TESIS DE DOCTORADO

CHANGES IN INNATE IMMUNITY IN CHRONIC MIGRAINE. ROLE OF TOLL-LIKE RECEPTORS 2 AND 4 AS KEY PLAYERS IN MIGRAINE CHRONIFICATION AND POTENTIAL THERAPEUTIC TARGETS

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CHANGES IN INNATE INMUNITY IN CHRONIC MIGRAINE. ROLE OF TOLL-LIKE RECEPTORS 2 AND 4 AS KEY PLAYERS IN MIGRAINE CHRONIFICATION AND POTENTIAL THERAPEUTIC TARGETS
(Alteración de la inmunidad innata en la migraña crónica. Papel de los receptores Toll-like 2 y 4 como elementos clave en la cronificación de la migraña y potenciales dianas terapéuticas)

D./Dña. CLARA DOMINGUEZ VIVERO

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Título de la tesis: **Alteración de la inmunidad innata en la migraña crónica. Papel de los receptores Toll-like 2 y 4 como elementos clave en la cronificación de la migraña y potenciales dianas terapéuticas.**

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A los que viven con dolor.

"Quién sabe de dolor, todo lo sabe"
Dante Alighieri

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Changes in innate immunity in chronic migraine. Role of Toll-like receptors 2 and 4 as key players in migraine chronification and potential therapeutic targets

RESUMO

A migraña é un trastorno multifactorial e complexo que implica varios mecanismos fisiopatolóxicos tales como a despolarización cortical propagada (CSD), a activación do sistema trixéminovascular (TVS), cambios no fluxo sanguíneo cortical (CBF) e inflamación. A activación dos *Toll-like receptors* (TLR) intervén na neuroinflamación, pero ata hoxe non se comprende completamente a súa importancia na migraña. Este estudo traslacional pretende investigar se a expresión dos Toll-like receptors 2 e 4 (TLR2 e TLR4) aumenta nos pacientes con migraña crónica (CM), e se o TLR4 está implicado na resposta vascular á CSD nun modelo animal de migraña. O estudio clínico ten un diseño transversal e mide a expresión de TLR2 e TLR4 en monocitos e neutrófilos en sangue periférica, así coma os niveis de ligandos de TLR (HSP60 e cFN), interleuquinas (IL-6, IL-10 e hsCRP) e biomarcadores relacionados coa activación do TVS (CGRP) e a disfunción endotelial (PTX3 e sTWEAK). No estudo experimental, comparouse a resposta á CSD entre animais WT e TLR4-KO, así como o efecto do bloqueo farmacolóxico dos TLR mediante a administración de TAK-242. Os suxeitos con CM mostraron unha maior expresión de TLR4 e TLR2 en monocitos e neutrófilos en sangue periférica. A expresión de TLRs correlacionouse cos niveis de ligandos, interleuquinas e biomarcadores de migraña. Ademáis, a expresión de TLR2 en monocitos e neutrófilos e a de TLR4 en monocitos foron quen de predicir o diagnóstico de CM. Os ratos TLR4 KO mostraron cambios na resposta á estimulación da CSD que se reproduciron ó bloquear TLR4 con TAK-242. Os nosos resultados suxiren que os TLRs 2 e 4 teñen un papel na fisiopatoloxía da migraña, proporcionando unha potencial nova estratexia para o seu tratamento.

PALABRAS CHAVE: *Toll-like receptors*, migraña crónica, inflamación, depresión cortical propagada, inmunidade innata.

RESUMEN

La migraña es un trastorno multifactorial y complejo que implica varios mecanismos fisiopatológicos tales como la despolarización cortical propagada (CSD), la activación del sistema trigeminovascular (TVS), cambios en el flujo sanguíneo cortical (CBF) e inflamación. La activación de los *Toll-like receptors* (TLR) interviene en la neuroinflamación, pero hasta la fecha no se ha estudiado su importancia en la migraña. En este estudio traslacional pretendemos investigar si la expresión de los Toll-like receptors 2 y 4 (TLR2 y TLR4) aumenta en los pacientes con migraña crónica (CM), y si el TLR4 está implicado en la respuesta vascular a la CSD en un modelo animal de migraña. El estudio clínico tiene un diseño transversal y mide la expresión de TLR2 y TLR4 en monocitos y neutrófilos en sangre periférica, así como los niveles de ligandos de TLR (HSP60 y cFN), interleuquinas (IL-6, IL-10 y hsCRP) y biomarcadores relacionados con la activación del TVS (CGRP) o la disfunción endotelial (PTX3 y sTWEAK). En el estudio experimental, se comparó la respuesta a la CSD entre animales WT y TLR4-KO, así como el efecto del bloqueo farmacológico de los TLR mediante administración de TAK-242. Los pacientes con CM mostraron una mayor expresión de TLR4 y TLR2 en monocitos y neutrófilos en sangre periférica. La expresión de TLRs se correlacionó con los niveles de ligandos, interleuquinas y biomarcadores de migraña. Además, los niveles de TLR2 en monocitos y neutrófilos y los niveles de TLR4 en monocitos fueron capaces de predecir el diagnóstico de CM. Los ratones TLR4 KO mostraron cambios en la respuesta a la estimulación de la CSD que se reprodujeron al bloquear TLR4 con TAK-242. Nuestros resultados sugieren que los TLRs 2 y 4 tienen un papel en la fisiopatología de la migraña, proporcionando una potencial nueva estrategia para su tratamiento.

PALABRAS CLAVE: Toll-like receptors, migraña crónica, inflamación, depresión cortical propagada, inmunidad innata.

ABSTRACT

Migraine is a multifactorial and complex disorder that involves several physiopathological mechanisms such as cortical spreading depolarization (CSD), trigeminovascular system (TVS) activation, changes in cortical blood flow (CBF) and inflammation. Toll-like receptors (TLRs) activation is involved in neuroinflammation, however, to date, the clinical significance of TLR in migraine is not completely understood. In this translational study we aim to investigate whether Toll-like receptors 2 and 4 (TLR2 and TLR4) expression is increased in chronic migraine (CM) patients, and if TLR4 is involved in the vascular response to CSD in an animal model of migraine. The clinical study has a cross-sectional design and measures TLR2 and TLR4 expression in peripheral blood monocytes and neutrophils, as well as levels of TLR ligands (HSP60 and cFN), interleukins (IL-6, IL-10 and hsCRP) and biomarkers related with TVS activation (CGRP) or endothelial dysfunction (PTX3 and sTWEAK). In the experimental study, CSD response between WT and TLR4 KO mice was compared followed by pharmacological blockade of TLRs using TAK-242. CM patients showed increased expression of TLR4 and TLR2 in peripheral blood monocytes and neutrophils. Expression of TLRs was correlated with levels of ligands, interleukins and migraine biomarkers. TLR2 levels in monocytes and neutrophils and TLR4 expression in monocytes were able to predict CM diagnosis. TLR4 KO mice showed changes in response to CSD stimulation that were reproduced after blocking TLR4 with TAK-242. Our results suggest that TLR2 and TLR4 may have a role in migraine pathophysiology, providing a potential novel strategy for treatment.

KEYWORDS: Toll-like receptors, chronic migraine, inflammation, cortical spreading depression, innate immunity.

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ABBREVIATIONS

AD: Alzheimer's disease
ALS: amyotrophic lateral sclerosis
AP-1: activator protein-1
APCs: antigen-presenting cells
ATF3: activating transcription factor 3
AU: fluorescence arbitrary units
AUC: area under the curve
BBB: blood-brain barrier
BDNF: brain-derived neurotrophic factor
BMI: body mass index
BP: blood pressure
CADASIL: Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy
CBF: cerebral blood flow
CBP: CREB binding protein
CEPBδ: enhancer-binding δ protein
cFN: celular fibronectin
CGRP: calcitonin gene-related peptide
CI: confidence interval
CM: chronic migraine.
CNS: central nervous system.
COPD: chronic obstructive pulmonary disease
CREB: cAMP response element binding
CSD: cortical spreading depolarization
CTN: caudal trigeminal nucleus
DAMPs: danger associated molecular patterns
DLP: dyslipidemia
DM: diabetes mellitus
DMSO: Dimethyl sulfoxide
DNA: deoxyribonucleic acid

DW-MRI: Diffusion-weighted magnetic resonance imaging
EDTA: Ethylenediamine tetraacetic acid
EEG: electroencephalogram
ELISA: enzyme-linked immunosorbent assay
EM: episodic migraine
FASPS: Familial Advanced Sleep Phase Syndrome
FHM: familial hemiplegic migraine
FSC: forward scattering signal
GWAS: genome-wide association studies
HBP: high blood pressure
HMGB-1: high mobility group box 1
hs-CRP: high-sensitivity C-reactive protein
HSP: heat shock protein
HSV: herpes simplex virus
ICER: inducible CAMP early repressor
ICHD: International Classification of Headache Disorders
IDO: Indoleamine 2,3-dioxygenase
IFN: interferon
IKK: I κ B kinase complex
IL: interleukin
IRAK: interleukin-1 receptor-associated kinase
IRF: interferon regulatory factor
KCl: potassium chloride
KO: knockout
LDL: low density lipoproteins
LP: lateral posterior nucleus (of the thalamus)
LPS: lipopolysaccharide
LRRs: leucine rich repeats
MAPK: Mitogen-Activated Protein Kinases
MELAS: Mitochondrial Encephalomyopathy Lactic Acidosis and Stroke-like Episodes Syndrome
MHC: major histocompatibility complex
MMP-9: matrix metalloproteinase 9
MPLA: monophosphoryl lipid A
MPSA: magnetic suppression of perceptual accuracy
MRI: magnetic resonance imaging

MS: multiple sclerosis
MwA: migraine with aura
MwoA: migraine without aura
MyD88: myeloid differentiation factor 88
NEMO: NF κ B essential modulator
NF κ B :nuclear factor κ B
NK: natural killer
NLRs: NOD-like receptors
NO: nitric oxide
NSAIDs: non-steroidal anti-inflammatory drugs
NSS: normal saline solution
NTG: nitroglycerin
ONABOTA: Onabotulinumtoxin A
OSA: obstructive sleep apnoea
ROS: reactive oxygen species
PAG: periaqueductal gray matter
PAMPs: pathogene associated molecular patterns
PB: parabrachial nucleus (medulla)
PBS: Phosphate-buffered saline
PET: positron emission tomography
PFO: patent foramen ovale
PGE2: prostaglandin E2
PNS: peripheral nervous system
Po: posterior nucleus (of the thalamus)
PPG: pterigopalatin ganglion
PRMs: pattern recognition molecules
PRRs: pattern recognition receptors
PTX3: pentraxin 3
Pul: pulvinar nucleus (of the thalamus)
RIP1: receptor interacting protein 1
RLRs: Rig-I-like receptors
RNA: ribonucleic acid
ROC: receiver operating characteristic
ROS: reactive oxigen species
SARM: Sterile α and armadillo motif containing protein.
SBU: standard beverage unit

SD: standard deviation
SNPs: single nucleotid polymorphisms
SP: substance P
STROBE: Strengthening the Reporting of Observational Studies in Epidemiology
SSC: side scattering signal
SSN: superior salivatory nucleus
sTWEAK: soluble tumor necrosis factor (TNF)-like weak inducer of apoptosis
TAB1: TAK 1 binding protein 1
TAB2: TAK 1 binding protein 2
TAK1: transforming growth factor β activating kinase
TBI: traumatic brain injury
TBK1: TANK binding kinase 1
TCC: trigeminal cervical complex
TFB-TBOA: 2S,3S-3-3-4-trifluoromethyl benzoylamino benzyloxy aspartate
TG: trigeminal ganglion
TIR: Toll/interleukin 1 receptor
TIRAP: TIR domain-containing adaptor protein.
TLR: toll-like receptor
TNF-α : tumour necrosis factor α
TRAF-6: tumor necrosis receptor-associated factor 6
TRAM: TRIF-related adaptor molecule
TRIF: toll/interferon response factor
TRPV1: vallinoid receptor type 1
TSI: tarjeta sanitaria individual
TVS: trigeminal-vascular system
USA: United States of America
VAS: visual analogic scale
VIP: vasoactive intestinal peptide
VPM: ventral postero-medial nucleus (of the thalamus)
WT: wild type
YLDs: years lived with disability

1. INTRODUCTION

1.1. EPISODIC MIGRAINE AND CHRONIC MIGRAINE

1.1.1. Concept and classification

Etymologically, the word migraine comes from the late Latin *hemicranĭa*, which in turn derives from the Greek *ἡμικρανία (hēmikranía)*, in allusion to the unilateral nature of this type of headache (Real Academia Española, 2014). Migraine is a primary or idiopathic headache characterized by repeated attacks of pain lasting between 4 and 72 hours. The pain is usually unilateral, pulsating, of moderate or high intensity, and worsens with physical activity. It is often associated with nausea, photophobia, and/or sonophobia (Headache Classification Committee of the International Headache Society. 2013).

The latest version of the International Classification of Headache Disorders (ICHD) (Headache Classification Committee of the International Headache Society. 2013) categorizes migraine according to two basic criteria: its frequency and the presence or absence of associated neurological deficits. Depending on the frequency of the attacks, a distinction is made between episodic migraine (EM) and chronic migraine (CM). Based on the symptoms of focal neurological dysfunction, a distinction is made between migraine with aura (MwA) and migraine without aura (MwoA). In addition, the classification refers to other entities such as "probable migraine", "migraine complications" and "migraine-associated syndromes".

The diagnosis of migraine requires at least 5 episodes of headache lasting from 4 to 72 hours, accompanied by nausea and/or vomiting or photophobia/sonophobia or aura symptoms (visual, sensory, speech, motor, brainstem, or retinal) with the duration and characteristics specified in section C of heading 1.2 of the ICHD (Headache Classification Committee of the International Headache Society. 2013). In CM there are 15 or more headache days per month for more

than three months, of which, in at least 8, the headache meets diagnostic criteria for migraine. If attacks are less frequent, we then talk about EM.

Table 1.1. Diagnostic criteria for EM without aura according to the ICHD, 3rd Edition (ICHD-3)

A. At least five attacks meeting criteria B-D
B. Headache of 4-72h duration (with no treatment or no response to treatment)
C. The headache has at least two of the following four characteristics: 1.Unilateral location 2.Pulsating character 3.Moderate or severe intensity 4.Aggravated by or resulting in avoidance of usual physical activities (e.g. walking or climbing stairs)
D. At least one of the following occurs during the headache: 1. Nausea and/or vomiting 2. Photophobia and sonophobia
E. Does not correspond with any other ICHD-3 diagnosis

Table 1.2. Diagnostic criteria for EM with aura according to the ICHD, 3rd Edition (ICHD-3)

A. At least 2 attacks meeting criteria B and C
B. One or more of the following aura symptoms, which are completely reversible 1.Visual 2.Sensitive 3.Language/Speech 4.Motor 5.Brainstem 6.Retina
C. At least three of the following six characteristics: - At least one of the aura symptoms spreads gradually over ≥5 minutes - Two or more aura symptoms occur consecutively - Each individual aura symptom lasts between 5 and 60 minutes - At least one of the aura symptoms is unilateral - At least one of the aura symptoms is positive - The aura is accompanied, or followed by, a headache within 60 minutes
D. Does not fit into any other ICHD-3 diagnostic category

Table 1.3. Diagnostic criteria for CM according to the IHCD, 3rd Edition (ICHD-3)

A. Headache (migraine or tension headache type) at ≥15 days per month for >3 months, meeting criteria B and C
B. It occurs in a subject who has had at least five attacks that meet

diagnostic criteria for MwA or MwoA.
C. At ≥8 days a month for 3 months, fulfilling any of the following: 1.Criteria C and D for MwoA 2.Criteria B and C of MwA 3.The patient thinks it is a migraine and the pain is relieved by a triptan or an ergotic
D. Does not fit into any other ICHD-3 diagnostic category

Although the term CM was not officially included in the ICHD until the 2000s, the concept of frequent or chronic migraine already existed in clinical practice. It was defined by Silberstein and Lipton in 1994 (Silberstein SD et al. 1994) and for some time coexisted with the term "transformed migraine" (Mathew NT et al.1987), both making reference to the progressive or evolutionary nature of the disorder. The ICHD has always preferred the term CM and included it for the first time in the second edition of its classification in 2004 (Headache Classification Subcommittee of the International Headache Society. 2004) as one of the complications of migraine. Initially, the definition was restricted to those patients in whom there was no abuse of medication, but in the latest version of the ICHD, the coexistence of medication abuse is admitted as long as the headache continues to meet diagnostic criteria for CM when medication overuse has resolved.

