

GRADO EN CIENCIAS BIOMÉDICAS

TRABAJO FIN DE GRADO

Escribir Título TFG en Castellano

Investigando los niveles de α - y β -CGRP en plasma: Hacia un Biomarcador Fiable para la Migraña Crónica

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Investigating α - and β -CGRP levels in plasma: Towards a Reliable Biomarker for Chronic Migraine

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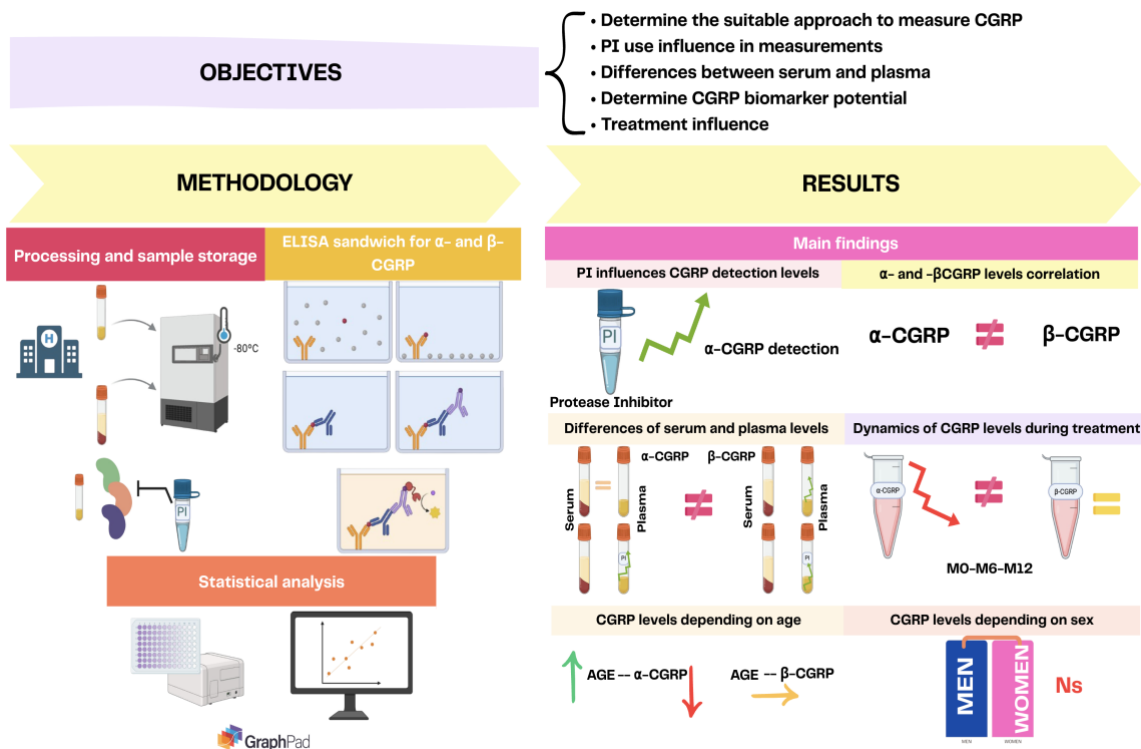
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GRAPHICAL ABSTRACT



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Investigating α - and β -CGRP levels in Plasma: Towards a Reliable Biomarker for Chronic Migraine

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RESUMEN

Introducción: la migraña es una enfermedad incapacitante común, especialmente en mujeres, relacionada con cambios hormonales. El neuropéptido CGRP, en particular α -CGRP, es clave en su fisiopatología. Aunque es diana terapéutica y potencial biomarcador, persisten inconsistencias en su medición. El objetivo del estudio es analizar factores que influyen en la medición de CGRP en plasma.

Métodos: Se reclutaron pacientes con migraña crónica en el Marqués de Valdecilla. Se recogieron muestras de sangre en distintos momentos del tratamiento y fueron procesadas y almacenadas. Los niveles de CGRP se midieron con test ELISA específicos para cada isoforma. Se aplicaron pruebas estadísticas para evaluar diferencias entre grupos.

Resultados: La adición de inhibidores de proteasa (PI) aumento la detección de α -CGRP, pero no de β -CGRP. No hubo correlación entre ambas isoformas en plasma. El plasma con PI mostró concentraciones más altas de CGRP comparado con suero. α -CGRP disminuyó sus concentraciones durante el tratamiento, mientras que β -CGRP permaneció estable. α -CGRP mostró una correlación negativa con la edad, pero sin diferencias según sexo.

Conclusión: Añadir PI mejoró la detección del CGRP en plasma. α -CGRP disminuyó con tratamiento y estuvo influenciado por la edad, sugiriendo su papel como biomarcador. β -CGRP se mantuvo estable, sugiriendo regulación independiente de ambas isoformas.

ABSTRACT

Introduction: migraine is a common disabling disease, especially among women, and is related to hormonal changes. The neuropeptide CGRP, especially α -CGRP is central in migraine pathophysiology. Although CGRP is a therapeutic target and potential biomarker, inconsistencies in measurement procedures need to be further studied for reliable results. This study aims to analyze factors influencing plasma CGRP measurement.

Methods: chronic migraine patients were recruited at Marqués de Valdecilla. Blood samples were collected at different times during treatment, processed and stored. Levels of CGRP were measured with isoform-specific ELISA tests. Statistical tests were used to assess concentration changes and group differences.

Results: protease inhibitors (PI) addition to plasma samples significantly increased α -CGRP levels detection, with a smaller effect on β -CGRP. No correlation was found between α - and β -CGRP in plasma. Plasma-PI samples showed higher CGRP concentrations than in serum. α -CGRP levels decreased during treatment while β -CGRP levels maintained stable. α -CGRP and age correlated negatively, but no sex-related differences were observed neither for α nor β -CGRP.

Conclusion: PI improved CGRP detection in plasma. α -CGRP levels decreased with treatment and were influenced by age, suggesting its potential role as a biomarker. In contrast, β -CGRP remained stable, suggesting independent regulation of both isoforms.

KEY WORDS

CGRP, migraine, protease inhibitors, biomarker, plasma, serum.

INTRODUCTION

Migraine is a highly prevalent and disabling neurological disorder which involves headache attacks as well as nausea, photophobia or phonophobia among other symptoms. This disorder continues to be the second cause of disability worldwide, and the first among young women according to the Global Burden Disease of 2019 ².

Its incidence is significantly higher in women than men, occurring from 3-4 times more often in females during puberty. The tendency decreases after menopause, suggesting that changes in ovarian hormones might play a key role in the physiopathology. In fact, several studies relate hormonal changes as a trigger to develop a migraine attack ³. For instance, chronic migraine (CM) (headaches occurring 15 or more days/month for 3 or more months) has a prevalence of 1,29% in females and only 0,48% in males ³.

Thus far, its pathophysiology remains poorly understood. Nevertheless, it is well established that migraine has a strong genetic component, as family and twin studies have detected a 30-60% of heritability of migraine and recent genomewide association studies have identified target genes of the calcitonin gene-related pathway (CGRP) (CALCA and CALB) ^{4,5}. In addition, it has been also described that the trigeminal vascular system (TVS) activation is required for the headache to develop ⁶. This activation leads to the release of vasoactive neuropeptides such as CGRP. This peptide has been highly associated to migraine for years ⁷, since it was the only peptide consistently elevated within a migraine attack in a study conducted in 1990 ⁸.

CGRP is a multifunctional neuropeptide of 37 amino acids with several functions that results from an alternative splicing of the calcitonin gene transcript (CALCA), which leads to the **alpha-CGRP** (α -CGRP) isoform, whereas a different gene (CALCB) encodes the **beta-CGRP** (β -CGRP) isoform of the peptide ⁹. Both isoforms are distributed all over the body and only differ in 3 out of the 37 amino acids. Nonetheless, α -CGRP has been described to be predominant in the nervous system (both central and peripheral) and highly abundant in pericerebral vessels and in the Gasserian ganglion ⁸, while β -CGRP is higher in the enteric nervous system and linked with the gastrointestinal effects of CGRP in humans ¹⁰. It has also been recently shown that β -CGRP levels are elevated in COVID-19 patients experiencing diarrhea ^{9,11}.

Because of these findings, several CGRP-based drugs have been developed such as the generation of monoclonal antibodies (mAb) against CGRP ligand or its receptor (CGRP-R). In fact, four monoclonal antibodies have been approved for migraine prevention (erenumab, eptinezumab, fremanezumab, galcanezumab) ^{9,12}, the first of them targeting the CGRP-R and the others targeting the CGRP ligand. Newly, small oral molecules called “gepants” have emerged to complement the monoclonal antibodies as they also block the CGRP receptor ¹³.

