Universitat Autònoma de Barcelona

Migraine molecular phenotyping through the study of the calcitonin gene-related peptide: towards precision medicine

Alicia Alpuente Ruiz

Migraine molecular phenotyping through the study of the calcitonin gene-related peptide: towards precision medicine

Alicia Alpuente Ruiz

Doctoral Thesis

Supervisors: José Álvarez Sabín, Patricia Pozo Rosich

Tutor: José Álvarez Sabín

PhD in Medicine, Department of Medicine, Universitat Autònoma de Barcelona

Barcelona, 2022

Vall d'Hebron Barcelona Campus Hospitalari

Universitat Autònoma de Barcelona

A mi madre,

por acompañarme en cada parte del camino

A mi mentora,

por inspirarme personal y profesionalmente

A Jorge,

porque su amor es el motor de mi día a día

Agradecimientos

Escribir esta parte no es tarea fácil pues tengo sentimientos encontrados. Por una parte, y aunque en más tiempo del deseado, estoy contenta de haber acabado mi tesis doctoral. Por otra, me entristece haber llegado al fin de una etapa en la que he aprendido y me he divertido formándome como investigadora.

Esta tesis se compone de 2 artículos, producto del esfuerzo coordinado entre mis compañeros de trabajo. Gracias al Dr. Álvarez-Sabín, por tutorizarme en esta etapa. No puedo agradecer lo suficiente el trabajo realizado a Laila. Encontrar la mejor manera de analizar la saliva fue un gran reto lleno de dificultades. Sin su paciencia y rigurosidad esto no habría sido posible. Tras varias desilusiones y teniendo en cuenta el impacto de la pandemia, ha conseguido superar los obstáculos y me enorgullece decir que, a día de hoy, es toda una experta en el análisis del CGRP en saliva. A Víctor, por hacer magia con sus análisis, y su optimismo a la hora de explicárnoslos. Gracias a Edoardo, mi neurólogo favorito, sin él no habría sido posible desarrollar el segundo proyecto de la tesis. A Lali, por ayudarme con la logística de ambos proyectos, ambas sabemos lo difícil que ha sido. A Marta, mi amiga y compañera de batallas, gracias por involucrarme en este proyecto cuando aún era residente. Y por último, un gracias de todo corazón a Patricia, mi mentora, por ser tan generosa conmigo, y ayudarme a crecer como médico y como persona.

Por otra parte, quiero dedicar un pequeño agradecimiento a mi familia. Gracias a mis padres, ya que me han ayudado a ser el médico que he querido ser. A mi amiga María, uno de mis grandes apoyos dentro del hospital. A Jorge, por tranquilizarme constantemente, recordarme que todo sale y ayudarme a ver las cosas desde un mejor punto de vista.

Y por último, MUCHAS GRACIAS a mis pacientes con migraña, que con gran motivación y ayuda desinteresada, han hecho posible esta tesis con una conclusión clara: hacer visible lo invisible.

Abbreviations

AD Alzheimer's disease

ADHD Attention deficit hyperactivity disorder

ALS Amyotrophic lateral sclerosis

Anti-CGRP mAbs Monoclonal antibodies targeting CGRP

AOPP Advanced oxidation protein products

Aβ42 Amyloid-β1-42 peptide

BBB Blood-brain barrier

BDNF Brain-derived neurotrophic factor

BTX-A OnabotulinumtoxinA

CALCA Calcitonin–CGRP gene

CALCRL Calcitonin receptor-like receptor

CAR C-reactive protein (CRP)/albumin

CGRP Calcitonin gene-related peptide

CGRP mAbs Monoclonal antibodies targeting CGRP or its receptor

CGRP-LI Calcitonin gene related peptide like immunoreactivity

CH Cluster Headache

CM Chronic migraine

CNS Central nervous system

CSD Cortical spreading depression

CSF Cerebrospinal fluid

d/mo days/month

DATscan Dopamine transporter imaging

DTI Diffusion tensor imaging

ELISA Enzyme-linked immunosorbent assay

EM Episodic migraine

FDA Food and Drug Administration

fMRI Functional MRI

FRAP Ferric reducing antioxidant power

FTD Frontotemporal dementia

GFC Gingival crevicular fluid

GLMM Generalized linear mixed model

GWAS Genome wide association study

GαS The α- subunit of the GS protein

HC Healthy controls

HFEM High-frequency episodic migraine

HIV human immunodeficiency virus

HRQoL The health-related quality of life

ICHD-3 International Classification of Headache Disorders

IGF-1 Insulin-Like Growth Factor-1

INR International normalized ratio

LFEM Low-frequency episodic migraine

LP Lumbar puncture

MCI Mild cognitive impairment

MDD Major depressive disorder

MLR Monocyte/lymphocyte ratio

MO Medication overuse

MOH Medication overuse headache

MRI Magnetic resonance imaging

MRS Magnetic Resonance Spectroscopy

MS Multiple Sclerosis

MwA Migraine with aura

MwoA Migraine without aura

NCI No cognitive impairment

NFL Neurofilament light

NFL Neurofilament light chain

NGF Nerve growth factor

NLR Neutrophil/lymphocyte ratio

NSAIDs Non-steroidal anti-inflammatory drugs

PACAP Pituitary adenylate cyclase-activating polypeptide

PD Parkinson’s disease

PiB Pittsburgh Compound-B

PLR Platelet/lymphocyte ratio

pNFH Phosphorylated heavy chain

PTX3 Pentraxin 3

RAMP1 Receptor activity-modifying protein 1

RCP Receptor coupling protein

RIA Radioimmunoassay

SBM Surface-based morphometry

SNPs single nucleotide polymorphism

SP Substance P

sTWEAK Tumor necrosis factor-like weak inducer of apoptosis

TG Trigeminal ganglia

TGV trigeminovascular system

WHO World Health Organization

Table index

Figure index

Index

SUMMARY

Nowadays, we acknowledge that **calcitonin gene-related peptide (CGRP)** plays an **essential role in the pathophysiology of migraine** and that its blockage represents **a clinically meaningful treatment of migraine**.

When we started this doctoral thesis project, **treatment with monoclonal antibodies targeting CGRP treatments was in its clinical development stage.** Specifically, phase 3 clinical trials with anti-CGRP monoclonal studies as preventive treatment for migraine were being carried out in Spain. However, the role of CGRP as a therapeutic target had been previously demonstrated with gepants, a class of CGRP receptor antagonists. However, clinical development of several gepants had been terminated, in part, due to a risk of liver toxicity with their long-term use. Additionally, **several studies quantifying CGRP levels had been published, mainly performed in plasma, with contradictory results.** The heterogeneity in the studies population, sample collection, assays used, matrix analyzed, amongst other factors, gave rise to a lack of reproducibility, which cast doubt on the possibility of considering CGRP as a migraine biomarker.

This disparity in the results of the studies prompted us to start a line of work focused on **the quantification of CGRP from a solid methodological base**. So, **given the dynamic nature of the disease, we needed a continuous surveillance of the migraine cycle**. So, we thought of finding a way to continuously monitor the dynamic nature of the disease through a non-invasive way, saliva, **a matrix that met the required technical features**. And that was how the first project of this thesis began. Our hypothesis was based on the dynamic changes of CGRP during ictal and interictal period and the lack of a global response to anti-CGRP treatments, with a goal of working towards a pathophysiological driven classification.

Thereby, in the first study of this doctoral thesis, we designed a methodology able to include the whole migraine cycle. **During 30 days, study participants collected daily saliva samples and extra samples during their migraine attacks**. We recruited a very homogeneous sample of **young women with low-**

frequency episodic migraine (LFEM). We developed the most appropriate methodology to collect saliva samples at home and quantified salivary CGRP though ELISA method. We encountered several methodological problems from which we gradually learned and eventually corrected. We also collected some plasma samples so we could compare saliva and plasma levels of CGRP. We were able to measure salivary CGRP in a kit primarily designed for plasma samples since there were no available kits for saliva. We found that **the quantification of CGRP in saliva was reliable**, probably due to its close distance to the trigeminovascular system, the cornerstone of the pathophysiology of migraine. We also found that **participants with migraine had higher levels of CGRP** than healthy controls and that **these levels fluctuated throughout the migraine attack**. The dynamic monitorization allowed us to start differentiating **types of patients** according to the CGRP pattern: those patients with attacks in which these levels were clearly elevated and those patients with attacks in which the opposite occurred. Interestingly, those patients defined as **"CGRP dependent"** associated canonical migraine symptoms: **photophobia and phonophobia**.

Coronavirus disease 2019 (COVID-19) started at the end of 2019 and quickly became a pandemic. Spain was hit in March 2020. At that time, all healthcare professionals had to attend COVID-19 patients. Obviously, normal medical activity and research was stopped. Erenumab and galcanezumab, two monoclonal antibodies targeting CGRP were approved in November 2019. We started treating patients despite of the COVID-19 pandemic although with less patients than we would have if the pandemic was not ongoing. Months after we progressively recovered normal activity and we were able to start the second project.

Thus, in this second project, we performed an internal validation of our CGRP quantification method, and, we focused on a different cohort to explore if saliva CGRP measurements were also meaningful in patients with a higher migraine frequency and, also, in men (not only women). We quantified **CGRP levels at baseline and after receiving 3 doses of erenumab treatment**. Our hypothesis was that levels of the neuropeptide could act as a predictor of response to

erenumab treatment. We found, first, that **the quantification of CGRP in saliva was reproducible**, and that **CGRP levels were higher as migraine frequency increased**. In addition, participants who had **depressive symptoms had higher CGRP levels**. We also found that **baseline CGRP levels were able to differentiate high frequency episodic migraine (HFEM) and chronic migraine (CM) from healthy controls** as well **as predicting response to erenumab treatment** in some patients, especially those with HFEM. Finally, we also showed that treatment with **erenumab** was able **to modulate the levels of this neuropeptide and, interestingly, stabilize them.**

For this reason, this doctoral thesis supports the quantification of **CGRP in saliva as a potential diagnostic biomarker in migraine and predictive of the therapeutic response to treatment with anti-CGRP monoclonal antibodies**. In addition, it supports the possibility of a **molecular classification of migraine** based on the CGRP pathophysiology. These results represent a step forward in **the development of both precision and personalized medicine in migraine**. Currently, the diagnosis of migraine is clinical and these target-driven specific treatments are reimbursed based on pharmacoeconomic conditions, not on their efficacy or characteristics of the patient. It will be necessary to continue this line of work by expanding the number and type of patients to confirm our results; as well as, plan on collecting long-term samples in order to be able to validate CGRP as a reliable molecular biomarker in migraine.

In accordance with these findings, this thesis gives insight into the fact that migraine patients are not all alike and opens new hypothesis about the link of migraine and depressive symptoms in these patients, and the role of neuroinflammation in the disease.

RESUMEN

Hoy sabemos que el **péptido relacionado con el gen de la calcitonina** (**CGRP**, por su acrónimo en inglés) tiene un **papel esencial en la fisiopatología de la migraña** y que el bloqueo del CGRP ha supuesto una revolución **clínicamente significativa en el tratamiento** de la migraña.

Cuando planteamos el inicio de esta tesis, **el tratamiento con anticuerpos monoclonales anti-CGRP se encontraba en su etapa de desarrollo clínico**. En concreto, en España se estaban llevando a cabo los ensayos clínicos fase 3 con anticuerpos monoclonales anti-CGRP como tratamiento preventivo de migraña. Sin embargo, el papel de CGRP como diana terapéutica ya se había demostrado gracias a los antagonistas del receptor de CGRP (gepantes). No obstante, el desarrollo clínico de varios gepantes terminó, en parte, debido al riesgo de toxicidad hepática con su uso a largo plazo. Se habían publicados **varios estudios que cuantificaban el CGRP, principalmente en plasma, con resultados variables**. La heterogeneidad en la población de estudio, en la forma de recoger las muestras, en los ensayos utilizados, entre otros factores, daba lugar a una falta de reproducibilidad, lo cual ponía en duda el papel del CGRP y su utilidad en migraña por parte de la comunidad científica. Incluso de aquellos que se dedican a la cefalea.

Esta diversidad en los resultados de los estudios fue lo que nos impulsó a iniciar una línea de trabajo centrada en la cuantificación del CGRP desde otro punto de vista metodológico. **Dada la naturaleza dinámica de la enfermedad, necesitábamos una monitorización continua del ciclo de la migraña.** Esto solo podría ser posible recopilando muestras repetidas durante el ciclo de la migraña. Por lo tanto, necesitábamos que los participantes recogieran **muestras en casa y de una manera práctica y no invasiva.** Encontramos **la saliva como matriz que cumplía con estas características técnicas**. Y así fue como comenzó el primer trabajo de esta tesis. Nuestra hipótesis se basó en los cambios dinámicos del CGRP, estudiando el impacto individual del CGRP en cada paciente y comenzando a trabajar en una clasificación fisiopatológica de la migraña.

Por ello, en el primer proyecto de esta tesis doctoral, diseñamos una metodología que fueses capaz de abordar todo el ciclo de la migraña. Por ello, **durante 30 días, los participantes del estudio recogieron muestras de saliva diarias y muestras adicionales durante los ataques.** Reclutamos **mujeres jóvenes con migraña episódica de baja frecuencia** (MEBF) y analizamos las muestras de saliva recogidas durante las diferentes fases del ciclo de la migraña. Desarrollamos la metodología más adecuada para que los participantes pudieran recoger muestras de saliva en casa y para cuantificar el CGRP salival a través del método ELISA. Encontramos varios problemas metodológicos de los cuales gradualmente aprendimos y finalmente corregimos. También recogimos muestras de plasma para poder comparar los niveles de CGRP en saliva y plasma. Pudimos medir el CGRP salival en un kit diseñado principalmente para muestras de plasma ya que no había kits disponibles para saliva. Encontramos que **la cuantificación de CGRP en saliva era fiable**, probablemente debido a su cercanía con el sistema trigeminovascular, la piedra angular de la fisiopatología de la migraña. También encontramos que los participantes con **migraña tenían niveles más altos de CGRP** que los controles sanos y que estos niveles **fluctuaban durante el ataque de migraña.** Esta monitorización dinámica nos permitió empezar a diferenciar **tipos de pacientes según el patrón CGRP**: aquellos pacientes con ataques en los que estos niveles estaban claramente elevados y aquellos pacientes con ataques en los que ocurría lo contrario. Curiosamente, aquellos pacientes definidos como **"dependientes de CGRP"** presentaban síntomas canónicos de migraña: **fotofobia y sonofobia.**

La enfermedad por coronavirus 2019 (COVID-19) comenzó a fines de 2019 y rápidamente se convirtió en una pandemia. España fue confinada en marzo de 2020. En ese momento, todos los profesionales de la salud debían atender a los pacientes con COVID-19. Erenumab y galcanezumab, dos anticuerpos monoclonales dirigidos contra CGRP, fueron aprobados en noviembre de 2019. Comenzamos a tratar pacientes a pesar de la pandemia, aunque con menos pacientes de los que tendríamos si la pandemia no continuara. Meses después recuperamos progresivamente la actividad normal y pudimos iniciar el segundo proyecto.

Por lo tanto, en este segundo proyecto, haciendo una validación interna del método de cuantificación de CGRP se aumenta el número de participantes con mayor variedad en términos de sexo (se incluyen hombres) y en frecuencia de migraña (se incluyen pacientes con migraña episódica de alta frecuencia (MEAF) y migraña crónica (MC)). Cuantificamos los niveles de **CGRP al inicio y después de recibir 3 dosis de tratamiento con erenumab.** Nuestra hipótesis fue que los niveles del neuropéptido podrían actuar como predictor de respuesta al tratamiento con erenumab. Encontramos, primero, que **la cuantificación de CGRP en la saliva era reproducible** y que **los niveles de CGRP eran más altos a medida que aumentaba la frecuencia de la migraña**. Además, los participantes que tenían **síntomas depresivos tenían niveles más altos de CGRP**. También descubrimos que **los niveles iniciales de CGRP podían diferenciar MEAF y MC de los controles sanos**, así como **predecir la respuesta al tratamiento con erenumab en algunos pacientes**, especialmente aquellos con MEAF. Finalmente, también demostramos que el tratamiento con anti-CGRP era capaz de **modular los niveles de este neuropéptido y, curiosamente, estabilizarlos.**

Por ello, esta tesis doctoral apoya la **cuantificación del CGRP en saliva como potencial biomarcador diagnóstico en migraña, predictivo y de respuesta terapéutica al tratamiento con anticuerpos monoclonales anti-CGRP**. Además, apoya una **clasificación molecular** de la migraña, basada en la fisiopatología. Esto representa un paso adelante en el desarrollo tanto en **la medicina de precisión como personalizada en migraña.** Actualmente el diagnóstico de la migraña es clínico y estos tratamientos específicos están indicados en función de unas condiciones económicas, que no de las características del paciente. Será necesario seguir la línea de trabajo ampliando el número y el tipo de pacientes, y la recogida de muestras a largo plazo para así poder ser capaces de validar el CGRP como biomarcador fiable.

Por estos hallazgos, parece que no todos los pacientes con migraña son iguales, y genera nuevas hipótesis sobre si los otras terapias anti-CGRP (gepantes) son también capaces de modificar los niveles de CGRP de la misma manera que

erenumab y sobre si la neuroinflamación, de momento controvertida, también juega un papel importante en la depresión.

1. INTRODUCTION

1.1. Migraine

1.1.1 Epidemiology and burden

Migraine is a chronic, complex neurological disorder that manifests as recurrent attacks of moderate to severe headache pain lasting 4–72 hours. The headache is typically unilateral, has a pulsating quality, is aggravated by routine physical activity and is associated with nausea and/or sensitivity to light and sound. In migraine with aura (MwA), the headache phase is preceded by reversible focal neurological symptoms, often visual or sensory, that usually develop gradually over 5–20 min and last for <60 min (1).

Migraine is a highly prevalent and a disabling disease, **affecting 1 billion people worldwide** (2). The World Health Organization (WHO) states that migraine is the third most prevalent disease and the second cause of disability (3). **It is first cause of disability in women aged under 50** (4). The prevalence in the general population is 12%, and interestingly, it has been stable over the last 20 years (2). Migraine is more common in women than in men, with a sex prevalence that varies according to age: 1.5**:**1 between 12-17 years and 3.25**:**1 between 18-29 years. Therefore, it affects 10% of children of school age and prepuberal age being the prevalence at this age slightly higher in boys than in girls. Although in half of the patients the onset of migraine occurs before the age of 20, it can also occur at an early age. **The highest prevalence is found between 25-55 years of age and coincides with the peak of disability** (5).

Approximately 6.8–7.8% of all patients with migraine have **chronic migraine (CM**), defined by headache occurring on 15 or more days/month (d/mo) for more than three months, where, on at least 8 d/mo, has the features of migraine headache (1)**.** CM has an estimated prevalence of 1.4–2.2% in the general population (6,7). Between 2.5-3% of people with episodic migraine (EM) in one year meet criteria for CM the following year (8). **The economic burden caused by CM,** including medical costs and work productivity, **is threefold higher than that caused by EM** (9). Notably, the prevalence of migraine is affected by age and sex. Female patients with CM experience higher levels of headache-related disability, including longer headache duration, higher frequency of attacks, and

more severely impacted efficiency at work (10). There are several modifiable risk factors for the development of CM, including attack frequency (1/week), excessive consumption of analgesics, caffeine, snoring or obesity. Other risk factors include female gender, allodynia, traumatic brain injury, low socioeconomic status, anxiety and comorbid pain conditions (11,12).

Migraine has substantial economic and humanistic burden that encompasses the acute attacks and time between them. The health-related quality of life (HRQoL) of patients with migraine is affected not only by the migraine attack but also by the interictal period (13). Patients with migraine usually experience negative effects between acute attacks because of fear and worry about the next attack (14).

1.1.2 Diagnosis

Migraine is diagnosed clinically, using the extensively field-tested International Classification of Headache Disorders (ICHD-3) criteria (1). The classification criteria for MwA and without aura (MwoA) and for CM is exhibited below:

Table 1. Diagnostic criteria for migraine without aura according to the ICHD-3

Migraine without aura
A. At least five attacks fulfilling criteria B–D
B. Headache attacks lasting 4–72 hours (when untreated or unsuccessfully treated)
C. Headache has at least two of the following four characteristics: 1. unilateral location 2. pulsating quality 3. moderate or severe pain intensity 4. aggravation by or causing avoidance of routine physical activity (e.g. walking or climbing stairs)
D. During headache at least one of the following:

1. nausea and/or vomiting 2. photophobia and phonophobia
E. Not better accounted for by another ICHD-3 diagnosis

Table 2. Diagnostic criteria for migraine with aura according to the ICHD-3

Migraine with aura
A. At least two attacks fulfilling criteria B and C
B. One or more of the following fully reversible aura symptoms: 1. visual 2. sensory 3. speech and/or language 4. motor 5. brainstem 6. retinal
C. At least three of the following six characteristics: 1. at least one aura symptom spreads gradually over ≥ 5 minutes 2. two or more aura symptoms occur in succession 3. each individual aura symptom lasts 5–60 minutes 4. at least one aura symptom is unilateral 5. at least one aura symptom is positive 6. the aura is accompanied, or followed within 60 minutes, by headache
D. Not better accounted for by another ICHD-3 diagnosis

Table 3. Diagnostic criteria for chronic migraine according to the ICHD-3

Chronic Migraine
A. Headache (migraine-like or tension-type-like1)on ≥15 days/month for >3 months, and fulfilling criteria B and C
B. Occurring in a patient who has had at least five attacks fulfilling criteria B–D for 1.1 *Migraine without aura* and/or criteria B and C for 1.2 *Migraine with aura*.
C. On ≥8 days/month for >3 months, fulfilling any of the following2: 1. criteria C and D for 1.1 Migraine without aura

2. criteria B and C for 1.2 Migraine with aura 3. believed by the patient to be migraine at onset and relieved by a triptan or ergot derivative
D. Not better accounted for by another ICHD-3 diagnosis.

It is worth mentioning that the ICHD-3 indirectly classifies migraine in a dichotomic manner: episodic (<15 d/mo) vs. chronic (≥15 d/mo), what does not cover the complexity of the disorder. EM and CM have been properly characterized on large population-based studies (15,16) determining them as different entities. However, both are part of a disease spectrum, which is not binary. In order to categorize this continuum Bigal and Lipton proposed a model to describe this transition dividing episodic into low-frequency episodic migraine (LFEM) and high-frequency episodic migraine (HFEM) (17). Later, it has been shown that **HFEM is as disabling as CM which focuses on the importance of offering effective preventive treatment to those which are equally disabled** (18).

Migraine is a dynamic disease and headache frequency can fluctuate over time, therefore a static diagnosis does not properly correlate with the nature of the disease. Longitudinal studies in migraine have found intra-participant natural fluctuations in headache frequency during a year, finding that some patients transitioned from CM to EM and vice versa when followed during three-month intervals (19) and that some patients have a cyclic phenotype, fitted by sinusoidal models and measured monthly, which has a clear impact on clinical evolution after a year (20) and who seem to benefit from preventive treatment to prevent cycles.

1.1.3 Migraine cycle

Migraine is a neurological disorder characterized by cyclic paroxysmal multiphase attacks of head pain and a myriad of neurological symptoms (21). These phases are well defined clinically. Each of these phases includes clinical symptoms that differ intra and between individuals and can even change during a lifetime, being a heterogeneous disease. These migraine phases are a

continuum and they are frequently overlapped or absent, even not recognized by patients mostly, representing an important challenge when studying the phases separately (22–24).

Migraine is expressed in two main timepoints: **ictal period**, known as migraine attack, and **interictal period**, which is the period between migraine attacks. **A migraine attack has 4 clinical phases** (Fig. 1): **the prodromal phase, headache phase, aura and postdromal phase.**

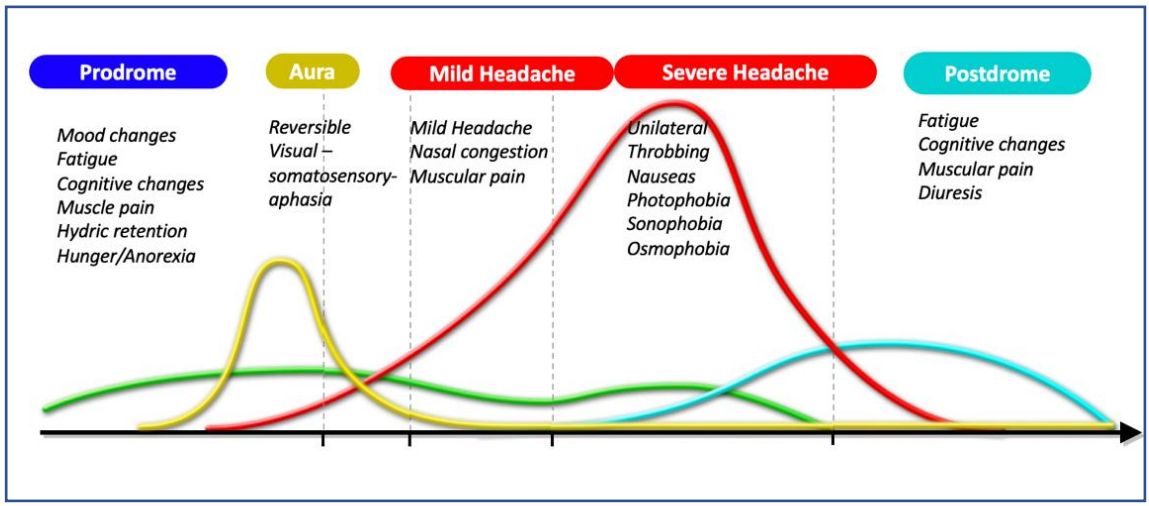

Figure 1. Natural course of a typical migraine attack. *Adapted from midolordecabeza.org*

The Prodromal Phase

Migraine attack starts with the prodromal phase, previously called the premonitory phase, first described in 1980 by Blau (25). Prodromal symptoms occur hours or days before the aura in migraine with aura and before the headache in migraine without aura. Its beginning and duration, however, are not clearly defined and there are no biomarkers to properly differentiate it. The ICHD-3 suggests **that prodromal symptoms may begin hours or a day or two before the other symptoms of a migraine attack, lasting up to 48 hours** (1). Functional changes observed in magnetic resonance imaging (MRI) studies performed prospectively during the whole migraine cycle support this time period (26,27).

The prodromal phase starts in the central nervous system (CNS). In particular, a MRI study of triggered and spontaneous attacks showed activation in the posterior and lateral regions of the hypothalamus and adjacent midbrain ventral tegmentum at the earliest stage of the prodromal phase (26). Activation of these regions and their central connections to the limbic system could explain why migraine is commonly triggered by alterations in homoeostasis (ie. changes in sleep–wake cycles, missed meals) and also some of the symptoms during the premonitory phase—ie. yawning, polyuria, food cravings.

These changes at the supratentorial level may be the initiating events for subsequent alterations that occur at the level of the brainstem (28). The periaqueductal grey (PAG) and dorsal pons, in the region of the noradrenergic locus coeruleus and serotonergic dorsal raphe nucleus, also show selective activation during the prodromal phase (29). These regions are key for modulating the intensity of sensory stimuli (ie. light, sound), cerebral blood flow, nociception, and the excitability of cortical and subcortical neurons and glial cells.

A 70.0% of patients suffering from MwA or MwoA experience prodromal symptoms, but not in every attack (30). Mood alterations, muscle pain, food cravings, cognitive changes, fluid retention, and yawning are the most common. Identification of prodromal symptoms could enable behavioral and treatment approaches that could mitigate or prevent the headache phase of migraine.

The Aura Phase

It occurs in 30% of patients with migraine. An aura involves focal, reversible neurologic symptoms that often precede the headache. It can rarely appear after the onset of pain. These symptoms can be very varied and usually last between 5 and 60 minutes. Visual aura occurs in about 90% of patients with MwA while paresthesia are the second most common symptom. Language dysfunction or symptoms of brainstem dysfunction, although less frequent, may occur. In a rare subtype of migraine, hemiplegic migraine, a motor deficit occurs.

Cortical spreading depression (CSD) is thought to be the underlying physiological cause of the aura phase of migraine (31). CSD is an extreme depolarization of glial and neuronal cell membranes that results in disruption of ionic gradients, a rise in extracellular potassium concentrations, release of glutamate, and a transient increase followed by a decrease in cerebral blood flow (32–36).

The Headache Phase

The characteristics of the headache are unilateral (60%), throbbing (50%), and aggravated by physical activity (90%). The location of the headache may change during the same attack or in others. The intensity is at least moderate or severe in most patients. The duration varies from 4-72 hours in adults and 2-48 hours in children, reaching the peak of intensity at one hour. Among the most common accompanying symptoms, **photophobia (94%), phonophobia (91%) and dizziness (72%) stand out.** Anorexia and nausea occur in half of the patients, while one third present vomit. A 70% of patients have visual symptoms unrelated to the aura, a third have osmophobia or hyperosmia. About 70% of patients have cutaneous allodynia, which may be predictive of a suboptimal response to triptans and a risk factor for progression to CM. Cervical stiffness (75%), sinus pain or pressure (40%) and cranial autonomic signs (50%) are other frequent accompanying symptoms.

Postdromal phase

This phase is defined from when the headache resolves until the individual fully recovers. It occurs in about 80% of people with migraine, and usually lasts less than 12 hours, but can persist for more than 24 hours in about 12% of patients. The most common symptoms during this phase include asthenia, fatigue, drowsiness, difficulty concentrating, photophobia, irritability, and nausea (37) These symptoms are more persistent in patients with CM due to the absence of pain-free intervals.

Although migraine is often described as a paroxysmal disorder with discrete attacks separated by pain-free intervals and without symptoms, a substantial

number of people with migraine may have very frequent attacks and interictal symptoms in the absence of pain. **People with CM are more likely to have these symptoms more persistently** (38).

1.1.4 Migraine pathophysiology: focusing on CGRP

Migraine is a complex disorder of brain function, its pathogenesis is favored by a combination of **genetic, epigenetic, and environmental factors** (39). Migraine has a strong (up to 50%) genetic component (40,41) with multifactorial, polygenic inheritance that may predispose patients with migraine to an increased susceptibility to cortical hyperexcitability (42). It is presumed that the migraine process is initiated **when the nervous system encounters an environment that exceeds its adaptive capabilities** (43,44).

Since this doctoral thesis is centered on CGRP**,** I will review **the role of CGRP in migraine pathophysiology.**

It is generally recognized that the development of migraine headache depends on the activation of sensory afferent fibers of the first branch of the trigeminal nerve (V1), although elucidation of the mechanism leading to the activation of the trigeminovascular system (TGV) is unclear and remains a major gap in the neurobiological understanding of migraine (45,46). **Upon activation of the TGV, neuropeptides such as calcitonin gene-related peptide (CGRP)** and vasoactive intestinal peptide (VIP) are **released by trigeminal ganglion neurons**, **leading to neurogenic inflammation in the dura** (43,47,48). Vasodilation of the meningeal vessels, and possibly cerebral endothelial dysfunction and permeability, results in plasma extravasation and in mast cell degranulation with secretion of other proinflammatory substances. Neurogenic inflammation may contribute to further activation and/or sensitization of the meningeal TGV afferents – a phenomenon described as peripheral sensitization (49) clinically represented by throbbing pain. The continuous and abnormal stimulation of the TGV system could promote central sensitization (clinically represented by cutaneous allodynia (50)): the sustained firing of sensitized meningeal nociceptors that leads to sensitization and activation of second-order

central TGV neurons in the trigeminal cervical complex (TCC) (in spinal nucleus caudalis) (47,49) resulting in increased sensitivity to incoming sensory stimuli. The second-order neurons within the TCC project to the brainstem and hypothalamic, subcortical (basal ganglia), thalamic, and cortical regions that process nociceptive signals from the TVS. Therefore, the nucleus caudalis serves as the door-step to the common final pathway for cortical awareness and cranial pain.

CGRP is the most potent and the most interesting of the neuropeptides which have been linked to the trigeminal system**. Due to the trigeminovascular system activation, CGRP is released from trigeminal afferents resulting in vasodilation and initiation of pain signaling from the periphery**. Thereby, **CGRP serves as a biological marker of trigeminovascular activation** (51). But CGRP involvement in migraine is thought to happen both centrally and peripherally (52). **It is implicated in the development of neurogenic inflammation and it is upregulated in conditions of inflammatory and neuropathic pain** (53,54); it may mediate vasodilation of cerebral and meningeal blood vessels, degranulation of dural mast cell, activation of trigeminal ganglion satellite glial cells, activation of second order neurons within the trigeminal nucleus caudalis, and activation of neurons in several nuclei and structures involved with pain modulation (39,48).

During acute migraine attacks, **CGRP secreted from peripheral trigeminal afferents is thought to mediate vasodilation and inflammatory events within the dura as well as trigeminal ganglion** (bringing about the characteristic throbbing headache associated with migraine) (55–57) while CGRP secretion centrally is believed to cause nociceptive and allodynic responses. Considerable evidence indicates that peripheral sensitization and its associated hyperalgesia are initiated and maintained in part by the actions of CGRP, and an enhanced release of CGRP at peripheral and central terminals of primary afferent fibers is associated with nociceptor sensitization and hyperalgesia. Upregulation of CGRP is believed to contribute to the development of central sensitization and enhanced pain in neuropathic and inflammatory pain states. Interactions between the TG neurons and satellite cells and with the meningeal vasculature can help maintain

the state of enhanced excitability by promoting the synthesis and release of both nitric oxide (NO) and CGRP, which act to promote each other's activities.

CGRP functions as a potent vasodilator and important mediator of pain transmission have put it in the crosshairs of anti-migraine therapies and strongly linked it to the migraine pathophysiology (58)**.** CGRP was first discovered in 1982 (59) and, from studies that have linked CGRP directly to migraine attacks to the information provided by data from clinical trials and real world evidence, we have a lot of information about the central role of this neuropeptide in migraine. It has been shown that triptans block CGRP release, the CGRP antagonists are effective for the acute (and preventive) treatment of migraine, and more recently, the anti-CGRP monoclonal antibodies (anti-CGRP mAbs) are effective for the preventive treatment of migraine (60) (Fig. 2).