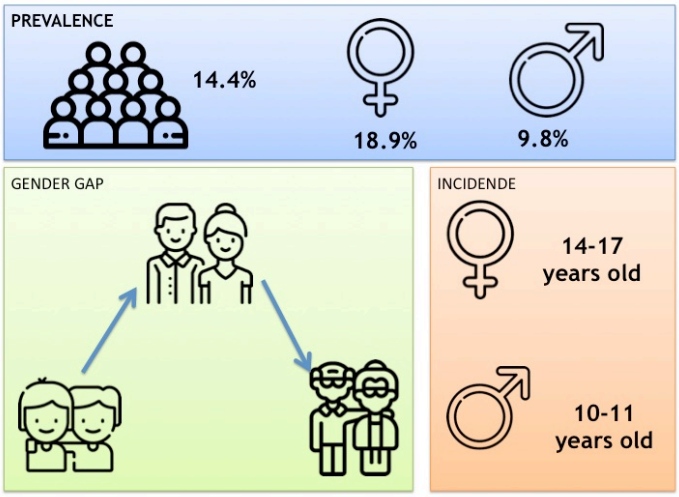
1.1.2.Epidemiology

1.1.2.1. Prevalence and incidence

Migraine is a very common neurological disorder. Prior to the publication of the Global Burden of Disease reports, prevalence estimates were very disparate. Thus, a 1992 study of 15,000 respondents revealed figures of 17.6% in women and 5.7% in men (Stewart WF et al. 1992), while a subsequent meta-analysis based on 24 studies concluded that, despite methodological differences, prevalence estimates ranged from 11.2% to 25% in women and 4% to 9.5% in men in Western countries (Stewart WF et al. 1995). A decade later, a new follow-up study showed prevalence figures of 18.2% in women and 6.5% in men (Lipton et al. 2001). According to the Global Burden of Disease Survey published in 2018 (GBD 2016 Headache

Collaborators. 2018), about 1 billion individuals suffer from migraine with an overall prevalence of 14.4%, a prevalence in women of 18.9% and in men of 9.8%. The cumulative lifetime prevalence is estimated at around 43% in women and 18% in men (Stewart WF et al. 2008). This gender difference decreases during childhood and after menopause. In terms of age, the most affected population group is women between 18 and 44 years of age, where the prevalence is 23.5% (Burch RC et al. 2015). With regard to incidence studies, the highest incidence in women occurs between 14 and 17 years of age, with 18.9 cases/100 000 persons/year, and in men a few years earlier, between 10 and 11 years of age, with 10 cases/100 000 persons/year (Stewart WF et al. 1992).

Figure 1.1. Epidemiology of migraine



The general prevalence of migraine (14.4%) and prevalence among females (18.9%) and males (9.8%). The gender gap in incidence and prevalence of migraine is bigger during adult life, while differences are smaller among children and the elderly. Maximum incidence in women occurs between 14 and 17 years of age and in men between 10 and 11 years of age.
Self-created image (FreePik. (nd). Retrieved from http://www.flaticon.com/free-icon).

1.1.2.2. Disability

Migraine is the seventh cause of disability quantified as the number of years lived with disability (YLDs). A migraine diagnosis involves a greater risk of developing other physical and psychiatric comorbidities that contribute to the impact of the disorder on quality of life. The incidence of comorbidities increases with increasing headache duration and frequency, making them much more frequent in CM (Vos T. et al. 2012).

1.1.2.3. Impact on social and health systems

Migraine has a great impact in health systems, although there are differences between countries and there is no homogeneity in the studies in this regard. According to the US National Center for Health Statistics, headache occupies a fourth place on the list of reasons for a consultation to the emergency services in the United States of America (USA) and represents 3.1% of total demands for emergency care. In Primary Care services, migraine represents 0.5% of visits (Burch RC et al. 2015). In Spain, headaches represent 1 out of every 5 consultations to a Neurology Service, generating 14 000 new consultations per month throughout the country, of which 50% are related to migraine (Gago Veiga AB et al. 2017).

1.1.2.4. Epidemiology of CM

CM, as a distinct disorder from EM, has its own epidemiological profile. Prevalence in the general population ranges between 0.9 and 5.1%, being similar to that of epilepsy. Prevalence is 2.5 to 6.5 times higher in women than in men (Natoli JL et al.1996). Approximately 40% of patients in a specialized headache clinic have CM (Pascual J et al. 2001), which is the most disabling form of chronic daily headache (Lanterini-Minet M et al. 2011). The International Burden of Migraine Study collects data on CM and EM patients in North America, Western Europe, Asia, and Brazil, confirming that healthcare expenditure is significantly higher in CM than in EM (Stokes et al.2011). In this study, productivity loss measured in days was 67.67 days over a 3 month period for CM versus 13.57 days for EM. In the case of the USA, CM patients were

less likely to get a full-time job. Domestic chores were also affected, with 58% of CM patients reporting deterioration in these activities. Use of healthcare resources was higher for CM patients, who used Emergency and Primary Care services more frequently and required admission or specialized treatment more often. CM is accompanied by several comorbidities: sleep disorders, fatigue, chronic pain, and other neurological, psychiatric, cerebrovascular, cardiovascular, and gastrointestinal diseases. In relation to patients with EM, people with CM are twice as likely to have depression, anxiety, and other forms of chronic pain, as well as bipolar disorder, respiratory pathologies (such as asthma or COPD), cardiac pathologies, and vascular risk factors (hypertension and dyslipidemia) (Schwedt TJ.2014).

The data presented so far show the importance of migraine on individual health and on health-care systems. The impact of migraine derives from its prevalence, its incidence, and the demographic profile of the individuals who suffer it, which conditions a greater social and occupational impact. Personal and community consequences are even greater for CM.

1.2. PHYSIOPATHOLOGY OF MIGRAINE

The pathophysiological mechanisms that trigger and perpetuate a migraine attack are not fully understood. It is generally accepted that migraine is a multifactorial disorder that affects cerebral excitability and sensory regulation and manifests itself eminently as a headache, although sometimes accompanied by certain neurological phenomena. Individual genetic susceptibility, together with various epigenetic factors, leads to the activation of a series of mechanisms that trigger attacks. These mechanisms involve some hypothalamic nuclei, the phenomenon of cortical spreading depression (CSD), sterile inflammation, activation of the trigeminal-vascular system (TVS), and the regulatory role of the brainstem nuclei in the transmission of pain. The clinical heterogeneity of migraine and the diversity of available treatments are proof of the variety of mechanisms involved in its origin. It is not easy to reach a unitary theory of migraine that includes all the different aspects affected in this disorder. Research into a possible generator of pain is extensive, although it has not yet been

possible to clarify whether any of the structures involved in the physiopathology of migraine plays a more prominent or causal role, or whether, on the contrary, the activation of all these elements is a consequence of the presence of pain (De Simone R. et al. 2013).

1.2.1. Genetic basis of migraine

Migraine has a hereditary component. In most cases, it is a disorder of multifactorial origin with a polygenic inheritance pattern. The implicated genes identified so far are known through association and GWAS studies and are related to several of the mechanisms underlying migraine, such as neuronal hyperexcitability, vascular changes, or glial dysfunction. Monogenic forms of migraine, which are very rare in clinical practice, have been crucial in the study of the mechanisms involved in migraine physiopathology.

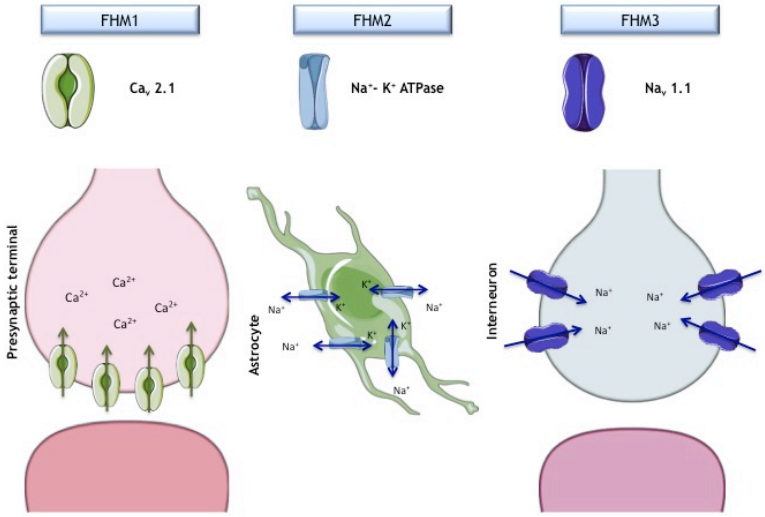
1.2.1.1. Monogenic migraine

Some rare forms of migraine have a monogenic, dominant, high-penetrance inheritance pattern (Ward TN. 2012). The importance of these disorders, which are rare in the population, is that they have facilitated the understanding of the molecular mechanisms underlying the most common forms of migraine.

In general, the mutations that cause familial forms of migraine lead to changes in cell membrane channels. In familial hemiplegic migraine (FHM), some regulatory proteins of ionic homeostasis are altered, resulting in variations in the concentration of glutamate in the synaptic terminal. Familial hemiplegic migraine type 1 (FHM1) is due to a nonsense mutation of CACNA1A on chromosome 19p13, which produces changes of the voltage-dependent Cav2.1 type P/Q calcium channels of the neuronal membrane and an increase in both pre-synaptic terminal calcium and extracellular potassium input and increased glutamate release (Ward TN. 2012). In familial hemiplegic migraine type 2 (FHM2), in contrast, there is a loss of function of the $Na^{+/}K^{+}$ astrocyte adenosine-triphosphatase derived from a nonsense mutation of the ATP1A2 gene on chromosome 1q23. This alteration prevents the optimal elimination of glutamate and K^{+} from the synapse by astrocytes (Ward TN. 2012) and leads to a state of

sustained hyperexcitability. Finally, familial hemiplegic migraine type 3 (FHM3) occurs as a result of a nonsense mutation of the SCN1A gene that causes a gain in the function of the neuron-dependent sodium channels, resulting in an increased release of glutamate into the extracellular space. Other mutations in these three genes are associated with syndromes in which ataxia or epilepsy predominate. In all three cases, changes in the ionic balance of the neuronal environment result in a higher concentration of glutamate and a lower threshold for the appearance of the phenomenon of CSD, which will be discussed later.

Figure 1.2. Functional roles of proteins encoded by genes involved in FHM.



Ca 2,1 channel, located in the presynaptic terminal, is affected in FHM1. Na^+-K^+ ATPase, located in astrocytes, is affected in FHM2. Na 1.1 channel, located in interneurons cell membrane, is affected in FHM3.

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In addition to the forms of FHM, there are other genetic modifications that give rise to much more complex clinical syndromes in which migraine is one of a wide range of signs and symptoms, such as, the mutation of the EAAT1 transporter (Kovermann P et al. 2017), in which hemiplegic migraine associates ataxia and seizures; MELAS (Mitochondrial Encephalomyopathy Lactic Acidosis and Stroke-like Episodes Syndrome), derived from a mutation in the NADH-dehydrogenase 4 gene (Pia S et al. 2018), the Familial Advanced Sleep Phase Syndrome (FASPS) due to a mutation in the CSNK1D gene or CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy) due to a NOTCH3 mutation (Liem MK et al. 2010). As in the forms of FHM, these genetic variations lead to changes of glutamatergic neurotransmission that result in a lowering of the threshold of cortical excitability, in short, a "more excitable" brain. This greater excitability does not only manifest itself during migraine attacks, but the affected individuals also present characteristic changes during inter-critical periods, such as poor habituation to repetitive stimuli, anomalies in the evoked potential tests (Wang W et al. 1998), and a low threshold for the provocation of phosphenes after transcranial magnetic stimulation (Aurora SK et al. 2003).

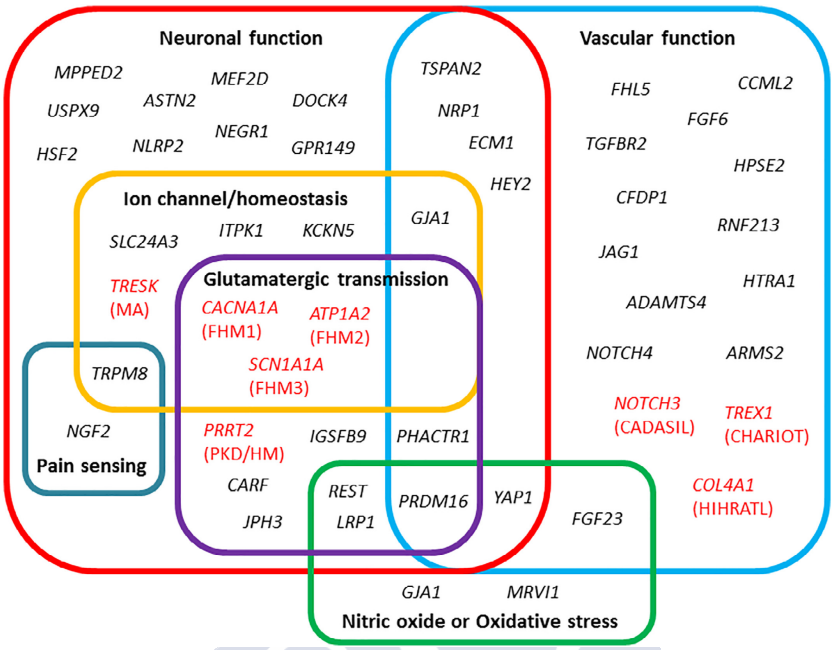
1.2.1.2. Polygenic migraine

Monogenic forms of migraine are very rare, although they have a great impact on affected individuals and their families and have contributed greatly to our current knowledge of the physiopathology of the disease. The study of the genetic component in the common forms of migraine, all characterized by polygenic inheritance and multifactorial origin, is much more complex. Our knowledge on this topic derives from association studies and GWAS studies.

In association studies, certain genes involved in the pathways assumed to participate in the physiopathology of the disorder are selected *a priori,* and differences in allele frequencies are sought between cohorts of patients and healthy controls. The effect of each allelic variant at each locus is small and very large sample sizes are

needed to achieve significant results. Association studies have been published on about 200 genetic polymorphisms related to migraine, whose robustness is questionable since many replication studies carried out later present contradictory findings (Sutherland HG et al. 2019). In GWAS studies, on the other hand, no *a priori* hypotheses are assumed and the entire genome is analyzed. So far there are five large GWAS studies on migraine that have found associations with 44 SNPs (single nucleotide polymorphisms) and 38 loci. The first studies identified variants of genes involved in different neuronal functions, such as LRP1 (regulation of glutamate receptors on the neuronal surface), TPRM8 (cationic channel related to pain), or PRDM16 (neurogenesis). Further studies found associations with genetic loci involved in vascular functions such as TGFBR2 (regulates neuronal but also endothelial function) and PHACTR1. A meta-analysis (Gormley P et al. 2016) combining 22 GWAS studies (59 674 migraine patients) confirms that the genes most likely to participate in migraine code proteins related to vascular and neuronal function (LRP1, PRDM16, ECM1, MEF2D, TGFBR2, ARHGEF26, REST, PHACTR1, NOTCH4, FHL5, GJA1, HEY2, NRP1, PLCE1, HTRA1, YAP1, FGF6, ZCCHC14, JAG1, and CCM2L) and are expressed mainly in vascular tissue. Other loci identified, less numerous, code ion channels (TRPM8, REST, KCNK5, SLC24A3) and proteins that participate in metal ion homeostasis (PRDM16, TGFBR2, REST, FHL5, NRP1, MMPED2, LRP1, ZCCHC14, RNF213, JAG1, SLC24A3).