The importance of CGRP extends beyond its use as a possible therapeutic target, as it has also been proposed as a biomarker for migraine and for its therapeutical response ⁴, particularly α -CGRP due to its predominance in both the central and peripheral nervous system as mentioned before. This is supported by a study in which treatment with mAbs progressively restored α -CGRP levels by 3 months and the data obtained supported a role of the neuropeptide as the first dynamic CM biomarker ¹⁰. Not only has it been highlighted as a biomarker in migraine, in fact it has also been proposed for early detection and tracking disease progression of various neurodegenerative diseases (NDDs) ¹⁴.

Although CGRP levels tend to be elevated in migraine, about one-third of migraine patients have CGRP levels similar to subjects without headache ⁸. In addition, the significance of blood measurements remains unclear due to the contradictory results among the different studies that have been published and the variability of biological fluids that have been used besides blood, like saliva, cerebrospinal fluid or tears ⁵. All these impede to confirm whether the elevation of this peptide can be consistently detected or used as a reliable biomarker for migraine ¹⁵. The reason of the discrepancies is most probably multifactorial, as it may be influenced by methodological and individual parameters such as hormonal changes due to menstrual cycle or other comorbidities that are not usually considered as they should.

Therefore, the aim of this study was to determine the suitable approach to measure CGRP concentrations, both α and β -CGRP isoforms, in plasma samples from patients with CM. Moreover, the effect of several aspects including measurements in different biological fluids (plasma or serum); the use of treatments or sex and age differences on CGRP levels was also analyzed to delve into the potential of this peptide to be actually used as the biomarker that has been proposed to be.

RESULTS

Effect of the use of Protease Inhibitors in plasma

The procedure of adding protease inhibitors (PI) to plasma samples was followed to determine if its presence may have an effect regarding the levels and stability of both CGRP isoforms, which might encourage its addition as a routine when processing the plasma samples.

In [Figure 1A](#) we can appreciate a strong positive correlation between α -CGRP plasma levels and PI-plasma levels (Spearman coefficient (rs)=0.9310, $p<0.0001$). Interestingly, we can see that α -CGRP plasma levels were significantly elevated in those plasma samples with PI added (PI-plasma), compared to the same samples without PI added (α -CGRP plasma with PI median= 38.29; α -CGRP plasma without PI median= 21.26; $p<0.0001$, [Figure 1B](#)).

Regarding β -CGRP, we can also observe a positive correlation between β -CGRP plasma levels and PI-plasma levels (rs=0.8390, $p<0.0001$, [Figure 1C](#)). However, with β -CGRP plasma levels there was no statistically significant increase in the concentrations of CGRP when PI was added into the samples (PI-plasma β -CGRP median= 6.909; plasma β -CGRP median= 6.200; $p<0.0001$, [Figure 1D](#)).

In summary, adding PI to plasma samples can make a difference in the detection of the α -CGRP because, although they show a high positive correlation, the absolute values are systematically different as we can see in [Figure 1B](#), an important discovery regarding plasma sample processing. Whereas not the same can be said for β -CGRP since the difference observed was not significant.

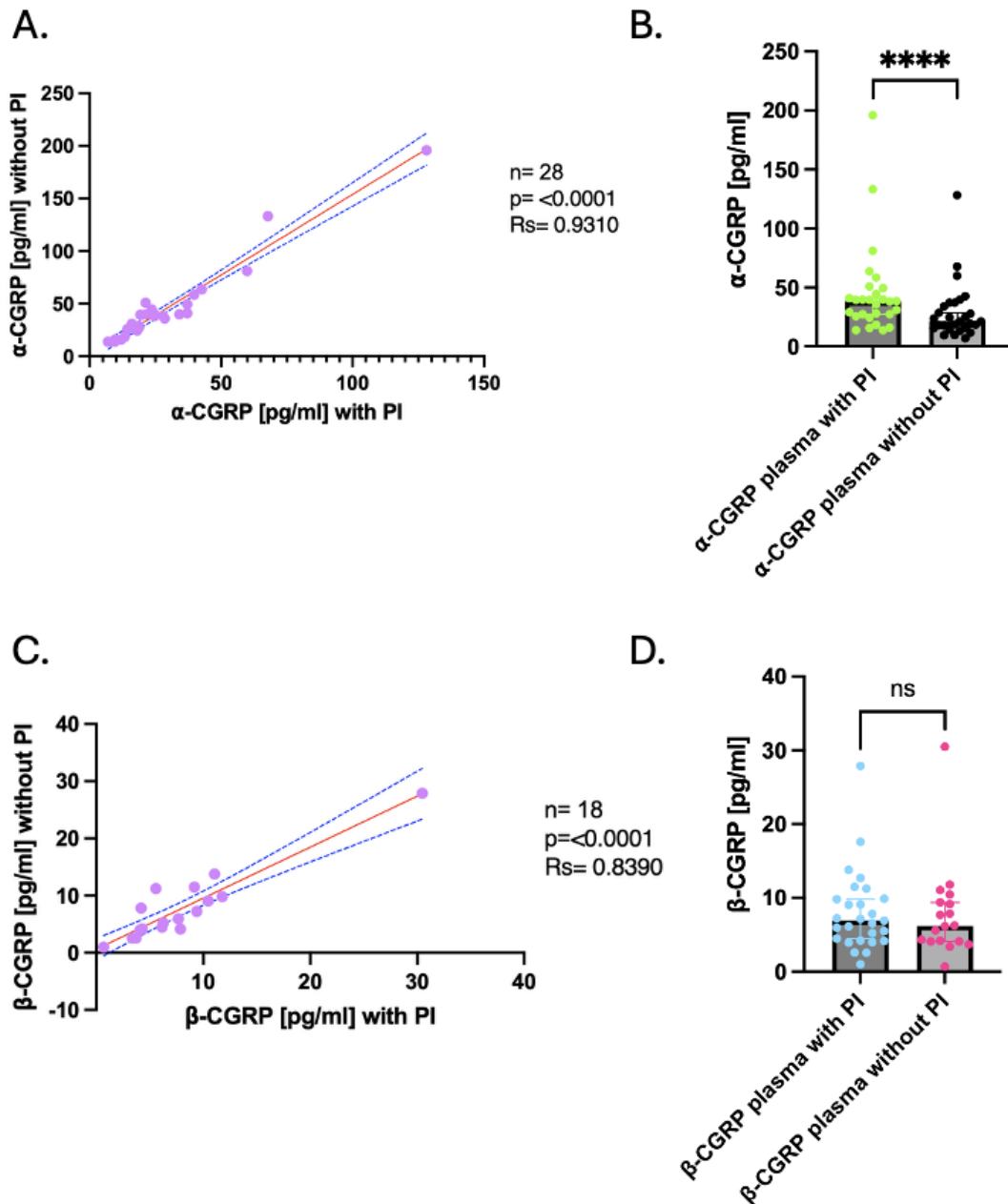


Figure 1. Differences of CGRP levels with and without PI.

(A) Correlation of α -CGRP levels in plasma with PI and plasma without PI. Red line represents the linear regression, and the dark blue dotted line represents the confidence interval (CI). (B) Difference of α -CGRP levels in plasma with PI (represented in green) vs plasma without PI (represented in black), data are shown as median with CI 95%. Comparison was made using the Wilcoxon Signed Rank test, (plasma with PI median= 38.28, plasma without PI median= 21.26, $p<0.0001$). (C) Correlation of β -CGRP levels in plasma with PI and plasma without PI. Red line represents the linear regression, and the dark blue dotted line represents the CI. (D) Difference of β -CGRP levels in plasma with PI (represented in light blue) vs plasma without PI (represented in pink), data are shown as median with CI 95%. Comparison was made using the Wilcoxon Signed Rank test, (plasma with PI median= 6.909, plasma without PI median= 6.200, $p=$ not significant).

Correlation of α - and β -CGRP levels

In previous published studies, our group observed that the levels of both isoforms of CGRP did not correlate in serum samples of patients¹⁵, so we decided to analyze if the same trend was maintained in plasma and PI-plasma or whether this aspect varied.

We performed a correlation analysis between α - and β -CGRP levels in plasma and, as shown in Figure 2, there was no statistically significant correlation between the concentration of both isoforms of the peptide in our cohort of patients ($n=115$, $r_s=0.05405$, $p=0.5662$, Figure 2A). Despite the reduced availability of PI-plasma, when comparing α - and β -CGRP levels in PI-plasma samples, we observed the same outcome, as there was no significant correlation either between α - and β -CGRP levels of the peptide in our subjects ($n=23$, $r_s=0.1542$, $p=0.4825$, Figure 2B).