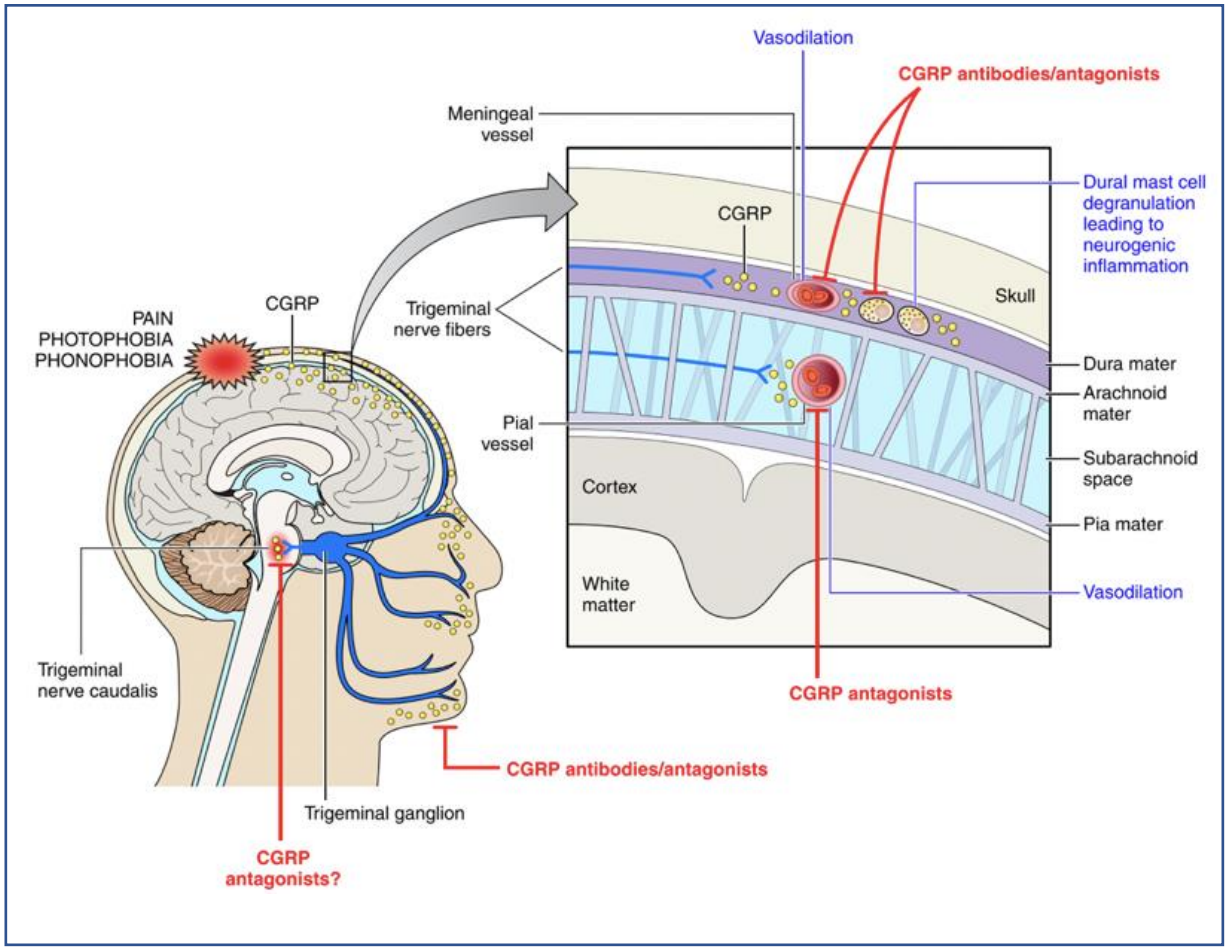

Figure 2. Components of CGRP transmission and sites of action for CGRP-related migraine therapies. *From Russell FA et al, Physiol Rev 2014*

Hereafter, I will review the **scientific evidence linking CGRP to migraine pathophysiology** (table 4).

CGRP during migraine attacks (ictal CGRP)

First studies, published by Goadsby, Edvinsson and Ekman, showed an **elevation in CGRP and substance P plasma levels during stimulation of the TG in humans** (61) **as well as an elevation in plasma CGRP levels in the jugular vein during the migraine attack** (61). A few years later, the same authors also found that **treatment attack with sumatriptan caused a normalization of CGRP levels in the cranial circulation during migraine attacks** in humans, and during stimulation of the trigeminal ganglion in cats (62,63). These observations demonstrated that trigeminal **CGRP release was a good indicator of the attack**, but the exact role of this process in headache generation was uncertain.

Further, several authors tried to replicate the results of Goadsby et al., with controversial results. On one hand, two studies measuring CGRP in jugular venous blood supported previous findings (64,65). The latter was the **first study able to find a correlation between migraine attack characteristics and CGRP levels.** They found that patients who responded to rizatriptan had unilateral, severe and throbbing pain. Otherwise, those who did not respond had bilateral and non-throbbing pain. CGRP levels measured before rizatriptan administration was significantly higher in responders than in non-responders. This finding supports the clinical evidence for increased trigeminal activation associated with a better response to triptan in patients with migraine. Otherwise, the poor response seems to be correlated with a lower degree of trigeminal activation, lower variations of the trigeminal neuropeptides after the administration of triptans. The authors suggested **that phenotyping migraine attacks, clinically and molecularly, would help predict response to treatment.**

On the other hand, two studies have shown opposite results. Tvedskov *et al,* using two different trials with intra-individual comparison, did not find an increase

in CGRP in jugular or cubital venous blood during migraine attack (66) nor did Friberg *et al* when measured in internal carotid and jugular venous arterial blood (67).

Due to the methodological difficulty of measuring CGRP in jugular blood, different studies looked for CGRP in cubital blood. Gallai *et al* replicated the initial findings by observing an increase in CGRP in young patients during a migraine attack (68).

Additional evidence that CGRP plays a role in headache comes from provocation studies. CGRP infusion induced a long-lasting migraine-like headache, suggesting that CGRP has a causal role in migraine symptoms (69,70). In addition, NO has a strong correlation with CGRP (71). Patients with migraine are very sensitive to NO (72) and the vascular effects of it are partly mediated by CGRP released from trigeminal nerve fibers, while at the level of the trigeminal system, NO synthase coordinates with NO production to release CGRP from trigeminal nerve fibers (73). Accordingly, intravenous infusion of NO produces a migraine-like headache with an associated increase in plasma CGRP levels (71).

CGRP between migraine attacks (interictal CGRP)

The first study that demonstrated **higher plasma CGRP levels in peripheral circulation of adults with migraine compared to controls** was conducted by Ashina *et al* (74). Later, the same group found similar results (75). These studies were contrary to what had been previously published, in which no differences were found in CGRP levels between migraine patients and controls (68). All these studies were carried out in patients with EM.

Regarding CM, there are two main studies suggesting the role of CGRP as a potential diagnostic biomarker, with opposite results. In the first of them, Cernuda-Morollón *et al* (76)**,** assessed interictal plasma levels of CGRP. They found that, compared to healthy women without a history of headache, **interictal CGRP levels were clearly elevated in peripheral blood in a large series of women with CM and, to a lesser extent, in women with EM.** They also showed

that CGRP levels in CM patients were higher than those of women with EM. In relation to the aura, they found that CGRP levels were higher in women with CM who had a history of attacks with aura. In contrast, Lee *et al* did not find an increase in the serum concentration of CGRP in patients with CM or EM compared to healthy controls (HC) (77) calling into question the role of CGRP as a diagnostic biomarker of migraine.

CGRP as a predictor of therapeutic response in patients who received treatment with onabotulinumtoxinA (BTX-A) was first tested by Cernuda-Morollon *et al* (78) and then supported by Domínguez *et al* (79) **finding a decrease in its levels after preventive treatment.** Previously, topiramate demonstrated inhibition of CGRP release in trigeminal neurons (80).

Table 4. Studies assessing interictal and ictal levels of CGRP

INTERICTAL				
	No differences	**Differences**		
Author (et al)	Gallai	Ashina	Fusayasu	Cernuda-Morollon
Year	1995	2000	2007	2013
Migraine	EM			CM
Sample	cubital			

	Increase					**No-increase**	
Author (et al)	Goadsby	Gallai	Sarchielli	Juhasz	Sarchielli	Friberg	Tvedskov
Year	1990	1995	2000	2003	2006	1994	2005
Migraine	EM	EM	EM	EM	EM	EM	EM
Sample	internal jugular	cubital	internal jugular	cubital	jugular	carotid, internal jugular	jugular and cubital

1.1.5 CGRP-related therapies: anti-CGRP mAbs

The goal of preventive treatment is to reduce the frequency, intensity, and duration of migraine attacks in patients with frequent attacks. According to Spanish Guidelines, it is indicated when patients have 3 or more attacks per month. Patients with less than 3 attacks per month that have several days per attack and with poor response or intolerance to symptomatic medication are also candidates for preventive treatment (81). There are different types of preventive treatments, including oral prophylactics, (BTX-A and more recently anti-CGRP mAbs and CGRP antagonists (gepants) (table 5).

Table 5. Pharmacological treatment in migraine

PREVENTIVE TREATMENT	
Oral prophylactics	• Beta-blockers propranolol, atenolol, metoprolol, nebivolol • Antiepileptic drugs sodium valproate, topiramate • Angiotensin receptor blockers and angiotensin-converting enzyme inhibitors lisinopril, candesartan • Calcio antagonists flunarizine • Antidepressants amitriptyline, venlafaxine
OnabotulinumtoxinA	-
Monoclonal antibodies targeting CGRP	• Erenumab • Galcanezumab • Fremanezumab • Eptinezumab
Gepants	• Atogepant • Rimegepant

Adapted from Spanish Guidelines in Headache 2020

Due to its relevance in the pathophysiology of migraine and its relation with this doctoral thesis, **treatment with anti-CGRP mAbs is detailed below.**

The development of CGRP-targeting drugs has ushered a new era of migraine therapy (82). CGRP was first discovered in 1982 (59) and, since then, studies of the trigeminovascular system have told us much of what we know about the role of CGRP in the cranial sensory nerves that are involved in migraine. Multiple components of CGRP transmission are now targeted by migraine therapies (58). The first anti-CGRP treatment, an intravenous CGRP-receptor antagonist or gepant, olcegepant, was described as effective acute treatment in humans in 2004 (83). Following olcegepant, other gepants underwent tested as acute treatment of migraine such as BI 44370 TA, telcagepant, MK-3207, rimegepant, and ubrogepant. However, it was reported hepatotoxicity associated with some of them (BI 44370 TA, telcagepant, and MK-3207), that resulted in discontinuation of development of those gepants (84). A second generation of gepants without liver toxicity concerns started to be proved in clinical trials. Both ubrogepant and rimegepant have met primary endpoints for efficacy as acute treatments of migraine in phase 3 clinical trials (85,86). For migraine prevention, both rimegepant and atogepant are being investigated in phase 3 clinical trials with positive results so far (87).

Anti-CGRP mAbs are the first specific target-driven treatment in migraine. To date, four anti-CGRP mAbs have been developed and approved, including one antibody against the CGRP receptor (erenumab) and three against the CGRP ligand (galcanezumab, fremanezumab, and eptinezumab). In Spain, erenumab started to be available in Headache Clinics in 2018 through a personalized access program from Novartis. In November 2019, erenumab and galcanezumab were approved for reimbursement when patients had 8 or more headache d/mo and had failed at least 3 previous preventive treatments being one of them BTX-A if CM (88). Fremanezumab was approved in August 2020, with the same prescribing conditions.

The fundamental role of CGRP in migraine pathophysiology has prompted the development of these CGRP treatments. The findings on the effectiveness of these therapies reinforce the link that had already been established over the previous years (58). These anti-CGRP mAbs have shown efficacy over placebo in the treatment of EM and CM (89,90,98,91–96,96,97). Unlike oral prophylactics, the response to these treatments starts early, already in the first week (99). The tolerability of these treatments is almost comparable to that of placebo. The treatment-emergent adverse effects which have been reported more commonly than placebo in all trials are an injection-site reaction, which includes erythema, pruritus, and pain, constipation and upper respiratory tract symptoms, such as nasopharyngitis or sinusitis (100). Given their large molecular weight, they practically do not cross the blood-brain barrier (BBB), so they do not give central nervous system effects and, as they do not undergo intracorporal metabolism (they are eliminated by the endoplasmic reticulum), drug interactions or hepatic/renal are not expected either as it occurs with classical drugs. In terms of safety, few adverse events have been described (100).

Site of action

Many questions remain unclear about how anti-CGRP mAbs act in the pathophysiology of migraine, since these drugs have reduced ability to cross the BBB (101) and therefore do not act at a central level, where many of the structures lie and have been correlated to the different migraine attack phases (46).

Some **animal studies** have shed light on these questions. For example, fremanezumab was able to prevent CSD-induced trigeminovascular activation and sensitivity and activation of Aδ-type but not C-type nociceptive meningeal fibers. It suggests that initiation of the headache phase of migraine depends on activation of meningeal nociceptors, and that for selected patients, activation of the Aδ-high threshold pain pathway may be sufficient for the generation of headache perception (102). Another study of the same group found that using standard electrocorticogram recording techniques in rats in which the BBB was intentionally compromised, fremanezumab did not prevent the induction,

occurrence, or propagation of CSD, which suggests that CGRP may not be involved in the initiation of CSD, at least not to the extent that it can prevent its occurrence (103).

Taking these data into account, **interrupting afferent traffic along peripheral sensory fibers could modulate the core networks that are responsible for generating a migraine attack**. Therefore, migraine attacks could be stopped and prevented by decreasing peripheral trigeminovascular transmission or directly modulating networks that control the upward transmission of nociceptive signals from central trigeminovascular neurons. These findings provide further evidence for the view that the mechanisms by which this class of drugs prevent migraine is mainly through their ability to directly alter headache-related peripheral functions in meningeal nociceptors, cerebral and meningeal blood vessels, and possible immune cells, which indirectly alter excitability and responsiveness of neurons in brain areas involved in migraine pathophysiology.

1.1.6 Comorbidities: depression

Migraine has been noted to be comorbid with a number of other illnesses in population-based and clinical studies (13,104), such as asthma, rhinitis, depression and anxiety,chronic pain disorders, and noncephalic pain disorders, identified as predictors of progression to CM (16) (table 6).

The Chronic Migraine Epidemiology and Outcomes (CaMEO) Study was a web-based survey study using cross-sectional modules with longitudinal follow-up assessments. Data from the CaMEO study were modeled using latent class analysis to identify subgroups of migraine based on comorbidity profiles. These subgroups differed in demographic profiles, disability, and headache characteristics (105). Some comorbidities could serve as predictors of progression from EM to CM when they are modelled (106).

Table 6. Migraine comorbidities

COMMON COMORBIDITIES IN MIGRAINE	
Psychiatric	**Cardiac**

• Depression • Anxiety • Panic disorder • Bipolar disorder	• Patent foramen ovale • Mitral valve prolapse • Atrial septum aneurysm
Neurologic	**Other**
• Epilepsy • Tourette's	• Snoring/Sleep apnea • Asthma/Allergy • Systemic lupus erythematosus • Chronic pain conditions
Vascular	
• Raynaud's phenomenon • Blood pressure • Ischemic stroke, white matter abnormalities	

Adapted from Bigal and Lipton, 2009

For the relevance in this thesis, depression is detailed below**.**

Psychiatric disorders are known to be comorbid with migraine, specially anxiety and depression. The clinical comorbidity between depression and migraine is well-established in epidemiological studies trough questionnaires in most studies (107–111). **Depression is approximately twice as present in CM than in EM** (16), in particular, it is more likely to occur from 7 d/mo (112). There is a linear relationship between the number of headache days and the presence and degree of depression and anxiety (112). However, when the number of headache days reaches the chronic variant, the linearity is lost and all patients suffer from a high impact of psychiatric impairment (113). Similarly, it has been demonstrated that similar to CM patients, HFEM patients show poor outcomes in emotional disability. seeming that the cut-off point for HFEM (≥10 headache days) could be a good option to better classify patients according to emotional disabilities (18).

Regarding the analysis of shared heritability in common disorders of the brain, common variant risk for psychiatric disorders was shown to correlate significantly, especially among attention deficit hyperactivity disorder (ADHD), bipolar disorder (BD), major depressive disorder (MDD), and schizophrenia. **By**

contrast, neurological disorders appear more distinct from one another and from the psychiatric disorders, except for migraine, which was significantly correlated to ADHD, MDD, and Tourette syndrome (41). Furthermore, there is evidence for **shared genetic factors that underlie these two disorders. A genetic overlap across migraine and MDD has been found**. Meta-analysis of results for 8,045,569 SNPs from a migraine genome wide association study (GWAS) and the top 10,000 single nucleotide polymorphism (SNPs) from a MDD GWAS implicated three SNPs (rs146377178, rs672931, and rs11858956) with novel genome-wide significant association to migraine and MDD. At gene-level, two genome-wide significant genes (ANKDD1B and KCNK5) were identified (114). A molecular evidence for such an association is lacking.

In recent years, relationship between the immune system and the presence of psychiatric disorders has gained interest and it would appear that **the severity of depressive symptoms is likely to be modulated by the degree of inflammation** (115)**.** While neurobiological correlates have only partially been elucidated, altered levels of CGRP like immunoreactivity (CGRP-LI) in animal models (116,117) and in the cerebrospinal fluid (CSF) of depressed patients has been reported (118), suggesting that **CGRP may be involved in the pathophysiology and/or be a trait marker of MDD**. Increased brain levels of CGRP have been found a well-established rat model of depression and, interestingly, antidepressants did not have effect on the brain level of this peptide (116). These rats were treated with escitalopram and nortryptiline and CGRP-LI was measured in selected brain regions. Interestingly, neither escitalopram nor nortriptyline significantly altered brain CGRP levels. However, this is in its first stages of study and needs further investigation to evaluate the role of CGRP and other neuroinflammatory biomarkers in depression (119).

If depression is a cause or it is more likely a consequence of migraine, especially migraine chronification remains unclear, even though studies would indicate that when depression occurs in patients with migraine it is more likely to be a consequence (120).

1.2 Calcitonin gene-related peptide

1.2.1 Biology of CGRP

CGRP is one of the most abundant neuropeptides in the peripheral and CNS. It is a **neuropeptide consisting of 37 amino acids found primarily in the C and Aδ sensory fibers arising from the dorsal root and trigeminal ganglia**, **as well as the CNS** (121–124) (Fig. 3). **In the trigeminal ganglia, CGRP is expressed in approximately 50% of the trigeminal C-fibers, mainly those innervating intracranial blood vessels** (125,126). It is synthesized in neurons through tissue-specific splicing of mRNA transcribed from the calcitonin–CGRP gene (CALCA) located on chromosome 11 (127).

Human CGRP has two isoforms: **α-CGRP (or CGRP1) and β-CGRP (or CGRP-2**) (128). β-CGRP differs by three amino acids from homologous human α-CGRP; and they are **very similar in their biological activities** (129). α-CGRP is widely distributed in the central and peripheral nervous systems. Most of the intracranial vasculature is innervated by α-CGRP-containing C and Aδ sensory nerve fibers. β-CGRP is located in the enteric nerve terminals and pituitary gland (130). **β-CGRP has been shown to be released alongside α-CGRP in the vascular system** (131). **Thus. it is now becoming clear that both isoforms can be expressed in the nervous system, depending on situation.**

Besides the CGRP-containing nerve fibers which originate in the trigeminal ganglion (TG), **in the periphery, CGRP-containing nerve fibers are often associated with smooth muscles such as**: (i) **most parts of the gastrointestinal tract, including the excretory ducts of the parotid gland**, over the epithelium of the fundic glands of stomach, endocrine cells of the duodenum and ileum and some myenteric ganglia, (ii) lungs, (iii) thyroid gland (close to C cells), (iv) splenic vein and sinusoids, (v) human skin, and (vi) pituitary gland (132–134).

CGRP receptor is a complex of several proteins: calcitonin receptor-like receptor (CALCRL), receptor activity-modifying protein 1 (RAMP1) and two cytoplasmic proteins (receptor coupling protein (RCP) and the α- subunit of the GS protein (GαS) (129). Cross-activation between calcitonin family ligands (CGRP, amylin, adrenomedullin and adrenomedullin 2) and receptors has been described in the scientific literature. CGRP can activate other receptors in the calcitonin receptor family in addition to the CGRP receptor itself (135) (Fig. 3). CGRP specifically binds to the CGRP receptor but also binds AMY1 with the same potency as amylin. **CGRP receptor is present in the nervous system (expressed in trigeminal Aδ-fibers)**, the cardiovascular system as well as other tissues such as thyroid gland, gastrointestinal tract, parotid gland, adrenals, pituitary, exocrine pancreas, kidneys, bones, skin and skeletal muscles (122). **Many of the sites of action of both CGRP and its receptors occur outside the BBB**. The CGRP receptor is also highly expressed in the meningeal vasculature, which is innervated by primary afferent fibers from the TG that express CGRP (125,136). Recent studies using **antibodies that specifically recognize the CGRP-binding site**, which was discovered by using a fusion protein of the extracellular domains of RAMP1 and CLR, **showed binding to human TG neurons and human vascular smooth muscle cells of the meningeal vasculature** (137).

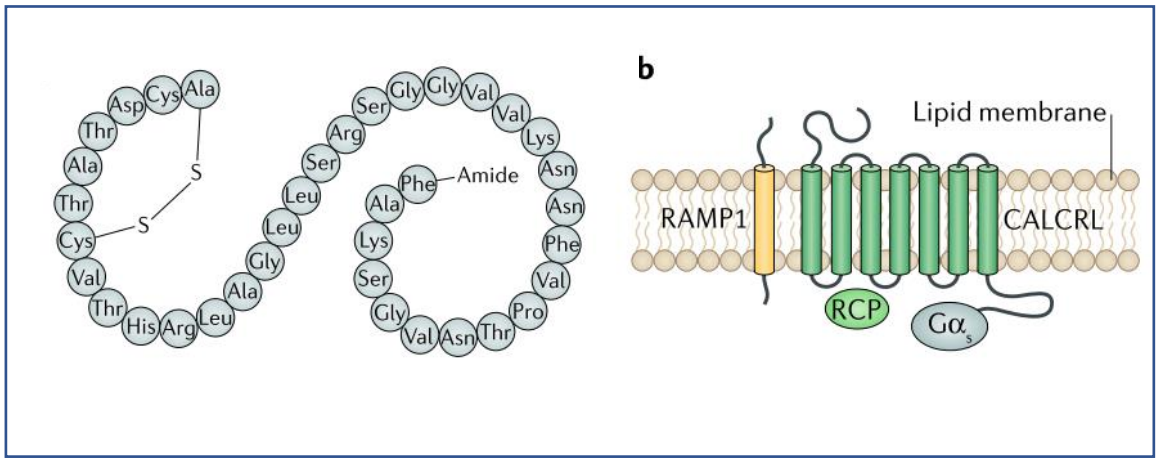

Figure 3. CGRP and its receptor. *From Edvinsson L et al. Nature Reviews Neurology, 2018*

The most pronounced action of CGRP in intracranial vasculature is vasodilatation. In cerebrovascular smooth muscle, elevation of cAMP upon

CGRP activation results in vasorelaxation and dilation of the blood vessels, which have no endothelium (138). On the other hand, **CGRP acts on receptors on Aδ-type sensory neurons and satellite glial cells to modulate pain sensitivity and nociceptive transmission within the ganglion** (139).

Besides the clear role of CGRP in the pathophysiology of migraine, there is some evidence regarding the role of CGRP in arthritis, skin conditions, diabetes, and obesity. However, CGRP's role in cardiovascular regulation is still speculative (122).

1.2.2 CGRP quantification

Nowadays there is no standardized and validated method to measure CGRP (140). The methodology used in each study is different, with the use of different assays (Radioimmunoassay (RIA), Enzyme-linked immunosorbent assay (ELISA), Western Blot), different biofluids, collection techniques or measurement units, among others. In addition, much of the information on the technique is scarce, not facilitating the reproducibility of experiments and results. The absence of a validated standardized method makes it difficult to obtain reproducible and reliable results. In order to overcome methodological limitations, recent studies are more complete, providing detailed information and methodological recommendations that could allow other authors to replicate the studies.

1.2.2.1 Blood

Historically, as explained in previous sections, CGRP has been measured in circulating blood (plasma or serum) where extracerebral tissues drain (internal jugular vein) and peripheral blood (antecubital vein). The presence of CGRP in the blood is generally attributed to spillover from sites of neuronal release, a hypothesis supported by experiments that demonstrated release of trigeminal CGRP into the rat jugular vein (141).

However, **it has been seen that plasma CGRP levels are usually in the low picomolar range, suggesting that measuring these peptides would be more efficient if performed closer to the effectors, e.g., near the**

trigeminovascular system (58). In addition it is likely that CGRP can mediate its biological effects without the need to circulate in plasma. it is considered that plasma CGRP is the result of an “overspill” from perivascular sensory neurons, and the major effects of CGRP are exerted locally, in the vessel wall, close to its site of release (122).

1.2.2.2 Cerebrospinal fluid

CGRP has been measured in cerebrospinal fluid (CSF) of CM patients in three different studies (142–144). There are no studies on CGRP concentrations in CSF from EM patients. The meta-analysis published in 2017 showed increased concentrations of CGRP in CM patients (145). Further studies were not performed possibly due to the invasiveness of the lumbar puncture (LP) and its limited accessibility.

1.2.2.3 Tears

More recent is the use of tears as a biofluid for the study of CGRP in migraine and other primary headaches such as cluster headache (146,147). CGRP has been measured in patients with EM, CM and HC in one study. Tear fluid CGRP concentrations were elevated in interictal migraine patients compared to controls. There was no difference in tear fluid CGRP levels between interictal episodic and CM patients and no correlation of tear fluid CGRP levels with headache frequency in interictal patients. Unmedicated ictal migraine patients had more elevated tear fluid CGRP levels than interictal migraine patients, while medicated ictal migraine patients had lower levels, which were undistinguishable from controls. In contrast to tear fluid, no significant group differences were found in plasma CGRP levels.

1.2.2.4 Saliva

1.2.2.4.1. Salivary CGRP

Saliva is a biofluid closer to the receptors than plasma and CSF. Thus, saliva as a substrate for the study of CGRP began to be used in the 1990s.

There is a rationale for measuring CGRP in saliva. **Several neuropeptides are part of the composition of human saliva**, including CGRP (table 7). These neuropeptides, synthesized in the cell body, are packaged in large vesicles and transported to the nerve terminals where they are depleted (148).**These sensory nerves are mainly located around the blood vessels and ductus.** In addition to their role in pain, these neuropeptides exert certain physiological effects such as significantly increasing blood flow in the salivary glands, causing salivation (149). The neuropeptides could also be released by agents such as capsaicin or nerve stimulation, since their demonstration in animal models (150,151) and in human models (152,153).

Table 7. Neuropeptides found in human saliva (154) (alphabetical order)

Salivary neuropeptides and growth hormones

- Beta-endorphin
- **Calcitonin gene-related peptide**
- C-flanking peptide of neuropeptide Y
- Endothelial growth factor
- Epidermal growth factor
- Epithelial growth factor
- Enkephalins
- Fibroblast growth factor
- Insulin-like growth factor
- Mesodermal growth factor
- Nerve growth factor
- Neural tube growth factor
- Neurokinin A and B (possible)
- Substance P
- Transforming growth factor alpha and beta
- Vasoactive intestinal peptide
- Wound contraction factor

The salivary glands (submandibular and sublingual) are **innervated by the third branch of the trigeminal nerve (V3).** The first (V1) and the second branch (V2) innervate different facial regions. **There is evidence that the activation of one branch of the trigeminal nerve can lead to the activation of other branches, resulting in pathophysiological changes** (155,156). In this way, it is possible to monitor changes in the levels of certain neuropeptides in saliva (V2 and V3) which reflect changes in the dura mater (V1). Since the salivary glands are partially innervated by sensory nerve fibers of the trigeminal nerve that contain CGRP (157,158) **CGRP levels in saliva can be interpreted as indicators of trigeminal nerve activation in patients with migraine** (159)**.**

In 1996, a Swedish group analyzed using RIA 5 neuropeptides (including CGRP) in saliva from HC and tested different saliva collection techniques (160). Although the CGRP had already been analyzed 6 years earlier in patients with migraine and cluster headache, this study was **the first one to evaluate only healthy subjects. Their results showed that these neuropeptides are continuously released into saliva and their amounts increase with stimulation, but are diluted by increasing saliva volume.** Specifically, the concentration of CGRP was higher in saliva at rest than in the other techniques, as will be explained below. CGRP has also been measured in saliva of HC in provocation studies. In this case, a higher concentration of CGRP was found after chronic treatment with anethole trithione, a substance used for conditions that cause dry mouth (149).

Over the years, the concept of analysis of neuropeptides in human saliva such as CGRP or VIP as markers of pathological conditions and therapeutic interventions and therefore as a clinical model for the study of the mechanisms involved in migraine has gained interest. Since 2006, the quantification of CGRP in human saliva began to be used in basal conditions and as a marker of response to specific treatments. Initially, in response to acute treatment: sumatriptan (159) and rizatriptan (161). Subsequently, CGRP levels were also measured in CM patients as a marker of disease state (162) and after BTX-A treatment (163).

Years after the publication of the capsaicin provocation model in patients diagnosed with cluster headache, another study was published in HC in order to consolidate the non-invasive study of the state of activation of the trigeminal nerve that innervates the salivary glands (164). They found that oral application of red chili homogenate was well tolerated and caused a dose-dependent release of CGRP in saliva, with no day-to-day effects on this response.

Although few studies exist that handle the correlation between salivary and plasma levels of neuropeptides, there are numerous reports that show certain biochemical, immunological and endocrine analytes in oral fluid and plasma demonstrate good correlation forming the basis of using saliva as an effective diagnosis tests. **The easy non-invasive nature of collection and the close relationship between oral fluid and plasma levels of such substances make oral fluid a valuable clinical tool** (165). Moreover, Parris *et al* reported that the salivary SP level of chronic pain patients were higher than its plasma level, showing that saliva may be a less invasive and more efficient diagnostic tool to measure markers that reflect pain states (166). In rats the major part of circulating CGRP is released from perivascular nerve terminals. Jang *et al* investigated the levels of sensory neuropeptides simultaneously in plasma and saliva samples in patients diagnosed with CM, finding a positive correlation (162). A positive correlation was also found in HC (167). Thus, possible changes of neuropeptide levels in blood and subsequently in saliva may reflect changes in their expression in the inflamed peripheral or CNS of CM patients.

1.2.4.4.2 Salivary CGRP in migraine

The first attempt of measuring neuropeptides on salivary CGRP was carried out in 1990 by Nicolodi *et al* (152). Saliva was collected with an electric drain. They used a previously validated RIA to measure sensory neuropeptides (CGRP and SP) as well as VIP in patients with migraine, cluster headache (CH), and HC, finding **higher levels of CGRP during migraine and cluster headache attacks compared to the interictal period and lower levels (ictal and interictal) than in HC** (168).

It was not until 2006 when CGRP was further analyzed in saliva samples from migraine patients during and between a migraine attack, as well as in response to therapeutic intervention (159). Some methodological improvements were made due to recommendations published in previous studies. Thus, using the 2% citric acid stimulated method collection, they found that **individuals suffering from multiple migraine attacks per month had elevated levels of salivary CGRP and VIP between attacks compared to HC.** However, no data on the migraine characteristics of the patients was published. Furthermore, treatment of the migraine attack with sumatriptan resulted in decreased levels of CGRP and VIP, which were correlated with symptom relief. Contrary to the first study mentioned above, CGRP levels in HC were lower.

In 2009, the same group showed that **CGRP levels were elevated in the premonitory period and during mild and moderate/severe headache and that a successful response to rizatriptan was correlated with the return to baseline of CGRP levels** in saliva (161) to values close to the baseline. For the first time, the correlation of CGRP levels across a migraine attack and clinical symptoms (premonitory symptoms now called prodromes and pain intensity) as well as predictors of response were examined.

Subsequently, CGRP levels in patients with CM, measured for the first time by ELISA, were investigated. In addition, **a correlation between CGRP levels in plasma and saliva and its association between pain intensity and concentration was studied.** Thus, they found that CM patients showed higher levels of CGRP in both plasma and saliva compared to HC and these levels were highly associated with pain intensity. Plasma levels of SP and CGRP were significantly correlated with their level in saliva (162).

More studies have been done in patients with CM. Cady *et al* (163) continued to study the levels of CGRP in saliva in patients diagnosed with CM, but this time, after treatment with BTX-A. On one hand, **no elevation of salivary CGRP was demonstrated during the attack nor was there a reduction of CGRP after acute treatment with a triptan or any other acute treatment**. Regarding CGRP levels change in response to BTX-A treatment, at months 2 and 3 after injections,

there **was a decrease in basal CGRP levels for the BTX-A group but not for the saline group**, missing statistical significance, which was probably due to the small number of subjects. On other hand, subjects who were classified as responders to BTX-A had a better response to acute treatment than those who did not respond to BTX-A or saline, regardless of the acute treatment used.

In 2018, another study (169) measured the CGRP content of gingival crevicular fluid (GCF) in CM patients and HC and determined whether there was a correlation between serum and GCF values of CGRP. They found that **CGRP levels were higher in CM patients compared with HC both in serum and GCF**. Furthermore there was a strong correlation between CGRP levels of the serum and GCF.

1.2.4.4.3. Experience in other headache disorders

One limitation would be whether the described increases in levels of some neuropeptides such as CGRP are specific for migraine versus other headaches. The specificity of CGRP in primary headaches is determined by two studies, one conducted in patients with cervicogenic headache (170) and another in patients with chronic tension headache (171) in which they found no differences in CGRP levels between days with pain and days without pain.

Regarding CH, **CGRP levels have been shown to increase during attacks in patients with CH**, but there is no published data showing a role for CGRP as biomarker of CH in interictal period (172). During CH attacks the trigeminal-autonomic reflex (an association between the trigeminal sensory system and the parasympathetic system—sphenopalatine and otic ganglia) is activated provoking vasodilation of cranial arteries by the release of vasodilatory molecules, including CGRP, vasoactive intestinal peptide (VIP) and Pituitary adenylate cyclase-activating polypeptide (PACAP) (172). An increase in salivary levels of CGRP-LI from basal during CH attacks (152) and after capsaicin application (153) has been seen. Interestingly, in the study by Bellamy *et al* the amount of CGRP was higher in patients with migraine and "sinus symptoms" (now called migraine with autonomic symptoms (159) between and during attacks

(173). Significantly higher levels of CGRP were found during allergic rhinosinusitis attacks and during attacks compared to controls (159). Interestingly, chronic CH patients were discovered to have lower plasma levels of CGRP than episodic CH patients, although very little is known on how the pathophysiology differs between these two conditions (174). Levels of CGRP are not elevated in saliva of patients with burning mouth syndrome (175).

Salivary neuropeptides related to nociception were suggested as a promise candidates to the chronic pain conditions such as rheumatoid arthritis, osteoarthritis or even fibromyalgia (154). However, evidence is scarce the best known role of CGRP is in migraine.

Table 8 summarizes studies assessing CGRP in biofluids closer to the TVS: saliva, gingival crevicular fluid and tears.