Figure 1.3. Genes involved in the physiopathology of migraine.



Migraine-related genes identified to date. Genes with more than one function appear in overlapping sections. Those genes related to monogenic forms of migraine appear in red.
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1.2.2. Trigeminovascular system

Pain is an essential symptom of migraine. Its appearance depends on the activation of what we call the trigeminovascular system (TVS). Although the cerebral parenchyma lacks nociceptive receptors, both the pia mater, the arachnoid, the dura mater, the dural blood vessels, and the venous sinuses have sensory and autonomic innervation. Current knowledge about pain perception in intracranial structures comes from experiments carried out in the 1940s (Penfield W.1940; Ray B et al.1940) in which it was shown that *in vivo* stimulation of the dura mater was capable of triggering headache with characteristics

and location similar to migraine. These studies proved that the activation of meningeal sensory fibers was responsible for pain in migraine and other primary headaches.

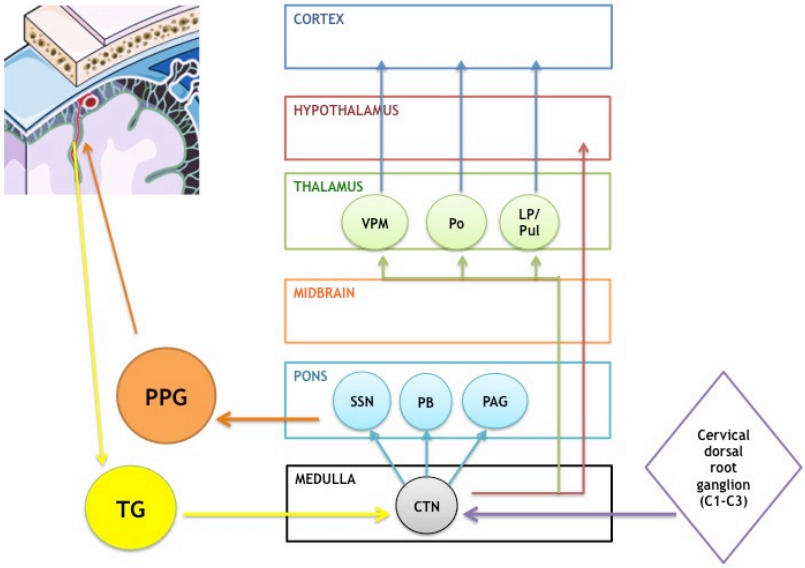
Supratentorial structures are innervated by the trigeminal nerve (cranial nerve V). This nerve is divided for anatomical study into three branches: V1 or ophthalmic nerve, V2 or maxillary nerve, and V3 or mandibular nerve. The infratentorial cranial structures are innervated by the cranial pairs VII, IX, and X and by sensory fibers of the cervical roots C2 and C3. All these structures make up the TVS (Ward TN. 2012). The amyelinic C fibers and the poorly myelinated Aδ fibers of the trigeminal nerve converge at the Gasser ganglion and are directed to the central nervous system (CNS) via the trigeminal tract, penetrate the pons and run in a caudal direction until they reach the caudal trigeminal nucleus (CTN). This nucleus extends to the third cervical medullary segment, where it gradually merges with the dorsal medullary columns. Some fibers coming from the cervical roots (C2 and C3) enter the CTN so this set is called the trigeminal-cervical complex (TCC). The vast majority of C and Aδ fibers end up in the superficial layers (*laminae* I and II) of this nucleus. The second-order neurons of the TCC ascend forming the qinto-thalamic tract until the posterior (Po) and ventral-medial (VPM) nuclei of the contralateral thalamus, which, in turn, sends projections towards the primary and secondary somatosensory cortex, the insula, and the anterior cingulate cortex (Ward TN. 2012).

The TCC emits and receives connections from the brainstem nuclei involved in pain regulation such as the ventral-medial rostral bulb, raphe nuclei, or periaqueductal gray matter (PAG), as well as from and to the hypothalamus (Espinosa-Sanchez JM et al. 2015).

The TCC also establishes connections with the superior salivatory nucleus (SSN), which is the link between the trigeminal nucleus and the cranial vasculature. Located in the pons, the SSN houses the nuclei of the parasympathetic neurons responsible for the trigeminal-auto reflex, and thus the cranial vasodilator response. Their efferences travel along the major petrous nerve and make synapses in the sphenopalatine ganglion from where they travel to the meningeal vessels, sinuses, and ocular structures (Goadsby PJ et al. 2002). The

activation of this nucleus is responsible for the parasympathetic symptoms that sometimes accompany migraine and that clinically define trigeminal-autonomic headaches (Goadsby PJ. 2002).

Figure 1.4. Trigeminal-vascular system



Inputs from meningeal dural and pial blood vessels travel to the trigeminal ganglion (TG) and then to the caudal trigeminal nucleus (CTN). The CTN, located in the medulla, has connections with the superior salivatory nucleus (SSN), parabrachial nucleus (PB), and periaqueductal gray (PAG), located in the pons. SSN sends effector parasympathetic fibers through the pterygopalatine ganglion (PPG). The CTN projects to several thalamic nuclei, such as the ventral posteromedial nucleus (VPM), the posterior nucleus (Po), the lateral posterior nucleus (LP), and the pulvinar nucleus (Pul), as well as to the hypothalamus. From the different thalamic nucleus projections head towards cortical areas relevant for pain processing: auditory cortex, entorhinal cortex, insular cortex, primary motor cortex, secondary motor cortex, parietal association cortex, retrosplenial cortex, primary somatosensory cortex, secondary somatosensory cortex, primary visual cortex, and secondary visual cortex.
Self-created image, created with Servier Medical Art images (Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License)

The mechanism by which nociceptive fibers of the trigeminal system are activated in migraine is not completely known. It is believed that the activation of certain areas of the cortex causes the release of hydrogen and potassium that stimulate the C-type fibers of the meninges (Scheller D et al. 1992). The hydrogenions act through vallinoid receptors or acid-sensitive receptors and the increased concentration of extracellular potassium results in direct depolarization of neurons (Caterina MJ et al. 1997; Waldmann R et al. 1997). Stimulation of the meningeal nociceptors leads to the transmission of pain signals to the CNS and causes C-type fibers to secrete several neuropeptides, such as calcitonin gene-related peptide (CGRP), substance P (SP), and neurokinin A (Ebersberger A et al. 1999). These mediators act on the endothelium of cranial vessels, which is surrounded by trigeminal fibers. The vessels become inflamed and dilated, and the plasma extravasation characteristic of neurogenic inflammation occurs (Moskowitz MA et al. 1993). The release of these neuropeptides produces additional effects: it activates the metalloproteases that modify the permeability of the blood-brain barrier (BBB) and stimulates mast cells, which degranulate, contributing to sterile inflammation (Ramachandran R. 2018). In turn, the excited trigeminal nerve, through its polysynaptic connections through the SSN, produces a reflex arc with the parasympathetic system and its fibers, which surround the meningeal vessels, and which release acetylcholine, nitric oxide (NO), and vasoactive intestinal peptide (VIP). This contributes to the ocular and vasomotor phenomena that sometimes accompany migraine attacks. The trigeminal nerve-SSN interaction constitutes another anatomical and physiological link between trigeminal fiber activation and vascular response (Ebersberger A et al. 2001).

Variations in the caliber of the meningeal vessels during migraine attacks underpinned the classical vascular theory, according to which these changes were the main cause and trigger of pain. Vascular theory dominated the research and clinical approach to migraine during the second half of the 20th century. In 1938, Graham and Wolff demonstrated how the reduction in the caliber of brain blood vessels following intravenous ergotamine infusion relieved migraine

pain (Graham JR et al.1938), turning into dogma the theory that the aura of migraine was secondary to vasoconstriction and headache to subsequent vasodilation. Today it is still debated whether vasodilation can stimulate vascular wall nociceptors but data against this hypothesis in both animal (Zhang X et al. 2013) and human (Rahmann A et al. 2008) experimentation are numerous. Current knowledge suggests that vascular alterations are phenomena accompanying trigeminal activation, and not a causal event (Ebersberger A et al.2001).

1.2.3. Brainstem and diencephalic structures involved in migraine

The neurons in TCC have connections with various nuclei in the brainstem and diencephalon that are involved in central pain processing and modulate activity in TCC. In the brainstem, TCC establishes connections with PAG, with certain medial areas of the ventral medulla, such as the raphe nuclei, and with other nuclei of the pons (Liu Y et al. 2009). At the diencephalic level, it communicates directly with the thalamus and hypothalamus through the trigeminal-hypothalamic tract (Benjamin L et al. 2004). SSN is connected to different cortical, limbic, and hypothalamic areas and direct connections of TCC to the amygdala and hippocampus are believed to exist, although they have only been demonstrated in animals (Jasmin L et al. 1997). These brainstem and diencephalic structures are activated after stimulation of the dura mater in experimental animals (Liu Y et al. 2009) and in humans during migraine attacks, as shown by functional neuroimaging studies (Bahra A et al. 2001). Such activation is not limited to headache attacks but persists after the pain is relieved by treatment, suggesting that it is not a simple response to pain but a much more complex mechanism.

1.2.3.1. Brainstem nuclei

Brainstem nuclei exert both facilitating and inhibiting effects on the spinal nuclei of pain (Fields HL et al. 1977, Porreca F et al. 2002). The brainstem structures most clearly involved in pain regulation are the PAG and the rostral-ventral medulla (*locus*

coeruleus and raphe nuclei). PAG is a serotoninergic nucleus with abundant connections with the hypothalamus and limbic structures, as well as with the medulla and spinal dorsal columns (Heinricher MM et al. 2009). Several studies have demonstrated the involvement of PAG in migraine and it has even been proposed as a "generator" of headache, although without sufficient evidence. The number of connections between PAG and spinal dorsal columns appears to increase in high-frequency migraine, while connections with pain-modulating limbic structures decrease (Mainero C et al. 2011); PAG remains active in patients with migraine even after pain relief (Weiller C et al. 1995) and structural changes, such as increases in iron deposits, have been described in CM patients (Dominguez C et al. 2019). Finally, the stimulation of PAG with electrodes can cause migraine attacks in subjects without previous headaches (Veloso F et al. 1998).

The main nuclei of the ventral-medial rostral medulla are the raphe nuclei (serotoninergic) and the *locus coeruleus* (adrenergic). Within these nuclei, there are "on" cells, which facilitate the transmission of pain, "off" cells, which inhibit it, and neutral cells (Gao K et al. 2001). The pain threshold varies according to the balance established between the activity of these cell nuclei. These systems play a central role in stress and pain control strategies and are the target of several analgesic treatments, such as opioids, cyclo-oxygenase inhibitors, and cannabinoids. The presence of vascular malformations in this area of the brainstem is sometimes accompanied by migraine-type headache (Obermann M et al. 2006).

1.2.3.2. Hypothalamus

Several areas of the hypothalamus are activated both during migraine attacks and interictal periods (Denuelle M et al. 2007). There are projections connecting the TCC, the SSN, and the paraventricular, anterior, lateral, and peripheral nuclei of the hypothalamus (Robert C et al. 2013). The hypothalamus is also connected with many other structures involved in the physiopathology of migraine, such as the nucleus of the solitary tract, the ventral-medial medullar nuclei, the PAG, in addition to the SSN and its caudal area. The hypothalamus

has a role in central sensitization; the hypothalamic nucleus A11, connected to the dorsal spinal columns, is particularly relevant since it appears to maintain a tonic inhibition of the trigeminal nucleus, so that its dysfunction may explain the hyperalgesia and allodynia associated with migraine (Charbit AR et al. 2009). The involvement of the hypothalamus in the physiopathology of migraine also helps to explain many of the prodromal and accompanying symptoms present in the disorder, such as sleep disturbances, thirst, or changes in appetite (Panda S et al. 2004).

1.2.3.3. Thalamus

As mentioned above, the thalamus is the structure that first collects the efferent tracts coming from the TCC. The nociceptive information generated at the trigeminal level is transmitted to third-order neurons in the thalamus through the Quinto thalamic tract; it is processed in the posteromedial ventral nucleus, the posterior lateral and dorsal lateral nuclei, the medial nucleus of the posterior complex, and the intralaminar thalamus (Veinante P et al. 2000). The involvement of thalamic nuclei in EM and CM has been demonstrated by PET (positron emission tomography), as well as functional and structural MRI (magnetic resonance imaging) techniques (Afridi SK et al. 2005; Schwedt TJ et al. 2014; Granziera C et al. 2014). The different thalamic nuclei project to specific areas of the cortex. Thalamic activation contributes to the extension of pain to the contralateral hemicranium and to the appearance of allodynia, as well as to the characteristic photophobia of the disorder (Noseda R et al. 2010). CGRP receptors have been found in the VPM nucleus of the thalamus and the administration of CGRP antagonists has been shown to inhibit nociceptive transmission to third-order thalamic neurons (Summ O et al. 2010). The thalamus is a core structure in the processing of pain and it belongs to the "pain matrix" or "pain network", along with the primary and secondary somatosensory areas, the anterior cingulate cortex, and the prefrontal cortex. All these structures are activated by pain and integrate the sensory, affective, and cognitive responses to it (Derbyshire SW et al. 1997).

1.2.3.4. Limbic lobe

Some authors include the PAG and the rostral-ventral medulla in the so-called limbic lobe, due to the numerous connections between these structures and the amygdala, cingulate cortex and pre-frontal cortex (Holstege G. 1992). Functional neuroimaging studies have shown hypometabolism of limbic areas in patients with migraine, and a relationship between the involvement of these areas and the duration of the disorder (Kim JH et al. 2010). The amygdala has direct projections to the thalamus and the trigeminal nucleus and can be affected by the phenomenon of CSD (Dehbandi S et al. 2008) and the elevation of CGRP levels (Sink KS et al. 2011). Some studies have shown changes in grey matter at the level of the amygdala and anterior cingulate cortex in patients with migraine (Valfre W et al. 2008). The involvement of the amygdala in the migraine neuronal network would partly explain the comorbidity between migraine, depression, and anxiety (Burstein R et al. 2009). The basal ganglia as a whole may play a role in the transformation of low-frequency migraine into a high-frequency migraine (Maleki N et al. 2011).

1.2.3.5. Cortex

Most nociceptive networks are under cortical control. There are direct projections from the cerebral cortex (mainly from the primary somatosensory and contralateral insular cortex) to the superficial and deep *lamellae* of the TCC (Kuypers HG. 1958). Patients with migraine show changes in several cortical regions related to pain modulation, either during migraine attacks and interictal periods. These shifts in the excitability of certain cortical areas may be a susceptibility factor for migraine (May A.2009), may result from repeated activation of the trigeminal system and consequent activation of these areas, or may derive from exposure of the cortex to repeated episodes of CSD.