We can conclude that the lack of correlation between α - and β -CGRP concentrations already observed in serum was maintained in plasma, no matter did we use untreated or PI-added plasma, suggesting that both isoforms of the peptide may be independently regulated in migraine.

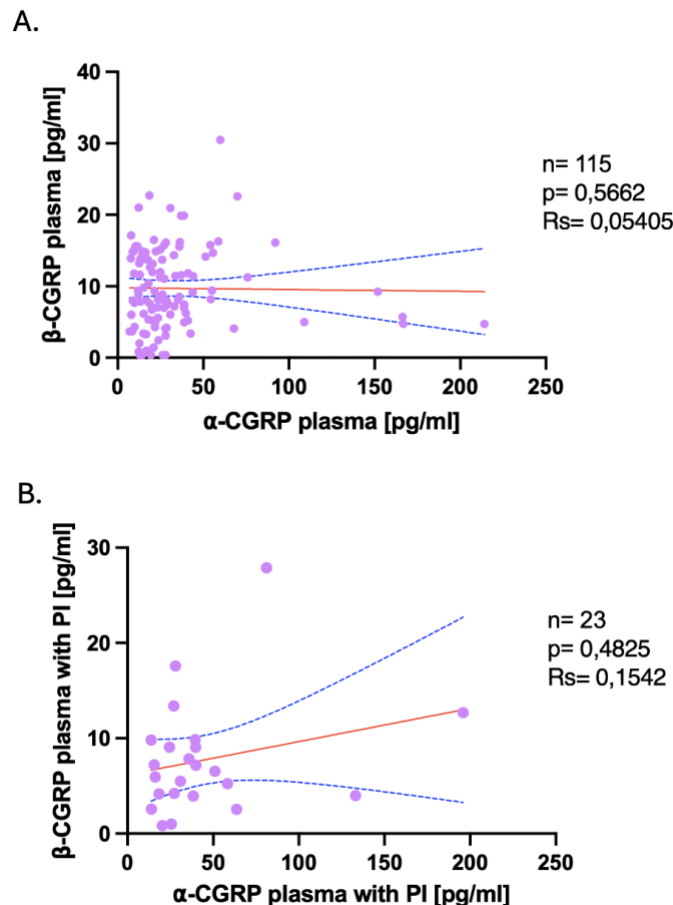


Figure 2. Correlation of α -CGRP and β -CGRP in plasma samples.

(A) Correlation of α -CGRP plasma levels vs β -CGRP plasma levels. Red line represents the linear regression, and the dark blue dotted line represents the CI. **(B)** Correlation of α -CGRP levels vs β -CGRP levels in PI-plasma. Red line represents the linear regression, and the dark blue dotted line represents the CI.

Differences of serum and plasma levels of α -CGRP

Although most studies have used plasma as the primary biological fluid to measure levels of CGRP, our group has specialised in the study of this peptide in serum^{10,15}. To determine whether levels in both fluids were correlated or not, measurements of CGRP levels in both types of samples from CM patients were compared.

Figure 3 presents a comparison of α -CGRP levels in serum and plasma. Although a slight positive tendency can be observed between α -CGRP levels in serum and plasma (Figure 3A, Spearman correlation), this difference is not significant ($r_s = 0.1522$, $p = 0.0677$), indicating that a clear relationship between α -CGRP levels in both fluids cannot be established. When comparing levels of α -CGRP between serum and plasma, we did not observe a significantly increase in serum than in plasma (Serum α -CGRP median = 24.32; Plasma α -CGRP median = 24.94; $p = ns$) (Figure 3B), what indicates no clear difference between both biological fluids.

Since the addition of PI appeared to significantly affect the peptide concentration, we subsequently performed a similar analysis, correlating serum with PI-plasma. In this case we were only able to analyze a smaller cohort since fewer PI-plasma samples were available. As with plasma, no significant correlation was found between α -CGRP in serum and PI-plasma, ($r_s = -0.02769$, $p = 0.8932$), suggesting that serum levels do not predict plasma levels in this patient group either. Despite the lack of correlation observed (Figure 3C), we found a significant difference in the mean concentrations (serum α -CGRP median = 13.63; PI-plasma α -CGRP median = 38.29; $p < 0.05$, Figure 3D), with higher values in PI-plasma than in serum this time, an important contrast to the results shown in Figure 3B.

Even though we cannot find a significant correlation between α -CGRP levels in serum and plasma, the concentrations differ significantly. In standard conditions, plasma shows slightly higher levels of the peptide when compared to serum but without statistical significance. Interestingly, when we add PI to the plasma, levels in plasma surpass serum levels significantly, indicating that the degradation of the peptide could be influencing measures.

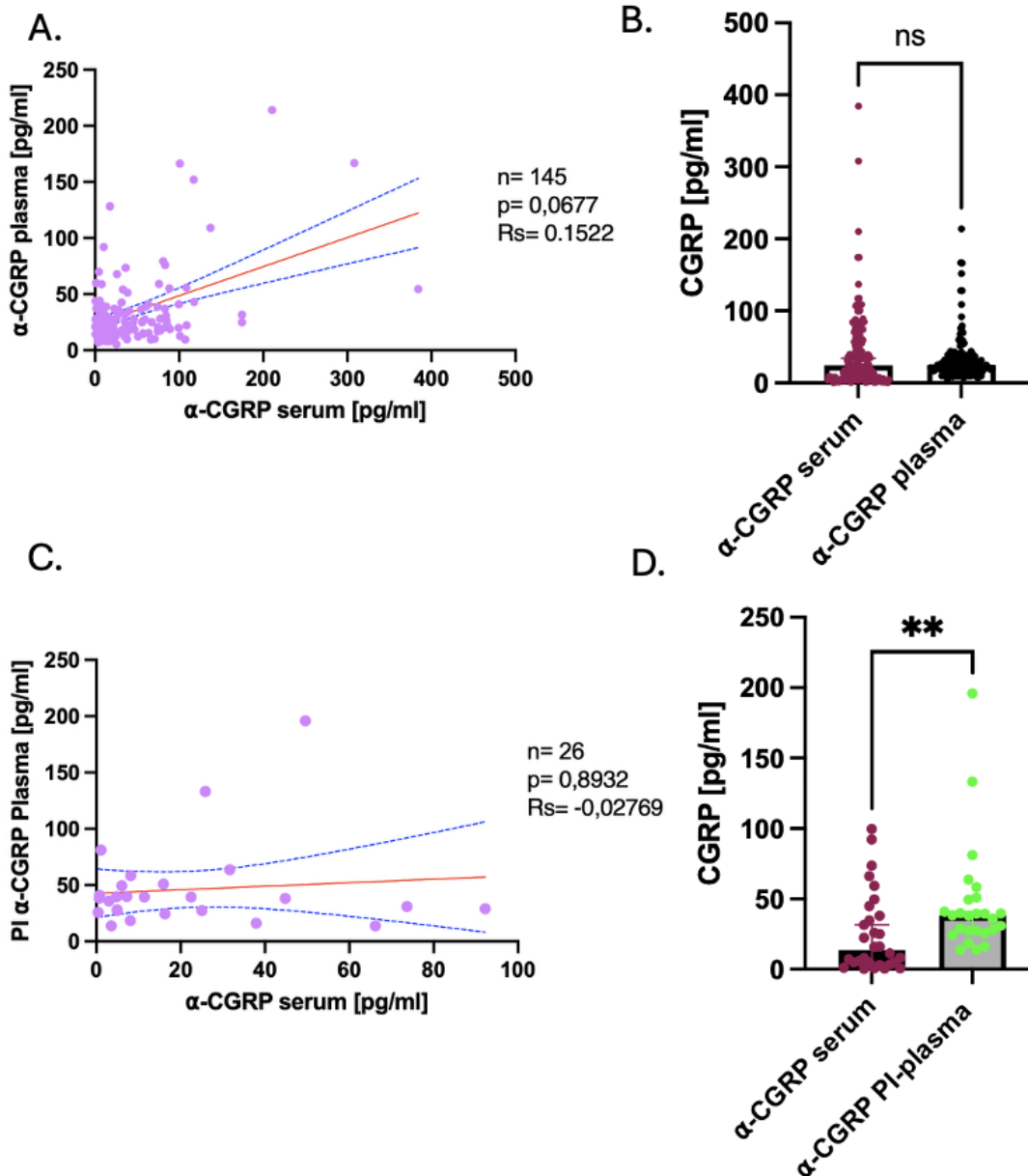


Figure 3. Comparison of α -CGRP levels in serum and plasma.