Table 8. Studies assessing CGRP in biofluids closer to the TVS: saliva, gingival crevicular fluid and tears

SALIVA											
		INTERVENTION			POPULATION						
Ref	BASAL	BTX	TRIPTANS	PROV	HC	EM (ictal)	CM (ictal)	Other (CH)	N	PLASMA*	ASSAY
(152)	14.3±2.5 pmol/l	-	-	-	22.02±1.7 pmol/l	27.3±2.9 pmol/l		53.7 ± 5.2 pmol/1 **(ictal)** 40.1±2.3 pmol/1 **(interictal)** 33.4±7.7 pmol/1 **(out of the cluster period)**	59	no	RIA
(176)	no data	-	-	-	no data	-	-	CH no data		no	RIA
(153)	7.6±1.5 picomol/l	-	-	capsaicin		-	-	CH 28.2±5.7 pmol/l	18	+	RIA
(160)	no data	-	-	-	≠data (M+F)	-	-	-	8	no	RIA
(149)	27.7±4.7 pg mL−1	-	-	anethole trithione	39.9±4.7 pg mL−1 (M)	-	-	-	6	no	EIA

(159)	53 pmol/mg total protein	-	Suma	-	10 pmol/mg total	65 pmol/mg total protein (M+F)	-	RS 24 pmol/mg total protein	25	no	RIA
(161)	58.2±1.6 pmol/mg total protein		Riza	-	-	(M+F)	-	-	22	no	RIA
(162)	431.6±272.8 pg/ml	-	-		301.5±188.9 pg/ml	-	(M+F)	-	69	+ CM 253.6±95.2 HC 136.2±92.5	EIA
(167)	-	-	-	pilocarpine	6492.1±86.1 pg/ml (M)	-	-	-	5	+ 7243.9± 2522.6 pg/ml	EIA
(177)	32±3 pmol/mg total protein	**X**	-	-	-	-	(M+F) No data	-	20	no	RIA
(164)				capsaicin	Depends on concentration						

	Depends on concentration	-	-	(M+F)	-	-	-	13	no	RIA
(178)	Depends on method	-	-	Depends on concentration (M+F)	-	-	-	20	not detected	WB
GINGIVAL CREVICULAR FLUID										
(169)	0.25±0.09 pg/µg	-	-	-	0.19 ± 0.07 pg/µg	-	-	-	-	+ CM 41±16 pg/mL HC 29±8 pg/mL	ELISA
TEAR FLUID										
(146)	EM 1.10±1.27 ng/ml CM 1.10±1.27 ng/ml	-	-	-	0.75±0.80 ng/ml (M+F)	1.92±1.84 ng/ml (unmedicated, M+F) 0.56±0.47 ng/ml (medicated, M+F))	-	141	+ 6.81±4.12 pg/ml	ELISA

BTX OnatobulinumtoxinA**; PROV** provocation; **HC** Healthy controls; **EM** Episodic migraine; **CM** Chronic migraine; **M** Males; **F** Females; **RS** Rhinosinusitis; **CH** Cluster Headache; **RIA** Radioimmunoassay; **WB** Western Blot; **ELISA** Enzyme-Linked ImmunoSorbent Assay; **Suma** Sumatriptan; **Riza** Rizatriptan

1.2.4.4.4. Saliva collection: methodology

For participants, **the collection of saliva samples provides a non-invasive and less stressful method**, rather than the collection of CSF or plasma. Other advantages are that the saliva can be collected at home, it does not require professional personnel (although it does require personnel experienced in collecting it, can be collected repeatedly and it is easily accessible and safer to handle).

There are different techniques to collect saliva. The disparity between methods is wide and the detailed description of the methodology is in general scarce. The first attempt to take it into consideration and measure neuropeptides was made by Dawidson *et al* (160), describing 4 different techniques: resting saliva, paraffin-chewing stimulated saliva, citric acid stimulated saliva and citric acid stimulated parotid saliva. They reported that salivary protein concentration varied inversely with salivary flow rate and therefore the resting whole saliva method was better. Similarly, the results of Jang *et al* (162) also show that neuropeptides concentrations fluctuate depending on salivary flow rate and let us know that in order to obtain reproducible results for follow-up studies, saliva must be collected under a repeatable standard measurement protocol. They also used the method of resting whole saliva.

Interestingly, Bellamy *et al* (159) introduced a new concept: collecting saliva at the participant's home, since it was shown that levels remain similar whether saliva was collected at home or in the clinic. They used the citric acid stimulated method. Contrary to previous studies, no differences were found between saliva flow and peptide concentration. They used this collection technique in the rest of their experiments, with some modifications. Other studies continued to use stimulated citric acid saliva (164).

The data suggest that resting methods will better differentiate individual salivary flow rates because the measurements are less variable. Thus, **the method of whole saliva at rest has been used in different studies** (167,179,180)

Comparative studies have found that different saliva collection methods provide clear differences in the salivary proteome and also in the relative amount of specific proteins. These results emphasize the importance of consistency when collecting saliva samples for proteomic analysis (181). In addition, the same group studied the ideal saliva collection technique to detect and measure pain-related biomarkers (178). Consequently, they tested 5 different techniques on HC (Fig. 4):

- **Unstimulated whole saliva**. Participants was instructed to sit upright and with their head slightly titled forward allow saliva to collect on the floor of the mouth and dribble into a 5 ml polypropylene tube,
- **Unstimulated sublingual saliva**. While blocking the Stensen's duct, sublingual saliva was collected from the floor of the mouth with a syringe every second minute. Samples from the first 2 minutes were discarded.
- **Stimulated parotid saliva**. Pure parotid saliva was collected using a modified Carlsson-Critten collector while actively stimulating salivary flow with citric acid solution as earlier described by Jasim *et al*, 2016
- **Stimulated sublingual saliva**. Saliva Bio Oral Swab® (Salimetrics) was placed for around 2 minutes under the tongue while stimulating with 2% Citric acid until the swab was fully covered in saliva. The fluid was then obtained by centrifugation.
- **Stimulated whole saliva**. Saliva was stimulated by chewing on paraffin tablets (Orion Diagnostica, Finland) as described earlier by Jasim *et al*, 2016.

Specifically, CGRP showed a large variation in expression and occurrence between different collection methods, although in this study the assay was performed by Western blot. They found that stimulated saliva expressed higher total CGRP compared to unstimulated, but the difference was only significant for

stimulated sublingual saliva. There were no significant differences in total CGRP expression between unstimulated whole and sublingual saliva.

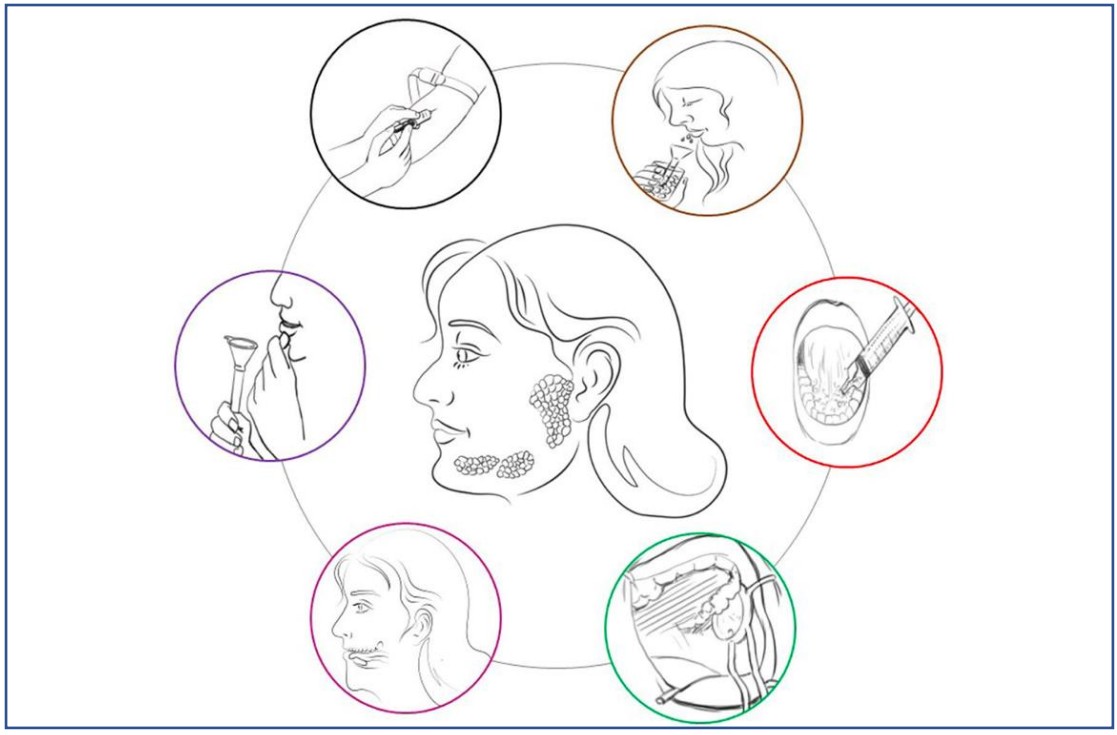

Figure 4. Illustrative overview of the main salivary glands and different collection approaches used in the study. *Adapted from Jasim H et al 2018*

1.3 Biomarkers

1.3.1 Definition and types

A biomarker is a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes or responses to an exposure or intervention (182). This definition encompasses therapeutic interventions and can be derived from molecular, histologic, radiographic, or physiologic characteristics.

Subtypes of biomarkers have been defined according to their putative applications. **Importantly, a single biomarker may meet multiple criteria for different uses, but it is important to develop evidence for each definition** (183).

Biomarkers are critical to the fabric of discovery science, medical product development, and healthcare for the individual and population.

Different subtypes of biomarkers according to their application are explained below:

- **Diagnostic biomarkers:** detects or **confirms the presence of a disease or condition of interest, or identifies an individual with a subtype of the disease.** Diagnostic biomarkers are extremely important in order to carry out **precision medicine.** Furthermore, such biomarkers may be used not only to identify people with a disease, but **to redefine the classification of the disease.** One goal is to define a method for validation that assures that the biomarker can be measured reliably, precisely, and repeatably at a low cost. All too often, assays are not validated, engendering misleading assumptions about the biomarker's value. Decision thresholds and clinical utility are becoming important measures for assessing the value of biomarkers for clinical application.
- **Monitoring biomarkers:** When a biomarker can be **measured serially to assess the status of a disease or medical condition** for evidence of exposure to a medical product or environmental agent, or to detect an effect of a medical product or biological agent. Monitoring biomarkers are also useful for measuring pharmacodynamic effects, to detect early evidence of a therapeutic response, and to detect complications of a disease or therapy.
- **Pharmacodynamic/response biomarkers:** When **the level of a biomarker changes in response to exposure to a medical product or an environmental agent.** This type of biomarker is extraordinarily useful both in clinical practice and early therapeutic development. It is therefore critically important to validate that the measured change in the pharmacodynamics/ response biomarker provides a reliable signal for the expected therapeutic response.
- **Predictive biomarkers:** defined by the finding that **the presence or change in the biomarker predicts an individual or group of individuals more likely to experience a favorable or or unfavorable**

effect from the exposure to a medical product or environmental agent. Proving that a biomarker is useful for this purpose requires a rigorous approach to clinical studies.

- **Prognostic biomarkers:** is used to identify the likelihood of a clinical event, disease recurrence, or disease progression in patients with a disease or medical condition of interest.
- **Susceptibility/risk:** which deal with association with the transition from healthy state to disease. The concept is similar to prognostic biomarkers, except that the key issue is the association with the development of a disease rather than prognosis after one already has the diagnosis. These types of biomarkers are foundational for the conduct of epidemiological studies about risk of disease.
- **Safety biomarker:** is measured before or after an exposure to a medical intervention or environmental agent to indicate the likelihood, presence, or extent of a toxicity as an adverse event.

One of the most modern type of biomarker and adjusted to the technological era we are living in are **digital biomarkers** (184)**.** Sensors and personal devices such as wearable sensors and smartphone apps provide us with a massive and continuous source of information about individuals such as physical and mental activity, cognitive abilities, behavior patterns, movement or sleep. The complexity of digital biomarkers lies not only in the need for more precise and reliable devices, but also in how we will be able to interpret and transform this large amount of data into interpretable results that are useful in health.

Taking into account both the biological complexity of the systems and the models used in biomarker research, the sole determination of one type of biomarker without the joint evaluation of the rest can lead to wrong conclusions. **The evaluation of composite biomarkers is as complex as it is necessary to allow a better prediction of the final result.** These suppose, then, the link between the measurement and the prediction of a clinical result, for which a biomarker is not equivalent to a clinical result.

1.3.2 Migraine biomarkers

There are no validated biomarkers in migraine. Diagnosis is based on ICHD-3 clinical criteria which do not fully capture the heterogeneity of migraine, including the underlying genetic and neurobiological factors. Furthermore, disease monitoring is based on headache diaries and preventive treatment is based on *trial and error approach.* Prognosis is based on clinical risk factors of chronification.

The lack of a biomarker in migraine has several implications. On one hand, it gives us an idea of **the complexity and dynamism involved in this disease**. On the other hand, it implies a lack of recognition both by society and by the medical and scientific community, due to its "invisible" character. In addition, due to the subjectivity involved in making a diagnosis based on the patient's history (language barrier, recall bias...) migraine can be underdiagnosed and undertreated. And this is directly related to the lack of use migraine-specific therapies (185,186).

During the past years several efforts have been made to identify reliable biomarker(s) for different purposes such as diagnosis, monitor disease activity and/or ascertain the response to a specific treatment (187)**.** A biomarker in migraine would be very useful in the diagnosis, with high sensitivity and specificity, in the monitoring of a disease that is cyclical and chronic, in the quantification of the progression and its severity, to predict the result of a therapeutic intervention, or to choose the best candidates for certain treatments or to be included in clinical trials (188)**.**

"Omics" is the part of biotechnology which analyzes the structure and functions of the whole makeup of a given biological function, at different levels (189). We remain at an early stage in combining data from the various -omics technologies (genomics, proteomics, metabolomics, transcriptomics, etc.) and linking this data to patient clinical information to optimize our search for disease-specific biomarkers linked to clinical phenotypes. **The development of genetic, molecular or imaging biomarkers, or a combination of them**

constitute the foundations of a new era for precision medicine in migraine (105,190,191). Understanding the mechanisms underlying disease expression could help with more accurate disease diagnosis, phenotype patient population to identify those that may best respond to the particular treatment, provide prognostic indications regarding disease progression, demonstrate a drug is "hitting its target" within the periphery or CNS, or predict treatment response.

I will briefly review molecular biomarkers in migraine (table 9). They are the most advanced and probably the most reliable biomarkers, **in particular, CGRP.** Data from functional and structural imaging have also provided promising and therefore potential disease biomarkers.

Molecular biomarkers

Potential biomarkers in different biofluids such as saliva, serum, and CSF have been investigated and implicated in migraine pathogenesis and chronification (192). Several circulating biomarkers have been proposed as diagnostic or therapeutic biomarkers in migraine, mostly related to migraine's inflammatory pathophysiological aspects (187,188,193–195). **At the present time, CGRP represents probably the most promising candidate,** and it has been separately reviewed above.

The other potential biochemical important biomarkers include glutamate, nerve growth factor (NGF), some inflammatory (C-reactive protein (CRP), tumor necrosis factor alpha (TNF-α), interleukins (IL)) and oxidative stress markers. Other molecules (including some neuropeptides such as and PACAP, cytokines, adipokines, vascular activation markers or neuroinflammation biomarkers) despite promising, showed inconsistent results and they do not possess the sufficient prerequisites to be considered as migraine biomarkers.

Inflammatory markers

TNF-α serum levels, one of the main proinflammatory marker, were associated to patients with both EM and CM (196). **Oxidative and inflammatory biomarkers in serum**, such as neutrophil/lymphocyte ratio (NLR),

monocyte/lymphocyte ratio (MLR), platelet/lymphocyte ratio (PLR), and C-reactive protein (CRP)/albumin (CAR) levels were proven to be biomarkers associated with migraine subtypes with different clinical features, such as migraine attack period, MwA, and patients with a family history of migraine (197). Moreover, Dini et al. described that effective prophylactic treatment for migraine can improve the levels of plasma oxidative stress biomarkers, e.g. advanced oxidation protein products (AOPP), ferric reducing antioxidant power (FRAP), thiolic groups, thereby confirming their potential role as migraine biomarkers (198).

Inflammatory biomarkers such as **CRP, IL-1β, and IL-6** were also investigated in the saliva of migraine and tension-type headache patients. IL-1β had the highest discriminative value between headache patients and controls (199).

Sensory neuropeptides

Several powerful vasodilator peptides are found in cell bodies within the trigeminal neurons that innervate blood vessels, including CGRP, substance P (SP), neurokinin A (NKA) and amylin.

SP and NKA were found to be released in the innervated tissues upon noxious stimulation and induce **neurogenic inflammation,** which, once it occurs in the cranial dura matter, was thought to underlie the generation of migraine pain (200,201) There have been very few studies of plasma SP in migraine patients, and these have provided contradictory results. Goadsby et al. reported that plasma CGRP level increased in migraine, but that SP did not change (56). Fusayusu et al. found increased interictal SP (and CGRP) levels in patients with migraine as compared to HC and suggested a role in migraine pathophysiology (75). The role of SP and NKA has not been studied in patients with CM.

Amylin is a 37-amino acid peptide structurally related to CGRP, with vasodilatory and pronociceptive actions (202). A study has shown that interictal amylin levels are elevated in peripheral blood in a series of CM patients, suggesting that this peptide could play a role in migraine chronification (203).

NGF is a neuropeptide responsible for activation and long-lasting sensitization and a key player in the inflammatory process related to the TVS. Higher NGF levels are often found in union with substance P in CSF of migraine patients. Jang et al. found that concentrations of NGF in plasma and saliva were increased in CM patients. NGF is not only a well-known growth factor but, following tissue injury, also an inducer of hyperalgesia via different peripheral mechanisms including mast cell degranulation (162).

Parasympathetic Neuropeptides

VIP is a polypeptide of 28 amino acid residues that belongs to a glucagon/secretin superfamily. Both the large cerebral and cortical pial vessels have a rich VIPergic innervation, which induces a powerful vasodilation in various species, including humans (204). **It seems that VIP is correlated with cranial parasympathetic symptoms** (172,205,206) Besides, VIP seems to be a therapeutic marker of triptan therapy response (64,159) and of BTX-A efficacy in CM patients (78). Contrary to CGRP, VIP levels were in the range of controls in a series of EM patients (56).

Data on PACAP studies has shown opposite results. PACAP-38 is a widely distributed neuropeptide involved in neuroprotection, neurodevelopment, nociception, and inflammation. Moreover, it is a potent inducer of migraine-like attacks (207). Contrary to VIP, which is expressed in sphenopalatine ganglia (208), PACAP is expressed in both the parasympathetic ganglia and in the human trigeminal ganglion (209). For instance, while PACAP levels were seen to be increasing in jugular samples during acute migraine attacks (210), decreased interictal PACAP levels (as compared to non-headache subjects or tension-type headache patients), which normalize during attacks, have been shown in EM patients (211,212) Contrary to CGRP and VIP, interictal serum levels of PACAP have been shown to be in the range of controls in a large series of CM (213). Therefore, serum levels of PACAP, as measured in cubital vein and by ELISA, do not seem to be a useful biomarker to test in this case the activity of the cranial parasympathetic arm of the TVS.

Glial neuropeptides

Trigeminal CGRP release activates satellite glial cells that then release nitric oxide and other proinflammatory cytokines that contribute to sensitization in migraine patients [61]. **S100 beta (S100B) is a calcium-binding protein, produced mostly and released by glial cells in the central nervous system in response to inflammatory stimuli**. Data on S100B serum levels in primary headaches are limited and inconsistent. Interictal S100B levels in episodic and CM patients are in the range of controls (214).

Endothelial dysfunction

Pentraxin 3 (PTX3) is a member of the long pentraxin family that **acts as an acute phase inflammatory glycoprotein.** Different studies have demonstrated higher plasma levels of PTX3 in migraine patients during attacks when compared to interictal periods (215) or HC (216) as well as higher plasma levels in CM patients interictally (217).

Plasma levels of soluble **tumor necrosis factor-like weak inducer of apoptosis (sTWEAK)** have been analyzed as potential biomarkers of cardiovascular disease and endothelial dysfunction in vascular and non-vascular diseases. It has been demonstrated higher levels of PTX3 and sTWEAK in patients with severe periodontitis and CM (218), higher plasma levels of sTWEAK in CM patients and that high plasma levels of PTX3 can predict a good response to BTX-A (79).

Other markers

Glutamate is the most abundant excitatory neurotransmitter in the CNS, and as such has been **implicated in aspects of migraine pathogenesis including CSD, trigeminal neuron activation, and central sensitization.** Interestingly, the elevation of glutamate level has been described in plasma, CSF, and saliva samples from migraine patients during the ictal and interictal periods. Salivary glutamate levels could be an indicator of CM (219); blood glutamate levels are elevated in migraine patients compared to HC (220) and plasma glutamate levels decrease intraindividually after prophylactic treatment with topiramate, amitriptyline, flunarizine, and propranolol (221).

Table 9. Molecular biomarkers which have been studied in migraine

Molecular Biomarkers					
		plasma	CSF	saliva	tears
Inflammatory markers	TNF-α	X	X		
	NLR	X			
	MLR	X			
	PLR	X			
	CAR	X			
	AOPP	X			
	FRAP	X			
	CRP	X			
	IL-1β	X	X		
	IL-6	X			
Sensory neuropeptides	CGRP	X	X	X	X
	SP	X	X		
	NKA	X	X		
	Amylin	X			
	NGF	X	X	X	
Parasympathetic Neuropeptides	VIP	X			
	PACAP	X			
Glial neuropeptides	S100B	X			
Endothelial dysfunction	PTX3	X			
	sTWEAK	X			
Other markers	Glutamate	X	X	X	

1.3.3 Biomarkers in other neurological diseases

The past decade has seen **an explosion in the number of studies to discover and ultimately validate diseases biomarkers** in the human patient population.

Clear examples of reliable and validated biomarkers are the use of troponin as an important biomarker for the diagnosis of acute myocardial infarction; CD4 cell counts as a monitoring biomarker of human immunodeficiency virus (HIV) plasma viral load; International normalized ratio (INR) used to monitor the dose of warfarin anticoagulation; or in the case of cancer patients with HER2 receptor positive assays as a predictive of response biomarker to treatment with herceptin.

Regarding neurological diseases, maybe the best example is **neurodegenerative diseases.** Biofluids, including blood and CSF, have been heavily investigated to identify candidate biomarkers for neurodegenerative diseases. Alzheimer disease (AD) has also investigated saliva (222). Differences in the acceptance of lumbar punctures (LP) have led some investigators to favor using other samples for biomarker studies, being blood the most popular. However, while this is much less invasive for the patient, blood represents a far more complex biofluid that contains proteins and RNAs derived from all tissue types and therefore most biomarkers are CSF-based. Neurodegenerative diseases that have had the greatest advancements in biomarkers are AD, Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) (223). Regarding **AD**, Biomarkers currently validated in AD are those derived from CSF. The three core biomarkers are the Amyloid-β1-42 peptide (Aβ42), total tau, and phospho-tau (p-tau). They are useful in the early diagnosis of AD and prediction of disease progression. They can be measured through standardized methods which result in marked increased inter-site reliability in data collected across multiple centers and reduced the coefficient of variation for each assay. It is worth mentioning that in **PD** dopamine transporter imaging (DATscan) can detect nigrostriatal degeneration and has been shown to have a positive impact on diagnosis of PD and clinical decision-making. DATscan received FDA approval to evaluate patients with suspected PD or Parkinson's syndrome. α-Synuclein remains in development stages. The biomarkers that have received most extensive validation across many labs in **ALS** are the neurofilament proteins. Neurofilament light (NFL) and phosphorylated heavy chain (pNFH) proteins in CSF are useful for diagnosis, prognostic for survival and pharmacodynamic for neuroprotection activity of drug.

Imaging techniques have also improved over the past decade and the use of CNS imaging has impacted the diagnosis and drug development pipelines for multiple neurodegenerative diseases.

Molecular biomarkers in other neurological diseases such as **epilepsy or stroke** are its initial stages and specificity and sensitivity for most biomarkers in most clinical situations are not known. Examples of biochemical markers that have been shown to have higher blood concentrations in study subjects with epilepsy include brain proteins like S100B or neuronal specific enolase, and neuroinflammatory proteins like IL, and TNF-α. Some of the blood biomarkers also seem to reflect seizure duration or frequency, and levels decrease in response to treatment with antiseizure medication. For most biomarkers, the literature contains seemingly conflicting results (224). Regarding **stroke**, for example, there is no reliable biomarker that can detect stroke with a high accuracy compared to troponin in the diagnosis of ischaemic heart disease. Individual biomarkers that have both sensitivity and specificity of more than 50% are S100B, glycogen phosphorylase isoenzyme BB (GPBB), NR2 peptide, matrix metalloproteinase-9 (MMP-9), Apolipoprotein A1 (APOA1), Parkinson disease protein 7 (PARK7), nucleoside diphosphate kinase A (NDKA) and heart-type fatty acid binding protein (H-FABP) (225).

Regarding **multiple sclerosis** (MS), there have been exciting advances with neurofilament light chain (NfL). It could be a good biomarker in predicting MS disease activity and progression. However, NfL levels can be difficult to use when clinically evaluating individual patients, due to many confounding variables, such as age, body mass index, and blood volume. Aditionally, NfL indicates neuronal damage and, thus, is nonspecific to MS. Elevated NfL also does not distinguish between patients with MS and those with minor head trauma, infection, other neurological diseases, or comorbidities, such as diabetes. When patients have a sudden spike in their NfL levels, it is usually indicative of inflammation and active lesions. Hence, increases in NfL levels may be more indicative of neuroinflammation than neurodegeneration in MS (226).

Therefore, clinical use of biomarkers requires continued efforts to validate them and demonstrate their disease specificity.

Table 10 summarizes potential biomarkers in neurological diseases.

Table 10. Molecular biomarkers in neurological diseases

Disease	Biomarker	Use
Alzheimer's disease	CSF: high total tau+ low $A\beta_{42}$/p-tau	Conversion of MCI to AD disease
	CSF: high NFL	Rapid AD progression and cognitive decline
	CSF $A\beta_{42}$	Differential diagnosis of AD from FTD
Parkinson's disease	Ratio of oligomeric to total α-synuclein in CSF	Diagnosis
	Plasma uric acid levels	Risk factor for PD, and prognostic indicator of disease progression
	Serum BDNF	Diagnosis and disease progression; correlation to cognitive impairment
	Serum IGF-1	Predicts progression of motor symptoms and executive function decline in PD patients
	p-Tau and p-Tau/$a\beta_{42}$ ratio	Predicts cognitive and executive function decline in levodopa treated PD patients
Amyotrophic lateral sclerosis	pNFH	Diagnosis, prognostic for survival; pharmacodynamic for neuroprotection activity of drug
	NFL	Diagnosis, prognostic for survival; pharmacodynamic for neuroprotection activity of drug
	Dipeptide repeat proteins (DPRs)	C9orf72 related ALS and ALS-FTD; pharmacodynamic for C9 treatments
	Serum creatinine; serum	Prognostic indicator of survival;

	creatinine/cystatin C ratio	pharmacodynamic biomarker of drug action
	Serum uric acid	Prognostic indicator of survival, with higher uric acid levels predicting longer survival in males
	CSF IL-8	Predicts disease duration
	Blood and CSF MCP-1	Predicts disease duration
Epilepsy	S100B neuronal specific enolase IL TNF-α	Diagnosis
Stroke	S100B GPBB NR2 peptide MMP-9 APOA1 PARK7 NDKA H-FABP	Diagnosis
Multiple Sclerosis	NfL	Predicts disease activity and progression

Adapted from Jeromin and Bowser 2017

2. HYPOTHESIS

Nowadays we acknowledge that CGRP plays a key role in migraine. We have evidence that CGRP is released during migraine attacks and that anti-CGRP therapies are clinically effective both as an acute and preventive treatment.

However, there is a bit of controversy, as “positive” and “negative” studies have been published. Moreover, **little is known on CGRP levels before and after treatment** (both acute and preventive) **or whether if CGRP levels are modified after CGRP therapies at a molecular level.** There are also questions on whether if it is a dynamic neuropeptide and on its behavior during the different migraine phases, and on the reasons of why not all patients respond equally to CGRP therapies. Therefore, more studies are needed in order to solve these.

Bearing in mind that migraine is a dynamic neurological disease with great clinical heterogeneity, **the hypothesis of this doctoral thesis was that CGRP levels change according to the phase of the migraine attack in most patients**. Furthermore, we hypothesized that **CGRP levels vary after treatment with anti-CGRP treatments, in particular with mAbs, and that these levels may also serve as a predictive treatment response biomarker to these target-driven migraine prevention therapies**.

Monitoring CGRP in human saliva could help us define **different migraine patient profiles and provide evidence towards establishing a** pathophysiological-driven classification. On one hand, it would help us choose which type of patient is the most suitable to receive the treatment; and on the other hand, it would help us manage the treatment response expectations. It would bring us closer to the practice of **precision medicine**.

3. OBJECTIVES

The **main objective** of this doctoral thesis is:

- To **monitor the temporal profile of salivary CGRP** longitudinally through the different migraine attack phases

Secondary objectives are:

- To assess salivary **CGRP as a potential diagnostic biomarker** in patients with migraine

- To evaluate salivary **CGRP levels as predictive treatment response biomarker** in patients with migraine treated with anti-CGRP monoclonal antibodies

- To analyze **changes in salivary CGRP levels before and** after treatment with anti-CGRP monoclonal antibodies

- To assess **saliva as a biofluid to measure CGRP** in patients with migraine

4. COMPENDIUM OF PUBLICATIONS

4.1. Article 1

Alpuente A, Gallardo VJ, Asskour L, Caronna E, Torres-Ferrus M, Pozo-Rosich P. **Salivary CGRP can monitor the different migraine phases: CGRP (in)dependent attacks.** Cephalalgia. 2022 Mar;42(3):186-196. doi: 10.1177/03331024211040467. Epub 2021 Oct 4. PMID: 34601944.

First article addresses main and two of the secondary objectives, focused on **monitoring the temporal profile of salivary CGRP** longitudinally through the migraine attack phases. Furthermore, it addresses **the role of CGRP as potential diagnostic biomarker** through the differentiation in its levels between patients and controls.

Methodology applied in both studies was alike.

Both projects received an Ethics Committee approval and all participants signed an informed consent.

All patients with migraine came from our Outpatient Clinic and therefore, they had a confirmed migraine diagnosis. Healthy control participants were carefully interviewed by a neurologist, discarding reasonably other headache conditions, as well as a personal or family history of migraine, and they were age- and sex-matched.

Saliva collection method was thoughtfully explained, in-person, to all participants. Additionally, all participants were given detailed verbal and written instructions for saliva collection.

Material used for saliva collection and kits used for CGRP extraction were alike.

In both projects, CGRP measurement was performed by the same person, trying to maintain the same conditions when it was possible to control them, and statistical analysis was performed by the same data analyst.

All patients included in the second study received erenumab 140 mg.

Original Article

Cephalalgia
An International Journal of Headache
International Headache Society

Cephalalgia
0(0) 1–11

DOI: 10.1177/03331024211040467
journals.sagepub.com/home/cep
SAGE

Salivary CGRP can monitor the different migraine phases: CGRP (in)dependent attacks

Alicia Alpuente[1,2], Victor J Gallardo[2], Laila Asskour[2], Edoardo Caronna[1,2], Marta Torres-Ferrus[1,2] and Patricia Pozo-Rosich[1,2]

Abstract

Background: CGRP plays a key role in the transmission and modulation of nociceptive signals and is a critical component in the pathogenesis of migraine.

Objective: To assess saliva as a substrate to measure CGRP by comparing interictal levels in patients with episodic migraine and controls; and to evaluate CGRP's temporal profile during migraine attacks.

Methods: This prospective observational pilot study included young women with episodic migraine and healthy controls. We monitored salivary CGRP-like immunoreactivity (CGRP-LI) during 30 consecutive days and during migraine attacks. We considered six timepoints for the analysis: interictal (72h headache free), preictal (PRE-24h before the attack), ictal (headache onset, after 2h, after 8h), postictal (POST-24h after the attack). CGRP levels were quantified by ELISA.

Results: 44 women (22 with episodic migraine, 22 healthy controls) were recruited. Differences in interictal salivary levels of CGRP between patients and controls (Me [IQR]: 98.0 [80.3] (95% CI 56.6, 124.0) vs. 54.3 [44.0] (95% CI 42.2, 70.1) pg/mL, $p = 0.034$) were found. An increase in CGRP levels during migraine attacks was detected (pre:169.0 [95% CI 104.2–234.0]; headache onset: 247.0 [181.9–312.0]; after 2h: 143.0 [77.6–208.0]; after 8h: 169.0 [103.5–234.0], post: 173.0 [107.8–238.0]). Patients were classified as having CGRP-dependent (79.6%) and non-CGRP dependent migraine attacks (20.4%) according to the magnitude of change between preictal and ictal phase. Accompanying symptoms such as photophobia and phonophobia were significantly associated to the first group.

Conclusions: Salivary CGRP-LI levels, which interictally are elevated in episodic migraine patients, usually increase during a migraine attack in the majority of patients. However, not every attack is CGRP-dependent, which in turn, might explain different underlying pathophysiology and response to treatment.

Keywords

Migraine, migraine phases, ELISA, CGRP, saliva

Date received: 31 March 2021; revised: 26 July 2021; accepted: 28 July 2021

Introduction

Migraine is a highly prevalent and a disabling neurological disease (1) and, so far, specific biomarkers have yet not been validated, which reflects its complexity and dynamic character (2–4). However, the most studied molecule is the calcitonin gene related peptide (CGRP), which has a clear implication in migraine pathophysiology (5,6), underpinned by the arrival of effective therapies targeting CGRP pathways (7). CGRP is implicated in the development of neurogenic

[1]Headache Unit, Neurology Department, Vall d'Hebron University Hospital, Barcelona, Spain.
[2]Headache and Neurological Pain Research Group, Vall d'Hebron Research Institute, Departament de Medicina, Universitat Autònoma de Barcelona, Barcelona, Spain.

Corresponding author:
Patricia Pozo-Rosich, 119-129 Passeig de la Vall d'Hebron, Barcelona, 08035, Spain.
Email: ppozo@vhebron.net

inflammation and it is upregulated in conditions of inflammatory and neuropathic pain (8,9), and it has been hypothesized as a potential diagnostic (10–12) and treatment predictive biomarker in migraine (13–16).

Saliva as a substrate to study biomarkers is a worthwhile approach because its collection is non-invasive and it allows the monitoring of neuropeptides because samples can be repeatedly obtained from subjects (17). The feasibility of CGRP detection in human saliva has been previously demonstrated and used as a marker of trigeminovascular activation in migraine (10,11,14,18–20).

