMD and MIDAS reduction, indicating that CGRP could serve as a predictor of less favorable outcomes with anti-CGRP therapy. Furthermore, in a linear regression model, baseline CGRP was independently associated with both MMD and MIDAS scores at six months, reinforcing its potential role as a predictive biomarker for treatment resistance.

These results differ from earlier studies that suggested a positive correlation between high CGRP levels and improved treatment outcomes. Such discrepancies could arise from methodological variations, including differences in biofluid sources (plasma *vs.* saliva), peptide isoforms analyzed, and assay techniques used to quantify CGRP concentrations. Overall, it is important to note that findings derived from blood and salivary samples are not directly comparable. In particular, the correlation between plasma and salivary concentrations of CGRP remains unexplored. Furthermore, various methodologies and different enzyme-linked immunosorbent assay (ELISA) kits have been employed across studies to quantify blood levels of circulating CGRP, and this could explain the varying data on CGRP plasma concentrations in the literature, even when employing the same techniques, such as enzyme-linked immunosorbent assays. (17,20,24) Furthermore, some studies investigated serum levels of the peptide and other studies the plasmatic ones. Nevertheless, all studies consistently underscored the use of CGRP levels as potential biomarkers for evaluating the therapeutic efficacy of anti-CGRP treatments in migraine prophylaxis.

Interestingly, plasma CGRP levels have also been suggested as a predictor of response to onabotulinumtoxin-A. (18) As CGRP assays are highly sensitive to methodological differences, standardizing assay protocols and differentiating between CGRP isoforms may be crucial for validating these findings and implementing CGRP as a reliable biomarker in clinical practice. In this direction, standardizing the methodology for quantifying CGRP concentrations could aid clinical practice and highlight the potential for personalized dosage strategies.

The association between high baseline CGRP levels and reduced response to mAb treatment suggests a state of trigeminovascular hyperexcitability, in which persistently elevated CGRP may reflect an upregulated or compensatory pain pathway that is less responsive to CGRP-targeted monoclonal antibodies. This observation aligns with findings that baseline CGRP levels in individuals with chronic migraine are often significantly elevated compared to those in episodic migraine and controls. (25) The presence of high CGRP levels may indicate an underlying physiological state where CGRP plays a more complex or compensatory role in migraine pathophysiology, potentially diminishing the efficacy of therapies that target a single neuropeptide pathway.

Our study also observed higher PACAP-38 concentrations in patients with migraine compared with controls. The values observed in our cohort are consistent with those previously reported in the literature, accounting for methodological differences across studies. A previous study reported increased PACAP-38 levels during migraine attacks compared with both the interictal phase and controls. (26) Further studies also reported elevated PACAP-38 concentrations in both pediatric and adult migraine populations compared with healthy subjects. (27,28)

We also analyzed the role of PACAP-38 as a biomarker for anti-CGRP therapy. In our cohort, baseline PACAP-38 levels showed no significant correlations with clinical outcomes, suggesting that PACAP-38 is not a reliable predictor of anti-CGRP mAbs efficacy. This aligns with preclinical studies indicating that the PACAP-38 pathway is independent of CGRP-mediated mechanisms. Recently, multiple migraine-relevant mouse models showed that the PACAP-38 pathway is distinct and independent from other migraine-provoking pathways. (29) Very recently, monoclonal antibodies against PACAP-38 have been demonstrated to be effective for migraine prophylaxis. (30) Our data indicate that the PACAP-38 levels did not directly influence the response to mAb therapy against CGRP, indirectly supporting a distinct pathogenetic pathway, and underscoring the importance of identifying distinct therapeutic targets for different patient subgroups.

Furthermore, the absence of significant correlations between plasma CGRP and PACAP-38 levels, both in the overall cohort and within migraine subgroups, further supports that these neuropeptides reflect distinct pathophysiological mechanisms. This finding is consistent with previous reports showing that CGRP and PACAP-38, although both implicated in trigeminovascular activation, are regulated independently and may contribute differently to migraine chronification and disability. (31) The lack of association in our data is therefore consistent with the view that CGRP and PACAP-38 are not redundant but rather complementary mediators, which could partly explain the heterogeneous clinical response to anti-CGRP therapies.

Taken together, our results and recent literature highlight the need for personalized strategies in migraine management. Measuring CGRP across multiple biofluids (plasma, saliva, tear fluid) could help stratify patients into likely responders and non-responders. Patients with persistently elevated CGRP despite therapy may require alternative or combined approaches, such as onabotulinumtoxin-A, higher mAb dosing, or therapies targeting other pathways.