(A) Correlation of α -CGRP serum levels vs α -CGRP plasma levels. Red line represents the linear regression, and the dark blue dotted line represents the CI. (B) Difference of α -CGRP levels in serum (represented in burgundy) vs plasma (represented in black), data are shown as median with CI 95%. Comparison was made using the Wilcoxon Signed Rank test, (serum median= 24.32, plasma median= 24.94, $p=ns$). (C) Correlation of α -CGRP serum levels vs α -CGRP plasma levels when using PI. Red line represents the linear regression, and the dark blue dotted line represents the CI. (D) Difference of α -CGRP levels in serum (represented in burgundy) vs plasma with PI (represented in green), data is shown as median with CI 95%. Comparison was made using the Wilcoxon Signed Rank test, (serum median= 13.63, PI-plasma median= 38.29, $p<0.01$).

Differences of serum and plasma levels of β -CGRP

As done with α -CGRP, β -CGRP levels in serum and plasma were compared. As observed in [Figure 4A](#), a significant positive correlation between serum and plasma levels of the β isoform was observed ($r_s = 0.4831$, $p < 0.0001$), indicating that β -CGRP levels in these two fluids are strongly associated across the sample population. Despite this positive correlation and, in contrast with α -CGRP findings, significantly higher levels in plasma samples were found ([Figure 4B](#)). The mean β -CGRP concentration in plasma was noticeably elevated relative to that in serum (serum β -CGRP median = 3.625; plasma β -CGRP median = 8.516; $p < 0.0001$).

Regarding PI-plasma samples, a positive tendency in the correlation between serum and PI-plasma was observed ([Figure 4C](#)), however, this had not statistical significance ($r_s = 0.4030$, $p = 0.0701$), a result that can be an outcome of the limited sample size or because of the variability in protease activity among individuals. Comparing the mean of β -CGRP levels in serum and PI-plasma samples ([Figure 4D](#)), we observed again higher levels in PI-plasma (Serum β -CGRP median = 1.716; PI-plasma β -CGRP median = 6.909; $p < 0.0001$), leading to the same suggestion that in [Figure 3B](#).

Collectively, these data indicate that plasma consistently yields higher β -CGRP levels than serum (an important difference compared to α -CGRP), suggesting that this biofluid might provide better sensitivity for β -CGRP detection. In addition, the use of PI may enhance peptide stability, particularly in studies requiring accurate quantification of β -CGRP levels.

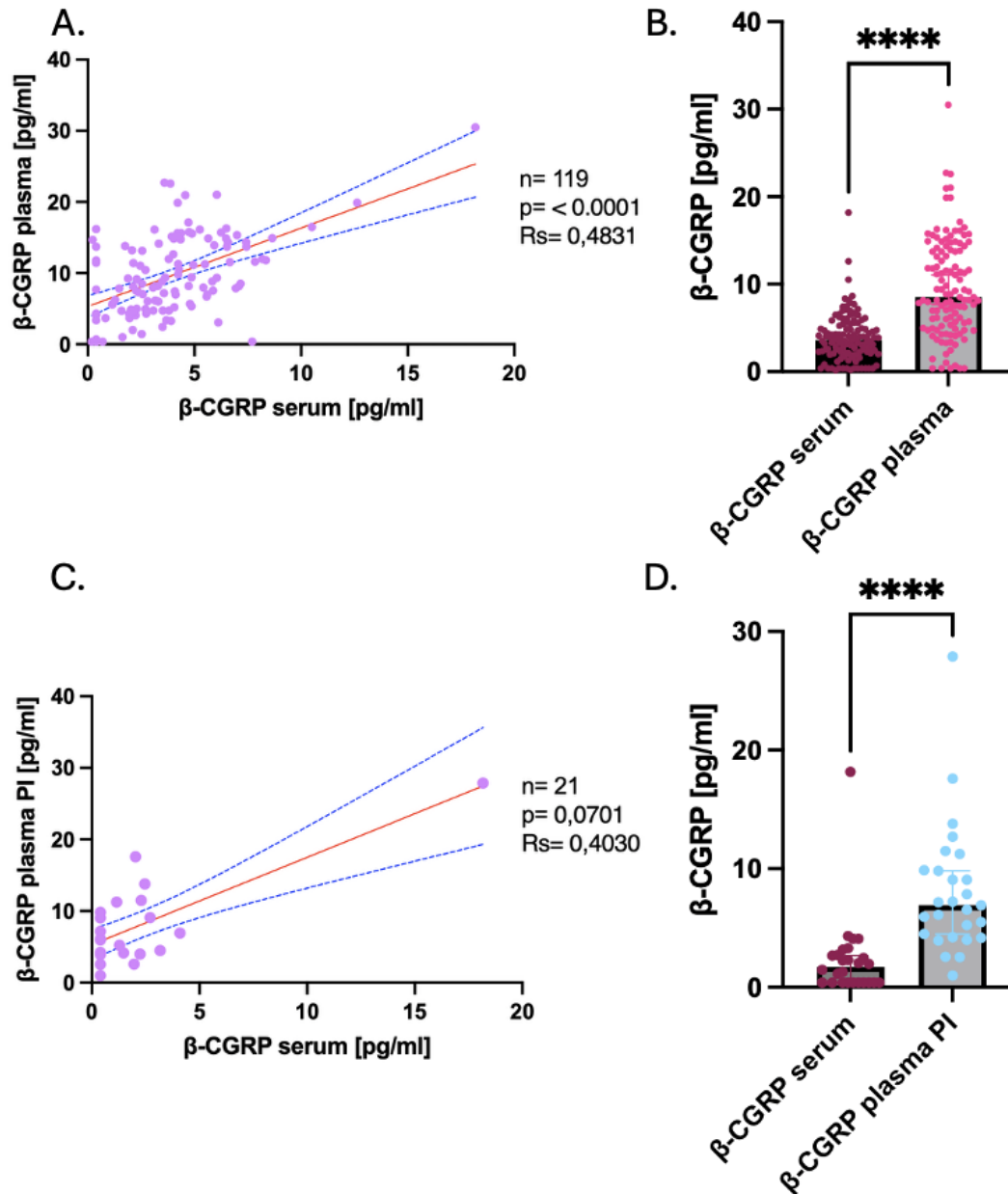


Figure 4. Comparison of β -CGRP levels in serum and plasma.

(A) Correlation of β -CGRP serum levels vs β -CGRP plasma levels, the red line represents the linear regression, and the dark blue dotted line represents the CI. (B) Difference of β -CGRP concentrations in serum (represented in burgundy) vs plasma (represented in pink), data is shown as median with CI 95%. Comparisons were made using the Wilcoxon Signed Rank test, (serum median= 3.625, plasma median= 8.516, $p<0.0001$). (C) Correlation of β -CGRP serum levels vs β -CGRP plasma levels when using PI, the red line represents the linear regression, and the dark blue dotted line represents the CI. (D) Difference of β -CGRP concentrations in serum (represented in burgundy) vs plasma with PI (represented in blue), data is shown as median with CI 95%. Comparisons were made using the Wilcoxon Signed Rank test, (serum median= 1.716, plasma with PI median= 6.909, $p<0.0001$).

Dynamics of CGRP levels during treatment

Patients enrolled in our study were prescribed with mAb anti-CGRP or anti-CGRP-R. CGRP levels were determined at some timepoints along treatment to analyze their potential role as a biomarker for the therapeutic response.

We studied both isoforms of CGRP independently. Patients were stratified based on the availability of plasma samples at different time points; those with samples collected at baseline (M0), and after 6 months of the treatment (M6); and those for whom samples were also available after 12 months (M12).

For the first group of subjects (n=27), we compared plasma α -CGRP at M0 and at M6. Statistical analysis using the Wilcoxon Signed Rank test indicated that there was a significant change in α -CGRP concentrations between these two time points ($p < 0.01$), with a higher predominance of negative rank sums, supporting a decreasing tendency in α -CGRP levels post-treatment initiation (Figure 5A).

In the group of patients with samples available at M0, M6 and M12 (n=16), we observed a similar trend in which α -CGRP concentrations tended to decrease at M6 and even more at M12. The Friedman test followed by Dunn's multiple comparisons test revealed significant differences among the three time points, with a significant reduction specially between M0 and M12 ($p < 0.05$, Figure 5B).

Regarding β -CGRP, we also compared concentrations at M0 and at M6 in the first group of patients (n=26). Even though we observed variability between subjects, we could not observe significant differences between M0 and M6 time points, as supported by the nonsignificant result of the Wilcoxon Signed Rank Test ($p = ns$, Figure 5C).