Variability between CGRP levels across the studies is probably due to methodological differences such as type of matrix, collection techniques, type of assay, time of sampling and heterogeneous study population. Standardized procedures in collection and analysis are mandatory in order to be able to use them as a valuable resource to gain scientific information in the migraine field. Therefore, the objectives of this study were: a) to compare interictal salivary CGRP levels between episodic migraine (EM) patients and healthy controls (HC) and, b) to assess the temporal profile of salivary CGRP during migraine attacks.

Methods

Participants and study design

This is a prospective longitudinal pilot study. Patients were recruited from the outpatient headache clinic and carefully interviewed by a headache specialist. Recruitment period was from March 2018 to November 2019.

Patients were women between 18–65 years old fulfilling the criteria for migraine with or without aura, according to the International Classification of Headache Disorders (ICHD-3) (21). Specifically, they had to report between 1–6 migraine days per month (d/mo). Participants with a smoking habit; medical diagnosis of anxiety/depression; medical diagnosis of chronic pain disorders; subjects taking medication affecting central nervous system; subjects taking migraine preventive medication in the past year prior to the study or history of any medical condition that could alter saliva content were excluded. Healthy controls (HC) were age-matched women with no personal or family history of migraine or headache, excluding sporadic tension-type headache, recruited from residency training program. At the screening visit, demographical and clinical data were collected.

All participants were given detailed verbal and written instructions for saliva collection. They were provided with appropriate material for saliva collection at home including pre-labelled tubes; diaries to register sample collection time and menstrual cycle; questionnaires to record migraine attack characteristics such as pain intensity and duration, accompanying symptoms and acute treatment used. During the study, subjects treated their migraine attacks as usual with an approval of the investigator at the initial visit (triptans and non-steroidal anti-inflammatory drugs were allowed). No migraine attack was treated before collecting the first sample of each attack.

Saliva collection

HC and EM patients collected saliva samples consecutively during a 30-day period (baseline samples). EM patients collected three extra saliva samples during the migraine attack (painful condition samples): headache onset, after 2h and after 8h.

Saliva collection was carried out with the resting unstimulated whole saliva method (17,19) using the following step-by-step indications:

- To collect saliva at the same time of the day, early in the morning, fasting condition.
- Not to eat, drink or brush their teeth before collection.
- To rinse their mouth with water, discarding initial saliva in order to avoid contaminated saliva with debris.
- To collect the fluid by spitting into a sterile tube of 5 mL of polypropylene material for 5 minutes with a minimum quantity of 3 mL. Each tube was used once only.
- Not to use citric acid since it could degrade CGRP.

After collection, all baseline saliva samples were kept in the freezer of participants at −18°C degrees. At the end of the study, the samples were carried to the laboratory on ice in order to avoid thawing. All samples were stored in the laboratory freezer at −80°C degrees.

Plasma collection

Plasma samples were also collected from each participant on day 1 in order to make a saliva-plasma correlation. It was noted if it was a free pain period (outside of an attack). Samples were collected from the subject's antecubital vein between 8–10 am and transferred to EDTA coated 10.8 mg tubes (BD Vacutainer System, K2E). All samples were centrifuged at 3500 rpm at 4°C for 15 min and supernatants were immediately stored at –80°C.

CGRP extraction

Saliva samples were thawed slowly at room temperature and then placed on ice. The samples were

centrifuged for 20 minutes at 3500 rpm −4°C, and the supernatant was aliquoted into 1.5mL centrifuge sterile and polypropylene Eppendorf tubes and stored at −80°C or immediately analyzed. Prior to Enzyme-linked Immunosorbent Assay (ELISA), samples were thawed slowly and then centrifuged for 5 minutes at 3500 rpm at room temperature to pellet cellular debris. −80°C storage helped avoid protein degradation and denaturalization. Moreover, to minimize degradation of unstable antigens, samples were kept in ice, and, repeated freeze-thaw cycles were avoided.

Assay protocol

Quantitative determination of plasma and salivary CGRP was measured by using human enzyme linked immunosorbent assay (ELISA) kits (Cusabio, detection range: 1.56–100 pg/ml, minimal detectable dose: 0.39 pg/ml). The assay was performed according to specification of the manufacturer. Intra-assay precision and inter-assay precision is declared with a coefficient of variation (CV) of <8%, respectively <10%. Duplicate measurements were performed for each sample. CGRP concentrations were determined from calibration curves using a 4PL fitting as implemented in Analysis software Gen 5 resulting in a fit with $R2 > 0.99$ in every case. The final CGRP level of each sample was calculated as the average of the two measurements. An internal validation of the test was performed, ensuring that the ELISA assay used was reliable (quality control was included in the kit). CGRP concentration from immunoassay procedure was corrected by inter and intra-assay coefficients of variability for each ELISA plate.

Regarding saliva samples we found important: to dilute samples 1:10 with sample dilutent; to homogenize the sample with the dilutent (repeated up-down cycles) and to discard blood contaminated samples. In plasma samples, it is important to discard lipemic and hemolyzed samples in order to reduce the effect on the ELISA results. Those samples were not diluted due to the low concentration of CGRP in plasma.

In order to analyze CGRP levels over the different migraine phases, we considered six timepoints: interictal (median value of five consecutive days, for migraine patients when they were headache-free for 72 hours), preictal (pre-24h before the migraine attack), ictal (headache onset, after 2h, after 8h) and postictal (post-24h after the migraine attack).

Statistical analysis

Nominal (categorical) variables were reported as frequencies (percentages) while mean ± standard deviation (age, disease evolution time, Headache Impact Test (HIT-6) and perceived stress scale [PSS]) or median and interquartile range (Me [IQR]) (Migraine Disability Assessment (MIDAS), Hospital Anxiety and Depression Scale [HAD]) and CGRP levels) were reported for continuous variables. For CGRP levels we also report 95% Confidence Interval (CI). Normality assumption of quantitative variables was checked through visual methods (Q-Q plots) and normality tests (Shapiro-Wilk test).

Statistical significance for intergroup variables was assessed by Pearson's chi-square when comparing categorical variables. In the case of having an expected count less than 5 in more than 20% of cells in the contingency table, Fisher's exact test was used. Linear trend chi-square was considered for ordinal variables. Independent t-test for continuous variables that followed a normal distribution (age and PSS) was used in order to assess differences between migraine patients and healthy controls and, Mann-Whitney U test was used for the rest variables that did not follow any normality assumption (HAD Scales and CGRP basal levels). The degree of association between interictal CGRP levels and clinical variables was computed by Spearman's rank correlation and summarized by Spearman's rho coefficient and related p-values.

We estimated the ictal log2Fold-Change (log_2FC_{ictal}) (Eq. 1) in patients with the aim of finding out whether all migraine attacks reflected an incremental change at salivary CGRP level between preictal phase (pre-24h) and ictal phase (headache onset). Hence, migraine attacks were classified into CGRP dependent (dCGRP) ($log_2FC_{ictal} > 0$) or non-CGRP dependent (nCGRP) ($log_2FC_{ictal} \leq 0$).

$$log_2FC_{ictal} = log_2[CGRP]_{attack\ onset} - log_2[CGRP]_{premonitory}$$

Equation 1. Ictal Fold-Change: Measure concept for the evaluation of the change of the CGRP salivary level between premonitory and the attack onset phases.

Finally, to determine the significance of CGRP intraindividual changes according to patient's migraine phase, we used non-linear mixed effects modeling fit by restricted maximum likelihood. Mixed models are an extension of simple models to allow both fixed and random effects, and are used when there is no independence in the data (e.g. different CGRP measurements from the same patient). Variance inflation factors (VIFs) for all the parameters were computed in order to estimate how much the variance of an estimated regression coefficient is inflated due to correlated variables so that we could avoid an overfitting problem in the model. The non-linear mixed effect models were generated using the lme function in the R package

nlme (22), with CGRP levels as a dependent variable, migraine cycle (time), age and stress score (PSS) as a fixed factors and patients as a random factor. To model the non-linear relationship between CGRP over the course of migraine attack, we added a cubic trend component for time. Post-hoc pairwise comparisons of estimated marginal means of CGRP was conducted between each subsequent time point (interictal, preictal, headache onset, after 2h, after 8h and postictal), adjusting for multiple comparisons using the false discovery rate (FDR) correction. Univariate linear mixed models, were used to test the interaction between time and dichotomized basal frequency (<5 d/mo or 6–10 d/mo) on the CGRP levels and treated attacks (None, NSAIDs or Triptans), again covarying for age and PSS. A statistical power calculation was not conducted prior to the study because the sample size was based on the available data for this exploratory analysis. However, effect size for each statistical test is reported. No adjustment for multiple comparisons was made to the statistical clinical inferences, but exact p-values were reported to allow post adjustments. P-values presented are for a two-tailed test and p-values <0.05 were considered statistically significant.

All statistical analysis were conducted in R v3.6.3 (23) and figures were produced using the package ggplot2 (24).

Approval for this study was obtained from the Vall d'Hebron Ethics Committee (PR(IR)292/2017). All participants gave their consent for data collection.

Results

Descriptive

In total, 22 EM and 22 HC with a mean age of 30.8 ± 10.2 years old were included (Table 1). EM patients had statistically significant higher proportion of perceived stress (95.5% vs. 68.2%; p = 0.046). Twelve patients (54.5%) reported headache frequency of ≤5 days/month and ten patients (45.4%) reported headache frequency of 6-10 days/month.

Interictal CGRP levels

Following results are expressed as CGRP-like immunoreactivity (CGRP-LI) due to the cross-reactivity. We found statistically significant higher interictal CGRP-LI salivary levels in EM compared to HC: EM, 98.0 [80.3] (95% CI 56.6, 124.0) vs. HC, 54.3 [44.0] (95% CI 42.2, 70.1) pg/mL (p = 0.034, Wilcoxon effect size r = 0.420) (Figure 1A). We did not find any correlation between CGRP-LI levels in regards to: age ($\rho = 0.192$, p = 0.493), HADS-A ($\rho = 0.190$, p = 0.217), HADS-D ($\rho = 0.259$, p = 0.162), or PSS score ($\rho = 0.202$,

Table 1. Baseline demographics, clinical characteristics and comorbidities of participants.

	HC (n = 22)	EM (n = 22)	p-value
Demographics and lifestyle			
Age, mean (SD), years old	31.2 (11.1)	30.4 (9.4)	0.805[a]
Physical Activity, n (%)			
Low	7 (32.8%)	6 (27.3%)	1.000[b]
Medium	11 (50.0%)	12 (54.5%)	
High	5 (18.2%)	4 (18.2%)	
Migraine characteristics			
Basal Headache Frequency, n (%)			
≤5 d/mo		12 (54.5%)	
6–10 d/mo		10 (45.5%)	
Evolution time, mean (SD), years		13.4 (11.0)	
Aura, n (%)		8 (36.4%)	
MIDAS, median [IQR]		20.0 [49.8]	
HIT-6, mean (SD)		63.7 (5.9)	
Comorbidities			
Anxiety (HADS ≥ 8), n (%)	8 (36.4%)	12 (54.5%)	0.364[c]
Depression, (HADS ≥ 8), n (%)	1 (4.5%)	4 (18.2%)	0.354[c]
Perceived stress (PSS ≥14), n (%)	15 (68.2%)	21 (95.5%)	0.046*[,c]

HC: Healthy Control; EM: Episodic Migraine; SD: Standard Deviation; IQR: Interquartile Range; HADS: Hospital Anxiety and Depression Scale; PSS: Perceived Stress Scale; d/mo: days/month.
[a]Independent T-test.
[b]Linear trend chi-squared.
[c]Fisher's exact test.
*p-value ≤0.05.

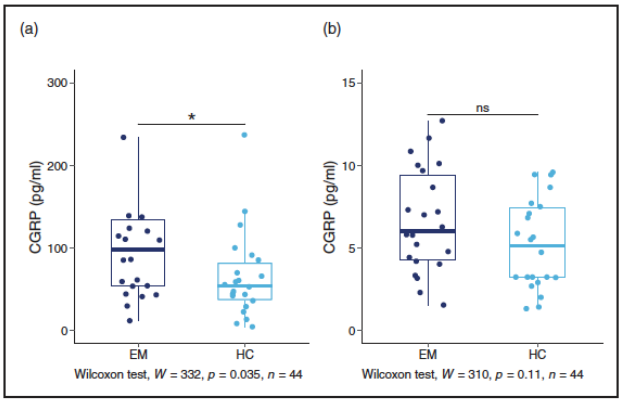

Figure 1. Basal salivary levels of CGRP. Box plots and 95% confidence intervals for interictal CGRP levels in saliva (A) and plasma (B). Basal salivary levels of CGRP were calculated as the median value of 5 consecutive random days in healthy controls. In migraine patients, interictal salivary CGRP levels were calculated as the median value of 5 consecutive days when they were headache-free for 72 hours (interictal period). Plasma CGRP values were extracted during the screening, patients did not report a migraine attack during plasma blood extraction.
*p value ≤0.05.

p = 0.189) in all participants. In regards to plasma CGRP-LI levels, we did not find statistically significant differences in this substrate between study groups (EM, 6.0[5.2] (95% CI 4.5, 8.4) vs. HC, 5.1[4.2] (95% CI 3.2, 7.1) pg/mL; p = 0.113, Wilcoxon effect size r = 0.241) (Figure 1B). There was not a correlation between salivary and plasma CGRP-LI levels.

Longitudinal analysis: salivary CGRP levels through migraine attack

A total of 49 migraine attacks were collected. We analyzed salivary CGRP-LI at each timepoint of the attack. We found that age and cubic trend of time (migraine cycle) drove statistically significantly changes on CGRP-LI concentration (Table 2). Post-hoc analysis showed statistically significantly higher concentration of CGRP-LI during headache onset. Estimated marginal means and 95% CI from the mixed-effect model were: 169.0 [95% CI 104.2–234.0] in the preictal; 247.0 [95% CI 181.9–312.0] during headache onset; 143.0 [95% CI 77.6–208.0] after 2h; 169.0 [95% CI 103.5–234.0] after 8h and 173.0 [95% CI 107.8–238.0] in the postictal (Figure 2).

Furthermore, we analyzed the possible interaction between time (migraine phase effect) and basal headache frequency, but this interaction did not reach the level of statistical significance (Table 2, p = 0.088). However, patients with higher basal frequency (>6–10 d/mo) presented a statistically significantly higher concentrations of CGRP in all time points (Table 2, Figure 2A). In regards to acute treatment, we also found no statistically significant interaction between migraine cycle and medicated attacks with neither non-steroidal anti-inflammatory drugs (NSAID) nor triptans (Table 2). However, post-hoc analysis revealed that attacks treated with triptans presented a statistically significantly reduction on CGRP after 2 h from headache onset (Figure 3B).

Subtypes of migraine patients

We found that 79.6% (39/49) of migraine attacks were CGRP dependent (dCGRP) and 20.4% (10/49) of migraine attacks were non-CGRP dependent (nCGRP). CGRP dependent group presented a statistically significant higher levels of CGRP-LI: dCGRP, 171.6 [130.8] (95% CI 160.0, 235.0) vs. nCGRP, 101.7 [153.9] (95% CI 39.8, 155.0); p = 0.009). In regards to accompanying symptoms, a statistically significant association between photophobia (dCGRP: 76.9% vs. nCGRP: 40.0%; p = 0.024) and phonophobia (dCGRP: 69.2% vs. nCGRP: 30.0%; p = 0.036) was found in dCGRP group. Dizziness (dCGRP: 30.8% vs. nCGRP: 70.0%; p = 0.047) was statistically significantly associated with nCGRP group (Table 3). When we analyzed migraine patients, 13 out of 22 patients only showed dCGRP migraine attacks; 3 out of 22 patients only showed nCGRP migraine attacks and 6 patients showed both types of migraine attacks.

Table 2. Results from the linear mixed effects model.

Fixed effects	Estimate	Standard Error	df	t-value	p-value
Overall CGRP changes					
Age	8.7695	3.0168	49	2.9069	0.0061**
PSS	−2.3417	3.0258	49	−0.7739	0.4439
Time (Cubic trend)	5.0403	2.1874	260	2.3042	0.0223*
Interaction of headache frequency and migraine cycle over CGRP changes					
Age	9.2867	2.9053	48	3.1964	0.0029**
PSS	−3.8710	2.9991	48	−1.2907	0.2050
Frequency (6–10d/mo)	152.4676	63.5269	48	2.4000	0.0217*
Time (Cubic trend)	−1.1222	0.5460	258	−2.0552	0.0312*
Time: Headache Frequency	1.2631	0.7362	258	1.7154	0.0878
Interaction of treated attacks and migraine cycle over CGRP changes					
Age	8.2983	3.3211	47	2.4986	0.0173*
PSS	−2.3424	3.1075	47	−0.7538	0.4560
Treatment (Triptans)	4.6847	79.5124	47	0.0589	0.9534
Treatment (NSAIDs)	−24.941	84.728	47	−0.2944	0.7702
Time (Cubic trend)	8.1491	3.4722	253	2.3479	0.0200*
Time: Triptans	−4.1030	5.0828	253	−0.8072	0.4205
Time: NSAIDs	−6.6753	5.5988	253	−1.1922	0.2346

*p-value ≤0.05.
**p-value ≤0.01.

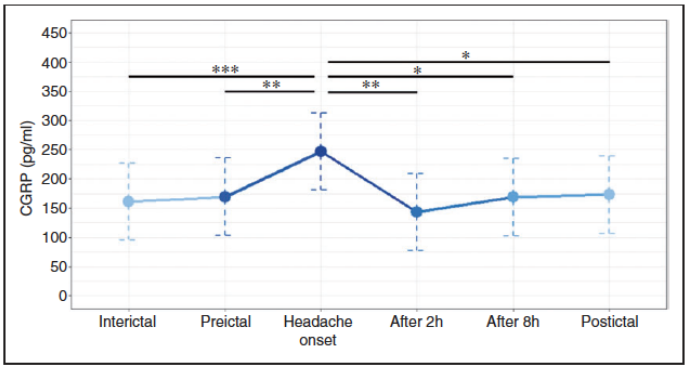

Figure 2. CGRP values changes through the different migraine phases.
CGRP levels (pg/ml) plotted across clinical assessment time points. Values are estimated marginal means from the linear mixed model with fixed effect of time (representing migraine phases), age and basal PSS score. Error bars represent 95% confidence intervals. Pairwise comparisons between time points with significant differences in mean CGRP level are shown with adjusted p-values (FDR correction).
*p value ≤0.05.
**p value ≤0.01.
***p value ≤0.001.

Regarding time of collection (circadian variations), we did not find statistical differences between two patterns: 69.4% of registered migraine attacks (34/49) occurred during afternoon (1–11PM): GCRP-dependent (30.8% AM and 69.2 PM) vs. non CGRP dependent (30.0% AM and 70.0% PM) (p = 0.962).

Discussion

In this study we performed an ELISA assay to measure and monitor salivary levels of CGRP in episodic and treatment naïve patients over the different migraine phases. With this longitudinal approach over 30 days we wanted to properly identify not only the onset, but

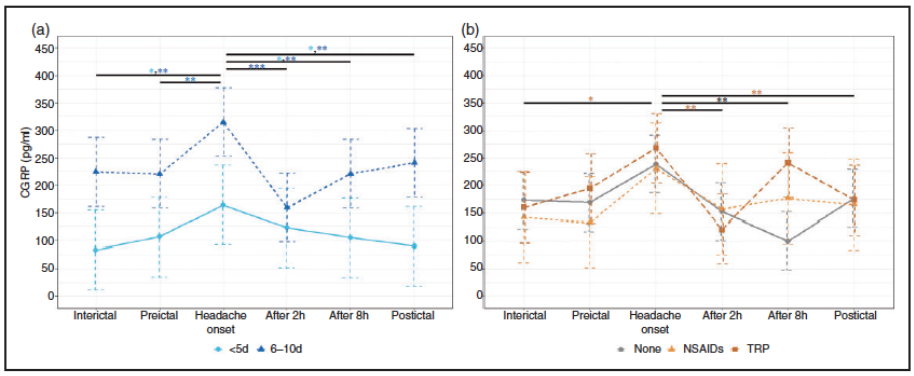

Figure 3. Changes in CGRP levels in the different migraine phases amongst (A) basal headache frequency or (B) acute treatment for migraine attacks.
CGRP levels are estimated marginal means from the linear mixed model with fixed effects of time, age and PSS score. Separate linear mixed models, with a repeated effect of time, were used to test the interaction of (A) dichotomized basal frequency (<5d/mo or 6–10d/mo) on the CGRP levels and (B) treated attacks (None, NSAIDs or Triptans), again covarying for age and PSS. Error bars represent 95% confidence intervals. Pairwise comparisons between time points with significant differences in mean CGRP level are shown with adjusted p-values (FDR correction).
*p value ≤0.05.
**p value ≤0.01.
***p value ≤0.001.

Table 3. Clinical and molecular parameters associated with CGRP dependent and non-CGRP dependent migraine attacks.

	Non-CGRP dependent (n = 10)	CGRP dependent (n = 39)	p-value
CGRP (Interictal)[a], median [IQR]	116.9 [337.6]	110.7 [79.8]	0.990
CGRP (Onset)[a], median [IQR]	101.7 [153.9]	171.6 [130.8]	0.009*
Treated attack[b], n (%)	6 (60.0%)	26 (66.7%)	0.721
Aura[b], n (%)	2 (20.0%)	6 (15.4%)	0.659
Unilateral Pain[b], n (%)	3 (30.0%)	10 (25.6%)	1.000
Nausea[b], n (%)	6 (60.0%)	23 (59.0%)	1.000
Dizziness[b], n (%)	7 (70.0%)	12 (30.8%)	0.047*
Vomiting[b], n (%)	0 (0.0%)	4 (10.3%)	0.569
Photophobia[b], n (%)	4 (40.0%)	30 (76.9%)	0.024*
Phonophobia[b], n (%)	3 (30.0%)	27 (69.2%)	0.036*
Allodynia[b], n (%)	8 (80.0%)	19 (48.7%)	0.076

[a]Mann-Whitney U test.
[b]Fisher's exact test.
*p-value ≤0.05.

also what happened right before and after the attacks, and also confirm that reliable interictal levels could differentiate patients from controls. Then, EM patients showed higher interictal CGRP levels compared to HC. Intraindividual CGRP levels can change over the course of a migraine attack. Moreover, EM patients with CGRP dependent attacks presented with classical migraine clinical symptoms, creating different phenotypes of migraine patients.

Our results show that interictal salivary levels of CGRP are significantly higher in patients with EM, even if they suffer from infrequent attacks, compared to HC. Bellamy et al. also found that patients with EM had elevated salivary levels of CGRP outside the attacks compared to controls (14). There is only one previous study that found higher CGRP levels in HC (10). Different results across the studies maybe lie in methodological aspects, in particular in the sample

collection time, since migraine is a cyclic disease. Other than saliva, previous studies using other substrates such as plasma (25,26) or tear fluid (27) also showed higher levels of CGRP outside the attacks in patients with EM. So, our finding endorses the presence of elevated CGRP interictal salivary levels in migraine patients. Moreover, we found an association between larger variations in CGRP concentration and higher headache frequency, which may support a greater activation of the trigeminovascular system in more severe forms of the disease (28,29). In this regard, Cernuda-Morollón et al. found that chronic migraine (CM) patients exhibited the highest levels of CGRP, followed by EM patients and HC (30). In contrast, Lee et al. did not find any significant differences between groups (31). In cerebrospinal fluid (CSF), there are 3 studies that found increased levels of CGRP in CM (32–34). There is no data on CGRP in CSF from EM patients.

As a cycling brain disorder, monitoring migraine over its different phases is important in order to understand the underlying mechanisms and pathogenesis (35) as it has previously been demonstrated in neuroimaging studies (36). At a molecular level, our study reveals that salivary levels of CGRP are dynamic and change over a migraine attack according to the timepoint analyzed: they were shown to increase as headache progressed from preictal to the ictal phase and decreased in the postictal phase at or below interictal levels. Our results are the first ones to see this gradual change of CGRP levels during an attack, and confirm previous studies which also showed an increase in salivary levels of CGRP during the ictal phase, interpreting it as a sign of trigeminovascular activation (10,15). This change reflects that CGRP is a dynamic neuropeptide in a disease that is not static; and, considering intraindividual change and not mean population CGRP levels as a baseline to start working on perhaps developing CGRP as a practical clinical biomarker.

Based on our fold-change (FC) analysis, we observed three different types of patients: those with CGRP dependent migraine attacks, those with non-CGRP dependent migraine attacks and those with two types of migraine attacks. The highest pain intensity peak occurred at the onset of headache in most migraine attacks. Some diagnostic migraine symptoms such as photo- and phonophobia were significantly related to presence of elevated CGRP. This could be interesting both to understand the link between CGRP and migraine symptoms (37); which could also help, if this is confirmed with larger cohorts in the future, to create an algorithm to clinically predict anti-CGRP treatment response according to the percentage of attacks with different symptoms. In this sense, it is worth mentioning the similarity found between the percentage of patients nCGRP dependent and those patients who are non-responders in anti-CGRP monoclonal antibodies clinical trials (38) or the percentage of patients who do not develop a migraine attack after provocation (39). Migraine diagnosis is currently based on clinical criteria according to the ICHD-3, which may result in misdiagnosis due to the recall bias. Then, our results may support the concept of classifying migraine from a pathophysiological point of view. This information might help us to start practicing precision medicine in migraine.

During the past decades, saliva has received growing attention as a substrate to study biomarkers in chronic pain disorders (40). Measuring CGRP closer to the afferents seems to be more effective than in plasma, easier and less-invasive, and is a reliable reflection of trigeminovascular activation (41). We have found that CGRP levels were higher in saliva than in plasma and there were no significant differences in CGRP plasma levels between EM and HC. Hence, serum perhaps is not the ideal matrix to measure CGRP levels since neuropeptides are circulating in low concentrations. Salivary glands are innervated by the third branch of the trigeminal nerve and therefore are closer to the trigeminovascular system. These CGRP-containing trigeminal nerves release this neuropeptide in conditions such as migraine and cluster headache (10,42). As in previous studies, we did not find a correlation between CGRP salivary and plasma levels (10,14,15).

Since the first demonstration of an increase in CGRP levels in the external jugular venous blood during a migraine attack (5), researchers have been seeking the most adequate technique to measure CGRP levels and, controversial results have been published possibly due to methodological differences (41,43). Differences lie in the type of matrix used (plasma, serum, saliva, CRF, tears), different type of collection methods, different immunoassays or brands, different timepoints of sample collection or phenotypically heterogeneous patients. It is worth mentioning that negative studies cannot be taken as evidence for the lack of importance of CGRP in migraine pathophysiology, as CGRP analysis can be challenging. When measuring CGRP there are several factors to take into account: age, gender, menstruation, fasting or circadian variation (44). However, with the approval of new therapies targeting CGRP or its receptor (45–47), the role of CGRP in migraine pathophysiology has been consolidated (48–50).

The study has some limitations. First, the small size of the sample. Some patients only collected one migraine attack so we need to be cautious when classifying patients into different types. However, we really wanted to include episodic migraine patients, in order to clearly differentiate the attack from the interictal period. Secondly, most of the migraine attacks were

treated. Thus, the majority of our CGRP levels were measured in medicated migraine attacks, proving that differences can be found even if we treat patients. Finally, if patients had a migraine attack far from home, they placed ictal samples in a fridge without temperature control which in turn could reduce the saliva quality; moreover, commercial freezers can vary slightly their freezer temperature between −18 and −20°C. However, this shows that in these conditions CGRP can be found in saliva.

Our study has a longitudinal approach collecting samples over a 30-day period, which in migraine is less frequent. Moreover, our study participants were strictly and carefully selected, resulting in a very homogeneous sample. Future research should be focused on finding a molecular, anatomical, genetical and physiological way of defining migraine which in turn could help to develop a pathophysiological driven classification.

Conclusions

In conclusion, our data confirm that CGRP levels vary according to the migraine phase; and, finds CGRP dependent and non-CGRP dependent migraine attacks. In the future, the CGRP migraine patient profile might allow clinicians to better phenotype patients, may help predict response to treatment and increases our understanding of migraine pathophysiology.

Article highlights

- CGRP varies intraindividually according to the migraine phase
- Patients with migraine can have CGRP dependent and/or non-CGRP dependent attacks, which is correlated with differences in clinical symptoms

Author contributions

AA, VJG, MTF and PPR made substantial contributions to the conception and design of the study. LA made substantial contribution to the methodology and she performed the ELISA protocol. AA wrote first draft of the paper. VJG performed statistical analysis. All authors participated in acquisition, analysis and/or interpretation of data. All authors have critically revised and finally approved the version to be published.

Acknowledgement

The authors thank the migraine patients who carefully followed all of our instructions for 30 days and did not take any acute treatment until the first sample of each migraine attack was collected.

Declaration of conflicting interests

The authors declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: AA has received honoraria as speaker for Abbie-Allergan and for education for Novartis and Eli Lilly. VJG and LA have nothing to disclose. EC has received honoraria as speaker for Novartis. MTF has received honoraria as a speaker for Abbie-Allergan, Chiesi, Eli Lilly and Novartis. PP-R has received honoraria as a consultant and speaker for Allergan, Almirall, Biohaven, Chiesi, Eli Lilly, Medscape, Neurodiem, Novartis and Teva. Her research group has received research grants from Allergan, AGAUR, la Caixa foundation, Migraine Research Foundation, Instituto Investigación Carlos III, MICINN, PERIS; and has received funding for clinical trials from Alder, Electrocore, Eli Lilly, Novartis and Teva. She is a trustee member of the board of the International Headache Society and a member of the Council of the European Headache Federation. She is in the editorial board of Revista de Neurologia. She is an associate editor for Cephalalgia, Frontiers of Neurology and Journal of Headache and Pain. She is a member of the Clinical Trials Guidelines Committee of the International Headache Society. She has edited the Guidelines for the Diagnosis and Treatment of Headache of the Spanish Neurological Society. She is the founder of www.midolordecabeza.org. PPR does not own stocks from any pharmaceutical company. In relation with this paper the authors have nothing to disclose.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was funded by a grant from Instituto de Salud Carlos III (ISCIII - PI16/01525) and Alicia Alpuente has been partially financed by a grant from the Headache Study Group of the Spanish Neurological Society 2018.

ORCID iDs

Alicia Alpuente https://orcid.org/0000-0001-5296-9401
Victor J Gallardo https://orcid.org/0000-0002-3042-2007
Edoardo Caronna https://orcid.org/0000-0001-5525-0267

References

1. GBD 2016 Headache Collaborators. Global, regional, and national burden of migraine and tension-type headache, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet Neurol* 2018; 17: 954–76.