1.2.3.6. Migraine unitary hypothesis

It is possible that the role of brainstem nuclei and diencephalic and cortical structures is limited to the regulation of the painful signals initiated at the peripheral level and transmitted through the TCC. However, the fact that some stimuli related to homeostatic control, such as hunger, changes in sleep, etc... trigger or precede migraine attacks, points to a possible origin of the disorder in brainstem structures, the hypothalamus, and the thalamus. Burstein and Jakubowski's so-called "unitary hypothesis of migraine" suggests that the SSN may be a key structure as it constitutes the link between the hypothalamus, the limbic lobe, the cortex, the trigeminal nucleus, and the parasympathetic efferences of the cranial vasculature (Burstein R et al. 2005). All the stimuli that have been identified as potential triggers of migraine attacks (hormonal, emotional, nutritional, and physiological) are initially processed in certain hypothalamic nuclei and other limbic areas, all of which are connected to the SSN. According to the unitary hypothesis of migraine, the activation of the SSN activates the sphenopalatine ganglion and triggers the vasodilation of meningeal vessels and the release of inflammatory mediators that stimulate the trigeminal meningeal fibers. Trigeminovascular projections towards several nuclei of the brainstem and diencephalon could generate some of the premonitory or accompanying symptoms. Two-way signals through this system would establish feedback loops that not only trigger pain but also contribute to its perpetuation. The dysfunction of this pain network would explain the individual predisposition to migraine and its chronification.

1.2.4. Cortical spreading depression

The phenomenon of CSD consists of a wave of transient depolarization of neurons and glia that spreads through the grey matter at a rate of 2-6mm/min. This wave associates a transient depression of local electrical activity and changes in blood flow. CSD is essential in most physiopathological theories of migraine. Much progress has been made in recent years in its characterization, but its

features in humans and its exact role in the chain of phenomena that trigger a migraine attack are still under study.

1.2.4.1. Historical aspects

CSD was described almost simultaneously by Lashley and Leão in the 1940s. Lashley (Lashley KS. 1941) studied the progression of the visual aura of his own migraine attacks and hypothesized that it corresponded to a phenomenon that moved through his visual cortex at a speed of 3mm/min. Shortly afterwards, Leão published four papers on the phenomenon of CSD in animals. In the first of these papers (Leão AAP. 1944), he described a wave of suppression of neuronal activity accompanied by changes in blood flow that moved at 3 mm per minute through the cerebral cortex of mice after focal electrical stimulation of the brain surface. This wave of suppression of neuronal activity was preceded by a wave of depolarization that manifested itself as a change in the direction of the current of the membrane potential (Leão AAP. 1944). Although the primary event was excitatory, the term spreading depression became established in the scientific community and is still used today, although the term cortical spreading depolarization is preferred. Leão's works showed that CSD can occur in any area of the cortex and that it is accompanied by changes in the caliber of pial vessels. He emphasized the particular speed of propagation of CSD and remarked the differences between CSD and the electrical phenomena associated with epilepsy: whereas an epileptic seizure is transmitted to the adjacent brain tissue in an asynchronous manner, CSD is initiated in one area of the brain, which is simultaneously activated in its entirety. Leão already pointed out at that time that the speed of propagation of CSD suggested that it was produced by non-synaptic mechanisms and proposed as an alternative mechanism the selective depolarization of neuronal dendrites or transmission mediated by astrocyte networks. These initial suspicions of Leão were confirmed in later works (Charles A. 1998).

1.2.4.2. CSD, aura, and pain

Lashley and Leão's findings led to the hypothesis that CSD could be the neurophysiological correlate of migraine aura. This hypothesis was initially met with skepticism, because of the differences between mice and human brain cortex: potential changes are difficult to detect in humans using surface electrodes and the threshold for CSD is much higher. The first experimental evidence of the phenomenon in humans was found in 1981 when the aura-associated changes in CBF were first recorded in vivo (Olesen J et al. 1981). The demonstration of the relationship between CSD and migraine was consolidated by showing the activation of trigeminal neurons ipsilateral to CSD: the expression of the c-fos gene increased in the neurons of the trigeminal nucleus ipsilateral to CSD, which suggested that neuroinflammatory mechanisms activated the TVS; CSD was, therefore, responsible not only for the aura but also for pain in migraine (Moskowitz MA et al. 1993). In the 2000s, several studies questioned the role of CSD as these results could not be replicated (Lambert GA et al. 1999), nor did CSD lead to increased plasma protein extravasation or increased CGRP secretion (Ebersberger A et al. 2001). On the other hand, from a theoretical point of view, CSD did not explain the origin of the disorder in cases of MwoA, which represent 80% of the total. Two key works have recently brought back to the table the role of CSD in the genesis of migraine. The first showed that the induction of CSD in rats generates an increase in the activation rate of meningeal nociceptors that begins 14 minutes after the depolarisation wave (Zhang X et al. 2010). In the second (Zhang X et al. 2011) it was definitively demonstrated that CSD produces activation of neurons in the superficial *laminae* (I and II) of the spinal trigeminal nucleus, precisely where Moskowitz had found increased expression of c-fos years earlier. Subsequent studies had focused on the deep *laminae* (III and IV), which is the reason why they probably could not replicate his findings. Finally, the clinical expression of these changes has been found in animal models: 20 minutes after induction of CSD there is a reduction in the pain threshold in non-anesthetized animal models and a dilation of the middle meningeal artery (Karatas et al. 2013).

Studies in therapeutics and pharmacology also support the role of CSD in migraine, since several preventive treatments for migraine (topiramate, valproate, propranolol, amitriptyline, and methysergide) reduce the frequency of CSD provoked by continuous stimulation in animals (Ayata C et al. 2006). The administration of topiramate or amiloride during the acute phase also inhibits CSD (Akerman S et al. 2005; Holland PR et al 2012). On the other hand, three drugs with similar mechanisms of action, but which are ineffective as a treatment for migraine, d-propranolol, oxcarbazepine, and carbamazepine, do not change the spread of CSD (Hoffmann U et al. 2011).

It is important to note, however, that the characteristic electrophysiological changes of CSD have never been recorded *in vivo* in a patient with migraine and that, therefore, the evidence in humans cannot be considered definitive (Charles AC et al. 2013). Magnetoencephalographic studies have demonstrated changes in cortical depolarization similar to CSD in cases of migraine with aura (Bowyer SM et al. 2001), but the study with surface-electrode-electroencephalogram (EEG) has not been able to capture changes (Lauritzen M et al. 1981). In patients with brain damage of vascular or traumatic origin, CSD waves have been recorded by electrocorticography and even by surface EEG, using a specific configuration and special signal processing methods (Drenckhahn C et al. 2012). It is possible, therefore, that if we were to use electrocorticography in patients with migraine we could record the phenomenon, which is much more subtle in the case of the aura than when derived from brain damage.

1.2.4.3. CSD in animal models of migraine

CSD is perfectly characterized in animal models and its validity as an experimental model of migraine is well-founded. Transgenic mice for FHM have a higher propensity to present CSD (van den Maagdenberg AM et al. 2004; Leo L et al. 2011), as well as mice with mutations in the enzyme casein kinase 1δ, one of the genetic variants associated with migraine of polygenic inheritance in humans (Brennan KC et al. 2013). On the other hand, the gender differences that characterize migraine in humans also exist in animal

models, in which females have a lower threshold for the development of CSD (Brennan KC et al. 2007). In animal models, the depolarization wave may be triggered by different stimuli (chemical, electrical, or mechanical) and propagates through the cerebral cortex but also the cerebellum, and hippocampus. In the beginning, there is a massive depolarization of neurons and glial cells, that comes with an increase in extracellular K^+, a reduction in extracellular Na^+, and an increase in transmembrane flows of other ions including protons, chlorine (Cl^-), magnesium (Mg^{2+}), and zinc (Zn^{2+}). Intracellular calcium increases and calcium-dependent depolarization waves propagate through the glia, producing changes in vascular tone. Ionic exchanges produce cellular edema and other changes in the composition of the extracellular space (Hansen AJ et al. 1981). These processes lead to changes of BBB that depend on type 9 matrix metalloproteinase, resulting in the extravasation of plasma proteins and the release of H^+, K^+, NO, and neurotransmitters into the extracellular space (Gursoy-Ozdemir Y et al. 2004). These neurotransmitters activate and sensitize trigeminal afferents. The propagation of CSD to subcortical nuclei can also affect the modulation of nociceptive signals by these structures.

CSD propagation, as already said, is different in animals and humans. The human cortex has more convolutions and a higher astrocyte/neuron ratio. For this reason, it is accepted that the threshold for depolarization is higher in humans, although their speed of propagation seems similar, according to neuroimaging studies and the speed of aura propagation (Santos E et al. 2014). Furthermore, CSD in humans usually affects the cortex in a more limited way, spreading only through some layers or over a limited area of the cortical surface, instead of a complete brain lobe, as in animal models (Dahlem MA et al. 2015).

1.2.4.4. CSD and vascular changes

The phenomenon of CSD is accompanied by a vascular response that has been fully characterized in animal models. These vascular changes present a multiphasic pattern: they begin with vasodilation that propagates ahead of the CSD wave, followed by

marked vasoconstriction of the vessels as the depolarization wave passes. Subsequently, either the vessels regain their normal caliber or go into a subtly dilated state, followed by prolonged vasoconstriction with reduced blood oxygenation that can last up to one hour (Piilgaard H et al. 2009; Chang JC et al. 2010). This sustained vasoconstriction occurs at the same time as the recovery of neuronal activity, which implies a dissociation of the normal relationship between brain activity and blood flow. Although, as already mentioned, CSD has never been recorded in humans in migraine, the associated neurovascular dissociation has been observed in patients during migraine attacks using MRI angiography, transcranial doppler, and functional imaging techniques (Cutrer FM et al. 2000).

1.2.4.5. CSD triggers

The triggers for CSD in migraine are unknown. Brain damage can trigger CSD in humans, as has been shown in cases of subarachnoid hemorrhage, stroke, or trauma (Dreier JP et al.2009; Hartings JA et al.2011), but it is not clear what mechanism initiates CSD in migraine. It has been observed that microemboli can trigger CSD in animal models of migraine. This could be the cause in some humans and also offers a mechanism for the well-known statistical correlation between patent foramen ovale (PFO) and MwA (Nozari A et al.2010). Another theory proposes that local increases in K^+ or other neurotransmitters may lead to the spontaneous appearance of CSD in susceptible individuals. This mechanism would relate the process to changes in the channels and membrane transporters characteristic of FHM1 and FHM2 (van den Maagdenberg AM et al. 2004; Leo L et al.2011), as well as some of the genes involved in polygenic migraine. However, there are still many unanswered questions regarding the cause of CSD in humans.

1.2.5. Inflammatory mechanisms and migraine

Inflammation is classically defined as the presence of flushing, heat, tumor, and loss of function. Acute inflammation is an adaptive response, but chronic inflammation is usually deleterious. It is unclear whether chronic inflammation plays a role in chronic pain, as it does

acute inflammation in acute pain, but it seems to be involved in mechanisms related to neuronal plasticity and pain chronification (Ji RR et al. 2016).

1.2.5.1. TVS and inflammation

The relationship between the TVS and inflammation was proposed 40 years ago by Moskowitz and his team (Moskowitz MA et al. 1979). When the TVS is activated, the nerve terminals release vasoactive neuropeptides (mainly VIP and CGRP) into the perivascular parasympathetic terminals. These neuropeptides produce vasodilation of the vessels, increased blood flow, plasma extravasation, and degranulation of plasma cells in the dura mater. Local inflammation, in turn, activates the meningeal nociceptors (Levy D et al.2010) in a positive feedback process. This sterile inflammatory reaction is known as "neurogenic inflammation" (Pietrobon D et al.2013). Nociceptors may remain persistently activated after contact with inflammatory mediators, promoting central sensitization of trigeminal and hypothalamic neurons. It has been shown experimentally that trigeminal ganglion stimulation triggers neurogenic inflammation mechanisms and that this effect is blocked by the administration of ergotics and triptans (Buzzi MG et al. 1995). There is a lack of large-scale clinical data to support the theory of neuroinflammation in humans, but a single-patient study demonstrated increased vascular permeability during a migraine attack (Knotkova H et al. 2007). Furthermore, the neuroinflammation hypothesis is supported by indirect evidence, such as the presence of inflammatory mediators in the cephalic venous blood of patients with migraine or the reduction of headache after treatment with corticosteroids and non-steroidal anti-inflammatory drugs (Sarchielli P et al. 2006).

1.2.5.2. Mast cells and inflammation

Mast cells are important agents in neuroinflammation. In human and other mammalian’s dura mater, there are significant populations of mast cells, which are mostly concentrated around trigeminal afferent terminals, those expressing substance P (SP) and

CGRP (Strassman AM et al. 2004). Activation of the trigeminal ganglion produces morphological changes in these mast cell populations and the release of SP and CGRP leads to their degranulation (Eftekhari S et al. 2013), releasing histamine, serotonin, tumor necrosis factor α (TNF-α) and proteases (Mekori YA et al. 2000). The release of substances contained in the dural mast cells produces as well activation of most of the meningeal nociceptors and the nociceptive neurons of the caudal trigeminal nucleus, contributing to the pain feedback loop (Levy D et al. 2007).

The triggers of these neuroinflammatory processes are unknown. Studies in animal models have shown how a single event of CSD leads to persistent vasodilation of the dural vessels and extravasation of plasma proteins (Bolay H et al. 2002), in addition to mast cell degranulation (Karatas H et al. 2013), so it is believed that CSD may be the initial trigger. This chain of events depends on the integrity of the trigeminal nerve and involves the activation of the sphenopalatine ganglion, including the parasympathetic system in the mechanism. It is possible that subjects predisposed to migraine have a greater number of dural mast cells, perhaps as a consequence of hormonal variations (Boes T et al. 2012), or that their mast cells are more easily activated. On the other hand, CSD can activate meningeal nociceptors very briefly when it passes through their receptive field; this brief activation could promote the neuroinflammatory cascade, which would subsequently produce a late but persistent activation of these same receptors (Levy D. 2012).

1.2.6. Mechanisms of migraine chronification

Chronic migraine usually develops after a slow increase in headache frequency over months or years, in a process that until recently was called "migraine transformation". Each year, about 2.5% of patients diagnosed with EM go on to meet diagnostic criteria for CM (Buse DC et al. 2012).

1.2.6.1.Risk factors for chronification

The chronification of pain has been extensively studied, in an attempt to determine the clinical and sociodemographic factors associated with it (Schwedt TJ. 2014). There is an increased risk of chronification associated with females, as well as the presence of co-morbidities such as obesity, sleep apnea-hypopnea syndrome, insomnia, psychiatric diseases, other types of chronic pain, and a history of head or neck trauma. Both medication abuse and caffeine abuse seem to lead to an increased tendency to become chronic. Medication abuse is a common disorder among CM patients; inappropriate use of barbiturates and opioids is associated with the greatest risk, followed by abuse of triptans (Lipton RB et al. 2013). In terms of headache characteristics, seizure frequency and the presence of allodynia are predictors of risk. The occurrence of major life events such as divorce, marriage, or a change of job as well as the low socioeconomic status may also contribute to the chronification of the disorder. CM is not a permanent state but fluctuates, and many patients return to or transition from the state of EM. Studies show that fewer headaches, absence of allodynia, adherence to preventive treatment, cessation of symptomatic medication abuse, and physical exercise are associated with a greater likelihood of reverting to the EM state (Manack A et al. 2011).