We acknowledge some limitations in our study. Firstly, the number of patients examined is relatively low and, due to drug reimbursement restrictions, we have investigated mainly patients with severe forms of migraine. Larger, multicenter trials are necessary to confirm the role of baseline CGRP levels in both high-frequency episodic migraine and chronic migraine, and to clarify whether their prognostic role differs across migraine subtypes. Secondly, another limitation of our study is the possible influence of MOH, which was present in a substantial proportion of CM patients at baseline. Thirdly, variability in assay techniques complicates comparisons with prior studies.

We used a specific ELISA kit designed to detect the bioactive mature form of CGRP, but it could not differentiate between the alpha and beta isoforms. This limitation highlights the need for standardized protocols to ensure consistency and reproducibility across studies. A further limitation is that a subset of patients had received acute migraine medication within 24 hours before blood sampling. Although CGRP and PACAP-38 levels did not differ between patients who did and did not take acute medication, a potential influence on peptide concentrations cannot be completely ruled out. Finally, we did not assess longitudinal changes in CGRP or PACAP-38 concentrations during treatment, which could have provided valuable information on their dynamic role as biomarkers.

Conclusions

Our findings indicate that higher baseline plasma CGRP levels predict a reduced clinical response to anti-CGRP mAbs in patients with CM and HFEM. In contrast, PACAP-38 plasma concentrations do not appear to influence treatment outcomes, underscoring the distinct roles of these neuropeptides in migraine pathophysiology. Standardized CGRP measurement protocols and further research on individualized treatment strategies are essential for optimizing the use of anti-CGRP therapies in clinical practice.

Materials and Methods

Study participants. This was a prospective, longitudinal, single-center study. A group of patients regularly followed at the Headache Center of the Department of Neuroscience, Città della Salute e della Scienza di Torino (Italy) was recruited for the study. The diagnosis of migraine with or without aura and chronic migraine was made according to the International Classification of Headache Disorders (ICHD)-3 criteria.[1] Adult patients (18-65 years of age) with high frequency episodic migraine (from 8 to 14 MMD) or chronic migraine, with or without MOH, and starting treatment with monoclonal antibodies anti-CGRP (fremanezumab, galcanezumab) or anti-CGRP receptor (erenumab) were included in the study. A complete physical and neurological examination was performed at baseline (T0). Patients were clinically re-evaluated at 3 months (T1) and 6 months (T2). To be included in the study, patients with HFEM and CM fulfill criteria for prescribing anti-CGRP/CGRP-R mAbs in Italy (no response/intolerance to at least 3 preventive treatments, >8 MMD in the last 3 months, MIDAS>11 points). Detailed headache characteristics were recorded using a questionnaire and were collected during the planned clinical examination. Clinical history was recorded, focusing on migraine characteristics, drug intake during attacks, and previous prophylactic therapies.

The recruited patients maintained a record of MMD and MMI using a diary. The pain level during each attack was self-reported by the patients in the same diary using the NRS. In addition, patients completed the MIDAS questionnaire. (32) Medication intake (triptans, nonsteroidal anti-inflammatory drugs [NSAIDs], or combination therapy) was recorded if it happened during the last 24 hours before the blood collection. Anxiety symptoms were assessed using STAI-Y1 and STAI-Y2, while depressive burden was evaluated with the BDI-II.

A group of healthy subjects (with no personal or family history of migraine or other primary headaches) was used as controls to evaluate the basal levels of CGRP and PACAP-38. They were voluntarily recruited from healthcare and research professionals.

The study protocol was approved by the Hospital Ethics Committee (Comitato Etico Interaziendale – A.O.U. Città della Salute e della Scienza di Torino – protocol 00307/2022). All participants gave written informed consent for their participation in the study and for data collection in accordance with the Declaration of Helsinki.

Biological samples. Sampling was performed in the ictal phase. Blood samples were collected in heparinized tubes, and within 30 minutes of collection were centrifuged at 3000 rpm at 4°C for 15 minutes. Plasma was immediately aliquoted into 200 µL tubes and frozen at –80°C. Samples were processed within a month to avoid loss of bioactivity and contamination.