2. Durham P and Papapetropoulos S. Biomarkers associated with migraine and their potential role in migraine management. *Headache* 2013; 53: 1262–77.
3. Ferroni P, Barbanti P, Spila A, et al. Circulating biomarkers in migraine. new opportunities for precision medicine. *Curr Med Chem* 2018; 26: 6191–6206.
4. Frederiksen SD. Promote biomarker discovery by identifying homogenous primary headache subgroups. *Headache* 2019; 59: 797–801.
5. Goadsby PJ, Edvinsson L and Ekman R. Vasoactive peptide release in the extracerebral circulation of humans during migraine headache. *Ann Neurol* 1990; 28: 183–7.
6. Edvinsson L, Haanes KA, Warfvinge K, et al. CGRP as the target of new migraine therapies – Successful translation from bench to clinic. *Nat Rev Neurol* 2018; 14: 338–50.
7. Charles A and Pozo-Rosich P. Targeting calcitonin gene-related peptide: a new era in migraine therapy. *Lancet* 2019; 394: 1765–74.
8. Iyengar S, Ossipov MH and Johnson KW. The role of calcitonin gene-related peptide in peripheral and central pain mechanisms including migraine. *Pain* 2017; 158: 543–559.
9. Edvinsson L, Haanes KA and Warfvinge K. Does inflammation have a role in migraine? *Nat Rev Neurol* 2019; 15: 483–490.
10. Nicolodi M and Bianco E Del. Sensory neuropeptides (substance p, calcitonin gene-related peptide) and vasoactive intestinal polypeptide in human saliva: their pattern in migraine and cluster headache. *Cephalalgia* 1990; 10: 39–50.
11. Jang MU, Park JW, Kho HS, et al. Plasma and saliva levels of nerve growth factor and neuropeptides in chronic migraine patients. *Oral Dis* 2011; 17: 187–93.
12. Nicolodi M. Nostril capsaicin application as a model of trigeminal primary sensory neuronal activation. *Cephalalgia* 1990; 14: 134–8.
13. Cady R, Turner I, Dexter K, et al. An exploratory study of salivary calcitonin gene-related peptide levels relative to acute interventions and preventative treatment with onabotulinumtoxinA in chronic migraine. *Headache* 2014; 54: 269–77.
14. Bellamy JL, Cady RK and Durham PL. Salivary levels of CGRP and VIP in rhinosinusitis and migraine patients. *Headache* 2006; 46: 24–33.
15. Cady RK, Vause CV, Ho TW, et al. Elevated saliva calcitonin gene-related peptide levels during acute migraine predict therapeutic response to rizatriptan. *Headache* 2009; 49: 1258–66.
16. Domínguez C, Vieites-Prado A, Pérez-Mato M, et al. CGRP and PTX3 as predictors of efficacy of onabotulinumtoxin type a in chronic migraine: an observational study. *Headache* 2018; 58: 78–87.
17. Jasim H, Carlsson A, Hedenberg-Magnusson B, et al. Saliva as a medium to detect and measure biomarkers related to pain. *Sci Rep* 2018; 8: 1–9.
18. Sicuteri F, Fanciullacci M, Nicolodi M, et al. Substance P theory: a unique focus on the painful and painless phenomena of cluster headache. *Headache* 1990; 30: 69–79.
19. Dawidson I, Blom M, Lundeberg T, et al. Neuropeptides in the saliva of healthy subjects. *Life Sci* 1996; 60: 269–78.
20. Nagano T and Takeyama M. Enhancement of salivary secretion and neuropeptide (substance P, α-calcitonin gene-related peptide) levels in saliva by chronic anethole trithione treatment. *J Pharm Pharmacol* 2001; 53: 1697–702.
21. Headache Classification Committee of the International Headache Society. The International Classification of Headache Disorders, 3rd edition. *Cephalalgia* 2018; 38: 1–211.
22. Pinheiro J, Bates D, DebRoy S, et al. Package 'nlme'. Linear and nonlinear mixed effects models, R package version 3.1-152.
23. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2021. Available at: http://www.R-project.org/ (accessed 2020).
24. Wickham, H. Elegant graphics for data analysis. *Media* 2009; 35: 10–1007.
25. Ashina M, Bendtsen L, Jensen R, et al. Evidence for increased plasma levels of calcitonin gene-related peptide in migraine outside of attacks. *Pain* 2000; 86: 133–8.
26. Fusayasu E, Kowa H, Takeshima T, et al. Increased plasma substance P and CGRP levels, and high ACE activity in migraineurs during headache-free periods. *Pain* 2007; 128: 209–14.
27. Kamm K, Straube A and Ruscheweyh R. Calcitonin gene-related peptide levels in tear fluid are elevated in migraine patients compared to healthy controls. *Cephalalgia* 2019; 39: 1535–43.
28. Seybold VS. The Role of Peptides in Central Sensitization. *Handb Exp Pharmacol* 2009; 451–91.
29. Strassman AM, Raymond SA and Burstein R. Sensitization of meningeal sensory neurons and the origin of headaches. *Nature* 1996; 384: 356–8.
30. Cernuda-Morollón E, Larrosa D, Ramón C, et al. Interictal increase of CGRP levels in peripheral blood as a biomarker for chronic migraine. *Neurology* 2013; 81: 1191–6.
31. Lee MJ, Lee SY, Cho S, et al. Feasibility of serum CGRP measurement as a biomarker of chronic migraine: a critical reappraisal. *J Headache Pain* 2018; 19: 53.
32. Gallai V, Alberti A, Gallai B, et al. Glutamate and nitric oxide pathway in chronic daily headache: Evidence from cerebrospinal fluid. *Cephalalgia* 2003; 23: 166–174
33. Sarchielli P, Alberti A, Floridi A, et al. Levels of nerve growth factor in cerebrospinal fluid of chronic daily headache patients. *Neurology* 2001; 57: 132–134.
34. Sarchielli P, Pini LA, Coppola F, et al. Endocannabinoids in chronic migraine: CSF findings suggest a system failure. *Neuropsychopharmacology* 2007; 32: 1384–1390
35. Dodick DW. A phase-by-phase review of migraine pathophysiology. *Headache* 2018; 58: 4–16.
36. Schulte LH and May A. The migraine generator revisited: Continuous scanning of the migraine cycle over

30 days and three spontaneous attacks. *Brain* 2016; 139: 1987–93.
37. Benarroch EE. CGRP: Sensory neuropeptide with multiple neurologic implications. *Neurology* 2011; 77: 281–7.
38. Broessner G, Reuter U, Bonner JH, et al. The spectrum of response to erenumab in patients with episodic migraine and subgroup analysis of patients achieving ≥50%, ≥75%, and 100% response. *Headache* 2020; 60: 2026–2040.
39. Guo S, Vollesen AL, Olesen J, et al. Premonitory and nonheadache symptoms induced by CGRP and PACAP38 in patients with migraine. *Pain* 2016; 157: 2773–2781.
40. Fischer HP, Eich W and Russell IJ. A possible role for saliva as a diagnostic fluid in patients with chronic pain. *Semin Arthritis Rheum* 1998; 6: 348–59.
41. Edvinsson L, Ekman R and Goadsby PJ. Measurement of vasoactive neuropeptides in biological materials: Problems and pitfalls from 30 years of experience and novel future approaches. *Cephalalgia* 2010; 30: 761–6.
42. Kamm K, Straube A and Ruscheweyh R. Baseline tear fluid CGRP is elevated in active cluster headache patients as long as they have not taken attack abortive medication. *Cephalalgia* 2020; 41: 69–77.
43. Tvedskov JF, Lipka K, Ashina M, et al. No increase of calcitonin gene-related peptide in jugular blood during migraine. *Ann Neurol* 2005; 58: 561–8.
44. Wimalawansa SJ. Circadian variation of plasma calcitonin gene-related peptide in man. *J Neuroendocrinol* 1991; 3: 319–22.
45. Silberstein SD, Dodick DW, Bigal ME, et al. Fremanezumab for the preventive treatment of chronic migraine. *N Engl J Med* 2017; 377: 2113–22.
46. Ho TW, Connor KM, Zhang Y, et al. Randomized controlled trial of the CGRP receptor antagonist telcagepant for migraine prevention. *Neurology* 2014; 83: 958–66.
47. Tepper S, Ashina M, Reuter U, et al. Safety and efficacy of erenumab for preventive treatment of chronic migraine: a randomised, double-blind, placebo-controlled phase 2 trial. *Lancet Neurol* 2017; 16: 425–34.
48. Edvinsson L and Warfvinge K. Recognizing the role of CGRP and CGRP receptors in migraine and its treatment. *Cephalalgia* 2019; 39: 366–73.
49. Edvinsson L. The trigeminovascular pathway: Role of CGRP and CGRP receptors in migraine. *Headache* 2017; 57: 47–55.
50. Iyengar S, Johnson KW, Ossipov MH, et al. CGRP and the trigeminal system in migraine. *Headache* 2019; 59: 659–81.

4.2. Article 2

Alpuente A, Gallardo VJ, Asskour L, Caronna E, Torres-Ferrus M, Pozo-Rosich P. **Salivary CGRP and Erenumab Treatment Response: Towards Precision Medicine in Migraine.** Ann Neurol. 2022 Nov;92(5):846-859. doi: 10.1002/ana.26472. Epub 2022 Aug 24. PMID: 36054144.

Second article addresses third and fourth objectives, focused on the role of **CGRP as potential predictive and therapeutic response biomarker** in migraine patients treated with monoclonal antibodies anti-CGRP mAbs (erenumab).

RESEARCH ARTICLE

Salivary CGRP and Erenumab Treatment Response: Towards Precision Medicine in Migraine

Alicia Alpuente, MD,[1,2] Victor J Gallardo, MSc,[2] Laila Asskour, MLT,[2] Edoardo Caronna, MD,[1,2] Marta Torres-Ferrus, MD, PhD,[1,2] and Patricia Pozo-Rosich, MD, PhD[1,2]

Objective: We aimed (1) to analyze salivary calcitonin gene-related peptide (CGRP) levels in patients with migraine, (2) to predict erenumab response from baseline CGRP levels, and (3) to evaluate CGRP change post-treatment.
Methods: This is a prospective observational study that measured salivary CGRP levels in healthy controls (HCs), patients with episodic migraine (EM) and patients with chronic migraine (CM). Participants collected saliva samples at baseline and, the patients who were candidates to receive erenumab, also collected saliva after 3 doses of treatment. We quantified CGRP-like immunoreactivity (CGRP-LI) by enzyme-linked immunosorbent assay (ELISA) and we performed an analysis at baseline and post-treatment through generalized linear mixed models.
Results: At baseline, a higher headache frequency was associated with higher CGRP levels, those being even higher in the presence of depressive symptoms. A cutoff point (mean, 95% confidence interval [CI]) of 103.93 (95% CI = 103.35–104.51) pg/ml was estimated to differentiate migraine from controls with an area under the receiver operating characteristic (ROC) curve (AUC, 95% CI) of 0.801 (95% CI = 0.798–0.804). We also found that higher pretreatment salivary CGRP levels were statistically significantly associated to a higher probability of having 50% or greater reduction in headache frequency in patients with EM, but not in patients with CM. After 12 weeks of treatment with erenumab, salivary CGRP levels from patients within all spectrum of migraine frequency converged to similar CGRP values. In contrast, in patients with concomitant depressive symptoms, this convergence did not happen.
Interpretation: Patients with migraine not only have higher CGRP levels compared with HCs, but also the presence of depressive symptoms seems to increase salivary CGRP levels and we have evidence, for the first time, that baseline salivary CGRP concentration is associated with treatment response to erenumab.

ANN NEUROL 2022;00:1–14

Migraine is a highly prevalent and a disabling neurological disease, affecting 1 billion people worldwide.[1] It is the second most disabling disorder across all age groups and first cause in women aged under 50 years.[2] Migraine is diagnosed using the extensively field-tested International Classification of Headache Disorders Third Edition (ICHD-3) criteria.[3] Currently, there is not a measurable validated biomarker, although there are some promising candidates.[4,5]

Calcitonin gene-related peptide (CGRP) is a neuropeptide clearly implicated in migraine pathophysiology, including neurogenic inflammation of trigeminal nerve fibers, dural vasodilation, and nociceptive transmission in the peripheral and central nervous system.[6,7] Several studies have measured CGRP in different substrates, in particular, plasma,[8–12] but also in cerebrospinal fluid,[13] tears,[14] and saliva.[15–18] However, its quantification is challenging and researchers have faced many methodological difficulties, such as rapid degradation and different commercial assays. Notwithstanding, these experimental studies hypothesized CGRP as a migraine biomarker with diagnostic[9–11,14,15,18] and treatment predictive purposes.[8,16,17]

View this article online at wileyonlinelibrary.com. DOI: 10.1002/ana.26472

Received Apr 27, 2022, and in revised form Aug 3, 2022. Accepted for publication Aug 5, 2022.

Address correspondence to Dr Pozo-Rosich, Headache Unit, Neurology Department, Vall d'Hebron University Hospital, 119-129 Passeig de la Vall d'Hebron, Barcelona, 08035, Spain. E-mail: ppozo@vhebron.net

From the [1]Headache Clinic, Neurology Department, Vall d'Hebron University Hospital, Barcelona, Spain; and [2]Headache and Neurological Pain Research Group, Vall d'Hebron Research Institute, Departament de Medicina, Universitat Autònoma de Barcelona, Barcelona, Spain

Experimental studies on CGRP are crucial in order to phenotype patients from a molecular perspective and move toward a pathophysiological-driven classification in migraine.

The development of CGRP-targeting drugs has ushered in a new era of migraine therapy,[19] and, since 2018, they have become available as the only target-driven treatments for migraine prevention.[20] Three monoclonal antibodies (mAbs) against the CGRP ligand (fremanezumab, galcanezumab and eptinezumab) and one against CGRP receptor (erenumab) have been clinically developed. The indications for reimbursement in Spain require that patients have 8 or more headache days/month and have failed at least 3 previous preventive treatments.[21] It is still to be shown if there is a correlation between baseline CGRP levels and prediction of response to these treatments or if CGRP levels are modified and how with treatment. Previous studies with nonspecific migraine preventive treatments (eg, onabotulinumtoxinA) have found an association between baseline CGRP levels and treatment response.[8,17]

We have previously demonstrated that measuring CGRP in saliva is feasible and a practical and reproducible way of measuring CGRP.[18] In the present study, we aimed to go one step further: (1) to study salivary CGRP levels in all the frequency-spectrum of migraine through an extension of our previous study,[18] and (2) to find a relationship between baseline salivary CGRP levels and mAb treatment response in an exploratory analysis including patients with migraine who were eligible to be treated with erenumab.

Methods

Participants and Study Design

This was a prospective longitudinal observational pilot study. Patients were recruited from the outpatient headache clinic and carefully interviewed by a headache specialist. The recruitment period was from March 2018 to December 2021. In this study, we also considered participants included in the previous analysis at baseline.[18]

Adults fulfilling the criteria for migraine and chronic migraine (CM) according to the ICHD-3 were recruited. Healthy controls (HCs) were age-matched adults with no personal or family history of migraine or headache, excluding sporadic tension-type headache. Patients taking other preventive treatments (including onabotulinumtoxinA) and participants having any medical condition that could alter saliva (including smoking habit, presence of chronic pain conditions, such as fibromyalgia or chronic fatigue syndrome, systemic disorders, such as Sjogren's syndrome, and oral pathology) were excluded.

Patients with high-frequency episodic migraine (HFEM) and CM received erenumab 140 mg subcutaneous every 4 weeks (because it was the first anti-CGRP mAb approved) according to the National Regulatory Agency considerations.[21]

Clinical Variables

Demographics (age and sex) and migraine characteristics were collected at baseline, including aura, disease evolution time (in years), monthly headache days (MHDs), monthly migraine days (MMDs), and monthly acute medication intake. Patients recorded the presence of headache, pain intensity using a 0 to 3 numerical scale (0 = no pain, 1 = mild pain, 2 = moderate pain, and 3 = severe pain), associated symptoms and use of acute medication using a web-based daily electronic diary (eDiary). A migraine day was defined as any day with moderate to severe headache lasting at least 4 hours or treated with analgesic. A headache day was defined as any headache lasting at least 30 minutes. The MHDs were considered as the sum of migraine days per month (MMDs) and non-migraine headache days per month.

Participants also completed the Migraine Disability Assessment (MIDAS) questionnaire,[22] the Headache Impact Test (HIT-6) score,[23] the Beck Depression Inventory (BDI-II),[24] and the Beck Anxiety Inventory (BAI).[25] Participants with ≥8 BAI score were classified as having anxiety symptoms and participants with ≥14 BDI-II score were classified as suffering from depression symptoms. Questionnaires were completed at baseline for all participants and after 3 doses of erenumab (week 0 and week 12). Treatment response was measured by 50% or greater reduction in MHD (response rate [RR]), classifying patients into responders (RR = ≥50%) and nonresponders (RR = <50%). All patients completed all the questionnaires regardless of their treatment response using REDCap surveys.

Saliva Collection and CGRP Quantification

Saliva collection procedure and CGRP-like immunoreactivity (CGRP-LI; from now on designated as "CGRP") quantification have been specified in more detail in the previous study.[18] For this protocol, all participants were instructed to collect saliva samples during 7 consecutive days at baseline and after 3 erenumab administrations if treated. The latter were collected 15 days after the third dose during 7 consecutive days as well.

Saliva collection was carried out with the resting unstimulated whole saliva method.[26,27] at the participant's home with a specific custom-kit for saliva extraction, and they were kept in the freezer at −18°C. After collection, samples were carried to the laboratory on ice and they

were stored at −80°C. At the moment of the CGRP extraction, samples were centrifuged for 20 minutes at 3500 rpm -4ºC, and the supernatant was aliquoted into 1.5 ml centrifuge sterile and polypropylene Eppendorf tubes and immediately analyzed. CGRP quantification was measured by using human enzyme linked immunosorbent assay (ELISA) kits (Cusabio; detection range = 1.56–100 pg/ml, minimal detectable dose = 0.39 pg/ml). Duplicate measurements were performed for each sample. CGRP concentrations were determined from calibration curves using a 4PL fitting (log scale concentration) as implemented in Analysis software Gen5 resulting in a fit with R2 > 0.99 in every case. The final CGRP level of each sample was calculated as the average of the 2 measurements. An internal validation of the test was performed. CGRP concentration from the immunoassay procedure was corrected by inter and intra-assay coefficients of variability for each ELISA plate.

Statistical Analysis

All statistical analysis were conducted in R version 4.1.1 and figures were produced using the package ggplot2.

Nominal variables (sex, aura, presence of anxiety or depression, and treatment response rate) were reported as frequencies (percentages), whereas the median and interquartile range (IQR) were reported for quantitative variables (age, disease evolution time, MHD, MMD, monthly acute medication intake, MIDAS, and HIT-6). Normality assumption of quantitative variables was checked through visual methods (Q-Q plots) and normality tests (Shapiro–Wilk test). Age and HIT-6 score were the only normally distributed quantitative variables.

At baseline, statistical significance between study groups (HCs, patients with episodic migraine [EM], and patients with CM) was assessed by Fisher's exact test when comparing categorical variables (sex, anxiety, and depression) and 1-way analysis of variance (ANOVA) was used to test statistical significance of age between study groups. In order to assess differences between patients with EM and patients with CM, independent *t* test for HIT-6 was used and the Wilcoxon rank-sum test was used for the other quantitative variables that did not follow any normality assumption. After 12 weeks of treatment, statistical significance pre-post treatment for continuous data was performed with paired *t* test or paired Wilcoxon signed-rank test, considering data distribution, and McNemar's test was performed for categorical data. In the comparison between treatment subgroups (responder vs non-responder), the independent *t* test was used for normal quantitative variables, Wilcoxon rank-sum test for not-normally distributed quantitative variables and Fisher's exact test when comparing categorical variables.

In this expanded study, 3 different analyses were performed in order to study salivary CGRP: (1) comparative study at baseline on salivary CGRP concentration between HCs and all the frequency-spectrum of patients with migraine; (2) a predictive study of treatment response according to the salivary CGRP at baseline, and (3) a longitudinal analysis of the CGRP change after 3 months of treatment (Fig 1). Generalized linear mixed models (GLMMs) were fitted because there was no independence in the data (different CGRP measurements from the same patient at baseline and after treatment). All independent variables were scaled and centered before model fitting and only random intercepts per participant were implemented as random effects. All models were also adjusted by age because it has been previously shown that there are age-dependent differences in CGRP levels.

For the first and third analysis, a GLMM was generated using the glmer function from the lme4 version 1.1–27.1 in the R package with salivary CGRP levels as a dependent variable. For the second analysis, a multivariate-logistic GLMM was performed in order to predict treatment response (50% MHD responders vs nonresponders) as dependent variable using glmer function (binomial family). The area under the receiver operating characteristic (AUC-ROC) curve for the multivariate-logistic GLMM was also computed in order to evaluate model's classification accuracy rate. We used the pROC version 1.18.0 in the R package.

For all 3 analysis, variable selection was performed from different predictors combination (full models) using the MuMIn version 1.43.17 in the R package. Then, best models were selected according to their minimum Akaike information criterion (AIC) and likelihood ratio tests were performed to ensure that best AIC model was better than the null model (model without predictors). The models were validated using leave-one-out cross-validation (LOOCV) clustered by patient. Variance inflation factors (VIFs) for all the parameters were computed in order to estimate how much the variance of an estimated regression coefficient is inflated due to correlated variables so that we could avoid an overfitting problem in the final models.

Finally, we computed the optimal cutoff point of salivary CGRP to classify patients with migraine (MIG) and HCs through the estimation of the Youden Index (*J*) from the ROC curve: salivary CGRP levels at baseline and study group (HCs vs. MIG). The *J* index was estimated using the cutoff point with a bootstrapping model validation through the cutpointr version 1.1.1 in the R package.

A statistical power calculation was not conducted prior to the study because the sample size was based on the available data for this exploratory analysis. However, a post hoc power analysis was performed using the simr

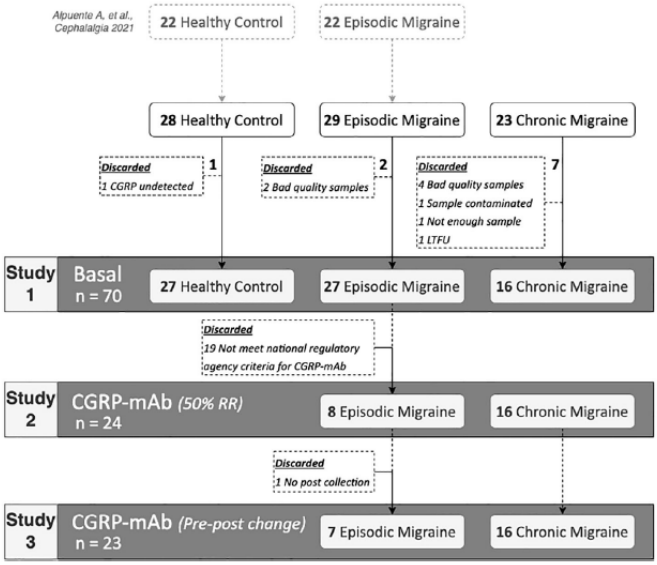

FIGURE 1: Study participants. CGRP-mAb = anti-calcitonin gene-related peptide monoclonal antibody; LTFU = lost to follow-up; RR = treatment response rate.

package (version 1.0.6) for the extended study following the next steps: (1) we simulated new values for the our response variable using the GLMM calculated; (2) we refitted the model to the simulated responses; and (3) we applied a statistical test to the simulated fit.[28] From each GLMM, statistically significant fixed effects terms were tested through a Parametric bootstrap test and power (±95% CI) was estimated.[29] The p values <0.05 were considered as statistically significant and are reported for a 2-tailed test.

Data Availability

Data not published within this article will be made available by request from any qualified investigator.

Standard Protocol Approvals and Patient Consent

All patients voluntarily signed consent forms for their participation in the study. Approval for this study was obtained from the Vall d'Hebron Ethics Committee PR(AG)590/2021. Approval for the first study was obtained from the Vall d'Hebron Ethics Committee PR(IR)292/2017.18. All participants gave their consent for data collection.

Results

Descriptive

Since March 2018, a total of 80 participants were recruited. From them, 12.5% (10/80) were excluded for different reasons (see Fig 1). Therefore, 70 participants (27 HCs, 27 patients with EM, and 16 patients with CM) were considered for the analysis being 94.3% (66/70) women with a median (IQR) age of 36.0 (IQR = 23.2 to 44.0) years old. No statistically significant differences were found in demographic variables (age or sex) between groups, although patients with CM reported greater anxiety percentages (HC = 25.9%, EM = 37.0%, and CM = 87.5%; p = 0.002) Median headache frequency at baseline for EM was 9.0 (6.5, 11.0) days/month and for CM 19.5 (17.8, 23.2) days/month. Comparing patients with migraine, we only found statistically significantly greater values in MHD, MMD, and acute medication use in patients with CM (Table 1).

Salivary CGRP Levels between Groups

The total number of basal salivary samples finally obtained was 180 (70 samples from HCs, 68 from patients with EM, and 42 from patients with CM). No statistically significant differences were found in the total number of samples collected among groups (balanced samples per patients). In the multiple GLMM adjusted by patient's

TABLE 1. Demographics, Comorbidities, and Migraine Characteristics at Baseline

Variable	HCs (n = 27)	EM (n = 27)	CM (n = 16)	Total (n = 70)	*p* value
Demographics					
Age, yr	32.0 (21.5, 41.0)	35.0 (24.5, 40.0)	41.5 (34.8, 48.5)	36.0 (23.2, 44.0)	0.052[a]
Female	26 (96.3%)	26 (96.3%)	14 (87.5%)	66 (94.3%)	0.543[b]
Comorbidities					
Anxiety	7 (25.9%)	10 (37.0%)	14 (87.5%)	31 (44.3%)	**0.002**[b]
Depression	2 (7.4%)	3 (11.1%)	6 (37.5%)	11 (15.7%)	0.069[b]
Migraine characteristics					
Duration of migraine disease, yr		14.0 (7.5, 26.5)	24.5 (14.8, 27.2)	19.0 (9.5, 27.0)	0.163[d]
Aura		10 (37.0%)	8 (50.0%)	18 (41.9%)	0.526[b]
Headache frequency (MHD), d/mo		9.0 (6.5, 11.0)	19.5 (17.8, 23.2)	12.0 (8.0, 18.0)	**<0.0001**[d]
Migraine frequency (MMD), d/mo		5.0 (3.0, 6.0)	14.0 (10.0, 16.5)	6.0 (4.0, 10.0)	**<0.0001**[d]
Acute medication frequency, d/mo		8.0 (6.0, 10.5)	11.0 (9.0, 15.0)	8.0 (6.0, 12.5)	**0.006**[d]
Migraine-related clinical burden					
Disability (MIDAS) score		26.0 (16.0, 51.0)	56.5 (28.5, 68.2)	31.0 (17.0, 60.5)	0.053[d]
Headache-related impact (HIT-6), score		63.0 (60.0, 66.0)	66.0 (62.8, 67.0)	63.0 (61.0, 66.5)	0.088[c]

Continuous data is represented in median (IQR) and categorical data in % (n).
CM = chronic migraine; EM = episodic migraine; HCs = healthy controls; HIT-6 = Headache Impact Test; IQR = interquartile range; MHD = monthly headache days; MIDAS = Migraine Disability Assessment; MMD = monthly migraine days.
Anxiety was considered when patients had ≥8 BAI score and depression, ≥14 BDI-II score.
Bold font indicates statistically significant variables.
[a]Statistical significance assessed with 1-way analysis of variance (ANOVA).
[b]Statistical significance assessed with Fisher's exact test.
[c]Statistical significance assessed with unpaired *t* test.
[d]Statistical significance assessed with unpaired Wilcoxon rank-sum test.

age, we found that basal MHD (β [SE]: 4.156 [SE = 2.589]; p = 0.002), depression (−75.691 [SE = 85.341]; p = 0.030) and the interaction between these two predictors for salivary CGRP levels (14.418 [SE = 5.349]; p = 0.007) were the independent variables that remained statistically significant in the final model (lower AIC, 2176.452; Table 2). Post hoc power analysis showed a simulated statistical power of 70.0% [60.0–78.8%] for the interaction term. In Figure 2 we plotted the final model (see Fig 2A) and we observed that the increase of MHD is associated to an increase of the CGRP levels at baseline. This increase was even higher in the presence of depression according to the BDI-II questionnaire (see Fig 2B). Pairwise comparison post hoc tests after false discovery rate (FDR) adjustment between migraine diagnosis and depression at baseline revealed that in absence of depression, both patients with EM (estimate mean difference [SE] = 198.90 [SE = 27.89] pg/ml; adjusted p = 0.007) and patients with CM (194.34 [SE = 43.75] pg/ml; adjusted p = 0.039) presented higher values of basal CGRP than HCs (75.97 [SE = 27.46] pg/ml; see Fig 2C) but when depressive symptoms strike, only patients with CM (460.69 [SE = 57.24] pg/ml) had statistically significantly higher basal CGRP levels than patients with EM (213.67 [SE = 78.27] pg/ml; adjusted p = 0.022) and HCs (90.82 [SE = 108.50] pg/ml; adjusted p = 0.001; see Fig 2D).

We also calculated the optimal cutoff point (*J* index) of salivary CGRP levels for classifying patients with migraine and HCs. At baseline, a cutoff point (mean [95% confidence interval [CI]) of 103.93 [95% CI = 103.35–104.51] pg/ml (with an 0.801 [95% CI = 0.798–0.804] AUC, 0.732 [95% CI = 0.728–0.737] sensitivity, and 0.916 [95% CI = 0.912–0.920] specificity) was able to classify

TABLE 1. Demographics, Comorbidities, and Migraine Characteristics at Baseline

Variable	HCs (n = 27)	EM (n = 27)	CM (n = 16)	Total (n = 70)	*p* value
Demographics					
Age, yr	32.0 (21.5, 41.0)	35.0 (24.5, 40.0)	41.5 (34.8, 48.5)	36.0 (23.2, 44.0)	0.052[a]
Female	26 (96.3%)	26 (96.3%)	14 (87.5%)	66 (94.3%)	0.543[b]
Comorbidities					
Anxiety	7 (25.9%)	10 (37.0%)	14 (87.5%)	31 (44.3%)	**0.002**[b]
Depression	2 (7.4%)	3 (11.1%)	6 (37.5%)	11 (15.7%)	0.069[b]
Migraine characteristics					
Duration of migraine disease, yr		14.0 (7.5, 26.5)	24.5 (14.8, 27.2)	19.0 (9.5, 27.0)	0.163[d]
Aura		10 (37.0%)	8 (50.0%)	18 (41.9%)	0.526[b]
Headache frequency (MHD), d/mo		9.0 (6.5, 11.0)	19.5 (17.8, 23.2)	12.0 (8.0, 18.0)	**<0.0001**[d]
Migraine frequency (MMD), d/mo		5.0 (3.0, 6.0)	14.0 (10.0, 16.5)	6.0 (4.0, 10.0)	**<0.0001**[d]
Acute medication frequency, d/mo		8.0 (6.0, 10.5)	11.0 (9.0, 15.0)	8.0 (6.0, 12.5)	**0.006**[d]
Migraine-related clinical burden					
Disability (MIDAS) score		26.0 (16.0, 51.0)	56.5 (28.5, 68.2)	31.0 (17.0, 60.5)	0.053[d]
Headache-related impact (HIT-6), score		63.0 (60.0, 66.0)	66.0 (62.8, 67.0)	63.0 (61.0, 66.5)	0.088[c]

Continuous data is represented in median (IQR) and categorical data in % (n).
CM = chronic migraine; EM = episodic migraine; HCs = healthy controls; HIT-6 = Headache Impact Test; IQR = interquartile range; MHD = monthly headache days; MIDAS = Migraine Disability Assessment; MMD = monthly migraine days.
Anxiety was considered when patients had ≥8 BAI score and depression, ≥14 BDI-II score.
Bold font indicates statistically significant variables.
[a]Statistical significance assessed with 1-way analysis of variance (ANOVA).
[b]Statistical significance assessed with Fisher's exact test.
[c]Statistical significance assessed with unpaired *t* test.
[d]Statistical significance assessed with unpaired Wilcoxon rank-sum test.

age, we found that basal MHD (β [SE]: 4.156 [SE = 2.589]; $p = 0.002$), depression (−75.691 [SE = 85.341]; $p = 0.030$) and the interaction between these two predictors for salivary CGRP levels (14.418 [SE = 5.349]; $p = 0.007$) were the independent variables that remained statistically significant in the final model (lower AIC, 2176.452; Table 2). Post hoc power analysis showed a simulated statistical power of 70.0% [60.0–78.8%] for the interaction term. In Figure 2 we plotted the final model (see Fig 2A) and we observed that the increase of MHD is associated to an increase of the CGRP levels at baseline. This increase was even higher in the presence of depression according to the BDI-II questionnaire (see Fig 2B). Pairwise comparison post hoc tests after false discovery rate (FDR) adjustment between migraine diagnosis and depression at baseline revealed that in absence of depression, both patients with EM (estimate mean difference [SE] = 198.90 [SE = 27.89] pg/ml; adjusted $p = 0.007$) and patients with CM (194.34 [SE = 43.75] pg/ml; adjusted $p = 0.039$) presented higher values of basal CGRP than HCs (75.97 [SE = 27.46] pg/ml; see Fig 2C) but when depressive symptoms strike, only patients with CM (460.69 [SE = 57.24] pg/ml) had statistically significantly higher basal CGRP levels than patients with EM (213.67 [SE = 78.27] pg/ml; adjusted $p = 0.022$) and HCs (90.82 [SE = 108.50] pg/ml; adjusted $p = 0.001$; see Fig 2D).

We also calculated the optimal cutoff point (*J* index) of salivary CGRP levels for classifying patients with migraine and HCs. At baseline, a cutoff point (mean [95% confidence interval [CI]) of 103.93 [95% CI = 103.35–104.51] pg/ml (with an 0.801 [95% CI = 0.798–0.804] AUC, 0.732 [95% CI = 0.728–0.737] sensitivity, and 0.916 [95% CI = 0.912–0.920] specificity) was able to classify

TABLE 2. Coefficient Estimates (β), Standard Errors (SE [β]), Associated Wald's T-Statistic (t) and Significance Level (*p* Value) for all Fixed Predictors in the Univariate and Multivariate Linear Mixed-Effect Models of Salivary CGRP at Baseline

Model	Variable[a]	β	SE [β]	t	p value[b]	AIC
Null	*Intercept*	165.90	20.80	7.978		2217.296
1	*Intercept*	50.31	62.62	0.803		2198.588
	Age, yr	1.27	1.80	0.703	0.481	
	MHD (Basal)	8.87	2.35	3.774	**<0.001**	
2	*Intercept*	171.42	21.39	8.016	0.27921	2207.292
	Sex (F vs M)	−97.16	89.77	−1.082		
3	*Intercept*	123.13	26.93	4.572	**0.017**	2204.529
	Anxiety (N vs Y)	96.54	40.46	2.386		
4	*Intercept*	138.50	21.27	6.511	**0.001**	2199.727
	Depression (N vs Y)	171.41	53.18	3.223		
5	*Intercept*	42.48	72.57	0.585		2186.188
	Age, yr	1.55	1.88	0.824	0.410	
	MHD (Basal)	5.02	4.13	1.215	**0.005**	
	Anxiety (N vs Y)	17.82	58.70	0.304	0.248	
	MHD:Anxiety	4.01	5.20	0.771	0.440	
6	*Intercept*	100.48	61.53	1.633		2176.452
	Age, yr	0.28	1.71	0.166	0.868	
	MHD (Basal)	4.16	2.59	1.605	**0.002**	
	Depression (N vs Y)	−75.69	85.34	−0.887	**0.030**	
	MHD:Depression	14.42	5.35	2.695	**0.007**	

AIC = Akaike Information Criterion; CGRP = calcitonin gene-related peptide; F = female; M = male; MHD = monthly headache days; N = No; Y = yes.
':' = symbol indicates interaction between 2 variables.
Bold font indicates statistically significant variables.
[a]All predictors were rescaled to a z-score metric (mean = 0, SD = 1) in the prediction model.
[b]Statistical significance assessed the analysis of deviance in each model (type II Wald F test).

participants including 7.4% of HC versus 72.1% of patients with migraine ($p < 0.0001$).

Salivary CGRP at Baseline and Erenumab Response

From all patients, 24 (8 patients with EM and 16 patients with CM) started erenumab 140 mg. After 3 months of treatment, there was a statistically significantly reduction (Δ_{W12}) in basal MHD (median [IQR]: −4.5 [IQR = −9.2 to −1.8] day/months; $p < 0.0001$), MMD (median = −4.0 [IQR = −8.5 to −1.8] day/months; $p < 0.0001$), MIDAS (median = −36.5 [IQR = −53.0 to −10.8] score; $p < 0.0001$), and HIT-6 (median = -11.0 [IQR = −20.0 to −3.5] score; $p < 0.0001$). There was also an improvement in depression but not in anxiety scores; ≥50% RR was observed in 41.7% (10/24) of the patients. However, we did not find any statistically significant difference in baseline clinical characteristics between responders (10/24, ≥50% MHD RR) and nonresponders (14/24, <50% MHD RR) to erenumab.