1.2.6.2. Mechanisms of chronification

To date, the physiopathological mechanisms underlying the chronification of migraine are not fully understood. Pain processing, peripheral and central sensitization, cortical hyperexcitability, and neurogenic inflammation are key elements in the process. Both clinical and experimental data suggest that chronification may be a consequence of the development of hyperexcitability along the pain transmission pathways. In this process, the downstream pathways facilitating pain transmission would be activated, while the inhibitory pathways would be suppressed. According to this hypothesis, repeated headaches produce a progressive dysfunction of the neuronal networks involved in the regulation of nociceptive signal

transmission. This abnormal pain modulation leads to a progressive increase in the frequency and duration of headache episodes (Cortelli P et al. 2003).

Neuroimaging studies have shown that structural and functional differences exist in areas of the brainstem and the medial and lateral pain transmission pathways in subjects with CM. Using voxel-based morphometry techniques, a reduction of gray and white substance in areas of the pain network has been found along with an increase in the volume of the nuclei of the brainstem (specifically of the PAG) (Chiapparini L et al. 2010). The presence of iron, a structural marker of inflammatory activity, in PAG is one of the structural changes present in the nociceptive network of subjects with CM (Dominguez C et al. 2019; Welch KM et al. 2011). Iron deposits result from repeated episodes of inflammation, but we do not know if they also contribute to the chronification of the inflammatory process. The rostral brainstem is activated during the migraine attack (PAG, raphe nuclei, and *locus coeruleus*) and changes in these areas may also lead to changes in pain perception (Aurora SK et al. 2011). In addition to structural modifications, functional neuroimaging studies show differences in regions of the brainstem of patients with CM that are correlated with the presence of allodynia, a symptom of central sensitization that affects second-order trigeminal neurons (Schwedt TJ et al. 2014).

Structural and functional changes affect as well the supratentorial structures of CM patients. A greater number of white substance lesions have been observed (Kruit MC et al. 2010), as well as reduction of the volume of grey substance and evident alterations in T2 sequence in the anterior cingulate, bilateral insula, and the prefrontal, motor/pre-motor, and right parietal cortex (Kim JH et al.2008). A functional neuroimaging (PET) study suggests that the inhibitory capacity of the cerebral cortex is reduced in CM (Aurora SK et al. 2007). The work done with transcranial magnetic stimulation shows important differences between EM and CM in terms of cortical hyperexcitability. The MPSA (magnetic suppression of perceptual accuracy) technique reveals a continuum of cortical excitability in patients with migraine, in which subjects with CM have greater

cortical excitability than those with EM, and the latter in turn have greater cortical excitability than healthy controls. This cortical hyperexcitability could be due to the absence of intracortical inhibition (Aurora SK et al. 2005). The frequency of pain and level of cortical hyperexcitability is associated with changes in several brain areas involved in sensory, affective discrimination, and cognitive processing of pain (Schwedt TJ et al. 2013), as shown by some studies suggesting changes in the frontal lobe in patients with CM (Mongini F et al. 2005).

Neuroinflammation also contributes to the persistence and chronification of pain, as well as to the development of comorbid conditions, such as fibromyalgia (Xanthos DN et al. 2014). Repeated episodes of acute inflammation or the presence of a prolonged state of subtle inflammation produce changes in the central and peripheral nervous system. Inflammation involves activation of glial cells and infiltration of nerve structures by leukocytes, with increased production of inflammatory mediators, as well as changes in the vascular endothelium that facilitate leukocyte infiltration. The increased expression of cytokines resulting from the activation of neurons and glial cells of the TVS also contributes to chronification.

In summary, neuronal, vascular, and inflammatory phenomena concur in migraine and its chronification. The physiopathology of the disorder is very complex and we do not fully understand it, but it is clear that not only neurons participate in it, but also endothelial cells and the immune system composed of glial cells and the molecules of the inflammatory cascade.

1.3. MIGRAINE AND IMMUNITY

For much of the 20th century, the CNS was considered an immune sanctuary, which is why the contribution of inflammation to neurological diseases was ignored for decades. Today we know that the CNS has its own immune system, represented mainly by microglia, and also that the lymphatic system is innervated and there is fluid communication between the immune and nervous systems (Buchanan MM et al.2010). In the last 20 years, the interactions between both systems have been the subject of constant research and

it has been proven that the activation of glia is essential in many CNS diseases, including chronic pain. In the specific case of migraine, work on the role of underlying immune mechanisms is still scarce.

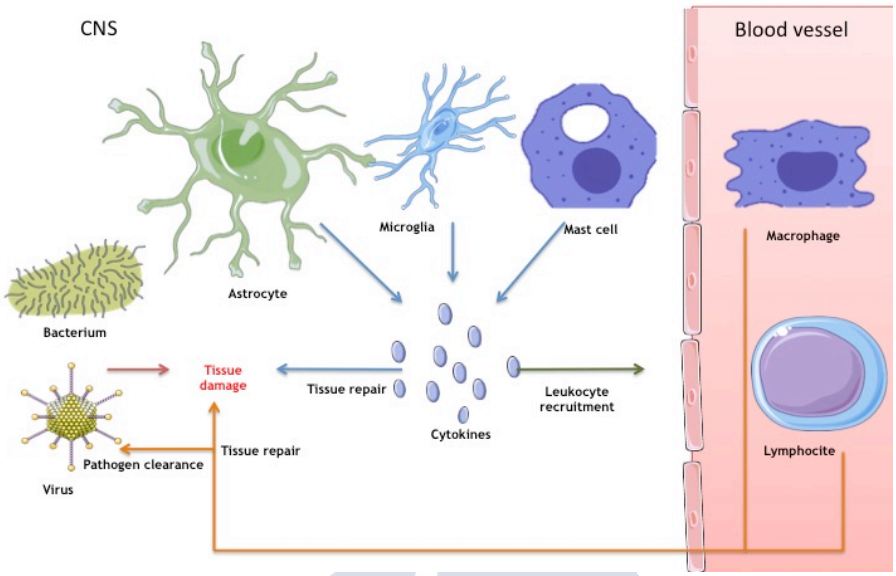
1.3.1.Innate and adaptive immunity

The body's response to infectious agents is of two types: innate and adaptive. The innate (also immediate) immune response is the first line of defense against invading microorganisms. It is the oldest from a phylogenetic point of view and some of its mechanisms are present from plants to higher mammals. The innate immune system has evolved to detect structures foreign to the organism and its mechanisms are triggered immediately after contact with the harmful agent (Medzhitov R et al. 2002). The first level of defense is constituted by passive barriers, such as skin, pH, or mucus, but if the invading agent trespasses these initial barriers, active mechanisms provided by resident phagocytic cells (neutrophils and macrophages) come into play. Inflammation is an essential mechanism in the innate response that contributes to the success of pathogen phagocytosis and the recruitment of a wide variety of immune cells. The innate immune response is characterized by the fact that it is triggered by all types of agents, regardless of previous exposure (Moresco EM et al. 2011).

Adaptive immunity, unlike innate immunity, is established more slowly (3-8 days after exposure to a foreign agent) and is modified by previous exposures to pathogens. It is highly specific at the molecular level, often to the extent of recognizing particular species of organisms. This specificity depends on the formation of receptors generated *ad hoc* by somatic DNA recombination. The adaptive response is relatively recent from an evolutionary point of view and exists only in vertebrate organisms (Medzhitov R et al. 2002).

In mammals, the innate and adaptive responses work in tandem. However, although there is coordination between the two mechanisms, each of them can develop independently.

Figure 1.5. Innate and adaptive immune response in the CNS



Mast cells, microglia, and astrocytes exert protective and restorative responses to CNS infection by virus and bacterium, and also against other types of injury like cellular necrosis or inflammation. Cytokines expressed by resident CNS defense cells help to recruit circulating lymphocytes and myeloid cells. Adapted from Ransohoff RM et al. 2012. (This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). Created with Servier Medical Art images (Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License)

1.3.2. Pattern Recognition Receptors (PRRs)

Both innate and adaptive responses depend on the ability to distinguish one's own from another's. This recognition is exercised by a range of receptors directed against highly conserved molecules characteristic of viruses, fungi, bacteria, and protozoa, such as lipoteichoic acids and bacterial lipopolysaccharides (Bartley J. 2009). These highly conserved ligands between microorganisms are called pathogen-associated molecular patterns (PAMPs) and the receptors that recognize these structures in the "invaded" organism are called pattern recognition receptors (PRRs).

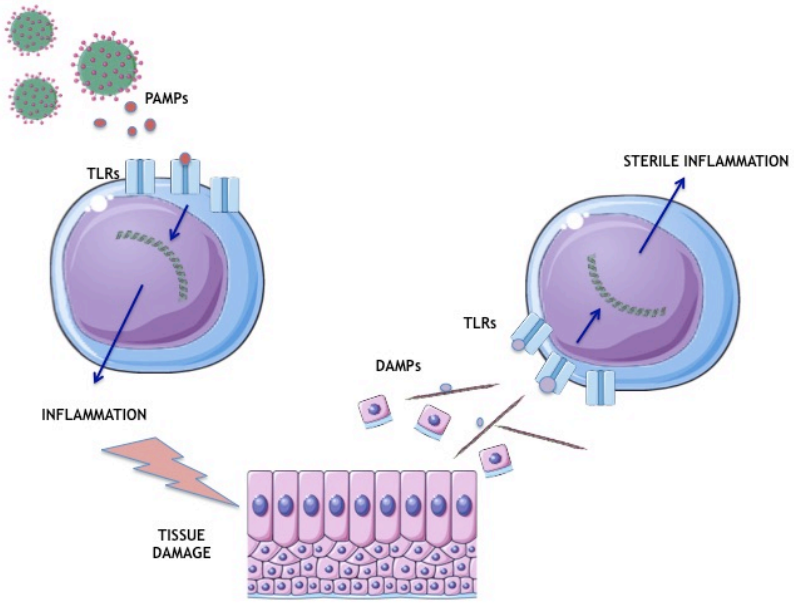
Several families of PRRs have been described, one consisting of soluble receptors (opsonins) and another consisting of cellular receptors, which includes three types: Rig-I-like receptors (RLRs), NOD-like receptors (NLRs), and Toll-like receptors (TLRs). Opsonins bind to microorganisms and facilitate their phagocytosis; they are the complement factors and molecules of the pentraxin family (Delneste Y et al. 2007), among others. RLRs and NLRs recognize cytosolic ligands, while TLRs recognize both extracellular and intracellular ligands (Victorino F et al. 2013). The family of TLRs represents the most important system of pathogen recognition in mammals. The other families of receptors mentioned are to some extent dependent on TLRs and are never able to compensate for the absence of TLRs, which produces severe immunodeficiency (Moresco EM et al. 2011).

The innate response is critical to a robust adaptive response (Victorino F et al. 2013). TLRs are involved in the activation of adaptive immunity, functioning as an interface between the pathogen recognition phase and the production of a complex immune response. For example, activation of TLRs is necessary to induce the expression of stimulator molecules on antigen-presenting cells, which then activate T-lymphocytes and regulate their function by producing and releasing IL-12 (Hoebe K et al. 2004). The role of TLRs in adaptive immunity is secondary, as opposed to innate response; there is no evidence that the absence or dysfunction of TLRs prevents the development of adaptive immunity. On the contrary, it has been proven that the adaptive response occurs equally after infection in animals lacking TLRs (Moresco EM et al. 2011).

PRRs are also key in non-infectious inflammatory diseases. In addition to PAMPs, TLRs can recognize a variety of endogenous molecules, which are normally isolated from contact with the immune response and are called danger-associated molecular patterns (DAMPs). DAMPs are endogenous markers of danger released after the death of host cells (Seong SY et al. 2004). These endogenous structures include hyaluronic acid (Taylor KR et al. 2004) and several molecules released by necrotic and apoptotic cells (Jiang D et al. 2005), such as heat shock proteins (Vabulas RM et al. 2002),

surfactant protein type A (Sato M et al.2003), high mobility group box 1 protein (HMGB-1) (Park JS et al. 2004), β-amyloid (Jana M et al. 2008), oxidized low-density lipoproteins (LDL) (Balogh S et al. 2009) and endogenous nucleic acids (Guiducci C et al 2010). The activation of PRRs by DAMPs produces what we call sterile inflammation, and generates a favorable environment for the repair of tissue damage, but also contributes to the emergence of long-term pathologies such as neoplasms, autoimmune diseases, or atherosclerosis (Jimenez-Dalmaroni MJ et al. 2016).

Figure 1.6. PRRs, PAMPs, and DAMPs



Inflammatory responses in innate immune cells can be derived from exogenous microbe-derived (PAMP) or endogenous host-derived molecules (DAMP). PAMPs from pathogens or commensal bacteria that act as TLR agonists induce the production of inflammatory cytokines in response to infection. DAMPs or alarmins released from dead or dying cells that act as TLR agonists mediate the induction of inflammatory cytokine production from tissue in response to injury or stress.

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Chronic inflammation underlies many human diseases and is responsible for much of the deleterious effects that occur during infectious processes. The activation of PRRs is key in this process, so understanding their signaling pathways and how they can be interrupted or modified may offer new therapeutic possibilities (Suresh R et al. 2013). PRRs also play an essential role in the maintenance of certain physiological environments and the modulation of the inflammatory response, for example, in the relationship with commensal microbiota (De Nardo D et al. 2015).

1.3.3. Toll-like receptors.

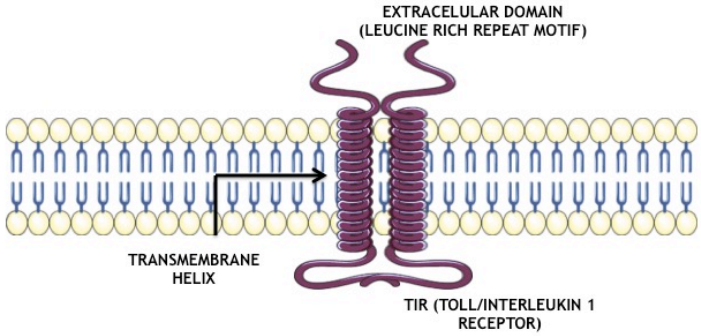
In 1980 a gene was discovered that, when mutated in the embryo of the fruit fly (Drosophyla melanogaster), produced modifications in the dorsal-ventral polarity of the embryo; this gene was called the Toll gene (Nusslein-Volhard C et al. 1980). In 1984 the protein "Toll" derived from the gene in Drosophyla melanogaster was described, and it was proved that it not only intervened in the formation of the embryo but also the recognition of the microbes and the initiation of the immune response (Steward R et al. 1984). It was later found that mutation in a Toll-related receptor, 18-wheeler, increased the incidence of fungal infections in this type of fly (Williams MJ et al. 1997).

In 1997, a receptor functionally similar to the toll protein, located on the cell surface, was described for the first time in humans (Medzhitov R et al. 1997). Later studies revealed that it was the receptor for lipopolysaccharide (LPS), the main immunogenic component of the external wall of gram-negative bacteria. Since then, 13 TLRs have been described in mice and 10 TLRs in humans (Liu Y et al. 2014). Types 1, 2, 4, 5, 6, and 11 are located on the cell surface, while types 3, 7, 8, and 9 are located inside the cell and recognize nucleic acids (Akira S. 2006). TLRs are expressed mainly in cells of the immune system, but also in some cell types that are not directly involved in immunity.

1.3.3.1. Molecular structure of TLRs

TLRs are type I transmembrane glycoproteins that are composed of an extracellular amino N-terminal domain with recognition function, a helical transmembrane domain, and an intracellular carboxy-C-terminal domain with signaling function (Jimenez-Dalmaroni MJ et al. 2016). The extracellular domain consists of 16-28 leucine-rich repeats (LRRs) and is responsible for recognizing PAMPs and DAMPs. The intracellular domain of TLRs is called TIR (Toll/interleukin 1 receptor) because it is very similar to the intracellular domain of the IL-1 receptor. It consists of about 200 amino acids. Once the extracellular domain recognizes the ligand, the intracellular domain is dimerized and signaling pathways are activated through the interaction of the TIR domains of the TLR with the TIR domain of various intermediary molecules.