CGRP analysis. We analyzed plasma samples using a commercial ELISA from Bertin Bioreagent targeting both α-CGRP and β-CGRP (Cat. No. A05481, Bertin Bioreagent, Montigny-le-Bretonneux, France, batch numbers 121 and 123). To minimize matrix effects, all calibration curves and quality controls (QCs) were prepared in CGRP-free plasma. This was achieved by incubating a pool of samples with CGRP antibodies (Cat No: A19482, Bertin Bioreagent, batch no. 0222) overnight at 4°C on a tilt shaker, followed by filtration in accordance with CGRP-kit instructions. Analysis of two replicates of the resulting CGRP-depleted plasma confirmed successful depletion (<2 pg/mL) in accordance with a previous study. (33)

The assay procedure was performed following the manufacturer's instructions. Briefly, after loading the samples, the tracer was added, and the plate was incubated overnight (20 hours at 4°C). Subsequently, Ellman's reagent was added, and the resulting color intensity was analyzed with a microplate spectrophotometer after 30 minutes of incubation. A calibration curve was included on each plate and plotted, from which data were interpolated. A spline line fitting was used to determine concentrations. Details about the standard curve are available upon request. The intra-assay and inter-assay coefficients of variation for CGRP were 5.2% and 4.3%. The limit of detection was 2 pg/mL as reported in the datasheet.

PACAP-38 analysis. A commercially available competitive ELISA kit for PACAP-38 (BlueGene Biotech Co., Pudong New District, Shanghai) was used to measure plasma concentrations. The PACAP-38 ELISA kit employs a competitive enzyme immunoassay technique, using an anti-PACAP antibody and a PACAP-horseradish peroxidase (HRP) conjugate. The optical density at 450 nm was measured spectrophotometrically using a microplate reader. The color intensity was inversely proportional to PACAP concentration, as PACAP in the samples and the PACAP-HRP conjugate compete for the anti-PACAP antibody binding site. A standard curve was used to determine sample concentrations. The intra- and inter-assay coefficients of variation were 4.4% and 5.6%, respectively, for PACAP-38. No pre-analytical or immunoreactivity detection issues were encountered.

Statistics. Continuous variables were described using the median and IQR, while categorical variables were presented as counts and percentages. Normality of variables was assessed with the Shapiro-Wilk test. Differences in continuous data between groups were analyzed using two-tailed independent-samples t-tests or Mann-Whitney U tests, while categorical variables were examined with Fisher's exact test. For comparisons involving repeated measures across three time points, a repeated-measures ANOVA was applied, followed by Tukey's HSD test for *post hoc* pairwise comparisons to assess changes. Parametric tests were used only when the normality assumption was met ($p<0.05$). Sample size estimation also considered CGRP and PACAP-38 plasma concentration differences between patients and controls, assuming a large effect size (Cohen's $d=0.9$) for an independent-samples t-test, with power ≥ 0.8 and $\alpha=0.05$. This

yielded required sample sizes of 36 patients and 15 controls (group ratio 2.5), corresponding to power = 0.819.

Clinical response in migraine patients was defined as a ≥50% reduction in monthly migraine days (responders), with additional exploratory thresholds of ≥30% and ≥75% thresholds explored (partial responders and super-responders, respectively). A ≥50% reduction in MIDAS score at 3 and 6 months was also considered a response criterion. Power analysis was conducted assuming a medium effect size (Cohen's d=0.5) for a paired t-test, with α=0.05 and power ≥0.8, yielding a required sample size of 34 pairs (power = 0.841). For correlations, a moderate strength (r/rho=0.45) was assumed, resulting in a sample size of 36 observations for both methods.

To investigate the potential association between baseline neuropeptide concentrations and clinical outcomes, we performed linear regression analyses. In these models, clinical variables at different time points were used as dependent variables, while baseline CGRP and PACAP-38 levels were entered as predictors, together with relevant patient characteristics as covariates. Additionally, to further investigate these relationships, a stratification based on whether the patient belonged to the HFEM or CM group was performed. Analyses were performed with R, version 4.2.3 (The R Project for Statistical Computing, Vienna, Austria) and Jamovi (The Jamovi Project, version 2.3.28). Graphics were obtained using GraphPad Prism 10.0.1. The level of statistical significance was defined at $p<0.05$.

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Contributions: ER and IR conceived and supervised the project; SB carried out the sample analysis; AM, EMP, EG, and FR were responsible for samples and data collection; AMC performed the statistical analyses; IR revised the manuscript for intellectual content; all authors drafted the manuscript.
All authors have read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

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Availability of data and materials: the datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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