From these patients, the total number of salivary samples that we obtained was 66. A multivariate logistic GLMM was fitted, predicting the probability of treatment response based on salivary CGRP levels at baseline and

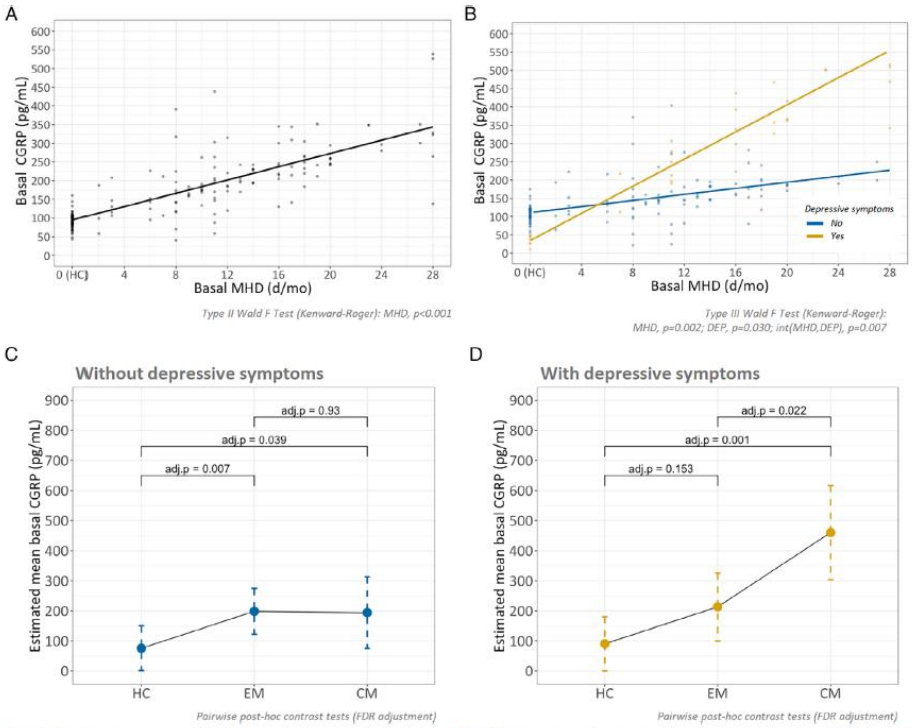

FIGURE 2: Basal salivary CGRP line plots according to basal MHD (A) and with the interaction between basal MHD and depression (B). Post hoc comparisons between basal salivary CGRP and migraine diagnosis in patients without (C) and with (D) depression. adj. = adjusted; CGRP-mAb = anti-calcitonin gene-related peptide monoclonal antibody; CM = chronic migraine; d = days; DEP = depression; EM = episodic migraine; FDR = false discovery rate; HC = healthy controls; int = interaction; MHD = monthly headache days; mo = months.

clinical data. The final model obtained to predict treatment response had a classification accuracy [95% CI] of 74.8% [95% CI = 65.0–82.9%] with an 87.7% sensitivity and a 57.1% specificity within the LOOCV. The AUC [95% CI] obtained was 0.678 (95% CI = 0.562–0.793; Fig 3A). Independent statistically significant predictors associated to treatment response in the model (corrected by the patient's age) were salivary CGRP at baseline (odds ratio [OR] = 1.091, 95% CI = 1.013–1.102; $p < 0.001$) and the interaction of salivary CGRP and MHD at baseline (OR = 0.997, 95% CI = 0.984–0.998; $p < 0.001$; see Fig 3B). Model interaction effect is plotted in Figure 4; surprisingly, higher basal CGRP levels were statistically significantly associated to a higher probability of having a ≥50% MHD improvement in patients with EM. However, in patients with higher headache frequency (CM), the likelihood to response to CGRP-mAbs was dramatically reduced, suggesting that salivary CGRP levels are not associated to treatment response in patients with CM.

Salivary CGRP Levels after 12 Weeks of Erenumab

Finally, we studied the change in salivary CGRP levels in patients treated with 3 doses of erenumab. In this analysis, we included 7 patients with EM and 16 patients with CM, with a total of 167 salivary samples (66 pretreatment and 105 post-treatment; see Fig 1).

In the multiple GLMM adjusted by patient's age, we found that the 3-way interaction of Time (week 0 vs week 12), depression at baseline (yes vs no), and headache frequency at baseline (MHD, days/month) was the only independent variable statistically significantly associated to the salivary CGRP levels change in the final model

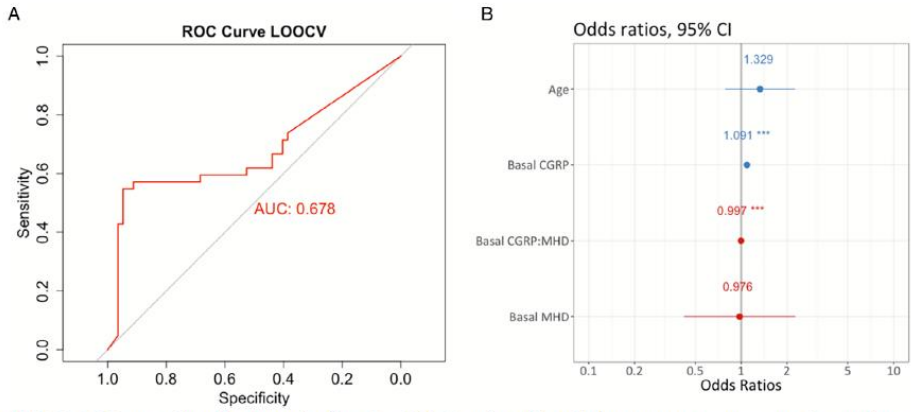

FIGURE 3: ROC curve (A) and estimated odds ratios, 95% CI and *p* values (B) for treatment response rate (responder vs nonresponders) statistically significant predictors of the final logistic GLMM. AUC = area under the ROC curve; CGRP-mAb = anti-calcitonin gene-related peptide monoclonal antibody; CI = confidence interval; GLMM = generalized linear mixed model; LOOCV = leave-one-out cross-validation; MHD = monthly headache days; OR = odds ratio; ROC curve = receiver operating characteristic curve. ':' = symbol indicates interaction between two variables. * The *p* values are <0.001. †All predictors were rescaled to a z-score metric (mean = 0, SD = 1) in the prediction model.**

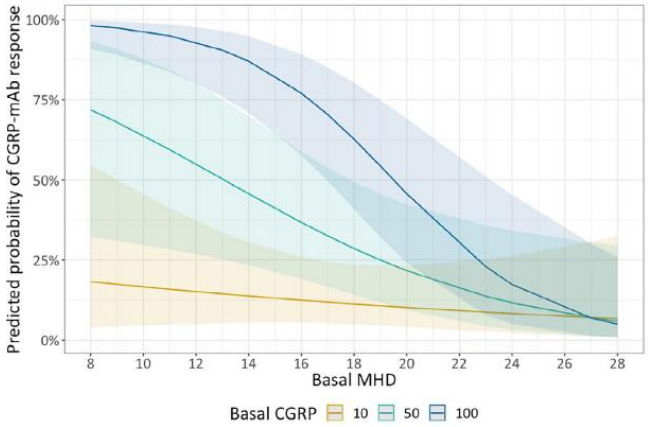

FIGURE 4: Effect plots of the salivary CGRP and headache frequency (MHD) interaction at baseline for treatment response rate (responder vs. non-responder) of the logistic GLMM. CGRP-mAb = anti-calcitonin gene-related peptide monoclonal antibody (erenumab); d = days; GLMM = generalized linear mixed model; MHD = monthly headache days; mo = months.

(β = −26.02, SE = 9.97; $p = 0.009$; Table 3). The interaction plot of the model is shown in Figure 5A, where we can observe that after 3 months of treatment, salivary CGRP levels in all frequency of patients with migraine (8, 15, and 25 MHD are exemplified in the plot) were converged to similar CGRP values. In presence of depression, CGRP levels did not reach such a convergence. Moreover, salivary CGRP levels did not decrease but seem to slightly increase after treatment, both in patients with or without depression.

Pairwise comparison post hoc tests after FDR adjustment of salivary CGRP post-treatment (in patients) among groups (HCs, EM, and CM groups) and the presence of depression showed similar results with values

TABLE 3. Coefficient Estimates (β), Standard Errors (SE [β]), Associated Wald's T-Statistic (t), and Significance Level (*p* Value) for all Fixed Predictors in the Multivariate Linear Mixed-Effect Models of Salivary CGRP after 3 Months

Model	Variable[a]	β	SE [β]	t	p value[b]	AIC
Null	*Intercept*	282.88	45.41	6.230		2202.709
1	*Intercept*	333.57	223.78	1.491		2147.045
	Age, yr	−0.21	4.88	−0.042	0.966	
	Time (W0 vs W12)	−96.50	80.75	−1.195	0.282	
	Depression (N vs Y)	−415.19	349.18	−1.189	0.116	
	MHD (Basal)	−7.77	10.11	−0.768	0.599	
	Time:Depression	469.63	197.33	2.380	0.655	
	Time:MHD	8.36	4.99	1.674	0.671	
	Depression:MHD	31.75	17.34	1.831	0.230	
	Time:Depression:MHD	−26.02	9.97	−2.610	**0.009**	

AIC = Akaike Information Criterion; CGRP = calcitonin gene-related peptide; MHD = monthly headache days; N = No; Y = yes.
':' = symbol indicates interaction between variables.
Bold font indicates statistically significant variables.
[a]All predictors were rescaled to a z-score metric (mean = 0, SD = 1) in the prediction model.
[b]Statistical significance assessed the analysis of deviance in each model (type II Wald F test).

obtained from pretreated patients: in absence of depression, both patients with EM (235.05 [SE = 59.32] pg/ml; adjusted $p = 0.031$) and patients with CM (256.34 [SE = 47.56] pg/ml; adjusted $p = 0.008$) presented higher values of basal CGRP with HCs (75.97 [SE = 27.46] pg/ml) but in the presence of depression, only patients with CM (446.99 [SE = 63.44] pg/ml) had statistically significantly higher basal CGRP with HC (90.82 [SE = 108.50] pg/ml; adjusted $p = 0.007$; see Fig 5B).

Discussion

In this study, first, we examined salivary levels of CGRP in patients with migraine over the headache frequency spectrum and HCs; we further studied CGRP as a predictor of response to erenumab in those who receive it, and, finally, we assessed change in CGRP levels after 12 weeks of treatment. CGRP can differentiate migraine from controls, even more in the presence of depressive symptoms, differentiating among migraine groups. CGRP is a molecular predictor of response to erenumab in some patients and its excess seems to be regulated after treatment, but not to the levels of controls, after 12 weeks of treatment.

First, our results showed that salivary levels of CGRP progressively increased as headache frequency worsened from low frequency EM to CM, without statistically significant differences among migraine groups. There are very few studies measuring CGRP levels over the headache frequency spectrum. These previous studies, albeit using different samples than saliva, showed different results. In the case of the Cernuda-Morollón et al study, and in line with our results, patients with CM exhibited the highest plasma levels of CGRP, followed by patients with EM and HCs.[11] In contrast, Lee et al did not find any significant differences among the groups.[30] However, the challenges in analyzing CGRP serum levels have been reflected in the literature.[31] Even if studies are not always able to reflect differences, it is clear that CGRP has a central role in migraine pathophysiology.[32] In our previous study, although only patients with EM were included, we already observed this trend whereby those patients with headache frequency between 6 and 10 days/month had higher CGRP levels compared to those with <5 days/month.[18] Therefore, CGRP seems to be a marker of disease burden.

Our results also contribute to increase the evidence which supports that baseline CGRP levels are higher in patients with migraine. In our study, CGRP levels were statistically significant higher in patients with migraine than in controls in the absence of depression. Kamm et al found increased tear fluid levels of CGRP in patients with

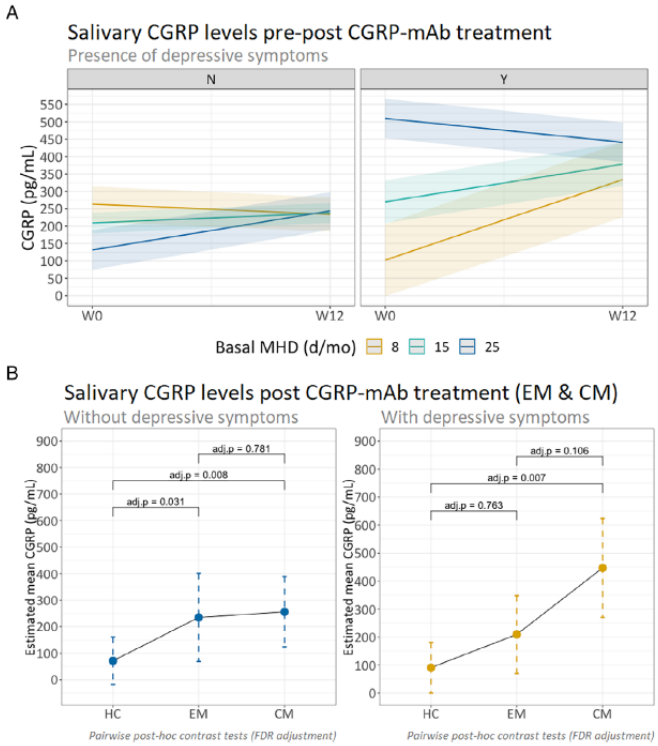

FIGURE 5: Salivary CGRP change after 12 weeks of treatment in patients with depression symptoms at baseline (A) and salivary CGRP levels comparison between controls and patients with migraine (EM and CM) post-treatment. HCs were included in the analysis as a reference salivary CGRP level. adj. = adjusted; CGRP-mAb = anti-calcitonin gene-related peptide monoclonal antibody (erenumab); CM = chronic migraine; d = days; EM = episodic migraine; FDR = false discovery rate; HCs = healthy controls; MHD = monthly headache days; W0 = week 0; W12 = week 12; mo = months.

migraine compared with healthy subjects also without differences between patients with EM and patients with CM.[14] It is important to highlight that in the presence of depression, levels of CGRP increase in all participants and statistically significant differences disappear between controls and patients with EM. Interestingly, an optimal salivary CGRP cutoff point of 103.75 pg/ml was found, which allows us to create levels of normality versus disease.

One of the most striking findings of the study, despite the small size, is the influence of depression on baseline CGRP levels. The clinical relationship between depression and migraine, in particular CM, is well-established in epidemiological studies.[33] Furthermore, there is a linear relationship between the number of headache days and the degree of depression.[34] There is evidence of shared genetic polymorphisms between migraine and depression as well.[35,36] Our study not only supports this linear relationship between headache frequency and depression but also states that CGRP could play an important role in this relation. Perhaps one could hypothesize in its neuroinflammatory central function,[37,38] for which anti-CGRP mAbs do not have as much access due to their very low permeability through the brain blood barrier.[39] Although neurobiological correlates have only partially been elucidated, altered levels of CGRP-LI in animal models[40,41] and in the cerebrospinal fluid of patients with depression were reported,[42] suggesting that CGRP may be involved in the pathophysiology and/or be a trait marker of depressive disorders. Increased brain levels of CGRP have been found to be a well-established rat model of depression and, interestingly,

antidepressants did not have an effect on the brain level of this peptide.[40] This relationship between the immune system and the presence of psychiatric disorders has gained interest in recent years and it would appear that the severity of depressive symptoms is likely to be modulated by the degree of inflammation.[43,44] However, the field of psychiatry and inflammation is in its first stages and needs further investigation to evaluate the role of CGRP and other neuroinflammatory markers in depression.[45]

The relationship between CGRP levels and CGRP-related migraine-specific therapies is an interesting matter of study because it might contribute to the development of precision medicine in migraine. We found that baseline CGRP and headache frequency were the only independent statistically significant predictors associated to erenumab response. Thereby, in patients with HFEM, higher CGRP levels at baseline were statistically significantly associated to a higher probability of response. Surprisingly, as headache frequency worsens and CM is reached, the likelihood to response based on CGRP levels is dramatically reduced, indicating again the fact that a peripheral regulation of CGRP is not enough in these patients. However, we already know from clinical trials that CM also responds to treatment but,[46] according to our results, the response in patients with CM is not as influenced by CGRP levels at baseline as is in patients with EM, and therefore it is clear that in CM there must be other biological or genetic components involved. On a practical clinical level, this supports, on one hand, the importance of treating patients with migraine with effective preventive treatments earlier in the development of the disease because it seems that there is a possibility of reverting migraine molecularly before it reaches a no-return turning point with the current therapeutic option. On the other hand, it is important to consider CGRP levels when disease burden is not so high. It is proven that, in CM, patients have a poor adherence to treatment due to adverse events or lack of efficacy among other factors.[47–50] In our study, we demonstrate that in migraine there is not only a clinical spectrum but also a molecular spectrum with a pathophysiological meaningful turning point of the disease, which is related to impact and treatment response. Furthermore, it seems to be a pharmacodynamic explanation for the fact that the higher CGRP concentration, the worse the treatment response. In a recent study in vitro, it has been shown that in the presence of human αCGRP there is a reduction in binding of erenumab to neuroblastoma cell line (SK-N-MC cells). This observed reduction may be due to competition for receptor binding and/or ligand-induced receptor downregulation.[51] Therefore, it seems that the excess of CGRP in the trigeminovascular system as headache frequency worsens might have an impact on erenumab response.

Finally, we studied the change in CGRP levels after treatment. Erenumab is the first human IgG2 monoclonal antibody developed for migraine.[52] It is directed to the CGRP binding site of the canonical CGRP receptor and therefore has differences in the CGRP pathway compared with the other CGRP agents.[53] We found that after 12 weeks of treatment, CGRP levels in all patients with headache frequency converged to similar CGRP values, whereas in the presence of depression, CGRP levels do not reach such a convergence. The effect of depression seems to be linked with the need for more time for the CGRP levels to converge as there is a trend. So, maybe with a longer time of treatment this CGRP could be regulated. Furthermore, it seems that salivary CGRP levels do not decrease but seem to be increasing after treatment in some patients, with or without depression. In line with this, it is suggested that long-term blockade of CGRP receptors could induce an increase in systemic CGRP levels via a classical upregulation mechanism.[54] In line with our results, a previous exploratory study showed that plasma CGRP levels were increased after 6 months of treatment, although without statistically significance probably due to the small size of the study.[55]

Saliva seems to be a worthwhile biofluid to study CGRP levels. Measuring CGRP closer to the afferents seems to be more effective than in plasma, easier and less-invasive, and is a reliable reflection of trigeminovascular activation. Saliva contains a wide variety of neuropeptides due to salivary gland innervations by the nerve terminals of the trigeminovascular system and could, therefore, provide a certain clue about nervous system pathophysiology.[56] Previous studies on serum CGRP levels showed contradictory results[9,11,12,18] and there was no correlation between CGRP salivary and plasma levels.[15,16,18,56]

Some limitations should be mentioned. First, there was the small size of the sample, in particular, in patients with CM. Second, depression was established according to a questionnaire, without a confirmation by a psychiatrist. However, most of the studies focused on the study of psychiatric comorbidities in migraine are also based on questionnaires. Third, because patients with CM have a persistent headache state, it was difficult to get samples in a real interictal state, in particular, at baseline. This study has several strengths, such as the prospective longitudinal approach in patients without any other preventive treatment. Participants in this study are well phenotyped, including all the spectrum of migraine frequency as well as psychiatric comorbidities, allowing the discovery of interesting interactions in the model and avoiding confounding factors. To the best of our knowledge, this is the

first study reporting the biological association among migraine, CGRP, and the presence of depressive symptoms; as well as the fact that CGRP could be perhaps started to be considered as a molecular biomarker predicting response to treatment in patients with HFEM. This study keeps completing our previous work, and represents a step forward because it includes a wider population of the study in terms of sex (including men), migraine frequency (including HFEM and patients with CM) with a presence of depressive symptoms; and also allowed us to link CGRP levels with erenumab treatment from a molecular point of view.

Several interesting questions arise henceforth. First, the analysis of the effect of other anti-CGRP treatments, such as gepants on CGRP levels. In addition, it will be necessary to better study patients with CM in order to disentangle which are the factors that influence treatment response. Finally, the study of central inflammation in patients with CM may be of essence to understand the relationship between migraine and depression.

In conclusion, saliva seems to be a reliable matrix to measure CGRP in patients with migraine. Patients with high-frequency and CM do not only have higher CGRP levels compared with controls, but (i) depressive symptoms seem to increase CGRP levels, creating a meaningful distinction between patients with EM and patients with CM, (ii) baseline CGRP levels can be used as an erenumab predictor of response in non-chronic patients, and (iii) CGRP heterogeneity within migraine frequency spectrum seems to be regulated and "normalized" by erenumab in such a manner that CGRP levels converge after 3 months of treatment, whereas depression seems to avoid this convergence.

Acknowledgment

This study was funded by a grant from Instituto de Salud Carlos III (ISCIII - PI16/01525) and partially financed by a grant from the Headache Study Group of the Spanish Neurological Society 2021.

Authors Contributions

A.A. and P.P.R. made substantial contributions to the conception and design of the study. L.A. made substantial contribution to the methodology and she performed the ELISA protocol. A.A. and E.C. contributed to data collection. A.A. wrote the first draft of the paper. V.J.G. performed statistical analysis. All authors participated in acquisition, analysis, and/or interpretation of data. All authors have critically revised and finally approved the version of the paper to be published.

Potential Conflicts of Interest

In relation with this paper the authors have nothing to disclose. A.A. has received honoraria as speaker from AbbVie and for education from Novartis and Eli Lilly. V.J.G. and L.A. have nothing to disclose. E.C. has received honoraria as speaker from Novartis. M.T.F. has received honoraria as a speaker for AbbVie, Chiesi, Eli Lilly, and Novartis. P.P.R. has received honoraria as a consultant and speaker for AbbVie, Amgen, Biohaven, Chiesi, Eli Lilly, Lundbeck, Medscape, Novartis, and Teva. Her research group has received research grants from AbbVie, Novartis, Teva, AGAUR, FEDER RIS3CAT, la Caixa foundation, Migraine Research Foundation, Instituto Investigación Carlos III, EraNet Neuron, International Headache Society, MICINN, and PERIS; and has received funding for clinical trials from Alder, Biohaven, Electrocore, Eli Lilly, Lundbeck, Novartis, and Teva. She is the Honorary Secretary of the Executive Board of the International Headache Society. She is in the editorial board of Revista de Neurologia. She is an associate editor for Cephalalgia, Frontiers of Neurology, Headache, Neurologia, and a scientific advisor of The Journal of Headache and Pain. She is a member of the Clinical Trials Guidelines Committee of the International Headache Society. She has edited the Guidelines for the Diagnosis and Treatment of Headache of the Spanish Neurological Society. She is the founder of www.midolordecabeza.org. P.P.R. does not own stocks from any pharmaceutical company.

References

1. GBD 2016 Headache Collaborators. Global, regional, and national burden of migraine and tension-type headache, 1990–2016: a systematic analysis for the global burden of disease study 2016. Lancet Neurol 2018;17:954–976.
2. Steiner TJ, Stovner LJ, Jensen R, et al. Lifting the burden: the global campaign against headache. Migraine remains second among the world's causes of disability, and first among young women: findings from GBD2019. J Headache Pain 2020;21:137.
3. Headache Classification Committee of the International Headache Society (IHS). The international classification of headache disorders. Cephalalgia 2018;38:1–211.
4. Ashina M, Terwindt GM, MAM A-K, et al. Migraine: disease characterisation, biomarkers, and precision medicine. Lancet 2021;39: 1496–1504.
5. Durham P, Papapetropoulos S. Biomarkers associated with migraine and their potential role in migraine management. Headache 2013; 53:1262–1277.
6. Goadsby PJ, Edvinsson L, Ekman R. Vasoactive peptide release in the extracerebral circulation of humans during migraine headache. Ann Neurol 1990;28:183–187.
7. Edvinsson L, Haanes KA, Warfvinge K, Krause DN. CGRP as the target of new migraine therapies - successful translation from bench to clinic. Nat Rev Neurol 2018;14:338–350.

8. Domínguez C, Vieites-Prado A, Pérez-Mato M, et al. CGRP and PTX3 as predictors of efficacy of Onabotulinumtoxin type a in chronic migraine: an observational study. Headache 2018;58:78–87.
9. Ashina M, Bendtsen L, Jensen R, et al. Evidence for increased plasma levels of calcitonin gene-related peptide in migraine outside of attacks. Pain 2000;86:133–138.
10. Fusayasu E, Kowa H, Takeshima T, et al. Increased plasma substance P and CGRP levels, and high ACE activity in migraineurs during headache-free periods. Pain 2007;128:209–214.
11. Cernuda-Morollón E, Larrosa D, Ramón C, et al. Interictal increase of CGRP levels in peripheral blood as a biomarker for chronic migraine. Neurology 2013;81:1191–1196.
12. Tvedskov JF, Lipka K, Ashina M, et al. No increase of calcitonin gene-related peptide in jugular blood during migraine. Ann Neurol 2005;58:561–568.
13. Van Dongen RM, Zielman R, Noga M, et al. Migraine biomarkers in cerebrospinal fluid: a systematic review and meta-analysis. Cephalalgia 2017;37:49–63.
14. Kamm K, Straube A, Ruscheweyh R. Calcitonin gene-related peptide levels in tear fluid are elevated in migraine patients compared to healthy controls. Cephalalgia 2019;39:1535–1543.
15. Bellamy JL, Cady RK, Durham PL. Salivary levels of CGRP and VIP in rhinosinusitis and migraine patients. Headache 2006;46:24–33.
16. Cady RK, Vause CV, Ho TW, et al. Elevated saliva calcitonin gene-related peptide levels during acute migraine predict therapeutic response to rizatriptan. Headache 2009;49:1258–1266.
17. Cady R, Turner I, Dexter K, et al. An exploratory study of salivary calcitonin gene-related peptide levels relative to acute interventions and preventative treatment with onabotulinumtoxinA in chronic migraine. Headache 2014;54:269–277.
18. Alpuente A, Gallardo VJ, Asskour L, et al. Salivary CGRP can monitor the different migraine phases: CGRP (in)dependent attacks. Cephalalgia 2021;42:119–129.
19. Charles A, Pozo-Rosich P. Targeting calcitonin gene-related peptide: a new era in migraine therapy. Lancet 2019;394:1765–1774.
20. Hou M, Xing H, Cai Y, et al. The effect and safety of monoclonal antibodies to calcitonin gene-related peptide and its receptor on migraine: a systematic review and meta-analysis. J Headache Pain 2017;18:1–12.
21. Agencia Española de Medicamentos y Productos Sanitarios. *Informe de posicionamiento terapéutico Erenumab*, 2019:1-8 ESPAÑA (SPAIN): Agencia Española de Medicamentos y Productos Sanitarios (AEMPS) Available from: https://www.aemps.gob.es/medicamentosUsoHumano/informesPublicos/docs/IPT-erenumab-Aimovig.pdf?x33378.
22. Stewart WF, Lipton RB, Dowson AJSJ. Development and testing of the migraine disability assessment (MIDAS) questionnaire to assess headache-related disability. Neurology 2001;56:S20–S28.
23. Yang M, Rendas-Baum R, Varon SF, et al. Validation of the headache impact test (HIT-6TM) across episodic and chronic migraine. Cephalalgia 2011;31:357–367.
24. Beck A, Steer R. Brown. Manual for the Beck depression inventory-II. In: Gellman MD, Turner JR, eds. *San Antonio Psychol Corp*. Springer New York: New York, NY, 1996.
25. Beck AT, Epstein N, Brown G, Steer RA. An inventory for measuring clinical anxiety: psychometric properties. J Consult Clin Psychol 1988;56:893–897.
26. Jasim H, Carlsson A, Hedenberg-Magnusson B, et al. Saliva as a medium to detect and measure biomarkers related to pain. Sci Rep 2018;8:1–9.
27. Dawidson I, Blom M, Lundeberg T, et al. Neuropeptides in the saliva of healthy subjects. Life Sci 1996;60:269–278.
28. Green P, MacLeod CJ. SIMR: an R package for power analysis of generalized linear mixed models by simulation. Methods Ecol Evol 2016;7:493–498.
29. Halekoh U, Højsgaard S. A kenward-roger approximation and parametric bootstrap methods for tests in linear mixed models–the R package pbkrtest. J Stat Softw 2014;2014:1–32.
30. Lee MJ, Lee SY, Cho S, et al. Feasibility of serum CGRP measurement as a biomarker of chronic migraine: a critical reappraisal. J Headache Pain 2018;19:53.
31. Edvinsson L, Ekman R, Goadsby PJ. Measurement of vasoactive neuropeptides in biological materials: problems and pitfalls from 30 years of experience and novel future approaches. Cephalalgia 2010;30:761–766.
32. Arulmani U, MaassenVanDenBrink A, Villalón CM, Saxena PR. Calcitonin gene-related peptide and its role in migraine pathophysiology. Eur J Pharmacol 2004;500:315–330.
33. Buse DC, Manack A, Serrano D, et al. Sociodemographic and comorbidity profiles of chronic migraine and episodic migraine sufferers. J Neurol Neurosurg Psychiatry 2010;81:428–432.
34. Ruscheweyh R, Müller M, Blum B, Straube A. Correlation of headache frequency and psychosocial impairment in migraine: a cross-sectional study. Headache 2014;54:861–871.
35. Yang Y, Zhao H, Boomsma DI, et al. Molecular genetic overlap between migraine and major depressive disorder. Eur J Hum Genet 2018;26:1202–1216.
36. The Brainstorm Consortium. Analysis of shared heritability in common disorders of the brain Science 2018;360:eaap8757.
37. Russell FA, King R, Smillie SJ, et al. Calcitonin gene-related peptide: physiology and pathophysiology. Physiol Rev 2014;94:1099–1142.
38. Edvinsson L, Haanes KA, Warfvinge K. Does inflammation have a role in migraine? Nat Rev Neurol 2019;15:483–490.
39. Yuan H, Lauritsen CG, Kaiser EA, Silberstein SD. CGRP monoclonal antibodies for migraine: rationale and Progress. BioDrugs 2017;31:487–501.
40. Angelucci F, Ellenbroek BA, El Khoury A, Mathé AA. CGRP in a gene-environment interaction model for depression: effects of antidepressant treatment. Acta Neuropsychiatr 2019;31:93–99.
41. Hashikawa-Hobara N, Ogawa T, Sakamoto Y, et al. Calcitonin gene-related peptide pre-administration acts as a novel antidepressant in stressed mice. Sci Rep 2015;5:1–14.
42. Mathé AA, Ågren H, Wallin A, Blennow K. Calcitonin gene-related peptide and calcitonin in the CSF of patients with dementia and depression: possible disease markers. Prog Neuro-Psychopharmacol Biol Psychiatry 2002;26:41–48.
43. Caldiroli A, Capuzzi E, Barkin JL, et al. Is there an association between inflammatory/anti-oxidant markers and the presence of psychotic symptoms or severity of illness in mood and psychotic disorders? A multi-centric study on a drug-free sample. Brain Behav Immun Health 2022;22:100453.
44. Buchmann A, Ritter C, Müller ST, et al. Associations between heart rate variability, peripheral inflammatory markers and major depressive disorder. J Affect Disord 2022;304:93–101.
45. Sanchez C, El Khoury A, Hassan M, et al. Sex-dependent behavior, neuropeptide profile and antidepressant response in rat model of depression. Behav Brain Res 2018;351:93–103.
46. Tepper S, Ashina M, Reuter U, et al. Safety and efficacy of erenumab for preventive treatment of chronic migraine: a randomised, double-blind, placebo-controlled phase 2 trial. Lancet Neurol 2017;16:425–434.
47. Lipton RB, Bigal ME, Diamond M, et al. Migraine prevalence, disease burden, and the need for preventive therapy. Neurology 2007;68:343–349.
48. Hepp Z, Dodick DW, Varon SF, et al. Adherence to oral migraine-preventive medications among patients with chronic migraine. Cephalalgia 2015;35:478–488.

49. Hepp Z, Bloudek LM, Varon SF, et al. Systematic review of mi- graine prophylaxis adherence and persistence. J Manag Care Pharm 2014; 20:22–33.

50. Bigal ME, Serrano D, Reed M, Lipton RB. Chronic migraine in the population: burden, diagnosis, and satisfaction with treatment. Neurology 2008;71:559–566.

51. Bhakta M, Vuong T, Taura T, et al. Migraine therapeutics differentially modulate the CGRP pathway. Cephalalgia 2021;41: 499–514.

52. Amgen Inc. Erenumab (Aimovig™):US prescribing information. 2018. https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/761077s000lbl.pdf. Accessed Feb 21, 2021.

53. Al-Hassany L, Goadsby PJ, Danser AHJ, et al. Calcitonin gene-related peptide-targeting drugs for migraine: how pharmacology might inform treatment decisions. Lancet Neurol 2022;21:284–294.

54. Tringali G, Navarra P. Anti-CGRP and anti-CGRP receptor monoclonal antibodies as antimigraine agents. Potential differences in safety profile postulated on a pathophysiological basis. Peptides 2019;116:16–21.

55. Tringali G, Vollono C, Calabresi P, Navarra P. A proof-of-concept study on CGRP plasma levels of migraineurs during a 6-month treatment with ERENUMAB. J Headache Pain 2020;21:20–21.

56. Nicolodi M, Del BE. Sensory neuropeptides (substance P, calcitonin gene-related peptide) and vasoactive intestinal polypeptide in human saliva: their pattern in migraine and cluster headache. Cephalalgia 1990;10:39–50.

5. OVERALL SUMMARY OF RESULTS

We now report a summary of the results, correlated with the objectives of this thesis.

5.1 Longitudinal CGRP levels throughout the migraine cycle

Daily collection of saliva samples allowed us to monitor CGRP through all the migraine cycle encompassing interictal and ictal period and the different phases of the migraine attack.

In the first study, a total of 49 migraine attacks were collected. We analyzed salivary CGRP-LI at each timepoint of the attack, besides the interictal period. We found that **age and cubic trend of time (migraine cycle) drove statistically significantly changes on CGRP-LI concentration.**

We found **statistically significantly higher concentration of CGRP-LI during headache onset.** Furthermore, **patients who referred higher basal frequency presented a statistically significantly higher concentrations of CGRP in all time points** of the migraine cycle. Interestingly, post-hoc analysis revealed that **attacks treated with triptans presented a statistically significantly reduction on CGRP after 2h from headache onset**.

5.1.1 Subtypes of migraine patients

This longitudinal approach allowed us to differentiate **different types of migraine patients: 79.6%** (39/49) of migraine attacks were **CGRP dependent** (dCGRP) and **20.4%** (10/49) were **non-CGRP dependent** (nCGRP). **CGRP dependent group presented a statistically significant higher levels of CGRP-LI** and a statistically significant association between **photophobia** and **phonophobia**. On the other hand, **dizziness was statistically significantly associated with nCGRP group**.

When we analyzed migraine patients, 13 out of 22 patients only showed dCGRP migraine attacks; 3 out of 22 patients only showed nCGRP migraine attacks and 6 patients showed both types of migraine attacks.

5.2. CGRP as a potential biomarker in migraine

5.2.1 Diagnostic biomarker

Whereas in the first study we included young women with very LFEM, in the second study we included patients with HFEM and CM. In both studies HC were included. This approach allowed us **to study CGRP as a potential diagnostic biomarker in all the migraine frequency spectrum**. The fact of recruiting only young women in the first study was intended to properly study a sample representative of the disease globally and having a homogeneous sample. Recruiting a wider sample including male and female adult patients with more burdensome forms of the disease was intended to represent population at the headache clinics.

The first study found statistically significant **higher interictal CGRP-LI salivary levels in EM compared to HC**. In regards to **plasma CGRP-LI** levels**, we did not find statistically significant differences in this substrate between study groups**. There was no correlation between salivary and plasma CGRP-LI levels.

In the second study we found that **headache frequency at baseline, depressive symptoms and the interaction between these two variables were predictors for salivary CGRP levels.** We observed that **the increase of MHD was associated to an increase of the CGRP levels at baseline**. In absence of depressive symptoms, **both EM and CM patients presented higher values of basal CGRP than HC.** This increase was even higher in presence of depressive symptoms according to the BDI-II questionnaire. **When depressive symptoms are present, only CM patients had statistically significantly higher basal CGRP levels** than EM patients.

Interestingly, we calculated the optimal cut-off point of salivary CGRP levels for classifying migraine patients and HC. A **cut-off point** (mean [95.0% CI) of **103.93** [103.35-104.51] pg/mL (with an 0.801 [0.798-0.804] AUC, 0.732 [0.728-0.737] sensitivity and 0.916 [0.912-0.920] specificity) was able to classify participants including 7.4% of HC vs. 72.1% of migraine patients (p<0.0001).