Figure 1.7. Structure of TLRs

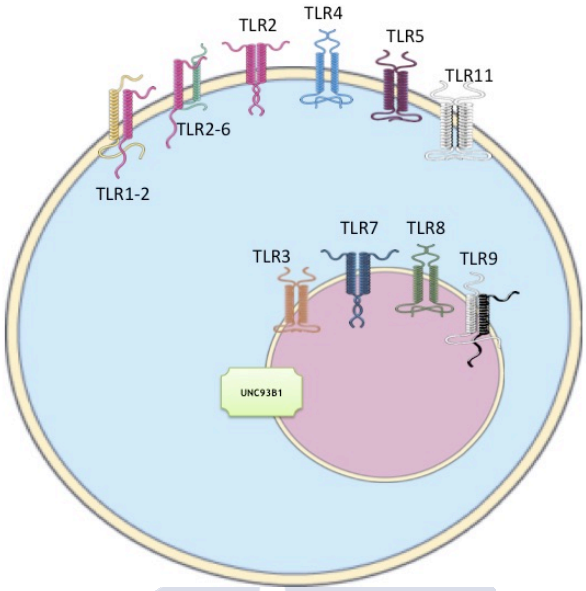


TLR structure consists of three basic components: (1) leucine-rich repeat (LRR) motif; (2) transmembrane helix; (3) intracellular TIR domain. TLRs normally form heterodimers.

1.3.3.2. Classification of TLRs

TLRs can be classified according to whether they are located on the cell surface or in the endosomal vesicles (endoplasmic reticulum, lysosomes, endolysosomes). TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11 appear on the cell membrane, while TLR3, TLR7, TLR8, and TLR9 are expressed on the endosome. TLR4 is expressed on the cell surface but it is internalized after activation. Surface TLRs recognize molecules located on the microbial membrane such as lipids, proteins, and lipoproteins and endosomal TLRs recognize microbial nucleic acids (De Nardo D. 2015). To perform this recognition, some TLRs need to form dimers, either with other TLRs or with adjuvant molecules. In the case of TLR2 and TLR4, the most important adjuvant molecules are CD14, which contributes to the recognition of ligands (Jiang Z et al. 2005), MD-2, which is essential for TLR4 to bind to bacterial LPS (Moresco EM et al. 2011), and CD36. CD14 is a 375 amino acid glycoprotein that may be present as a soluble form in the blood or as glycosylphatidylinositol anchored to the myeloid cell membrane. CD36 participates in the activation of TLR2 by negatively charged microbial ligands (Jimenez-Dalmaroni MJ et al. 2009) and the activation of TLR4 and TLR6 by endogenous ligands (Stewart CR et al. 2010).

Figure 1.8. Classification of TLRs.



TLRs are divided into two groups based on their localization in the cell. TLRs 1, 2, 4-6, and 11 are surface TLRs, and TLRs 3 and 7-9 are located in the endosomal compartments (intracellular TLRs). Cell surface TLRs bind microbial membrane materials (lipids, lipoproteins, and proteins), whereas intracellular TLRs recognize nucleic acids from bacteria and viruses. Adapted from: Goulopoulou S et al. 2016 with permission of ASPET. Created with Servier Medical Art images (Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License).

Surface TLRs respond to PAMPs from the bacterial surface:

- TLR1: works in conjunction with TLR2 and interacts with the N-acyl chain of ligands.
- TLR2: is the TLR that recognizes the largest number of ligands. It works together with TLR1 and TLR6. It recognizes glycolipids like the lipoteichoic acid of gram-positive bacteria (Jimenez-Dalmaroni MJ et al. 2015), the lipoarabinomannan of mycobacteria (Underhill DM et al. 1999), and the GPI

anchorage structures of *Trypanosoma cruzi* (Ropert C et al. 2004). Besides, it acts jointly with other co-receptors of the cell surface for the recognition of PAMPs.

- TLR4: the most studied TLR, it recognizes the LPS of the bacterial wall, as well as proteins of the respiratory syncytial virus, *Streptococcus pneumoniae,* and some drugs (paclitaxel). In order to work, TLR4 needs MD2, a soluble protein that anchors to the extracellular domain of TLR4 and is essential for its expression on the cell surface (Nagai Y et al. 2002) as well as for the glycosylation of TLR4 during its synthesis process. In KO mice for MD2, TLR4 does not reach the cell surface and accumulates in the Golgi apparatus.
- TLR5: recognizes the bacterial flagellin (Hayashi F et al. 2001) and is located in the basolateral face of the intestinal epithelium. It also participates in the transport of *Salmonella typhimurium* from the intestinal tract to the mesenteric lymph nodes.
- TLR6: it is part of the receptor complex with TLR2.

Endosomal TLRs recognize viral and bacterial nucleic acids that are not found in the extracellular space but appear during intracellular replication or after phagocytosis. For these endosomal TLRs to be functional, the molecule UNC93B1 must be present, which allows them to leave the endoplasmic reticulum.

- TLR3: recognizes double viral chain RNA (Chattopadhyay S et al. 2014) and participates in the immunity against Nile virus (Wang T et al. 2004), herpes simplex, and influenza virus (Liu Y et al. 2012). It triggers the immune response by producing interferon and inflammatory cytokines. Human TLR3 deficiency or UNC93B1 deficiency, which makes it difficult to function, implies susceptibility to herpes virus type 1 infections (Casrouge A et al. 2006).
- TLR7 and TLR8: TLR7 and TLR8 recognize single-stranded RNA from virus. TLR7 in particular causes antigen-presenting cells to produce more interferon and especially cytokines in response to viral infections. TLR8 is mainly expressed in

monocytes and its expression increases after bacterial infections (Heil F et al. 2004).

- TLR9: TLR9 recognizes DNA residues present in bacteria and viruses, that are very rarely found in mammalian cells (Hemmi H et al. 2000); it plays a major role in the recognition of HSV1, HSV2, and the insoluble crystal hemozoin, generated by *Plasmodium falciparum*.

1.3.3.3. Cell expression of TLRs

TLRs are expressed mainly in cells responsible for the innate immune response, i.e. macrophages, neutrophils, and dendritic cells, but also in cells involved in the adaptive immune response (B and T lymphocytes). Likewise, many non-immune cells, such as epithelial cells, neurons, astrocytes, and fibroblasts express TLRs and respond to their activation. In general, the activation of TLR signaling pathways triggers cytotoxic activities of immune cells (Delneste Y et al. 2007), but the effects are not the same in all tissues; in the digestive epithelium, for example, it induces cell proliferation, while in other locations it more closely resembles the classical inflammatory response (Moresco EM et al. 2011).

In the specific case of the innate immune system, the activation of TLRs triggers a variety of responses (Kumar H et al. 2009). Macrophages and dendritic cells are non-specialized antigen-presenting cells (APCs). In response to contact of TLRs with their ligands, macrophages intensify their phagocytic activity and produce numerous pro-inflammatory cytokines and chemokines. Dendritic cells are the main APCs, capable of activating virgin T-lymphocytes. The recognition of ligands by TLRs causes the dendritic cell to internalize the microorganism and mature, migrate to the peripheral nodes and present the microbial components to the T-lymphocytes for recognition. In addition, dendritic cells express various co-stimulatory molecules and chemokines that attract immune cells to the site of infection. Natural killer (NK) cells induce the production of antimicrobial peptides (defensins) and immunostimulatory cytokines. Polynuclear neutrophils are activated and undergo degranulation in response to the stimulation of TLRs. The expression of almost all

types of TLRs in mast cells, which are key to the innate immune response, has been confirmed in animals and humans. Mast cells are preferably located in well-vascularized connective tissue and are very numerous at the barriers between the body and the environment, such as the skin or the periphery of the vessels. They are the first defense cells to confront invading organisms. Mast cells are a source of inflammatory mediators such as proteases, histamine, metalloproteinases, arachidonic acid metabolites, cytokines, and chemokines (Agier J et al. 2018)

TLRs are also expressed in the cells that make up adaptive immunity (B-lymphocytes and T-lymphocytes) and are very important in making their response faster. Regulatory $CD4^+$ and $CD25^+$ T-lymphocytes express TLRs and play a key role in inhibiting many autoimmune or inflammatory manifestations (Liu G et al. 2007). The regulation of TLR expression in lymphocytes has a great therapeutic potential in autoimmune pathologies.

The stimulation of TLRs in endothelial cells leads to the production of immunostimulatory cytokines and adhesion molecules involved in the recruitment, activation, and migration of immune cells. Fibroblast activation also leads to the production of cytokines, chemokines, and antimicrobial peptides. The expression of TLRs in CNS cells will be discussed later in this section.

Table 1.4. Distribution of TLRs in major immune response cells

Toll-like receptor	Mono-cyte	NK	B Lymph	T Lymph	Neutro-phil	Eosino-phil	Baso-phil
TLR-1	++	++	++	+	+	+	-
TLR-2	++	+	±	±	++	+	+
TLR-3	-	+	±	±	-	+	-
TLR-4	++	±	±	±	++	+	+
TLR-5	+	+	±	+	+	+	-
TLR-6	+	+	++	±	+	+	-
TLR-7	±	±	+	±	+	+	-
TLR-8	+	±	±	±	+	-	-
TLR-9	±	±	+	±	+	-	-
TLR-10	±	±	+	±	+	-	+

1.3.3.4. Ligands of TLRs

TLRs recognize microbial ligands and endogenous ligands. The most relevant ligands in the case of human TLRs are displayed in the following table (Yu L et al. 2010).

Table 1.5. Main ligands of TLRs.

TLR	Microbial ligands (PAMPs)	Endogenous ligands (DAMPs)
TLR1	Bacterial lipopeptides	
TLR2	Lipomannan Lipoic acids Bacterial lipopeptides (peptidoglycan) Lipopolysaccharide	HSP60, HSP70, HSP96, HMGB-1, gp96, biglycan, SP-D, endoplasmin, cardiac myosin, hyaluronic acid, uric acid crystals
TLR4	Lipopolysaccharide	Biglycan, HSP60, HSP70, HSP22, HSP96, fibrinogen, fibronectin, hyaluronic acid, HMGB-1, OxLDL, ß-amyloid, ß-defensin, endoplasmin, heparan sulfate, resistin, s100, surfactant
TLR5	Flagelline	Unknown
TLR3	dsRNA (virus)	mRNA (necrotic cells), DNA
TLR6	Bacterial lipopeptides	
TLR7	ssRNA (virus)	ssRNA, imiquimod
TLR8	ssRNA (virus)	ssRNA, microRNAs
TLR9	CpG DNA (bacteria, virus)	Self-DNA, HMGBI1

Adapted from Yu L et al. 2010, with the permission of John Wiley and Sons (LICENSE NUMBER: 4939330642078).

Several molecular mechanisms increase the range of ligands capable of stimulating TLRs, such as the formation of heterodimers and the cooperation of accessory proteins and co-receptors. A good example of this type of process is the recognition of β-amyloid by the CD36 receptor, which later forms a complex with the TLR4-TLR6 dimer (De Nardo D. 2015).

Immediately after their discovery, TLRs were related to the recognition of foreign molecules; however, the context in which these

receptors were identified already suggested the existence of some kind of interaction with endogenous molecules. Toll played a role in the dorsal-ventral development of the nervous system in *Drosophyla melanogaster*; since its expression was generalized in the cells of the organism, there had to be a ligand in the dorsal-ventral axis responsible for its activation at that level. Finally, this ligand was found, called "spaätzle". Although no equivalent of this ligand has been found in mammals, several endogenous molecules have been identified that bind to TLRs: components of the extracellular matrix and intracellular proteins and nucleic acids released into the extracellular space after necrosis. These molecules, which we call "alarmins", can stimulate TLRs (O'Neill LA et al. 2009) and generate a sterile inflammatory response. This allows the immune system not only to defend the organism from invading agents but also to participate in tissue repair mechanisms (Yu L et al. 2010).

"Alarmins" are molecules of diverse origin and structure, ranging from compounds derived from cell damage to inflammatory mediators and oxidized lipids. They interact mainly with TLR2 and TLR4, but some of them can stimulate other TLRs. Among them are heat shock proteins (HSPs 22, 60, 70, 90, and 96), intercellular matrix products, DNA, HMGB-1, hyaluronic acid, fibronectin, uric acid crystals, and β-defensin (Matzinger P. 2002). Most endogenous TLR ligands are known from ischemia and reperfusion models, in which massive tissue damage and enormous release of DAMPs occur (Yu L et al. 2010), although it has subsequently been shown that they can be released in many other CNS diseases (Marshak-Rothstein A. 2006). The identification of endogenous TLR2 and TLR4 ligands was complex because of the ubiquity of LPS, its main exogenous ligand, that could contaminate the samples. In fact, some study groups still question whether "alarmins" are capable of stimulating TLRs by themselves or whether their effect derives from an increase in the susceptibility of cells to PAMPs or other inflammatory mediators (Erridge C. 2010). The main molecules included in the group of "alarmins" are detailed in the following table:

Table 1.6. Endogenous ligands of TLRs

Molecular family	DAMPs
Proteins and peptides	B-defensin, fibrinogen, fibronectin, HMGB1, HSP, human cardiac myosin, resistin, s100 protein, surfactant protein A, tenascin-C
Polysaccharides and proteoglycan	Biglycan, CD138, heparan sulfate, hyaluronic acid, and hyaluronic acid fragments.
Nucleic acids	DNA, RNA, mRNA
Phospholipids	OxPAPC
Small organic molecules	Monosodium urate crystals

The most relevant endogenous ligands at the CNS level are heat shock proteins, HMGB1, and fibronectin.

Heat shock proteins (HSPs) are present in almost all cell types, where they function as chaperones that maintain the protein configuration. The HSP family includes several members such as HSP60, HSP70, and HSP90 (gp96). Heat shock proteins are released into the extracellular space during necrosis, to a lesser extent during apoptosis, and also under conditions of cellular stress by exocytosis mechanisms. Once in the extracellular space, they can stimulate the innate immunity receptors of neighboring cells. HSP60 and HSP70 are the most important endogenous ligands of TLR2 and TLR4. Under conditions of stress or damage, their production increases, and they leak into the extracellular space where they induce the immune and inflammatory response (Wang Y et al. 2013). When neurons, in response to stress, produce HSP60, a vicious circle is initiated in which TLR4 is increasingly activated with the consequent production of neurotoxic effectors (Buchanan MM et al. 2010). It has been shown that blocking either HSP60 or TLR2 and TLR4 suppresses the inflammatory response in serum-cultured cells from patients with ischemic stroke, making this molecule a possible therapeutic target in stroke and other neuroinflammatory processes (Brea D et al. 2011).

HMGB1 belongs to the family of non-histone DNA-binding nuclear proteins. This group of proteins is responsible for maintaining the configuration of DNA in the cell nucleus and for regulating gene transcription. In situations of stress or damage, they leak into the extracellular space and activate the inflammatory response after binding to TLR2 and TLR4 (Yu L et al. 2010). High levels of HMGB1 have been found in stroke, multiple sclerosis, and other inflammatory diseases such as rheumatoid arthritis. HMGB1 is released at necrosis, but not during apoptosis (Sloane JA et al. 2010). It is a very potent inflammatory signal that induces the secretion of TNFα, IL-1β, IL-10, and IL-6 by monocytes and macrophages, facilitates the maturation of dendritic cells, and also has a relevant role in the immune response against tumor cells. Some cells, such as neurons, secrete HMGB1 in their basal stage (Bianchi ME. 2007).