For the group of patients with M0, M6 and M12 samples available (n= 11), although some patients showed increases or decreases in the concentration of β -CGRP, the Friedman test followed by the Dunn's multiple comparisons test did not show statistically significant differences among none of the compared time points ($p = ns$, Figure 5D).

All results together show that α -CGRP levels significantly decreased during migraine treatment whilst β -CGRP levels do not show statistical changes with migraine treatment.

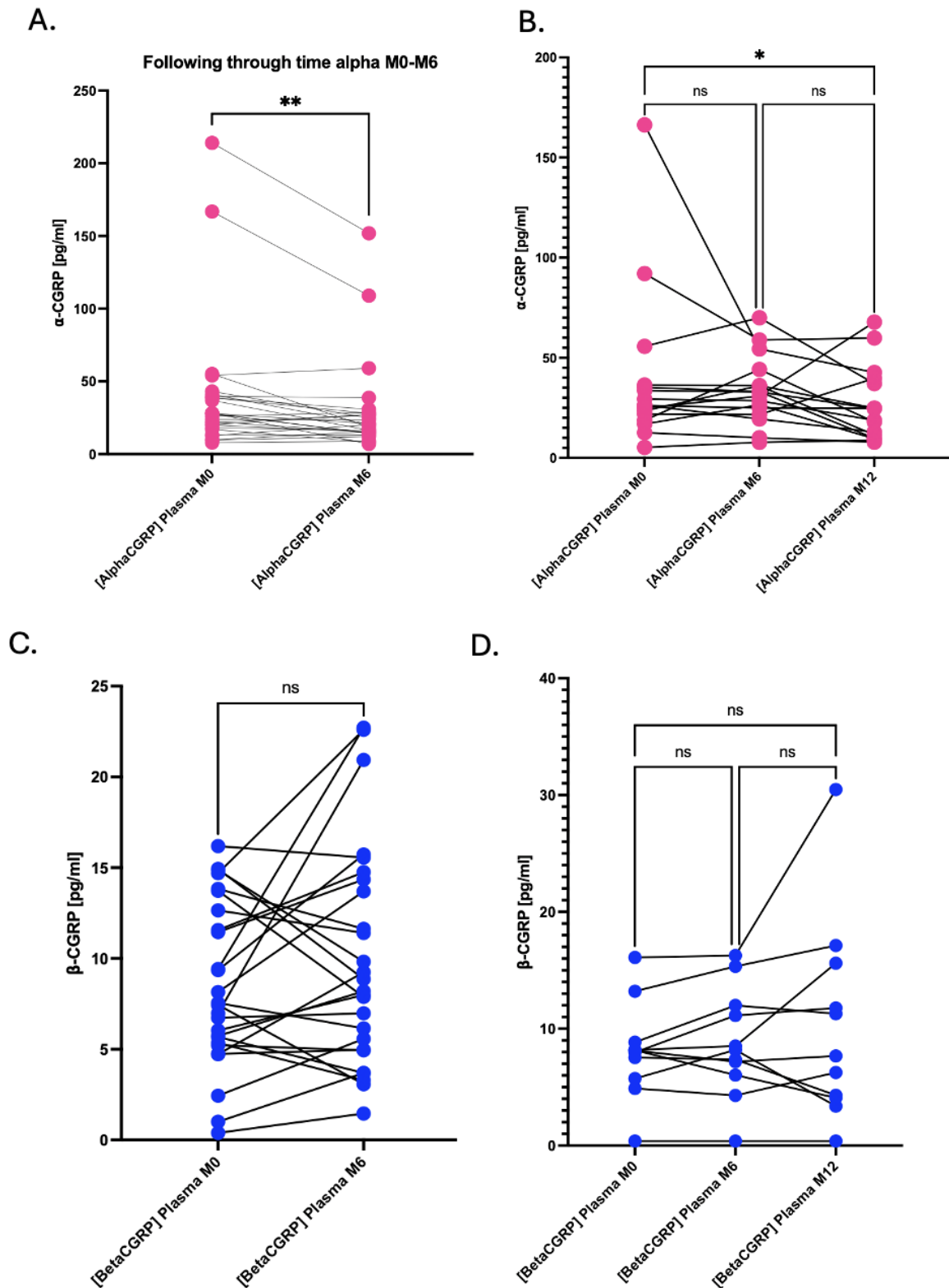


Figure 5. Dynamics of CGRP levels along migraine treatment

(A) Changes in α -CGRP concentrations of patients' plasma before starting the treatment (M0), 6 months after starting the chronic migraine treatment (M6); Wilcoxon Signed Rank Test ($p < 0.01$) and (B) after 12 months of the beginning of the treatment; Friedman test followed by Dunn's test. (C) Changes in β -CGRP levels of patients' plasma before starting the treatment (M0), 6

months after starting the chronic migraine treatment (M6); Wilcoxon Signed Rank test (ns) and (D) after 12 months of the beginning the treatment; Friedman test followed by Dunn's test. * $p < 0.05$ ** $p < 0.01$ ns= nonsignificant.

CGRP levels depending on demographic variables

Some key aspects of migraine include its high prevalence among women and its occurrence across a wide range of ages. To understand better the effect of these aspects on CGRP levels, we conducted several comparisons in our patients' plasma samples.

We observed a significant negative correlation between α -CGRP plasma levels and age, where the levels of the peptide tended to go downwards as age increased ($r_s = -0.2317$, $p = 0.0045$, [Figure 6A](#)). In PI-plasma samples, we could not observe that clear correlation, but α -CGRP levels also presented a light decrease as age increased ($r_s = -0.3864$, $p = 0.0564$, [Figure 6B](#)).

On the other hand, for β -CGRP we did not notice a significant correlation between peptide levels and age ($r_s = -0.02512$, $p = 0.7963$, [Figure 6C](#)). Neither could we appreciate a significant correlation of PI-plasma with age ($r_s = -0.03777$, $p = 0.8780$, [Figure 6D](#)).

Afterwards, plasma levels were compared depending on patients' sex. When α -CGRP plasma levels were compared between men and women, we could not observe statistical differences in our group of patients (men median= 23.31; women median= 26.09; $p = ns$, [Figure 6E](#)). The same happened in PI-plasma samples, where we could neither observe significant differences between men and women (men median=18.35; women median = 37.07; $p = ns$, [Figure 6F](#)).

Comparing β -CGRP plasma levels, we observed similar results, with no significant differences on plasma levels (men median= 8.350; women median= 8.849; $p = ns$, [Figure 6G](#)) and neither comparing PI-treated plasma samples (men median= 6.980; women median= 7.034; $p = ns$, [Figure 6H](#)).