5.2.2 Predictive biomarker

In the second study we also aimed to study whether CGRP levels predicted an individual or group of individuals more likely to experience a favorable or unfavorable effect to erenumab 140 mg.

Patients treated with erenumab 140 mg showed a statistically significantly reduction in headache frequency and improvement in patient-related outcomes after 3 months of treatment. There was also an improvement in depression but not in anxiety scores. ≥50% RR was observed in 41.7% patients. However, we did not find any statistically significant difference in baseline clinical characteristics between responders (10/24, ≥50% MHD RR) and non-responders (14/24, <50% MHD RR) to erenumab.

From these patients, the total number of salivary samples that we obtained was 66. A multivariate logistic GLMM was fitted, predicting the probability of treatment response based on salivary CGRP levels at baseline and clinical data. The final model obtained to predict treatment response had a classification accuracy [95% CI] of 74.8% [65.0-82.9%] with an 87.7% sensitivity and a 57.1% specificity within the LOOCV. The AUC [95% CI] obtained was 0.678 (0.562-0.793). **Independent statistically significant predictors associated to treatment response** in the model (corrected by patient's age) were **salivary CGRP at baseline and the interaction of salivary CGRP and headache frequency at baseline**. Surprisingly, higher basal CGRP levels were statistically significantly associated to a higher probability of having ≥50% RR improvement in EM patients. However, in patients with CM, the likelihood to response to CGRP-mAbs was reduced.

5.2.3 Therapeutic response biomarker

In the second study we also aimed to study changes in CGRP level in response to exposure to a medical product, in this case, erenumab 140 mg. Then, we studied the change in salivary CGRP levels in patients treated with 3 doses of erenumab. In this analysis, we included 7 EM patients and 16 CM patients, with a total of 167 salivary samples (66 pre-treatment and 105 post-treatment).

In the multiple GLMM adjusted by patient's age, we found that **the three-way interaction of Time** (w0 vs. w12), **depression at baseline** (yes vs. no) and **headache frequency at baseline** (MHD, d/mo) was the only independent variable statistically significantly **associated to the salivary CGRP levels change** in the final model.

After treatment, **salivary CGRP levels in patients within all spectrum of migraine frequency were converged to similar CGRP values.** In presence of **depressive symptoms, CGRP levels do not reach such a convergence**.

Pairwise comparison post-hoc tests after FDR adjustment of salivary CGRP post-treatment between groups (HC, EM and CM) and the presence of depressive symptoms showed similar results than values obtained from pre-treated patients: in absence of depression, both EM and CM patients presented higher values of basal CGRP than HC but in presence of depressive symptoms, only CM patients had statistically significantly higher basal CGRP than HC.

6. OVERALL SUMMARY OF THE DISCUSSION

With this doctoral thesis we have been able to monitor CGRP levels during a whole month in a non-invasive way through saliva samples. This allowed to differentiate different types of migraine attacks and therefore to start working in a **molecular classification of migraine** based on the pathophysiology.

Additionally, salivary CGRP levels were measured in all the migraine frequency spectrum and before and after treatment with erenumab. Results obtained support the quantification of **CGRP in saliva as a potential diagnostic biomarker in migraine and predictive of the therapeutic response to treatment with anti-CGRP monoclonal antibodies**.

These results represent a step forward in **the development of both precision and personalized medicine in migraine**.

6.1 Saliva as biofluid to measure CGRP

Saliva is as a safer, readily accessible and noninvasive method. It has already been used as a diagnostic tool to study the activation of trigeminal nerves in migraine conditions (154). In addition, **it contains a wide variety of neuropeptides due to salivary gland innervations by the nerve terminals of the trigeminovascular system and could, therefore, provide a certain clue about nervous system pathophysiology** (152). These suitable characteristics allowed us to have repeated samples from patients.

Several conditions must be taken into account when choosing saliva as a substrate to measure CGRP and they are explained in detail in the first paper. Standardized procedures in collection and analysis are mandatory in order to be able to use them as a valuable resource to gain scientific information in the migraine field.

6.2 CGRP over the migraine frequency spectrum

Taking together information from participants included in the first and second study allowed us to study salivary CGRP levels over the migraine frequency spectrum.

In our first study we carefully recruited young women with very LFEM and without comorbidities. This allowed us to properly study which we could consider as “pure migraine” in the interictal period. Our results showed that **interictal salivary levels of CGRP were significantly higher in patients with EM**, even if they suffer from infrequent attacks, compared to HC. Bellamy et al. also found that patients with EM had elevated salivary levels of CGRP outside the attacks compared to controls (159). There is only one previous study that found higher CGRP levels in HC (152). Other than saliva, previous studies using other substrates such as plasma (74,75) or tear fluid (146) also showed higher levels of CGRP outside the attacks in patients with EM.

In the second study we found **an association between greater CGRP levels and higher headache frequency, which may support a greater activation of the trigeminovascular system in more severe forms of the disease** (227,228). These levels progressively increase as headache frequency worsened from low frequency EM to CM, without statistically significant differences in CGRP levels between migraine groups. Previously, there are very few studies measuring CGRP levels over the headache frequency spectrum. These previous studies, albeit using different samples than saliva, showed different results. In the case of Cernuda-Morollón et al. study, and in line with our results, CM patients exhibited the highest plasma levels of CGRP, followed by EM patients and HC (76). In contrast, Lee et al. did not find any significant differences between groups (77). Kamm et al. found increased tear fluid levels of CGRP in migraine patients compared to healthy subjects also without differences between episodic and CM patients (146). Interestingly, a salivary CGRP cut-off point of 103.75 pg/mL was found, which allows us to create levels of normality vs disease with a threshold that gives a 72% of possibility of suffering from migraine when surpassed, and only includes a 7% of controls.

Despite the small size, we found that presence of **depressive symptoms had an impact on baseline CGRP levels**. The clinical relationship between depression and migraine, in particular CM, is well-established in epidemiological studies (16). Furthermore, there is a linear relationship between the number of headache days and degree of depression (113). There is evidence of shared genetic polymorphisms between migraine and depression as well (41,114). Our study not only supports this linear relationship between headache frequency and depression but also states that CGRP could play an important role in this relation. Perhaps one could hypothesize in its neuroinflammatory central function, (54,122) for which anti-CGRP mAbs do not have as much access due to their very low permeability through the BBB (101). While neurobiological correlates have only partially been elucidated, altered levels of CGRP-LI in animal model (116,117) and in the CSF of depressed patients were reported (118), suggesting that CGRP may be involved in the pathophysiology and/or be a trait marker of depressive disorders. Increased brain levels of CGRP have been found a well-established rat model of depression and interestingly, antidepressants did not have effect on the brain level of this peptide (116). This relationship between the immune system and the presence of psychiatric disorders has gained interest in recent years and it would appear that the severity of depressive symptoms is likely to be modulated by the degree of inflammation (115,229). However, the field of psychiatry and inflammation is in its first stages and needs further investigation to evaluate the role of CGRP and other neuroinflammatory markers in depression (119).

In summary, CGRP can differentiate migraine from controls, even more in presence of depressive symptoms. **Taking all these findings together, CGRP levels can support migraine diagnosis** in patients in which diagnostic criteria are not so clear or it exist barriers to optimal communication or recall bias, also prompting us to recruit ideal participants for clinical trials.

6.3 CGRP during migraine attacks

At a molecular level, our first study revealed that **salivary levels of CGRP are dynamic and change over a migraine attack**. Our study is the first one to see this gradual change of CGRP levels during a complete attack, and confirm previous studies which also showed **an increase in salivary levels of CGRP during the ictal phase, interpreting it as a sign of trigeminovascular activation** (152,177). This change reflects that **CGRP levels fluctuate in a disease which is dynamic and has different states or phases** considering intraindividual change.

Based on our FC analysis, we observed three **different types of patients**: those with **CGRP dependent migraine attacks**, those with **non-CGRP dependent migraine attacks** and those with two types of migraine attacks. **Some diagnostic migraine symptoms such as photo and phonophobia were significantly related to presence of elevated CGRP**. Biochemically it seems that CGRP may also have a role in photophobia because of the findings provided by animal models. Genetically engineered mice with elevated expression in nervous tissue of the human receptor RAMP1 (an important and required subunit of the canonical CGRP receptor), spend less time in light environments than control littermates. In addition, intracerebroventricular administration of CGRP causes a significant increase in light aversion, compared with those that received vehicle, a response that is prevented with simultaneous treatment with the human CGRP receptor antagonist olcegepant (230). CGRP injection in control mice also caused the development of an aversion to strong light, a response that is attenuated by a triptan (231), indicating that activation of endogenous CGRP receptor can drive this hypersensitive response. One possible mechanism would be the of a trigeminal nociceptive pathway by the bright light (232). These studies combined demonstrate **the likely neural pathways involvement in the development of symptoms such as photophobia, and that CGRP, which is released in migraine, can contribute to these symptoms**, although it is not clear from which loci the effect is driven.

However, CGRP may not be increased in all migraine patients, or it may be that, as for rodents, it depends on the particular individual's gene expression for how

susceptible they are to the effects of CGRP (123). Thus, CGRP is not the only neuropeptide involved in migraine pain generation and maintenance.

To sum up, **intraindividual CGRP levels can change over the course of a migraine attack**. Moreover, EM patients with CGRP dependent attacks presented with classical migraine clinical symptoms, creating different phenotypes of migraine patients. So, our results may support the concept of classifying migraine from a pathophysiological point of view at a theoretical level. This information might help us to start practicing precision medicine in migraine.

6.4 CGRP levels before erenumab treatment

Relationship between CGRP levels and CGRP-related migraine-specific therapies is an interesting matter of study since it might contribute to the development of precision medicine in migraine. We found that, **pre-treatment headache frequency and CGRP levels were the only independent statistically significant predictors associated to erenumab response.** Thereby, in patients with HFEM, higher CGRP levels at baseline were statistically significantly associated to a higher probability of response. Surprisingly, as headache frequency worsens and CM is reached, the likelihood to response based on CGRP levels is reduced, indicating again the fact that a peripheral regulation of CGRP is not enough in these patients**.** However, we already know from clinical trials that CM also responds to treatment but (98) according to our results, the response in CM patients is not as influenced by CGRP levels at baseline as is in EM patients, and therefore it is clear that in CM there must have other biological or genetic components involved. On the other hand, it is important to note the importance of prescribe preventive treatments earlier in the development of the disease since it seems that there is a possibility of reverting migraine molecularly before it reaches a no-return turning point with the current therapeutic option. In our study we demonstrate that in migraine there is not only a clinical spectrum but also a molecular spectrum with a pathophysiological meaningful turning point of the disease, which is related to impact and treatment response. Furthermore, there seems to be a pharmacodynamic explanation for

the fact that the higher CGRP concentration, the worse the treatment response. In a recent study *in vitro*, it has been shown that in presence of human αCGRP there is a reduction in binding of erenumab to SK-N-MC cells. This observed reduction may be due to competition for receptor binding and/or ligand-induced receptor down-regulation (233). Therefore, it seems that the excess of CGRP in the trigeminovascular system as headache frequency worsens might have an impact on erenumab response.

6.5 CGRP levels after erenumab treatment

Finally, we studied change in CGRP levels after treatment. Erenumab is the first human IgG2 monoclonal antibody developed for migraine. It is directed to the CGRP binding site of the canonical CGRP receptor, made up by the RAMP1 and CLR subunits, and therefore has differences in the CGRP pathway compared to the other CGRP agents (234,235). **Change in CGRP levels after treatment was related to the interaction between time, depression and headache frequency at baseline.** Thus, after 12 weeks, CGRP levels in patients within all spectrum of headache frequency converged to similar CGRP values whereas in presence of depressive symptoms, CGRP levels do not reach such a convergence. The effect of **depression seems to be related with the need for more time** to the aforementioned convergence and maybe with longer time of treatment this CGRP could be regulated.

Furthermore, it is suggested that long-term blockade of CGRP receptors could induce an increase in systemic CGRP levels via a classical up-regulation mechanism (236). In line with our results, a previous exploratory study showed that plasma CGRP levels were increased after 6 months of treatment although without statistically significance probably due to small size of the study (237).

6.6 Strengths and limitations

This doctoral thesis has several strengths. Our studies have a longitudinal approach collecting samples over a 30-day period in the first study and over 7 days in the second one, which in migraine is less frequent. Moreover, our study participants were strictly and carefully selected, without any other preventative treatment, resulting in a very homogeneous sample. Participants in this study are well phenotyped and diagnosed by a specialized neurologist, including all the spectrum of migraine frequency as well as psychiatric comorbidities, allowing for the discovery of interesting interactions in the model and avoiding confounding factors.

To the best of our knowledge, these are the first results reporting the biological association between migraine, CGRP and the presence of depressive symptoms; as well as the fact that CGRP could be perhaps started to be considered as a molecular biomarker predicting initial response to treatment in patients with HFEM.

Studies included in this thesis have some limitations. First, the small size of the sample, in particular in patients with CM. Secondly, depression was assessed through depressive symptoms according to a questionnaire, without a confirmation by a psychiatrist. However, The BDI-II is widely used as an assessment tool by healthcare professionals and researchers in a variety of settings. BDI-II is a validated questionnaire that measures severity of depression. Thirdly, since CM patients have a persistent headache state, it was difficult to get samples in a real interictal state in particular at baseline. Finally, the short period of treatment (3 months), which might not give a full explanation of what happens with patients who have depressive symptoms.

6.7 Challenges of considering CGRP as a biomarker

There are some inherent challenges of CGRP as a molecule; such as, its short half-life and the low circulating plasma levels (picomole range). In addition, migraine is a very heterogeneous disorder and clinical manifestations can vary

intra and interindividually. This disease is likely caused by a spectrum of genetic, epigenetic, and environmental factors, and, for that reason, it remains a diagnostic and therapeutic challenge to clinicians.

There are also some methodological issues. The generation of sensitive and reliable assays to accurately measure neuropeptides remains problematic. The current assays to measure these neuropeptides are developed for “research-use only”, and do not meet more stringent regulatory requirements of clinical diagnostic-grade assays. For example, assays used in CGRP studies are extremely variable and not well validated. Suboptimal assay validation leads to an inability to confidently determine whether the assay only detects the biomarker of interest. For example, ELISA assays are used to detect CGRP, but these assays could also detect close relatives, such as αCGRP versus βCGRP versus amylin (~40% identical sequence to CGRP). Most assays (ie, RIA or ELISA) use antibodies to detect peptides but antibodies can often detect both peptide fragments and the intact peptide. Each assay must initially be validated through a rigorous process that accounts for sensitivity, specificity, interassay and intra-assay variability, and the effect of matrix interference (ie, serum or plasma). Further methodologic improvements or generation of novel antibodies may be required to facilitate assay development for this class of biomarkers. The way of measuring CGRP today is slightly different; there are sandwich methods that have antibodies directed towards both the N- and C-terminals, hence the intact molecule is measured. This is clearly a step in the right direction.

Regarding the kit (Cusabio®), it was intended for the following samples types: serum, plasma, cell culture supernates and tissue homogenates. However, we found it reliable to measure salivary CGRP. In line with this issue, a previous study used the same kit for measuring CGRP in tears (46).On the other hand, it measured beta-type CGRP theoretically, although the company did not mention this initially in their kit, so, cross-reactivity could not be discarded (according to manufacturer specifications). It is necessary to say that at the time we performed the first study there were no kits that specifically distinguished between different components of CGRP (alpha and beta). However, CGRP isoforms differ in two amino acids from each other and they are very similar in their biological activities

(129). β-CGRP has been shown to be released alongside α-CGRP in the vascular system (131). Thus. it is now becoming clear that both isoforms can be expressed in the nervous system, depending on situation. Nowadays there exists more specific kits able to specifically measure and differentiate alpha and beta types (238).

An absence of standardized methods for samples and data collection hampers comparisons between studies. There are many aspects of sample processing that can affect results, such as use of different substrates (plasma, serum, saliva..), time delays, presence of protease inhibitors (which can interfere in assays), composition of storage tubes, and freeze–thaw cycles. All samples must also fall within the linear range of the assay. Researchers should follow appropriate guidance documents (ie. Bioanalytical Method Validation by the US Food and Drug Administration) and adequately report their methods. Commercially available assays infrequently have sufficient validation to give confidence in the results (194).

Finally, published studies, including ours, have small number of patient samples, and independent cohorts require validation in larger independent clinical cohorts, which are sufficiently statistically powered.

7. CONCLUSIONS

1. Salivary CGRP levels vary intraindividually according to the migraine cycle and define CGRP dependent and non-CGRP dependent migraine attacks.

2. Salivary CGRP levels are higher in migraine patients and they increase proportionally over the migraine frequency spectrum. In presence of depressive symptoms, CGRP levels increase even more and can differentiate episodic and chronic migraine patients.

3. Higher CGRP levels at baseline are associated with a higher probability of having 50% or greater reduction in migraine frequency after 3-months of erenumab treatment.

4. Heterogeneity in salivary CGRP is regulated by erenumab in such a manner that CGRP levels converge after 3-months of treatment, whilst presence of depressive symptoms does not allow this convergence.

5. Saliva is a reliable biofluid to measure CGRP in patients with migraine. The methodology used is reproducible and the quantified CGRP levels can be interpreted as a reflection of the trigeminovascular activation.

8. FUTURE RESEARCH

The results of this thesis have had a relevant impact in the scientific community and in society according to the press interest, providing a different methodology to measure CGRP in saliva of migraine patients, suggesting the possibility of developing a migraine deep phenotyping based on the molecular profile of patients, driving CGRP as a potential molecular biomarker with different possible purposes such as diagnostic, predictive and therapeutic response biomarker to treatment with anti-CGRP mAbs. Future research should be focused on finding a molecular, anatomical, genetical and physiological way of defining migraine which, in turn, could help develop a pathophysiological driven classification.

One of the gaps in the development of a biomarker for its use in clinical diagnostics is to fill the gap between biomarker discovery and verification/validation. This thesis has laid the ground to continue expanding this and has found a practical, stable, reproducible way of measuring CGRP through saliva.

Other interesting questions arise after this thesis. In our future research, we will try to disentangle which are the non-CGRP mechanisms in those patients with “non-CGRP dependent” migraine. Since the other mAbs (galcanezumab, fremanezumab, eptinezumab) act against the ligand rather than the receptor, it will be interesting to study whether CGRP levels also serve as a predictive biomarker response in these treatments. Furthermore, we will try to elucidate whether the other CGRP therapies (gepants) are linked with CGRP levels in the same way as mAbs, since they are able to cross the BBB. We will keep on studying the relationship between depression, migraine and CGRP as neuroinflammation in both diseases is still controversial. Lastly, we will try to shed light on the correlation between the presence of CGRP SNPs and migraine clinical manifestations.

9. BIBLIOGRAPHIC REFERENCES

1. Headache Classification Committee of the International Headache Society. The International Classification of Headache Disorders, 3rd edition. *Cephalalgia* 2018;38:1–211.
2. Ashina M, Katsarava Z, Do TP, Buse DC, Pozo-Rosich P, Özge A, et al. Migraine: epidemiology and systems of care. *Lancet* 2021;397:1485–95.
3. Vos T, Abajobir AA, Abbafati C, Abbas KM, Abate KH, Abd-Allah F, et al. Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990-2016: A systematic analysis for the Global Burden of Disease Study 2016. *Lancet* 2017;390:1211–59.
4. Steiner TJ, Stovner LJ, Jensen R, Uluduz D, Katsarava Z; Lifting The Burden: the Global Campaign against Headache. Migraine remains second among the world's causes of disability, and first among young women: findings from GBD2019. *J Headache Pain* 2020;21:137.
5. Buse DC, Loder EW, Gorman JA, Stewart WF, Reed ML, Fanning KM, et al. Sex differences in the prevalence, symptoms, and associated features of migraine, probable migraine and other severe headache: Results of the American Migraine prevalence and prevention (AMPP) study. *Headache* 2013;53:1278–99.
6. Natoli JL, Manack A, Dean B, Butler Q, Turkel CC, Stovner L, et al. Global prevalence of chronic migraine: A systematic review. *Cephalalgia* 2010;30:599–609.
7. Buse DC, Manack AN, Fanning KM, Serrano D, Reed ML, Turkel CC, et al. Chronic migraine prevalence, disability, and sociodemographic factors: Results from the American migraine prevalence and prevention study. *Headache* 2012;52:1456–70.
8. Bigal ME, Serrano D, Buse D, Scher A, Stewart WF, Lipton RB. Acute migraine medications and evolution from episodic to chronic migraine: A longitudinal population-based study. *Headache* 2008;48:1157–68.
9. Lipton RB, Manack Adams A, Buse DC, Fanning KM, Reed ML. A Comparison of the Chronic Migraine Epidemiology and Outcomes (CaMEO) Study and American Migraine Prevalence and Prevention (AMPP) Study: Demographics and Headache-Related Disability.

Headache 2016;56:1280–9.

10. Tsai C-K, Tsai C-L, Lin G-Y, Yang F-C, Wang S-J. Sex Differences in Chronic Migraine: Focusing on Clinical Features, Pathophysiology, and Treatments. *Curr Pain Headache Rep* 2022;26:347–55.
11. Lipton RB. Tracing transformation: Chronic migraine classification, progression, and epidemiology. *Neurology* 2009;72(5 Suppl):S3-7.
12. Lipton RB, Bigal ME. Migraine: Epidemiology, impact, and risk factors for progression. *Headache* 2005;45 (Suppl 1):S3-S13.
13. Burch RC, Buse DC, Lipton RB. Migraine: Epidemiology, Burden, and Comorbidity. *Neurol Clin* 2019;37:631–49.
14. Brandes JL. The migraine cycle: Patient burden of migraine during and between migraine attacks. *Headache* 2008;48:430–41.
15. Katsarava Z, Buse DC, Manack AN, Lipton RB. Defining the differences between episodic migraine and chronic migraine. Curr Pain Headache Rep. 2012;16(1):86–92.
16. Buse DC, Manack A, Serrano D, Turkel C, Lipton RB. Sociodemographic and comorbidity profiles of chronic migraine and episodic migraine sufferers. *J Neurol Neurosurg Psychiatry* 2010;81:428–32.
17. Bigal ME, Lipton RB. Clinical course in migraine: conceptualizing migraine transformation. *Neurology* 2008;71:848–55.
18. Torres-Ferrús M, Quintana M, Fernandez-Morales J, Alvarez-Sabin J, Pozo-Rosich P. When does chronic migraine strike? A clinical comparison of migraine according to the headache days suffered per month. *Cephalalgia* 2017;37:104–13.
19. Serrano D, Lipton RB, Scher AI, Reed ML, Stewart WBF, Adams AM et al. Fluctuations in episodic and chronic migraine status over the course of 1 year: implications for diagnosis, treatment and clinical trial design. *J Headache Pain* 2017;18:1–12.
20. Gallardo VJ, Alpuente A, Pozo-Rosich P. Association of a Cyclical Migraine Phenotype With Disease Progression: A One-Year Time Series Analysis. *Neurology* 2022; 20;99:e1326-e1334.
21. Dodick DW. Migraine. *Lancet* 2018;391:1315-1330.
22. Blau JN. Migraine: theories of pathogenesis. *Lancet* 1992;339:1202–7.
23. Charles A. The evolution of a migraine attack - A review of recent evidence.

Headache 2013;53:413-9.

24. Linde M, Mellberg A, Dahlöf C. The natural course of migraine attacks. A prospective analysis of untreated attacks compared with attacks treated with a triptan. *Cephalalgia* 2006;26:712–21.
25. Blau JN. Migraine prodromes separated from the aura: complete migraine. *Br Med J* 1980;281:658–60.
26. Schulte LH, May A. The migraine generator revisited: Continuous scanning of the migraine cycle over 30 days and three spontaneous attacks. *Brain* 2016;139:1987–93.
27. Schulte LH, Mehnert J, May A. Longitudinal Neuroimaging over 30 Days: Temporal Characteristics of Migraine. *Ann Neurol* 2020;87:646–51.
28. Cady R, Schreiber C, Farmer K, Sheftell F. Primary headaches: A convergence hypothesis. *Headache* 2002;42:204–16.
29. Maniyar FH, Sprenger T, Monteith T, Schankin C, Goadsby PJ. Brain activations in the premonitory phase of nitroglycerin-triggered migraine attacks. *Brain* 2014;137:232–41.
30. Giffin NJ, Ruggiero L, Lipton RB, Silberstein SD, Tvedskov JF, Olesen J, et al. Premonitory symptoms in migraine: An electronic diary study. *Neurology* 2003;60:935–40.
31. Pietrobon D, Moskowitz MA. Pathophysiology of migraine. *Annu Rev Physiol* 2013;75:365–91.
32. Schain AJ, Melo-Carrillo A, Strassman AM, Burstein R. Cortical spreading depression closes paravascular space and impairs glymphatic flow: Implications for migraine headache. *J Neurosci* 2017;37:2904–15.
33. Zhang X, Levy D, Kainz V, Noseda R, Jakubowski M, Burstein R. Activation of central trigeminovascular neurons by cortical spreading depression. *Ann Neurol 2011*;69:855–65.
34. Burstein R, Strassman A, Moskowitz M. Can cortical spreading depression activate central trigeminovascular neurons without peripheral input? Pitfalls of a new concept. *Cephalalgia* 2012;32:509–11.
35. Ayata C, Lauritzen M. Spreading depression, spreading depolarizations, and the cerebral vasculature. *Physiol Rev* 2015;95:953–93.
36. Noseda R, Burstein R. Migraine pathophysiology: Anatomy of the trigeminovascular pathway and associated neurological symptoms, cortical

spreading depression, sensitization, and modulation of pain. *Pain* 2013; 154 Suppl 1:10.

37. Giffin NJ, Lipton RB, Silberstein SD, Olesen J, Goadsby PJ. The migraine postdrome: An electronic diary study. *Neurology* 2016;0:309–13.
38. Lampl C, Thomas H, Stovner LJ, Tassorelli C, Katsarava Z, Laínez JM, et al. Interictal burden attributable to episodic headache: findings from the Eurolight project. *J Headache Pain* 2016;17:1–10.
39. Goadsby PJ, Lipton RB, Ferrari MD. Migraine--current understanding and treatment. *N Engl J Med* 2002;346:257–70.
40. Hautakangas H, Winsvold BS, Ruotsalainen SE, Bjornsdottir G, Harder AVE, Kogelman LJA, et al. Genome-wide analysis of 102,084 migraine cases identifies 123 risk loci and subtype-specific risk alleles. *Nat Genet* 2022;54:152–60.
41. Anttila V, Bulik-Sullivan B, Finucane HK, Walters RK, Bras J, Duncan L, et al. Analysis of shared heritability in common disorders of the brain. *Science* 2018;360:eaap8757.
42. Polderman TJC, Benyamin B, De Leeuw CA, Sullivan PF, Van Bochoven A, Visscher PM, et al. Meta-analysis of the heritability of human traits based on fifty years of twin studies. *Nat Genet* 2015;47:702–9.
43. Pietrobon D, Striessnig J. Neurobiology of migraine. *Nat Rev Neurosci* 2003;4:386–98.
44. Magis D, Lisicki M, Coppola G. Highlights in migraine electrophysiology: Are controversies just reflecting disease heterogeneity? *Curr Opin Neurol* 2016;29:320–30.
45. Ashina M, Hansen JM, Do TP, Melo-Carrillo A, Burstein R, Moskowitz MA. Migraine and the trigeminovascular system—40 years and counting. *Lancet Neurol* 2019;18:795–804.
46. Goadsby PJ, Holland PR, Martins-Oliveira M, Hoffmann J, Schankin C, Akerman S. Pathophysiology of migraine: A disorder of sensory processing. *Physiol Rev* 2017;97:553–622.
47. Vecchia D, Pietrobon D. Migraine: A disorder of brain excitatory-inhibitory balance? *Trends Neurosci* 2012;35:507–20.
48. Moskowitz MA. Neurogenic inflammation in the pathophysiology and treatment of migraine. *Neurology* 199;43(6 Suppl 3):S16-20. 49.

Pietrobon D. Migraine: New molecular mechanisms. *Neuroscientist* 2005;11:373–86.

50. Burstein R, Yarnitsky D, Goor-Aryeh I, Ransil BJ, Bajwa ZH. An association between migraine and cutaneous allodynia. *Ann Neurol* 2000;47:614–24.
51. Arulmani U, MaassenVanDenBrink A, Villalón CM, Saxena PR. Calcitonin gene-related peptide and its role in migraine pathophysiology. *Eur J Pharmacol* 2004;500:315–30.
52. Pozo-Rosich P, Storer RJ, Charbit AR, Goadsby PJ. Periaqueductal gray calcitonin gene-related peptide modulates trigeminovascular neurons. *Cephalalgia* 2015;35:1298–307.
53. Iyengar S, Ossipov MH, Johnson KW. The role of calcitonin gene-related peptide in peripheral and central pain mechanisms including migraine. *Pain* 2017:158:543–59.
54. Edvinsson L, Haanes KA, Warfvinge K. Does inflammation have a role in migraine? *Nat Rev Neurol* 2019;15:483–90.
55. Goadsby PJ, Edvinsson L. The trigeminovascular system and migraine: Studies characterizing cerebrovascular and neuropeptide changes seen in humans and cats. *Ann Neurol* 1993;33:48–56.
56. Goadsby PJ, Edvinsson L, Ekman R. Vasoactive peptide release in the extracerebral circulation of humans during migraine headache. *Ann Neurol* 1990;28:183–7.
57. Edvinsson L, Uddman R. Neurobiology in primary headaches. *Brain Res Rev* 2005;48:438–56.
58. Edvinsson L, Haanes KA, Warfvinge K, Krause DiN. CGRP as the target of new migraine therapies - Successful translation from bench to clinic. *Nat Rev Neurol* 2018;1:338–50.
59. Amara SG, Jonas V, Rosenfeld MG, Ong ES, Evans RM. Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. *Nature* 1982;298:240–4.
60. Durham PL. CGRP-Receptor Antagonists — A Fresh Approach to Migraine Therapy? *N Engl J Med* 2004;350:1073–5.
61. Goadsby PJ, Edvinsson L, Ekman R. Release of vasoactive peptides in the extracerebral circulation of humans and the cat during activation of the trigeminovascular system. *Ann Neurol* 1988;23:193–6.

62. Goadsby PJ, Edvinssont L. Sumatriptan reverses the changes in calcitonin gene-related peptide seen in the headache phase of migraine. *Cephalalgia* 1991;11:3–4.
63. Goadsby PJ, Edvinsson L. The trigeminovascular system and migraine: Studies characterizing cerebrovascular and neuropeptide changes seen in humans and cats. *Ann Neurol* 1993;33:48–56.
64. Sarchielli P, Pini LA, Zanchin G, Alberti A, Maggioni F, Rossi C, et al. Clinical-biochemical correlates of migraine attacks in rizatriptan responders and non-responders. *Cephalalgia* 2006;26:257–65.
65. Sarchielli P, Alberti A, Codini M, Floridi A, Gallai V. Nitric oxide metabolites, prostaglandins and trigeminal vasoactive peptides in internal jugular vein blood during spontaneous migraine attacks. *Cephalalgia* 2000;20:907–18.
66. Tvedskov JF, Lipka K, Ashina M, Iversen HK, Schifrer S, Olesen J. No increase of calcitonin gene-related peptide in jugular blood during migraine. *Ann Neurol* 2005;58:561–8.
67. Friberg L, Olesen J, Olsen TS, Karle A, Ekman R, Fahrenkrug J. Absence of vasoactive peptide release from brain to cerebral circulation during onset of migraine with aura. *Cephalalgia* 1994;14:47–54.
68. Gallai V, Sarchielli P, Floridi A, Trequattrini A, Franceschini M, Codini M, et al. Vasoactive Peptide Levels in The Plasma of Young Migraine Patients With and Without Aura Assessed Both Interictally and Ictally. *Cephalalgia* 1995;15:384–90.
69. Lassen LH, Haderslev PA, Jacobsen VB, Iversen HK, Sperling B, Olesen J. CGRP may play a causative role in migraine. *Cephalalgia* 2002;22:54–61.
70. Hansen JM, Hauge AW, Olesen J, Ashina M. Calcitonin gene-related peptide triggers migraine-like attacks in patients with migraine with aura. *Cephalalgia* 2010;30:1179–86.
71. Juhasz G, Zsombok T, Modos EA, Olajos S, Jakab B, Nemeth J, et al. NO-induced migraine attack: Strong increase in plasma calcitonin gene-related peptide (CGRP) concentration and negative correlation with platelet serotonin release. *Pain* 2003;106:461–70.
72. Thomsen LL, Olesen J. Nitric oxide in primary headaches. *Curr Opin Neurol* 2001;14:315–21.

73. Akerman S, Williamson DJ, Kaube H, Goadsby PJ. Nitric oxide synthase inhibitors can antagonize neurogenic and calcitonin gene-related peptide induced dilation of dural meningeal vessels. *Br J Pharmacol.* 2002;137:62–8.
74. Ashina M, Bendtsen L, Jensen R, Schifter S, Olesen J. Evidence for increased plasma levels of calcitonin gene-related peptide in migraine outside of attacks. *Pain* 2000;86:133–8.
75. Fusayasu E, Kowa H, Takeshima T, Nakaso K, Nakashima K. Increased plasma substance P and CGRP levels, and high ACE activity in migraineurs during headache-free periods. *Pain* 2007;128:209–14.
76. Cernuda-Morollón E, Larrosa D, Ramón C, Vega J, Martínez-Camblor P, Pascual J. Interictal increase of CGRP levels in peripheral blood as a biomarker for chronic migraine. *Neurology* 2013;81:1191–6.
77. Lee MJ, Lee SY, Cho S, Kang ES, Chung CS. Feasibility of serum CGRP measurement as a biomarker of chronic migraine: a critical reappraisal. *J Headache Pain* 2018;19:53.
78. Cernuda-Morollõn E, Martínez-Camblor P, Ramõn C, Larrosa D, Serrano-Pertierra E, Pascual J. CGRP and VIP levels as predictors of efficacy of onabotulinumtoxin type A in chronic migraine. *Headache* 2014;54:987–95.
79. Domínguez C, Vieites-Prado A, Pérez-Mato M, Sobrino T, Rodríguez-Osorio X, López A, et al. CGRP and PTX3 as Predictors of Efficacy of Onabotulinumtoxin Type A in Chronic Migraine: An Observational Study. *Headach* 2018;58:78–87.
80. Durham PL, Niemann C, Cady R. Repression of stimulated calcitonin gene-related peptide secretion by topiramate. *Headache* 2006;46:1291–5.
81. Grupo de estudio de Cefaleas. Manual de Práctica Clínica en Cefaleas. Recomendaciones diagnóstico-terapéuticas la SEN. 2020.
82. Charles A, Pozo-Rosich P. Targeting calcitonin gene-related peptide: a new era in migraine therapy. *Lancet* 2019;394:1765–74.
83. Olesen J, Diener H-C, Husstedt IW, Goadsby PJ, Hall D, Meier U, et al. Calcitonin Gene–Related Peptide Receptor Antagonist BIBN 4096 BS for the Acute Treatment of Migraine. *N Engl J Med* 2004;350:1104–10.
84. Negro A, Martelletti P. Gepants for the treatment of migraine. *Expert Opin Investig Drugs* 2019;28:555–67.