Fibronectin is a ubiquitous component of the extracellular matrix capable of binding to TLR4 (Marshack-Rothstein A. 2006). During tissue repair, fibronectin is key to regulate cellular processes and direct tissue organization. Fibronectin interacts with multiple binding partners, including cell surface receptors as TLRs. There are many isoforms of fibronectin, but they are mainly grouped in plasma fibronectin (pFN) and cellular fibronectin. pFN is secreted mainly by hepatocytes to plasma and its levels increase after trauma or inflammation. cFN, on the contrary, is produced by many different types of cells, such as endothelial cells. Isoforms of cFN change from one cell type to another. Overexpression of cFN has been described in wound healing as well as in fibrosis and tumorigenesis (To WS et al. 2011).

The remaining endogenous ligands reflected in Table 1.6 are less relevant in the CNS. Uric acid produces inflammation during gout attacks by a mechanism mediated by TLR2 and TLR4. TLR3 is activated after contact with RNA released in cell necrosis in rheumatoid arthritis. Single-stranded guanine residue complexes activate TLR7 by inducing secretion of TNFα, IFNα, and IL-6. TLR9 and TLR7 recognize the body's DNA and detect the anti-DNA complexes circulating in lupus (Matzinger P. 2002). In addition to fibronectin, other components of the extracellular matrix are released

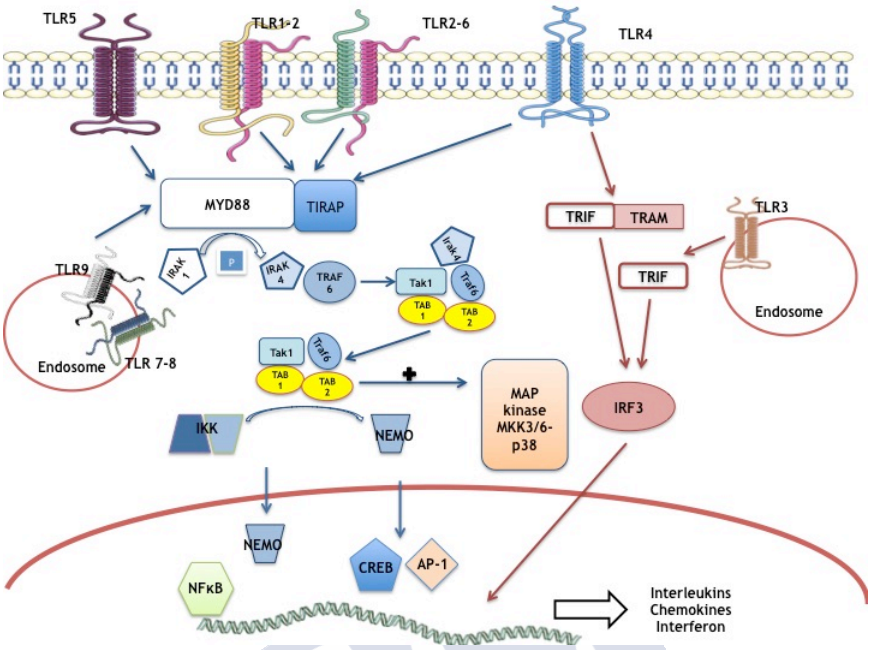
and fragmented during tissue damage due to enzymatic digestion, oxidative stress, or the direct effect of mechanical forces. Among all these molecules, only some can activate TLRs: low molecular weight hyaluronic acid (TLR4 and TLR2) and heparan sulfate (TLR4).

1.3.3.5. Signalling pathways

When TLRs join a ligand a conformational change occurs: the extracellular domain of the receiver takes the form of an M and the intracellular domain forms a dimer that gives rise to the structure we call TIR. This transformation leads to the coupling of a molecular adapter, which also has a TIR domain, in the internal part of the receptor.

Five types of TIR domains have been described so far: MyD88 (myeloid differentiation factor 88), TIRAP (TIR domain-containing adaptor protein, also known as MAL), TRIF (toll/interferon response factor), TRAM (TRIF-related adaptor molecule), and SARM (Sterile α and armadillo motif-containing protein) (Wang Y et al. 2013). Depending on the domain that is activated, one signaling cascade or another is triggered. In summary, the signaling pathways of TLRs can be divided into MyD88-dependent and TRIF-dependent. These two pathways originate from two different cellular locations, the cell surface (MyD88) and the endosome (TRIF), and generate an inflammatory response producing cytokines (MyD88), or an antiviral response, producing IFN-β (TRIF/TRAM) (Liaunardy-Jopeace A et al. 2014). The TRIF-dependent pathway is called "slow or late activation," while activation through MyD88 is called "rapid or early activation". TLR4 is the only TLR that has the ability to combine both mechanisms (Buchanan MM et al. 2010). It is important to highlight the role of nuclear factor κB (NFκB), the transcription factor in which both pathways culminate, as a regulator of a wide range of processes in the central nervous system such as synaptic plasticity, neurogenesis, and differentiation (Sarnico I et al. 2009). Activation of NFκB may protect neurons from oxidative stress or ischemic degeneration, but at the same time may contribute to inflammatory reactions and cellular apoptosis after brain damage and stroke (Caso JR et al. 2008).

Figure 1.9. TLR signaling pathways.



Toll-like receptors and signaling pathways, with their adaptor and intermediate signaling proteins.
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TLR1, TLR2, TLR5, TLR6, TLR7, TLR8, and TLR9 use the MyD88 dependent pathway. TLR3 works through TRIF. TLR4 can use both pathways at the same time, probably in a strategy to maximize the inflammatory response. TLR2 and TLR4 need an extra molecule to interact with MyD88, called TIRAP/MAL, and TLR4 also needs an accessory molecule for the TRIF-dependent pathway, TRAM, which also allows the transfer of the complex to the endosome (Kawai T et al. 2007). SARM was the last domain described; in humans, it seems to have an inhibitory function on the TLR3 pathway.

The different signaling pathways of TLRs and other PRRs communicate with each other. A single pathogen can express many ligands, which in turn can be recognized by several TLRs and activate different signaling pathways. TLRs interact with other PRRs and with their signaling pathways (De Nardo D. 2015).

1.3.3.5.a. MyD88 pathway

MyD88 has two domains, the TIR domain, which joins the TIR domain of the TLR, and a dead domain. The dead domain recruits IRAK-1 (interleukin-1 receptor-associated kinase), which is phosphorylated and transformed into IRAK4, to which TRAF-6 (tumor necrosis receptor-associated factor 6) is attached. This complex formed by IRAK4 and TRAF6 dissociates from the TLR and forms a complex with TAK1 (transforming growth factor β activating kinase), TAB1 (TAK 1 binding protein 1), and TAB2 (TAK 1 binding protein 2). The formation of this complex leads to the phosphorylation of TAK1 and TAB2. IRAK4 is degraded and the complex formed by TRAF6, TAK1, TAB1, and TAB2 is displaced to the cytosol where TRAF6 ubiquitination and TAK1 activation occurs. Activated TAK1 modulates the IκB kinase complex (IKK), producing the release of its NEMO subunit (NFκB essential modulator) and its translocation to the nucleus. TAK1 also activates the MAP kinase MKK3/6-p38 cascade leading to the activation of the nuclear transcription factor CREB (cAMP response element-binding), and the transcription factor activator protein-1 (AP-1). AP-1 and NEMO activate the expression of proinflammatory cytokines, chemoquins, and major histocompatibility complex stimulating molecules that play a central role in inflammation (Jimenez-Dalmaroni MJ et al. 2016).

The activation of TLR4 and the MyD88 pathway induces the expression of several pro-inflammatory substances: reactive oxygen species (nitrous oxide, hydrogen peroxide, and superoxides) and pro-inflammatory cytokines such as IL-1, IL-6, INF-α, IFN-β. It also stimulates the expression of CD40, CD80, CD86, and MHC-II (major histocompatibility complex) in immature APCs. MyD88 may also activate transcription of interferon regulatory factor 7 (IRF7), which induces powerful antiviral cytokines such as type 1 interferons

(Moresco EM et al. 2011). Activation of TLR4 also modulates the release of IFN-β, an anti-inflammatory molecule (Kagan JC et al. 2008).

1.3.3.5.b. TRIF pathway

The MyD88 independent pathway, or TRIF pathway, is responsible for the production of inflammatory cytokines and interferon after activation of TLR3 and TLR4. The interaction between TLR4 and TRIF requires an intermediary protein called TRAM. Once TLR4 is activated, it first starts the MyD88 pathway, and then it is internalized by endosomes, coupled to TRAM, and forms a dimer with TRIF. Once this dimer is formed, IKKε, TBK1 (TANK binding kinase 1), and TRAF3 are also joined. RIP1 (receptor-interacting protein 1), after contacting the carboxy-terminal region of TRIF, activates NFκB (NEMO). TBK1 phosphorylates IRF3, which, together with p300 and CBP (CREB binding protein), triggers the expression of interferon-inducible genes, IP-10 and RANTES. TRIF can also bind to TRAF6 and activate the late production of inflammatory cytokines. The TRIF-dependent pathway through TRAF3 (as opposed to TRAF6) leads to the synthesis of IFN-β, which has anti-inflammatory and antiapoptotic effects and constitutes an endogenous system of control of the innate immune response (Buchanan MM et al. 2010).

1.3.3.6. Regulation of the innate response mediated by TLRs

The signaling cascades of the TLRs allow a great amplification of the signals since a great number of kinases and ubiquitinases are part of them. Thus, the activation of only a few TLRs is capable of producing enormous transcriptional and post-transcriptional changes in a cell (Moresco EM et al. 2011). For this reason, the activation of TLRs is a very sensitive process and is strictly regulated to avoid aberrant signals.

If we consider the regulation of the response, the innate immune response can be divided into three phases. In each of these phases, different factors act, with stimulating and inhibiting effects, whose

aim is to produce a response proportionate to the stimulus at the right time (Victorino F et al. 2013).

- Phase 1: PRRs, signal transduction proteins, and transcription factors such as NFκB initiate the response to the PAMP or DAMP stimulus. The production of new proteins participating in the second phase is induced.
- Phase 2: It occurs 2-8 hours after initial activation and is mediated by transcription factors that induce or inhibit the response, such as CEPBδ (binds to the IL6 promoter and induces its transcription) or ATF3 (inhibits IL6 production and limits the inflammatory response).
- Phase 3: A chromatin remodeling of the genes related to inflammation occurs, aimed at maintaining the response over time or ending it permanently.

The most basic mechanism of regulation in these pathways is the internalization of TLRs. When the LPS activates the TLRs they are internalized, their presence on the cell surface is reduced and the cells lose their capacity to respond to the stimuli (Liaunardy-Jopeace A et al. 2014). Endosome-activated receptor endocytosis has two fundamental consequences, the activation of the TRAM/TRIF pathway and the termination of the signal.

At this level of the transcription pathway, the other basic mechanism in the regulation of TLRs takes place: the ubiquitination of TRAF3. If the TRIF pathway is activated, TRAF3 is activated, which is critical for IFN production; conversely, if the MyD88 pathway is activated, there is a degeneration of TRAF3 that leads to the induction of pro-inflammatory proteins by MAPK activation. Some studies are investigating the inmunomodulatory role of partial TLR4 agonists that only activate the TRIF pathway since they would function as potent adjuvants of the immune response but with low inflammatory toxicity (Bohannon JK et al. 2013). An example is MPLA (monophosphoryl lipid A) which activates the TRIF-dependent pathway and is a component of papilloma and hepatitis vaccines.

Several molecules are involved in inhibiting molecular signaling in subsequent levels of their signaling pathways (Liew FY et al.

2005). These molecules target receptors, adaptive molecules, or kinases. MyD88s, SOCS (suppressor of cytokine-signaling-1), IRAK-M, A20, MRSAs, and ADAM15 act on adaptor molecules such as MyD88, TIRAP, and TRIF. Tyrosine phosphatase SHP-1, IRAK-M, and Toll-interacting protein (Tollip) inactivate IRAK-1, which acts as an intermediate in the signaling cascade; A20, USP25, and Sky act on TRAF. ATF3 acts as a regulator at the transcriptional level. Also, the molecules that regulate the traffic of the receptors responsible for innate immunity have a very important role in its regulation. Mutations have been described in Unc93b, which facilitates the trafficking of TLR3, 7, 8, and 9 in patients with recurrent herpes virus type 1 encephalitis, in Griscelli syndrome, lupus, and Crohn's disease (Victorino F et al. 2013). Certain isoforms of the extracellular domains of TLR2 and TLR4 are secreted in saliva, plasma, and breast milk and function as inhibitors of the immune response by binding to and blocking TLR ligands in plasma (Raby AC et al. 2009). Activation of TLR10 also has an inhibitory effect, preventing the immune response to certain pathogens and slowing the progression of autoimmune diseases (Sallusto et al. 2009).

Table 1.7. Accessory molecules that regulate TLR4 activity.

Accessory molecules	Role in TLR regulation
PRAT4A	TLR4 folding in the endoplasmic reticulum
Gp96	TLR4 folding in the endoplasmic reticulum
MD2	Glycosylation of TLR4 and accessory molecule for LPS recognition
CD14	LPS co-receptor on the cell surface and promotes LPS-activated receptor endocytosis
TMED7	Transport of TLR4 to the cell surface
Rab10	Induces transport of TLR4 to the cell surface after stimulation
Rab7b	TLR4 degradation in the lysosome

MyD88	Adaptor for intracellular signal transduction
Mal	Adaptor for intracellular signal transduction
TRIF	Adaptor for intracellular signal transduction
TRAM	Adaptor for intracellular signal transduction
SARM	Inhibits TLR4 activation
CD11b	Inhibits TLR4 activation

An interesting phenomenon in terms of regulation of the inflammatory response is that of endotoxin tolerance. Exposure to low or moderate doses of LPS or lipid A makes the body less reactive to the next LPS stimulus. This state of tolerance allows us to survive a second contact with the LPS, which would be lethal. This is achieved by decreasing the production of inflammatory mediators such as TNFα, IL6, and IFN and increasing the production of anti-inflammatory mediators such as IL-10, TGFβ, and the IL-1 receptor agonist. This mechanism of tolerance also attenuates the production of inflammatory cytokines in other situations such as Gram-positive bacteria contact, ischemia-reperfusion damage, or hemorrhagic shock (Bohannon JK et al. 2013). Different cross-reactions between TLR agonists may also induce this tolerance. The molecular mechanisms that generate tolerance are varied, affecting both the receptor, adaptive proteins, signaling molecules, and transcription factors, but most of them act on the MyD88 pathway. At the nuclear level, changes in histone methylation, acetylation, and ubiquitination produce changes in gene transcription.

1.3.4. Immunity and TLRs in the CNS

Contrary to the traditional views on CNS pathology, immunity and inflammation are key players in neurological disease. In general, we can say that acute inflammation in the CNS is beneficial, helps to

eliminate pathogens, cellular debris, and contributes to tissue repair; prolonged inflammation, however, is neurotoxic. A prolonged inflammatory state inflicts damage on most tissues, but this damage is greater in the CNS, where there is an irreversible loss of neurons due to their high susceptibility to the toxins generated during the innate immune response and the absence of regeneration mechanisms (Lehnardt S. 2010). Inflammatory mediators released during acute inflammation promote neurogenesis, but inflammatory mediators released during prolonged inflammation inhibit it (Withney NP et al. 2009).

1.3.4.1. Expression of TLRs in the CNS

Innate immunity and PRRs, specially TLRs, are essential in CNS diseases, either infectious and non-infectious. Many inflammatory and neurodegenerative diseases activate mechanisms of innate immunity, without requiring the involvement of the adaptive system. The activation of innate immunity in these diseases is indistinguishable from that secondary to microbial exposure and occurs at almost the same rate. Glia is responsible for the immediate immune response in the CNS.