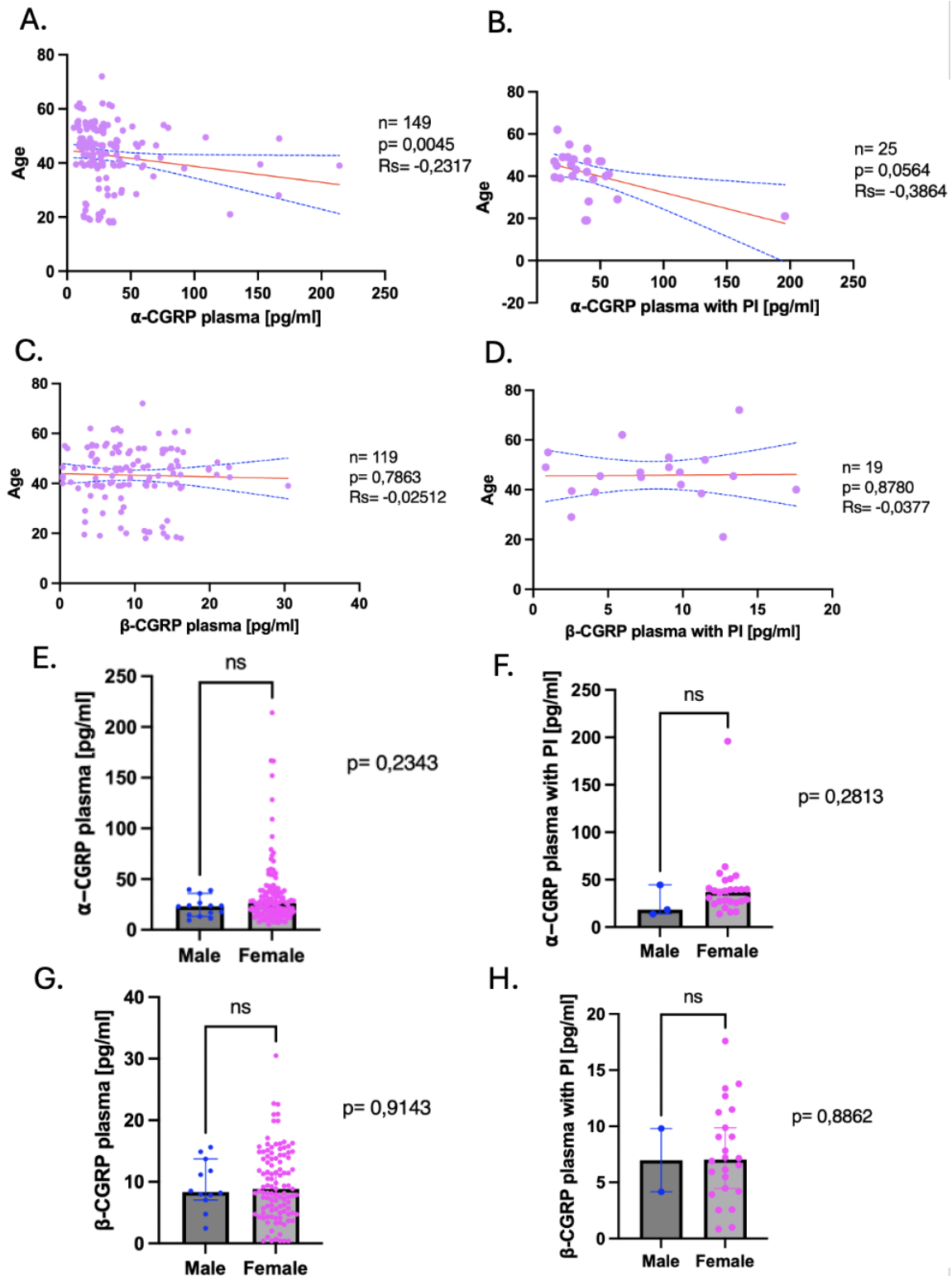


Figure 6. Analysis of CGRP levels in plasma depending on demographic variables

(A) Correlation of α -CGRP plasma levels and age. Red line represents the linear regression, and the dark blue dotted line represents the CI. (B) Correlation of α -CGRP PI-plasma levels and age. Red line represents the linear regression, and the dark blue dotted line represents the CI. (C) Correlation of β -CGRP plasma levels and age. Red line represents the linear regression, and the

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dark blue dotted line represents the CI. **(D)** Correlation of B-CGRP PI-plasma levels and age. Red line represents the linear regression, and the dark blue dotted line represents the CI. **(E)** Comparison of α -CGRP plasma levels from patients sorted by sex (column A median= 23.31; column B median= 26.09; p= ns). **(F)** Comparison of α -CGRP PI-plasma levels from patients sorted by sex. **(G)** Comparison of β -CGRP plasma levels by sex, (column A median= 8.350; column B median= 8.849; p=ns). **(H)** Comparison of β -CGRP PI-plasma levels sorted by sex. Data are shown as median with CI 95%. The comparisons in E, F, G, H were made using Mann-Whitney U test, ns: non-significant.

DISCUSSION

Based on the results obtained in our study, we can draw several conclusions. First, PI did improve CGRP detection, as its addition to plasma samples increased significantly the levels of α -CGRP, even if it did not improve that much β -CGRP isoform detection. It is known that CGRP is degraded by peptidases in the blood plasma. With a half-time of about 7-10 minutes, only a fraction of the original concentration will appear in the cubital veins, what may alter its original concentration, one of the main challenges that could be found when determining the peptide ¹⁶. This result might suggest that CGRP degradation in untreated plasma could be leading to an underestimation of the true levels of the alpha isoform of the peptide. With these findings we can confirm that the addition of PI during the processing of plasma samples improves CGRP stability, something critical for accurate quantification, especially in those studies involving fast degradation of peptides like CGRP.

As it had been done in previous papers, we analyzed if there was a correlation of both CGRP isoforms in the same individual, and we could see that there was no significant correlation found between α - and β -CGRP levels in plasma (neither with nor without PI), which supports the idea that both isoforms may be independently regulated in this disease context, a hypothesis that should be further investigated with functional studies.

Regarding the differences between serum and plasma, α -CGRP levels were not significantly different comparing serum with plasma but were significantly lower in serum than in PI-added plasma, implying that the peptide degradation in plasma could be accounting for lower levels. In contrast, β -CGRP concentrations were consistently higher in plasma than in serum, no matter plasma had PI added or not. Because of these results, measuring β -CGRP levels could be better done in plasma rather than in serum since the levels detected are higher in the first biological fluid. A suggestion is that the difference between both isoforms of the peptide could be due to different release mechanisms of each of them or because of the varying stability of CGRP. This suggestion is supported by recent studies in serum that do not observe correlation between both isoforms of the peptide, meaning that measuring α - or β -CGRP is not interchangeable and could lead to opposite conclusions as they may have different behaviours in the same disorder ¹⁵.

We also studied the changes of CGRP levels during CM treatment and obtained varied results. α -CGRP concentrations decreased over the course time of migraine treatment, which might support its potential utility as a biomarker for treatment response in CM. To confirm this, further studies including responders and non-responders should be performed. However, β -CGRP levels did not change significantly during treatment, which could imply different regulatory mechanisms or roles of both isoforms of the peptide in migraine's pathology. According to a published study, the response in patients with CM is not as influenced by CGRP levels at baseline as in patients with episodic migraine, what lead us to believe that there must be other biological or genetic components involved ¹⁴. Treatment effect has also been tested in serum in a previous article, and similar effects were shown, as α -CGRP levels were lower three months after the first dose of treatment but the same could not be said for β -CGRP levels, as no significant difference was observed ¹⁰.

The influence of demographic factors' was also studied in this study as several articles had mentioned its importance for developing the disease. α -CGRP plasma levels

showed a significant negative correlation with age, indicating reduced peptide levels in older patients. This tendency also persisted with PI-treated plasma samples although it was less pronounced. In contrast, β -CGRP levels did not show a significant relationship with age, highlighting that both isoforms do not correlate. With these findings, age might be considered when analysing CGRP levels as a possible biomarker as perhaps the values should be adjusted by age. Regarding sex differences, no significant variation was observed for either α - or β -CGRP concentrations no matter it had PI added or not.

Despite the outcomes of our study, several limitations should be considered, as our sample size for sex distribution was small and we did not have enough men to obtain a significant result. Apart from that, we consider that PI conditions should be standardized in order to have reliable results of the peptide levels, especially for α -CGRP taking our results into account. Another limitation might be that we did not measure serum and plasma at the same time, so differences between ELISA plates may have influenced the results obtained.

As for future work perspectives, larger sex-balanced cohorts should be selected to obtain significant results regarding these clinical aspects. We also suggest that a study including patients that are known to be responders to treatment and those who are also known to be non-responders, could be included in a stratified study in order to measure the real use of CGRP as a therapeutic response biomarker. Apart from that, as it has been studied that CGRP could also be a biomarker for NDDs ¹⁴, a study comparing the levels of CGRP of patients of both migraine and different NDDs could be done to determine the specificity of CGRP in CM.

In conclusion, with these findings we can support the importance of sample processing conditions since adding PI did change CGRP levels for an accurate quantification of the peptide, especially for α -CGRP. Our data also suggested that α and β isoforms of CGRP could be independently regulated and may undertake distinct biological roles in CM disease, supporting what has already been mentioned in the literature, in which α -CGRP isoform relates more with CM and the nervous system whereas β -CGRP is more related with the gastrointestinal system ¹¹. The consistent decline of α -CGRP with treatment, highlights its potential as a reliable biomarker for monitoring therapeutic response, although further studies should be carried out. Additionally, demographic factors like age could affect CGRP levels as it was shown in our results, however, we did not find a statistical difference of sex as had been said in previous articles published in the literature, probably because we had a small cohort of men, as we know that migraine oftenly affects a major number of women, and our results could not reflect a reliable result.

MATERIALS AND METHODS

Recruitment of study participants

Patients included in this study were recruited from the Headache Unit at the University Hospital Marqués de Valdecilla. In order to be included in the study, subjects had to be older than 17 years old and meet criteria for mAb anti-CGRP/CGRP-R treatment prescription.

Regarding the exclusion criteria, those who were pregnant or breast-feeding women, had any psychiatric disease, were alcohol-dependent individuals or were taking daily-medication for other medical reasons, were left out of the study.

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 December 11. All participants gave written informed consent for their inclusion in the study ¹⁰.

Blood samples extraction and processing of plasma

Blood samples were extracted from subjects with CM at any of three different time points during treatment: either before starting treatment (M0), six months after the beginning of the treatment (M6) and a year after its initiation (M12).