85. Lipton RB, Croop R, Stock EG, Stock DA, Morris BA, Frost M, et al. Rimegepant, an Oral Calcitonin Gene–Related Peptide Receptor Antagonist, for Migraine. *N Engl J Med* 2019;381:142–9.
86. Voss T, Lipton RB, Dodick DW, Dupre N, Ge JY, Bachman R, et al. A phase IIb randomized, double-blind, placebo-controlled trial of ubrogepant for the acute treatment of migraine. *Cephalalgia* 2016;36:887–98.
87. Tao X, Yan Z, Meng J, Wang W, Dai Q, Zhou Q, et al. The efficacy and safety of atogepant for the prophylactic treatment of migraine: evidence from randomized controlled trials. *J Headache Pain* 2022;23:1–12.
88. Agencia Española de Medicamentos y Productos Sanitarios. Informe de posicionamiento terapéutico Erenumab 2019;1–8. Available from: *https://www.aemps.gob.es/medicamentosUsoHumano/informesPublicos/docs/IPT-erenumab-Aimovig.pdf?x33378*
89. Silberstein SD, Dodick DW, Bigal ME, Yeung PP, Goadsby PJ, Blankenbiller T, et al. Fremanezumab for the Preventive Treatment of Chronic Migraine. *N Engl J Med* 2017;377:2113–22.
90. Goadsby PJ, Reuter U, Hallström Y, Broessner G, Bonner JH, Zhang F, et al. A Controlled Trial of Erenumab for Episodic Migraine. *N Engl J Med* 2017;377:2123–32.
91. Detke HC, Goadsby PJ, Wang S, Friedman DI, Selzler KJ, Aurora SK. Galcanezumab in chronic migraine: The randomized, double-blind, placebo-controlled REGAIN study. *Neurology* 2018;91:E2211–21.
92. Skljarevski V, Matharu M, Millen BA, Ossipov MH, Kim BK, Yang JY. Efficacy and safety of galcanezumab for the prevention of episodic migraine: Results of the EVOLVE-2 Phase 3 randomized controlled clinical trial. *Cephalalgia* 2018;38:1442–54.
93. Dodick DW, Silberstein SD, Bigal ME, Yeung PP, Goadsby PJ, Blankenbiller T, et al. Effect of Fremanezumab compared with placebo for prevention of episodic migraine a randomized clinical trial. 2018;319:1999–2008.
94. Dodick DW, Ashina M, Brandes JL, Kudrow D, Lanteri-Minet M, Osipova V, et al. ARISE: A Phase 3 randomized trial of erenumab for episodic migraine. *Cephalalgia* 2018;38:1026–37.
95. Ashina M, Saper J, Cady R, Schaeffler BA, Biondi DM, Hirman J, et al.

Eptinezumab in episodic migraine: A randomized, double-blind, placebo-controlled study (PROMISE-1). *Cephalalgia* 2020;40:241–54.

96. Lipton RB, Goadsby PJ, Smith J, Schaeffler BA, Biondi DM, Hirman J, et al. Efficacy and safety of eptinezumab in patients with chronic migraine: PROMISE-2. *Neurology* 2020;94:E1365–77.
97. Stauffer VL, Dodick DW, Zhang Q, Carter JN, Ailani J, Conley RR. Evaluation of galcanezumab for the prevention of episodic migraine: The EVOLVE-1 randomized clinical trial. *JAMA Neurol 2018*;75:1080–8.
98. Tepper S, Ashina M, Reuter U, Brandes JL, Doležil D, Silberstein S, et al. Safety and efficacy of erenumab for preventive treatment of chronic migraine: a randomised, double-blind, placebo-controlled phase 2 trial. *Lancet Neurol* 2017;16:425–34.
99. Schwedt TJ, Kuruppu DK, Dong Y, Standley K, Yunes-Medina L, Pearlman E. Early onset of effect following galcanezumab treatment in patients with previous preventive medication failures. *J Headache Pain* 2021;22:1–9.
100. Yang C-P, Zeng B-Y, Chang C-M, Shih P-H, Yang C-C, Tseng P-T, et al. Comparative Effectiveness and Tolerability of the Pharmacology of Monoclonal Antibodies Targeting the Calcitonin Gene-Related Peptide and Its Receptor for the Prevention of Chronic Migraine: a Network Meta-analysis of Randomized Controlled Trials. *Neurother J Am Soc Exp Neurother* 2021;18:2639–50.
101. Yuan H, Lauritsen CG, Kaiser EA, Silberstein SD. CGRP Monoclonal Antibodies for Migraine: Rationale and Progress. *BioDrugs* 2017;31:487–501.
102. Melo-Carrillo A, Strassman AM, Nir RR, Schain AJ, Noseda R, Stratton J, et al. Fremanezumab—a humanized monoclonal anti-cgrp antibody—inhibits thinly myelinated (Aδ) but not unmyelinated (c) meningeal nociceptors. *J Neurosci* 2017;37:10587–96.
103. Melo-Carrillo A, Schain AJ, Stratton J, Strassman AM, Burstein R. Fremanezumab and its isotype slow propagation rate and shorten cortical recovery period but do not prevent occurrence of cortical spreading depression in rats with compromised blood-brain barrier. *Pain* 2020;161:1037–43.
104. Bigal ME, Lipton RB. The Epidemiology, Burden, and Comorbidities of

Migraine. *Neurol Clin* 2009;27:321–34.

105. Lipton RB, Fanning KM, Buse DC, Martin VT, Reed ML, Manack Adams A, et al. Identifying Natural Subgroups of Migraine Based on Comorbidity and Concomitant Condition Profiles: Results of the Chronic Migraine Epidemiology and Outcomes (CaMEO) Study. *Headache* 2018;58:933–47.
106. Lipton RB, Fanning KM, Buse DC, Martin VT, Hohaia LB, Adams AM, et al. Migraine progression in subgroups of migraine based on comorbidities: Results of the CaMEO Study. *Neurology* 2019;93:e2224–36.
107. Breslau N, Schultz LR, Stewart WF, Lipton RB, Lucia VC, Welch KMA. Headache and major depression: Is the association specific to migraine? *Neurology* 2000;54:308–13.
108. Breslau N, Lipton RB, Stewart WF, Schultz LR, Welch KMA. Comorbidity of migraine and depression: Investigating potential etiology and prognosis. *Neurology* 2003;60:1308–12.
109. Patel N V., Bigal ME, Kolodner KB, Leotta C, Lafata JE, Lipton RB. Prevalence and impact of migraine and probable migraine in a health plan. *Neurology* 2004;63:1432–8.
110. Modgill G, Jette N, Wang JL, Becker WJ, Patten SB. A population-based longitudinal community study of major depression and migraine. *Headache* 2012;52:422–32.
111. Mongini F, Keller R, Deregibus A, Raviola F, Mongini T, Sancarlo M. Personality traits, depression and migraine in women: A longitudinal study. *Cephalalgia* 2003;23:186–92.
112. Zwart JA, Dyb G, Hagen K, Ødegård KJ, Dahl AA, Bovim G, et al. Depression and anxiety disorders associated with headache frequency. The Nord-Trøndelag Health Study. *Eur J Neurol 2003*;10:147–52.
113. Ruscheweyh R, Müller M, Blum B, Straube A. Correlation of headache frequency and psychosocial impairment in migraine: A cross-sectional study. *Headache* 2014;54:861–71.
114. Yang Y, Zhao H, Boomsma DI, Ligthart L, Belin AC, Smith GD, et al. Molecular genetic overlap between migraine and major depressive disorder. *Eur J Hum Genet 2018*;26:1202–16.
115. Caldiroli A, Capuzzi E, Barkin JL, Grassi S, Esposito CM, Auxilia AM, et al. Is there an association between inflammatory/anti-oxidant markers and the

presence of psychotic symptoms or severity of illness in mood and psychotic disorders? A multi-centric study on a drug-free sample. *Brain, Behav Immun Health* 2022;22:100453.

116. Angelucci F, Ellenbroek BA, El Khoury A, Mathé AA. CGRP in a gene-environment interaction model for depression: Effects of antidepressant treatment. *Acta Neuropsychiatr* 2019;31:93–9.
117. Hashikawa-Hobara N, Ogawa T, Sakamoto Y, Matsuo Y, Ogawa M, Zamami Y, et al. Calcitonin gene-related peptide pre-administration acts as a novel antidepressant in stressed mice. *Sci Rep* 2015;5:1–14.
118. Mathé AA, Ågren H, Wallin A, Blennow K. Calcitonin gene-related peptide and calcitonin in the CSF of patients with dementia and depression: Possible disease markers. *Prog Neuro-Psychopharmacology Biol Psychiatry* 2002;26:41–8.
119. Sanchez C, El Khoury A, Hassan M, Wegener G, Mathé AA. Sex-dependent behavior, neuropeptide profile and antidepressant response in rat model of depression. *Behav Brain Res* 2018;351:93–103.
120. Breslau N. Psychiatric comorbidity in migraine. *Cephalalgia* 1998;18:56–61.
121. Gibson SJ, Polak JM, Bloom SR, Sabate IM, Mulderry PM, Ghatei MA, et al. Calcitonin gene-related peptide immunoreactivity in the spinal cord of man and of eight other species. *J Neurosci* 1984;4:3101–11.
122. Russell FA, King R, Smillie SJ, Kodji X, Brain SD. Calcitonin gene-related peptide: physiology and pathophysiology. *Physiol Rev* 2014;94:1099–142.
123. Benarroch EE. CGRP: Sensory neuropeptide with multiple neurologic implications. *Neurology* 2011;77:281–7.
124. Ishida-Yamamoto A, Tohyama M. Calcitonin gene-related peptide in the nervous tissue. *Prog Neurobiol* 1989;33:335–86.
125. Eftekhari S, Salvatore CA, Calamari A, Kane SA, Tajti J, Edvinsson L. Differential distribution of calcitonin gene-related peptide and its receptor components in the human trigeminal ganglion. *Neuroscience* 2010;169:683–96.
126. Edvinsson L, Goadsby PJ. Neuropeptides in the Cerebral Circulation: Relevance to Headache. *Cephalalgia* 1995;15:272–6.
127. Emeson RB, Hedjran F, Yeakley JM, Guise JW, Rosenfeld MG. Alternative

production of calcitonin and CGRP mRNA is regulated at the calcitonin-specific splice acceptor. *Nature 1989*;341:76–80.

128. Tippins JR, Di Marzo V, Panico M, Morris HR, MacIntyre I. Investigation of the structure/activity relationship of human calcitonin gene-related peptide (CGRP). *Biochem Biophys Res Commun* 1986;134:1306–11.
129. Poyner D, Marshall I. CGRP receptors: Beyond the CGRP1-CGRP2 subdivision? *Trends Pharmacol Sci* 2001;22:223.
130. Mulderry PK, Ghatei MA, Bishop AE, Allen YS, Polak JM, Bloom SR. Distribution and chromatographic characterisation of CGRP-like immunoreactivity in the brain and gut of the rat. *Regul Pept* 1985;12:133–43.
131. Li D, Chen BM, Peng J, Zhang YS, Li XH, Yuan Q, et al. Role of anandamide transporter in regulating calcitonin gene-related peptide production and blood pressure in hypertension. *J Hypertens* 2009;27:1224–32.
132. Hagner S, Haberberger R V., Overkamp D, Hoffmann R, Voigt KH, McGregor GP. Expression and distribution of calcitonin receptor-like receptor in human hairy skin. *Peptides* 2002;23:109–16.
133. Hagner S, Knauer J, Haberberger R, Göke B, Voigt K, McGregor GP. Calcitonin receptor-like receptor is expressed on gastrointestinal immune cells. *Digestion* 2002;66:197–203.
134. Hagner S, Stahl U, Knoblauch B, McGregor G, Lang R. Calcitonin receptor-like receptor: Identification and distribution in human peripheral tissues. *Cell Tissue Res* 2002;310:41–50.
135. Walker CS, Eftekhari S, Bower RL, Wilderman A, Insel PA, Edvinsson L, et al. A second trigeminal CGRP receptor: Function and expression of the AMY1 receptor. *Ann Clin Transl Neurol* 2015;2:595–608.
136. Eftekhari S, Edvinsson L. Possible sites of action of the new calcitonin gene-related peptide receptor antagonists. *Ther Adv Neurol Disord* 2010;3:369–78.
137. Miller S, Liu H, Warfvinge K, Shi L, Dovlatyan M, Xu C, et al. Immunohistochemical localization of the calcitonin gene-related peptide binding site in the primate trigeminovascular system using functional antagonist antibodies. *Neuroscience* 2016;328:165–83.

138. L Edvinsson, B B Fredholm, E Hamel, I Jansen CV. Perivascular peptides relax cerebral arteries concomitant with stimulation of cyclic adenosine monophosphate accumulation or release of an endothelium-derived relaxing factor in the cat. *Neurosci Lett* 1985;58:213–7.
139. Poyner DR. Calcitonin gene-related peptide: Multiple actions, multiple receptors. *Pharmacol Ther* 1992;56:23–51.
140. Edvinsson L, Ekman R, Goadsby PJ. Measurement of vasoactive neuropeptides in biological materials: Problems and pitfalls from 30 years of experience and novel future approaches. *Cephalalgia* 2010;30:761–6.
141. Hoffmann J, Wecker S, Neeb L, Dirnagl U, Reuter U. Primary trigeminal afferents are the main source for stimulus-induced CGRP release into jugular vein blood and CSF. *Cephalalgia* 2012;32:659–67.
142. Sarchielli P, Pini LA, Coppola F, Rossi C, Baldi A, Mancini ML, et al. Endocannabinoids in chronic migraine: CSF findings suggest a system failure. *Neuropsychopharmacology 2007*;32:1384–90.
143. Gallai V, Alberti A, Gallai B, Coppola F, Floridi A, Sarchielli P. Glutamate and nitric oxide pathway in chronic daily headache: evidence from cerebrospinal fluid. *Cephalalgia* 2003;23:166–74.
144. Sarchielli P, Alberti A, Floridi A, Gallai V. Levels of nerve growth factor in cerebrospinal fluid of chronic daily headache patients. *Neurology* 2001;57:132–4.
145. Van Dongen RM, Zielman R, Noga M, Dekkers OM, Hankemeier T, Van Den Maagdenberg AMJM, et al. Migraine biomarkers in cerebrospinal fluid: A systematic review and meta-analysis. *Cephalalgia* 2017;37:49–63.
146. Kamm K, Straube A, Ruscheweyh R. Calcitonin gene-related peptide levels in tear fluid are elevated in migraine patients compared to healthy controls. *Cephalalgia* 2019;39:1535–43.
147. Kamm K, Straube A, Ruscheweyh R. Baseline tear fluid CGRP is elevated in active cluster headache patients as long as they have not taken attack abortive medication. *Cephalalgia* 2021;41:69-77.
148. Mansson B, Nilsson BO, Ekstrom J. Effects of repeated infusions of substance P and vasoactive intestinal peptide on the weights of salivary glands subjected to atrophying influences in rats. *Br J Pharmacol* 1990;101:853–8.

149. Nagano T, Takeyama M. Enhancement of salivary secretion and neuropeptide (substance P, α-calcitonin gene-related peptide) levels in saliva by chronic anethole trithione treatment. *J Pharm Pharmacol* 2001;53:1697–702.
150. Tamatani M, Senba E, Tohyama M. Calcitonin gene-related peptide- and substance P-containing primary afferent fibers in the dorsal column of the rat. *Brain Res* 1989;495:122–30.
151. Saria A, Gamse R, Petermann J, Fischer A, Theodorsson-Norheim E LJ. Simultaneous release of several tachykinins and calcitonin gene-related peptide from rat spinal cord slices. *Neurosci Lett* 1986;63:310–4.
152. Nicolodi M, Bianco E Del. Sensory Neuropeptides (Substance P, Calcitonin Gene-Related Peptide) and Vasoactive Intestinal Polypeptide in Human Saliva: Their Pattern in Migraine and Cluster Headache. *Cephalalgia* 1990;10:39–50.
153. Nicolodi M. Nostril capsaicin application as a model of trigeminal primary sensory neuronal activation. *Cephalalgia* 1990;14:134–8.
154. Fischer HP, Eich W, Russell IJ. A possible role for saliva as a diagnostic fluid in patients with chronic pain. *Semin Arthritis Rheum* 1998;6:348–59.
155. D Haley, E Schiffman, C Baker MB. The comparison of patients suffering from temporomandibular disorders and a general headache population. *Headache* 1993;33:210–3.
156. Schiffman E, Haley D, Baker C, Lindgren B. Diagnostic Criteria for Screening Headache Patients for Temporomandibular Disorders. *Headache* 1995;35:121–4.
157. Proctor GB, Carpenter GH. Regulation of salivary gland function by autonomic nerves. *Auton Neurosci Basic Clin* 2007;133:3–18.
158. Endoh T. Modulation of voltage-dependent calcium channels by neurotransmitters and neuropeptides in parasympathetic submandibular ganglion neurons. *Arch Oral Biol* 2004;49:539–57.
159. Bellamy JL, Cady RK, Durham PL. Salivary levels of CGRP and VIP in rhinosinusitis and migraine patients. *Headache* 2006;46:24–33.
160. Dawidson I, Blom M, Lundeberg T, Theodorsson E, Angmar-Månsson B. Neuropeptides in the saliva of healthy subjects. *Life Sci* 1996;60:269–78.
161. Cady RK, Vause C V., Ho TW, Bigal ME, Durham PL. Elevated saliva

calcitonin gene-related peptide levels during acute migraine predict therapeutic response to rizatriptan. *Headache* 2009;49:1258–66.

162. Jang MU, Park JW, Kho HS, Chung SC, Chung JW. Plasma and saliva levels of nerve growth factor and neuropeptides in chronic migraine patients. *Oral Dis* 2011;17:187–93.
163. Cady R, Turner I, Dexter K, Beach ME, Cady R, Durham P. An exploratory study of salivary calcitonin gene-related peptide levels relative to acute interventions and preventative treatment with onabotulinumtoxinA in chronic migraine. *Headache* 2014;54:269–77.
164. Van Oosterhout WPJ, Schoonman GG, Garrelds IM, Danser AHJ, Chan KY, Terwindt GM, et al. A human capsaicin model to quantitatively assess salivary CGRP secretion. *Cephalalgia* 2015;35:675–82.
165. Choo RE, Huestis MA. Oral fluid as a diagnostic tool. *Clin Chem Lab Med* 2004;42:1273–87.
166. W C Parris, B V Sastry, J R Kambam, R J Naukam BWJ. Inmunoreactive Substance P in Human Saliva. *Ann N Y Acad Sci* 1993;694:308–10.
167. Sato Y, Itoh H, Suzuki Y, Tatsuta R, Takeyama M. Effect of pilocarpine on substance P and calcitonin gene-related peptide releases correlate with salivary secretion in human saliva and plasma. *J Clin Pharm Ther* 2013;38:19–23.
168. Maggi CA, Santicioli P, Geppetti P, Patacchini R, Frilli S, Astolfi M, et al. Simultaneous release of substance P- and calcitonin gene-related peptide (CGRP)-like immunoreactivity from isolated muscle of the guinea pig urinary bladder. *Neurosci Lett 1988*;87:163–7.
169. Fekrazad R, Sardarian A, Azma K, Karami M, Borhani-Haghighi A, Gharesi-Fard B, et al. Interictal levels of calcitonin gene related peptide in gingival crevicular fluid of chronic migraine patients. *Neurol Sci* 2018;39:1217–23.
170. Frese A, Schilgen M, Edvinsson L, Frandsen E, Evers S. Calcitonin gene-related peptide in cervicogenic headache. *Cephalalgia* 2005;25:700–3.
171. Ashina M, Bendtsen L, Jensen R, Schifter S, Jansen-Olesen I, Olesen J. Plasma levels of calcitonin gene-related peptide in chronic tension-type headache. *Neurology* 2000;55:1335–40.
172. Goadsby PJ, Edvinsson L. Human in vivo evidence for trigeminovascular

activation in cluster headache. Neuropeptide changes and effects of acute attacks therapies. *Brain* 1994;117:427-34.

173. Ziegeler C, May A. Facial presentations of migraine, TACs, and other paroxysmal facial pain syndromes. *Neurology 2019*;93:E1138–47.
174. Snoer A, Vollesen ALH, Beske RP, Guo S, Hoffmann J, Fahrenkrug J, et al. Calcitonin-gene related peptide and disease activity in cluster headache. *Cephalalgia* 2019;39:575–84.
175. Zidverc-Trajkovic J, Stanimirovic D, Obrenovic R, Tajti J, Vécsei L, Gardi J, et al. Calcitonin gene-related peptide levels in saliva of patients with burning mouth syndrome. *J Oral Pathol Med* 2009;38:29–33.
176. Sicuteri F, Fanciullacci M, Nicolodi M, Geppetti P, Fusco BM, Marabini S, et al. Substance P Theory: A Unique Focus on the Painful and Painless Phenomena of Cluster Headache. H*eadache* 1990;30:69–79.
177. Cady R, Turner I, Dexter K, Beach ME, Cady R, Durham P. An exploratory study of salivary calcitonin gene-related peptide levels relative to acute interventions and preventative treatment with onabotulinumtoxinA in chronic migraine. *Headache* 2014;54:269–77.
178. Jasim H, Carlsson A, Hedenberg-Magnusson B, Ghafouri B, Ernberg M. Saliva as a medium to detect and measure biomarkers related to pain. *Sci Rep* 2018;8:1–9.
179. Nagano T, Ikawa K, Takeyama M. Anethole trithione raises levels of α-calcitonin gene-related peptide in saliva of healthy subjects. *Pharm Pharmacol Commun* 1998;4:459–63.
180. Navazesh M, Christensen CM. A Comparison of Whole Mouth Resting and Stimulated Salivary Measurement Procedures. *J Dent Res* 1982;61:1158–62.
181. Jasim H, Olausson P, Hedenberg-Magnusson B, Ernberg M, Ghafouri B. The proteomic profile of whole and glandular saliva in healthy pain-free subjects. *Sci Rep* 2016;6:1–10.
182. Robb MA, McInnes PM, Califf RM. Biomarkers and surrogate endpoints: Developing common terminology and definitions. J*AMA* 2016;315:1107–8.
183. Califf RM. Biomarker definitions and their applications. *Exp Biol Med* 2018;243:213–21.
184. Insel TR. Digital phenotyping: Technology for a new science of behavior.

JAMA 2017;318:1215–6.

185. Dodick DW, Loder EW, Manack Adams A, Buse DC, Fanning KM, Reed ML, et al. Assessing barriers to chronic migraine consultation, diagnosis, and treatment: Results from the Chronic Migraine Epidemiology and Outcomes (CaMEO) study. *Headache* 2016;56:821–34.
186. Lipton RB, Serrano D, Holland S, Fanning KM, Reed ML, Buse DC. Barriers to the diagnosis and treatment of migraine: Effects of sex, income, and headache features. *Headache* 2013;53:81–92.
187. Ferroni P, Barbanti P, Spila A, Fratangeli F, Aurilia C, Fofi L, Egeo G GF. Circulating Biomarkers In Migraine. New Opportunities For Precision Medicine. *Curr Med Chem* 2019;26:6191-6206.
188. Durham P, Papapetropoulos S. Biomarkers associated with migraine and their potential role in migraine management. *Headache* 2013;53:1262–77.
189. Committee on the Review of Omics-Based Tests for Predicting Patient Outcomes in Clinical Trials; Board on Health Care Services; Board on Health Sciences Policy; Institute of Medicine. Evolution of Translational Omics: Lessons Learned and the Path Forward. Micheel CM, Nass SJ, Omenn GS, editors. Washington (DC): National Academies Press (US); 2012 Mar 23.
190. Frederiksen SD. Promote Biomarker Discovery by Identifying Homogenous Primary Headache Subgroups. *Headache* 2019;0:1–5.
191. Lipton RB, Serrano D, Pavlovic JM, Manack AN, Reed ML, Turkel CC, et al. Improving the classification of migraine subtypes: An empirical approach based on factor mixture models in the american migraine prevalence and prevention (AMPP) study. *Headache* 2014;54:830–49.
192. Pozo-Rosich P, Coppola G, Pascual J, Schwedt TJ. How does the brain change in chronic migraine? Developing disease biomarkers. *Cephalalgia* 2020;119–29.
193. Riesco N, Cernuda-Morollón E, Pascual J. Neuropeptides as a Marker for Chronic Headache. *Curr Pain Headache Rep* 2017;21.
194. Ashina M, Terwindt GM, Al-Karagholi MAM, de Boer I, Lee MJ, Hay DL, et al. Migraine: disease characterisation, biomarkers, and precision medicine. *Lancet* 2021;397:1496–504.
195. Ferreira KS, Dhillon H, Velly AM. The role of a potential biomarker in

patients with migraine: review and new insights. *Expert Rev Neurother* 2021;21:817–31.

196. Martami F, Razeghi Jahromi S, Togha M, Ghorbani Z, Seifishahpar M, Saidpour A. The serum level of inflammatory markers in chronic and episodic migraine: a case-control study. *Neurol Sci* 2018;39:1741–9.
197. Yazar HO, Yazar T, Aygün A, Kaygisiz Ş, Kirbaş D. Evaluation of simple inflammatory blood parameters in patients with migraine. *Ir J Med Sci* 2020;189:677–83.
198. Dini E, Mazzucchi S, De Luca C, Cafalli M, Chico L, Gerfo A Lo, et al. Plasma levels of oxidative stress markers, before and after BoNT/A Treatment, in chronic migraine. *Toxins (Basel)* 2019;11:1–7.
199. Bougea A, Spantideas N, Galanis P, Katsika P, Boufidou F, Voskou P, et al. Salivary inflammatory markers in tension type headache and migraine: the SalHead cohort study. *Neurol Sci* 2020;41:877–84.
200. Goadsby PJ. Autonomic nervous system control of the cerebral circulation. *Handb Clin Neurol* 2013;117:193-201.
201. Tajti J, Szok D, Majláth Z, Tuka B, Csáti A, Vécsei L. Migraine and neuropeptides. *Neuropeptides* 2015;52:19–30.
202. Edvinsson L, Goadsby PJ, Uddman R. Amylin: localization, effects on cerebral arteries and on local cerebral blood flow in the cat. *ScientificWorldJournal* 2001;1:168–80.
203. Irimia P, Martínez-Valbuena I, Mínguez-Olaondo A, Domínguez-Vivero C, Sánchez-Arias JA, Martínez-Vila E, et al. Interictal amylin levels in chronic migraine patients: A case-control study. *Cephalalgia* 2021;41:604–12.
204. Edvinsson L, Ekman R. Distribution and dilatory effect of vasoactive intestinal polypeptide (VIP) in human cerebral arteries. *Peptides* 1984;5:329–31.
205. Riesco N, Cernuda-Morollón E, Martínez-Camblor P, Pérez-Alvarez AI, Verano L, García-Cabo C, et al. Relationship between serum levels of VIP, but not of CGRP, and cranial autonomic parasympathetic symptoms: A study in chronic migraine patients. *Cephalalgia* 2017;37:823–7.
206. Cernuda-Morollón E, Martínez-Camblor P, Alvarez R, Larrosa D, Ramón C, Pascual J. Increased VIP levels in peripheral blood outside migraine attacks as a potential biomarker of cranial parasympathetic activation in

chronic migraine. *Cephalalgia* 2015;35:310–6.

207. Schytz HW, Birk S, Wienecke T, Kruuse C, Olesen J, Ashina M. PACAP38 induces migraine-like attacks in patients with migraine without aura. *Brain* 2009;132:16–25.
208. Tajti J, Uddman R, Möller S, Sundler F, Edvinsson L. Messenger molecules and receptor mRNA in the human trigeminal ganglion. *J Auton Nerv Syst* 1999;76:176–83.
209. Edvinsson L, Hara H, Uddman R. Retrograde tracing of nerve fibers to the rat middle cerebral artery with True Blue: Colocalization with different peptides. *J Cereb Blood Flow Metab* 1989;9:212–8.
210. Zagami AS, Edvinsson L, Goadsby PJ. Pituitary adenylate cyclase activating polypeptide and migraine. *Ann Clin Transl Neurol* 2014;1:1036–40.
211. Tuka B, Helyes Z, Markovics A, Bagoly T, Szolcsányi J, Szabó N, et al. Alterations in PACAP-38-like immunoreactivity in the plasma during ictal and interictal periods of migraine patients. *Cephalalgia* 2013;33:1085–95.
212. Han X, Dong Z, Hou L, Wan D, Chen M, Tang W, et al. Interictal plasma pituitary adenylate cyclase-activating polypeptide levels are decreased in migraineurs but remain unchanged in patients with tension-type headache. *Clin Chim Acta* 2015;450:151–4.
213. Cernuda-Morollón E, Riesco N, Martínez-Camblor P, Serrano-Pertierra E, García-Cabo C, Pascual J. No Change in Interictal PACAP Levels in Peripheral Blood in Women With Chronic Migraine. *Headache* 2016;56:1448–54.
214. Riesco N, Cernuda-Morollón E, Martínez-Camblor P, Pérez-Pereda S, Pascual J. Peripheral, Interictal Serum S100B Levels are Not Increased in Chronic Migraine Patients. *Headache* 2020;60:1705–11.
215. Gokdemir MT, Nas C, Gokdemir GS. Pentraxin 3 level in acute migraine attack with aura: Patient management in the emergency department. *Am J Emerg Med* 2020;38:38–42.
216. Ceylan M, Bayraktutan OF, Becel S, Atis Ö, Yalcin A, Kotan D. Serum levels of pentraxin-3 and other inflammatory biomarkers in migraine: Association with migraine characteristics. *Cephalalgia* 2016;36:518–25.
217. Domínguez-Vivero C, Leira Y, López-Ferreiro A, Saavedra M, Rodríguez-

Osorio X, Sobrino T, et al. Pentraxin 3 (PTX3): A molecular marker of endothelial dysfunction in chronic migraine. *J Clin Med 2020*;9:1–11.

218. Leira Y, Ameijeira P, Domínguez C, López-Arias E, Ávila-Gómez P, Pérez-Mato M, et al. Severe periodontitis is linked with increased peripheral levels of sTWEAK and PTX3 in chronic migraineurs. *Clin Oral Investig* 2020;24:597–606.
219. Nam JH, Lee HS, Kim J, Kim J, Chu MK. Salivary glutamate is elevated in individuals with chronic migraine. *Cephalalgia* 2018;38:1485–92.
220. Campos F, Sobrino T, Pérez-Mato M, Rodríguez-Osorio X, Leira R, Blanco M, et al. Glutamate oxaloacetate transaminase: A new key in the dysregulation of glutamate in migraine patients*. Cephalalgia* 2013;33:1148–54.
221. Ferrari A, Spaccapelo L, Pinetti D, Tacchi R, Bertolini A. Effective prophylactic treatments of migraine lower plasma glutamate levels. *Cephalalgia* 2009;29:423–9.
222. Sayer R, Law E, Connelly PJ, Breen KC. Association of a salivary acetylcholinesterase with Alzheimer's disease and response to cholinesterase inhibitors. *Clin Biochem* 2004;3:98–104.
223. Jeromin A, Bowser R. Biomarkers in Neurodegenerative Diseases. *Adv Neurobiol* 2017;15:491-528.
224. Banote RK, Akel S, Zelano J. Blood biomarkers in epilepsy. *Acta Neurol Scand* 2022;146:362-368.
225. Liu Z, Yang C, Wang X, Xiang Y. Blood-Based Biomarkers: A Forgotten Friend of Hyperacute Ischemic Stroke. *Front Neurol* 2021;12:634717.
226. Yang J, Hamade M, Wu Q, Wang Q, Axtell R, Giri S, Mao-Draayer Y. Current and Future Biomarkers in Multiple Sclerosis. *Int J Mol Sci* 2022;23:5877.
227. Seybold VS. The role of peptides in central sensitization. *Handb Exp Pharmaco* 2009:451-91.
228. Strassman AM, Raymond SA, Burstein R. Sensitization of meningeal sensory neurons and the origin of headaches. *Nature 1996*;384:356–8.
229. Buchmann A, Ritter C, Müller ST, Haynes M, Ghisleni C, Tuura R, et al. Associations between heart rate variability, peripheral inflammatory markers and major depressive disorder. *J Affect Disord* 2022;304:93–101.

230. Russo AF, Kuburas A, Kaiser EA, Raddant AC, Recober A. A potential preclinical migraine model: CGRP-sensitized mice. *Mol Cell Pharmacol* 2009;1:264–70.
231. Kaiser EA, Kuburas A, Recober A, Russo AF. Modulation of CGRP-induced light aversion in wild-type mice by a 5-HT1B/D agonist. *J Neurosci* 2012;32:15439–49.
232. Okamoto K, Tashiro A, Chang Z, Bereiter DA. Bright light activates a trigeminal nociceptive pathway. *Pain 2010*;149:235–42.
233. Bhakta M, Vuong T, Taura T, Wilson DS, Stratton JR, Mackenzie KD. Migraine therapeutics differentially modulate the CGRP pathway. *Cephalalgia* 2021;41:499–514.
234. Al-Hassany L, Goadsby PJ, Danser AHJ, MaassenVanDenBrink A. Calcitonin gene-related peptide-targeting drugs for migraine: how pharmacology might inform treatment decisions. *Lancet Neurol* 2022;21:284–94.
235. Amgen Inc. Erenumab (AimovigTM):US prescribing information. 2018. https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/761077s000lbl.pdf (accessed Feb 21, 2021).
236. Tringali G, Navarra P. Anti-CGRP and anti-CGRP receptor monoclonal antibodies as antimigraine agents. Potential differences in safety profile postulated on a pathophysiological basis. *Peptides* 2019;116:16–21.
237. Tringali G, Vollono C, Calabresi P, Navarra P. A proof-of-concept study on CGRP plasma levels of migraineurs during a 6-month treatment with ERENUMAB. *J Headache Pain* 2020;21:20–1.
238. Messlinger K, Vogler B, Kuhn A, Sertel-Nakajima J, Frank F, Broessner G. CGRP measurements in human plasma – a methodological study. *Cephalalgia* 2021;41:1359–73.

10. ANEX: FUNDING

The studies included in this doctoral thesis have been funded by a grant from Instituto de Salud Carlos III (ISCIII - PI16/01525), and partially by a grant from the Headache Study Group of the Spanish Neurological Society 2018 and 2021.