Plasma samples were obtained from vein using EDTA-K tubes and centrifuged for 10 minutes at 3500 rpm. Aliquots of 500 μ l were prepared and stored at -80°C until the day of testing. Some plasma samples were added with 10% of PI ¹⁷.

Laboratory and ELISA procedures

α -CGRP and β -CGRP levels in plasma were measured using commercial enzyme-linked immunosorbent assays (ELISA) tests; using Abbexa CGRP1 (CALCA) ELISA kits for α -CGRP ¹⁸, and Cusabio Human Calcitonin Gene Related Peptide ELISA kits for β -CGRP ¹⁹, following the manufacturers' instructions.

Briefly, standards and test samples were added and incubated in the ELISA plate, which was already pre-coated with the capture antibody, at 37°C. After that, the biotin-conjugated reagent was added to the wells and incubated. After some washing steps, HRP-conjugated reagent was added and the plate incubated. Washing steps were again required and TMB substrate was used to quantify the HRP enzymatic reaction. After TMB-adding, only wells with enough CALCA (for the Abbexa kit) and CALCB (for the CUSABIO kit), would produce a blue coloured product that would change to yellow after acidic stop solution adding. The intensity of yellow was proportional to the CALCA/CALCB amount bound on the plate in each case. The optical density (OD) was measured spectrophotometrically at 450 nm in a microplate reader, from which the concentration of CALCA and CALCB could be afterwards calculated ^{18,19}. Concerning the last step of the incubation of the TMB substrate, in which the manufacturers leave an open range of time in both Abbexa and Cusabio kits and do not specify the exact time of incubation, we incubated the TMB substrate for 10 minutes for α -CGRP and 20 minutes for β -CGRP.

All the plasma samples were measured in duplicate, and the results of the concentrations were obtained generating a standard curve using a 4-parameter logistic regression,

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setting blank as standard data and subtracting the minimum OD value (of blank and standards) from all other ODs. The results obtained were saved in a database to organize the analysis done afterwards.

Data and Statistical analysis

Arigo's Elisa calculator²⁰ was used to calculate the concentrations of the CGRP peptide as well as Excel software and GraphPad Prism version 10, which helped us run all the statistical tests and comparisons and designed graphics for the results.

Continuous variables were reported as mean with standard deviation (SD) for normally distributed data and as median with 95% CI for non-normally distributed data, unless it is stated differently in the text.

The normality of the variables was checked using normality tests (D'Agostino & Pearson test, Shapiro-Wilk test, Kolmogorov-Smirnov test) as well as visually by using a QQplot in GraphPad.

Correlation relationships were evaluated by Spearman's correlation. Statistical significance differences between groups for non-normally distributed data and independent groups, was checked with the Mann-Whitney U test, whereas for non-normally distributed paired samples, the Wilcoxon Signed Rank test was used.

For multiple group comparisons among the same individuals at different timepoints, the Wilcoxon matched-pairs signed rank test was used when only M0 and M6 samples were available, whereas comparisons between M0, M6 and M12 samples were checked using the Friedman test followed by Dunn-s test to study specifically between which timepoints there was a statistical change. The p values presented are for two-tailed testing, and it was considered a $p < 0.05$ to prove statistical significance.

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To my parents and brother for supporting me throughout this journey and for encouraging me to believe that I could achieve whatever I wanted, even when I did not believe that I would be able to do so.

To my friends, for being side by side with me as they have made each step of the path more manageable, and a special mention to the incredible people that made these 4 years special.

And least but not last, I dedicate this to the person I love most, my grandfather Lorenzo. His strength and resilience in the face of illness were the reasons that inspired me to pursue a path in biomedical sciences, with the hope of one day helping others as I wish I could have helped in.

BIBLIOGRAPHY

1. Shiz Aoki, K.S. and R.M. (2017). Biorender. <https://www.biorender.com/>.
2. Aguilar-Shea, A.L., and Diaz-de-Teran, J. (2022). Migraine review for general practice. *Aten Primaria* 54. <https://doi.org/10.1016/j.aprim.2021.102208>.
3. Pavlovic, J.M., Akcali, D., Bolay, H., Bernstein, C., and Maleki, N. (2017). Sex-related influences in migraine. Preprint at John Wiley and Sons Inc., <https://doi.org/10.1002/jnr.23903> <https://doi.org/10.1002/jnr.23903>.
4. Juhasz, G., Gecse, K., and Baksa, D. (2023). Towards precision medicine in migraine: Recent therapeutic advances and potential biomarkers to understand heterogeneity and treatment response. *Pharmacol Ther* 250. <https://doi.org/10.1016/j.pharmthera.2023.108523>.
5. Alpuente, A., Gallardo, V.J., Asskour, L., Caronna, E., Torres-Ferrus, M., and Pozo-Rosich, P. (2022). Salivary CGRP and Erenumab Treatment Response: Towards Precision Medicine in Migraine. *Ann Neurol* 92, 846–859. <https://doi.org/10.1002/ana.26472>.
6. Khan, J., Asoom, L.I. Al, Sunni, A. Al, Rafique, N., Latif, R., Saif, S. Al, Almandil, N.B., Almohazey, D., AbdulAzeez, S., and Borgio, J.F. (2021). Genetics, pathophysiology, diagnosis, treatment, management, and prevention of migraine. Preprint at Elsevier Masson s.r.l., <https://doi.org/10.1016/j.biopha.2021.111557>.
7. Kamm, K. (2022). CGRP and Migraine: What Have We Learned From Measuring CGRP in Migraine Patients So Far? Preprint at Frontiers Media S.A., <https://doi.org/10.3389/fneur.2022.930383>.
8. Santos-Lasaosa, S., Belvís, R., Cuadrado, M.L., Díaz-Insa, S., Gago-Veiga, A., Guerrero-Peral, A.L., Huerta, M., Irimia, P., Láinez, J.M., Latorre, G., et al. (2022). Calcitonin gene-related peptide in migraine: from pathophysiology to treatment. Preprint at Spanish Society of Neurology, <https://doi.org/10.1016/j.nrl.2019.03.013>.
9. Russo, A.F., and Hay, D.L. (2023). CGRP PHYSIOLOGY, PHARMACOLOGY, AND THERAPEUTIC TARGETS: MIGRAINE AND BEYOND. Preprint at American Physiological Society, <https://doi.org/10.1152/physrev.00059.2021>.
10. Gárate, G., González-Quintanilla, V., González, A., Pascual, M., Pérez-Pereda, S., Madera, J., and Pascual, J. (2023). Serum Alpha and Beta-CGRP Levels in Chronic Migraine Patients Before and After Monoclonal Antibodies Against CGRP or its Receptor. *Ann Neurol* 94, 285–294. <https://doi.org/10.1002/ana.26658>.
11. Gárate, G., Pascual, M., Olmos, J.M., Fariñas, C., Rivero, M., Crespo, J., and Pascual, J. (2022). Increase in Serum Calcitonin Gene-Related Peptide β (CGRP β) Levels in COVID-19 Patients with Diarrhea: An Underlying Mechanism? Preprint at Springer, <https://doi.org/10.1007/s10620-022-07473-0> <https://doi.org/10.1007/s10620-022-07473-0>.

12. Puledda, F., Silva, E.M., Suwanlaong, K., and Goadsby, P.J. (2023). Migraine: from pathophysiology to treatment. *J Neurol* 270, 3654–3666. <https://doi.org/10.1007/s00415-023-11706-1>.
13. de Vries, T., Villalón, C.M., and MaassenVanDenBrink, A. (2020). Pharmacological treatment of migraine: CGRP and 5-HT beyond the triptans. Preprint at Elsevier Inc., <https://doi.org/10.1016/j.pharmthera.2020.107528>.
14. Sarkar, S., Porel, P., Kosey, S., and Aran, K.R. (2025). Unraveling the role of CGRP in neurological diseases: a comprehensive exploration to pathological mechanisms and therapeutic implications. *Mol Biol Rep* 52, 436. <https://doi.org/10.1007/s11033-025-10542-y>.
15. Gárate, G., Pascual, J., Pascual-Mato, M., Madera, J., Martín, M.M.S., and González-Quintanilla, V. (2024). Untangling the mess of CGRP levels as a migraine biomarker: an in-depth literature review and analysis of our experimental experience. *Journal of Headache and Pain* 25. <https://doi.org/10.1186/s10194-024-01769-4>.
16. Messlinger, K., Vogler, B., Kuhn, A., Sertel-Nakajima, J., Frank, F., and Broessner, G. (2021). CGRP measurements in human plasma – a methodological study. *Cephalalgia* 41, 1359–1373. <https://doi.org/10.1177/03331024211024161>.
17. Merck Protease Inhibitor cocktail powder (PI). Saint Louis, MO 63103, USA. <https://www.sigmaaldrich.com/ES/es/product/sigma/p2714>.
18. Abbexa Ltd. (2013). ELISA kit for α -CGRP. <https://www.abbexa.com/calcitonin-gene-related-peptide-elisa-kit-1?srsId=AfmBOordfUMK6-UJ9E1A5FLKW8g3ERFymJHI-glPvRdcS08abgL5HUk8>.
19. Wuhan Huamei Biotech Co., LTD. (2007). ELISA kit for β -CGRP. <https://www.cusabio.com/ELISA-Kit/Human-calcitonin-gene-related-peptideCGRP-ELISA-Kit-70882.html>.
20. Arigo Biolaboratories Corp. (2014). Arigo's Elisa calculator. <https://www.arigobio.com/elisa-calculator>.
21. Mehkri, Y., Hanna, C., Sriram, S., Lucke-Wold, B., Johnson, R.D., and Busl, K. (2022). Calcitonin gene-related peptide and neurologic injury: An emerging target for headache management. *Clin Neurol Neurosurg* 220. <https://doi.org/10.1016/j.clineuro.2022.107355>.

Supplementary information

Investigating α - and β -CGRP levels in Plasma: Towards a Reliable Biomarker for Chronic Migraine

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TRIGEMINAL NERVE CGRP RELEASE

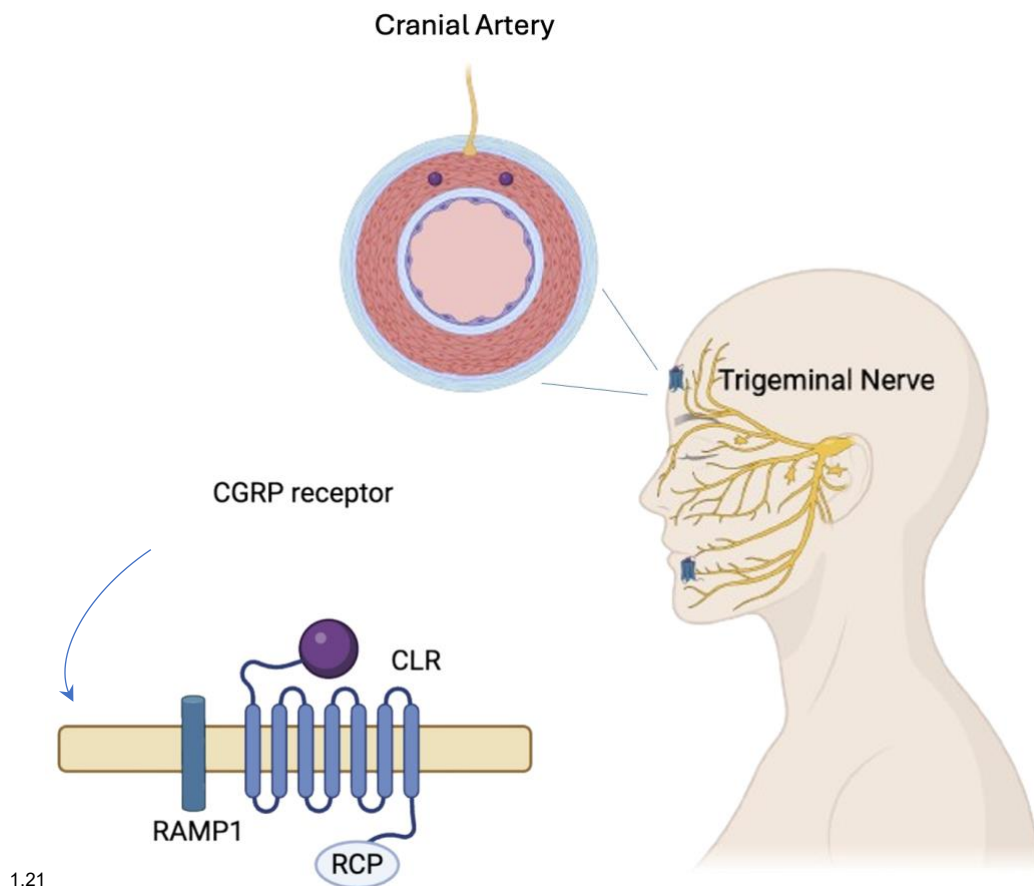


Figure S1- CGRP pathway release

Trigeminal nerve is activated during a migraine attack and releases CGRP among other peptides. Cranial arteries are closely associated with the trigeminal nerve and are dilated during the migraine, contributing to pain. CGRP receptor (a complex of three components; CLR (main receptor unit), RAMP1 and RCP (Receptor Component Protein)) is activated by CGRP and initiates the vasodilatation and pain cascade.

ELISA SANDWICH STEPS

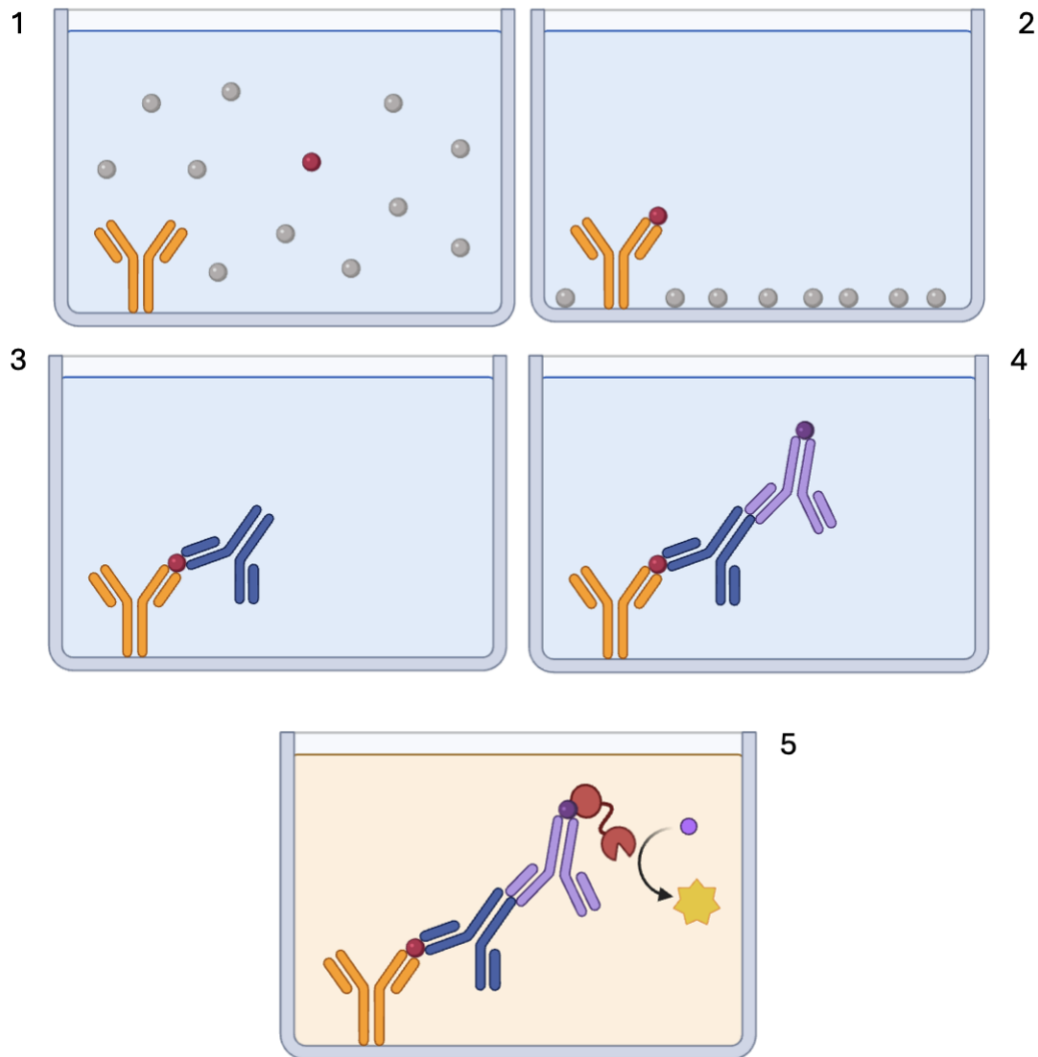


Figure S2- ELISA sandwich steps.

(1) Wells pre-coated with capture antibody. (2) Antigen binds to the capture antibody on the plate. (3) The detection antibody binds to the analyte. (4) Biotin-labelled antibody binds to the detection antibody. (5) Streptavidin-HRP binds to biotin and the enzyme HRP catalyses the enzymatic colour reaction in yellow.