Glial cells are present in the central and peripheral nervous system in greater numbers than neurons, constituting 70% of the CNS. The term includes three types of cells: microglia (myeloid lineage cells representing the mononuclear-phagocytic system resident in the CNS), astrocytes (responsible for modulating neuronal activity and maintaining homeostasis around neurons), and oligodendrocytes, which provide the myelin sheath of neurons. The innate immune response at the level of the nervous system mainly involves the activation of the microglia, the appearance of reactive astrocytosis, and increased production of pro-inflammatory cytokines (Olson JK et al. 2004).

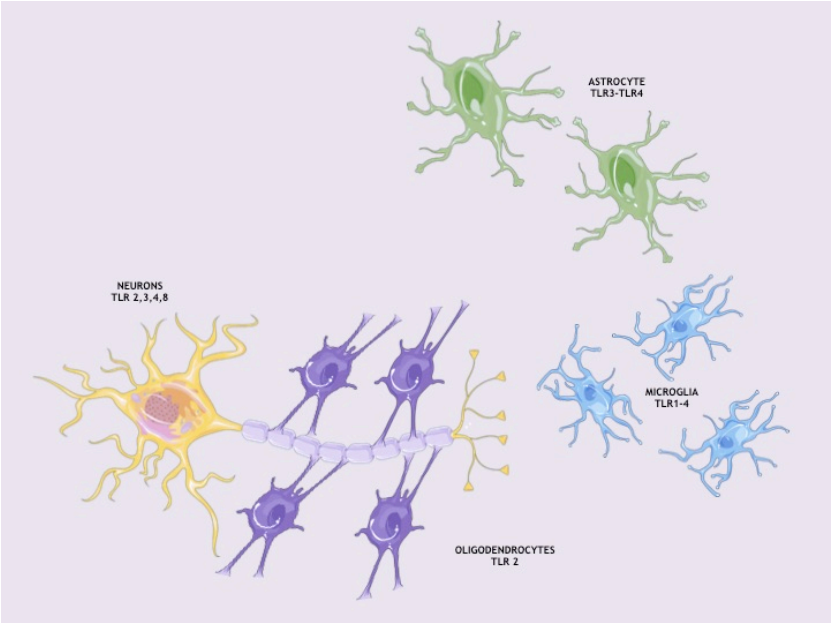
Microglia expresses a wide repertoire of TLRs (1-9), but TLR2, TLR3, and TLR4 are by far the most numerous (Bsibsi M et al. 2002). The levels of expression of TLRs in microglia in vivo appear to remain low under physiological conditions, but increased expression has been reported in animal models of AD and spinal cord injury, as

well as in various neurological pathologies. Stimulation of microglia by TLR agonists in animal models leads to increased production of inflammatory cytokines such as IFN-α, IFN-β, IL-1β, IL-6, IL-12, IL-18, TNF-α, and NO (Laflamme N et al. 2003). Other CNS cells that independently express TLRs require prior activation of the microglia to respond to ligand stimulation. The role of TLRs in the CNS is not univocal; microglia also regulates neuronal development, differentiation, and survival through mechanisms mediated by TLRs (Trotta T et al. 2014). TLR4 contributes significantly to neurotoxicity through prolonged inflammation and excitation (Block ML et al. 2007), as neurons and oligodendroglia are especially fragile under inflammatory conditions (Lehnardt S et al. 2003). If the TLR4 pathway is activated erroneously or disproportionately the inflammatory response can induce neuronal necrosis or apoptosis resulting in neurodegeneration. Concerning this, it has been shown that the administration of LPS is capable of causing extensive neural and axonal damage, which does not occur in animals lacking TLR4 (Trotta et al. 2014). On the other hand, TLR4-dependent signaling pathways may have neuroprotective effects as demonstrated in studies in stroke (Marsh B et al.2009) or AD (Tahara K et al.2006), through the secretion of NFκB and IFN-β, that block inflammation (Kagan JC et al. 2008).

Compared to the microglia, astrocytes express a more limited range of TLRs, mainly TLR3 and to a much lesser extent TLR1, 2, 4, 5, and 9 (Trotta T et al. 2014). The expression of TLR3 is clearly related to the role of astrocytes in the defense against viral infections (Farina C et al. 2006). The expression of TLR4 by astrocytes is in question, although low levels of constitutive expression of TLR4 have been detected, which increase after cell activation (Lehnardt S et al. 2002). It seems that TLR4 activation induces differentiation and proliferation of astrocytes while TLR2 activation inhibits it (Sloane JA et al. 2010).

Finally, oligodendrocytes can only express some TLRs as TLR2 and TLR3, and their function is reparative.

Figure 1.10. TLRs in the CNS



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Although in the nervous system TLRs are expressed predominantly in glial cells, they also appear in several non-purely "immunocompetent" cells such as endothelium and neurons. Their role in these cells is related to neurogenesis and brain development, as well as to the immune response (Trotta T et al. 2014). Neurons express TLRs 2, 3, 4, and 8, although evidence is scarce in vivo, probably because in the absence of pathology their level of expression is undetectable. In pathological states, however, TLR4 expression has been detected, for example, in neurons from areas of cortical dysplasia in temporal lobe epilepsy (Zurolo E et al. 2011). TLR3 and TLR8 are expressed in axons and the neuronal soma, but TLR2 and TLR4 are expressed only in the soma. TLR3 is expressed in hippocampal, cortical, and sensory neurons and functions as a neurite growth inhibitor, as does TLR8

(Sloane JA et al. 2010). Activation of TLR4 blocks the differentiation of neurons in the adult, while activation of TLR2 promotes it.

Endothelial cells present in the CNS also express TLR2 and TLR4, along with subventricular and subgranular progenitor cells (Jou I et al. 2006). The functional relevance of TLR expression in these cells is unknown.

1.3.4.2. TLRs and neurological pathology

The first studies on the role of TLRs in CNS pathology focused on cases of infectious origin and acute course, given the importance of TLRs in the recognition of pathogens and the initiation of the innate immune response. It was soon found that the role of TLRs went far beyond the initial phases of response: animal models of bacterial sepsis showed how LPS induces a chronic inflammatory response in the CNS that requires expression of TLR4 and is activated by mechanisms independent of both systemic inflammation and peripheral cytokine levels (Chakravarty S et al. 2005). These animal models of LPS-induced inflammation also experience progressive neurodegeneration, while mice that do not express TLR4 do not experience long-term neuronal loss despite being infected (Buchanan MM et al. 2010). Sustained microglia activation has deleterious effects and causes neuronal death, BBB damage, brain edema, etc. TLRs, particularly TLR2 and TLR4, participate in inflammation in non-infectious CNS pathologies by binding to endogenous ligands. These receptors play a role in chronic and degenerative CNS processes such as traumatic encephalopathy, Parkinson's disease, Alzheimer's disease, multiple sclerosis, or alcoholic encephalopathy, in which alcohol activates the microglia by binding to TLR4 and produces sustained neuroinflammation (Trotta T et al. 2014). The role of TLRs is not only deleterious, as they can also promote repair processes. The "signal strength" hypothesis postulates that the neuroprotective or deleterious effects of the microglia depend on the concentration of TLR agonists. These mechanisms are also present in the peripheral nervous system (PNS0 and are important in the physiopathology of chronic pain (Kielian T et al. 2009). TLR polymorphisms (which mainly affect the extracellular domain) are of

great importance in the response to infection and inflammation, although at the moment the relationship between the dozens of polymorphisms described and the different pathologies is not clear (Trotta T et al. 2014). Based on the theory that inhibition of inflammation can stop or slow down the neurodegenerative process, several therapeutic approaches in CNS based on the inhibition of TLR4 have been proposed. The best strategy consists of developing molecules or mechanisms that allow antagonizing the production of inflammatory mediators without affecting other functions of TLR4 such as phagocytic activity. Next, we review the role of TLRs in the main infectious, inflammatory, and neurodegenerative pathologies of the CNS.

- TLRs and bacterial meningitis: Bacterial PAMPs bind to TLR4 and TLR2 and trigger an immediate immune response. Both bacterial toxins and pro-inflammatory cytokines resulting from immune activation contribute to neurological damage. These cytokines, such as TNF-α, IL-1, and IL-8, are released after activation of glial cells and other defense cells attracted to them. Several TLR polymorphisms have been associated with different levels of susceptibility to develop bacterial meningitis, mainly by *Mycobacterium tuberculosis* (Carty M et al. 2011).
- TLRs and viral meningitis: The effects of TLRs following a viral infection are variable and complex and depend on the interaction between receptors. In humans, TLRs 3, 7, 8, and 9 have a role in protecting against CNS viral infections. In knock-out animal models for TLR2 and TLR4, inoculation of HSV-1 results in lethal encephalitis. However, activation of TLR2 seems to have a pernicious effect after HSV-1 infection (Schachtele SJ et al. 2010), increasing neuroinflammation, but becomes neuroprotective if TLR2 and TLR9 are activated together.
- TLRs and post-traumatic encephalopathy: Trauma produces primary damage, caused by the biomechanical impact, and secondary damage in which inflammation plays an essential role. TLR4 activation in astrocytes is essential for the

activation of NF-κB and the release of inflammatory mediators after a head injury. Inhibition of the TLR4 and HMGB1 pathway reduces long-term histological and functional damage after TBI (Chen CC et al. 2012)

- TLRs and multiple sclerosis: MS is an autoimmune disease primarily mediated by CD4+ T cells directed against myelin. Glial cells play a role in the pathogenesis after DAMP-mediated stimulation and contribute to the activation of T-lymphocytes. Stimulation of TLR4 also causes deterioration in oligodendrocyte function through activation of NOS (Yao S et al. 2010).
- TLRs and stroke: activation of TLR4 after ischemic injury worsens stroke's prognosis (Bianchi ME. 2007) and a better evolution of infarction has been demonstrated in animal models KO for TLR4 (Hua F et al. 2007). However, this is not the case for TLR3 and TLR9, which have a regulatory effect on the inflammatory response. Activation of the microglia by stimulation of TLRs releases numerous inflammatory mediators that attract immune cells to the site of injury. In the case of stroke, this effect is increased by disruption of the BBB and access of DAMPs to receptors on peripheral blood cells. In hemorrhagic stroke, increased expression of TLR2 and TLR4 in peripheral blood monocytes has been associated with poor functional outcome and greater residual hematoma volumen (Rodríguez-Yáñez M et al. 2012).
- TLRs and Alzheimer's Disease: The pathway through which amyloid plaques induce neurodegeneration is unclear, but several histological and clinical findings suggest that inflammation plays an important role. The β-amyloid plaques are surrounded by activated microglia and astrocytes and their presence is accompanied by increased levels of complement, pro-inflammatory factors, and proteases (Akiyama H et al. 2000). Overproduction of these inflammatory substances contributes to the neurodegenerative process, neurotoxic inflammation, and neuronal death. Patients with AD are known to express more CD14, more TLR4, and more TLR2.

The expression of TLR4 and some of its ligands, such as HSPs, is associated with increased metabolism and elimination of β-amyloid, and the absence of TLR4 leads to increased amyloid deposits and cognitive deficits. Paradoxically, a genetic polymorphism associated with hypofunction of TLR4s protects against AD (Minoretti P et al. 2006) and aberrant TLR signaling contributes to neuroinflammation in AD (Tan L et al. 2008). Blocking of TLR4 and TLR2 by antibodies reduces B-amyloid-induced production of NO, IL-6, and TNF-α. The effects of TLR activation in AD are therefore still unclear and it is not known whether these effects depend only on glial activation or also on TLR-mediated neuronal stimulation (Walter S et al. 2007).

- TLR and Parkinson's disease (PD): Elevated levels of inflammatory mediators have been found in the substantia nigra and striatum of PD patients (Whitney NP et al. 2009), as well as increased expression of TLR4 in the α-synucleinopathies as a whole (Letiembre M et al. 2009). In animal models, the blockade of TLR4 prevents phagocytosis of α-synuclein deposits and produces a clinical worsening (Cookson MR. 2009), so it seems that this inflammatory hyperactivation may be beneficial in this context.
- TLRs and glioma: TLRs can have both pro- and anti-tumor effects. Gliomas produce an immune suppression in their environment that allows them to grow. Injection of TLR9 agonists (CpG-28) reduces glioma growth by 80% in animal models and also prevents recurrence (Alizadeh D et al. 2010).
- TLR and amyotrophic lateral sclerosis (ALS): Microglia is important in the pathogenesis of sporadic and genetic forms of ALS (Zhang R et al. 2011). Overexpression of TLRs has been demonstrated in people who carry the SOD gene, which produces the familial forms of ALS, specifically microglial TLR2 and TLR4 and neuronal TLR4 (Casula M et al. 2011). The mutant form of the SOD1 protein binds to CD14 and activates the MyD88-dependent pathway, which can be

blocked with antibodies against TLR2, TLR4, and CD14 (Zhao W et al. 2010).

1.3.4.3. TLRs and chronic pain

For a long time pain and its chronification mechanisms were considered to depend only on neurons. As the physiopathology of pain became better understood, it was evident that this exclusively neuronal model was not explanatory. Glial cells, in particular microglia, are key in the onset and persistence of pain. The number of studies on this subject, in both animal and human models, has increased considerably since the 1990s (Haight ES et al. 2019).

Pain is usually associated with tissue injury, but there are situations where it persists beyond the initial damage, either because of inflammation, damage to peripheral nerves, or unknown mechanisms, in what is called dysfunctional pain, such as in the case of fibromyalgia or irritable bowel. Both neuropathic and dysfunctional pain appear to be the result of an abnormal amplification of nociceptive signals at the central and peripheral nervous system level. Models of peripheral nerve injury have demonstrated the importance of microglia. Microglia is the first cell type to be activated after peripheral nerve injury and the onset of pain after peripheral nerve injury depends on the expression of several glial genes. Astrocytes are involved at a later stage and they are activated about 4 days after microglia, but their activation persists for longer - about 12 weeks - so it seems to have more weight in the maintenance of pain over time (Rojewska E et al. 2018). Several substances that inhibit microglial activation, such as minocycline, propentofylline, and pentoxifylline, limit the development of neuropathic pain by reducing microglial activation and reducing cytokine secretion (Mika J et al. 2009).

Pain sensitization depends on both neurons and glia, and especially on the communication of these cells with each other and with other strains such as leukocytes or endothelial cells (Lacagnina MJ et al. 2018). We know, for example, that astrocytes release proinflammatory factors and alter the function of glutamate carriers, contributing to the induction and maintenance of chronic pain (Milligan ED et al. 2009), and that microglia can change its phenotype

irreversibly in response to trauma or a hostile environment, a process called "glial priming" (Frank MG et al. 2007). Overexpression of TLR4 is one of the markers of "glial priming" and therefore contributes to the transition from acute to chronic or pathological pain (Nicotra L et al. 2012).

Numerous studies in animal models have demonstrated the contribution of TLRs to the genesis and maintenance of neuropathic pain. One of the first studies showed how a section of a lumbar nerve increased mRNA expression of TLR4 in the bone marrow of mice; this TLR4 expression was essential for the activation of glia and the appearance of thermoalgesia (Tanga FY et al. 2005). It was also found that KO mice for TLR4 showed less pain-associated behavior (Jancalek R et al. 2010). Studies in rats proved that inducing pain through compression of the sciatic nerve increased the expression of mRNA and proteins of HMGB1, TLR4, TNF-α, and IL-1β. These changes disappeared when TLR4 was blocked, with a drastic reduction in pain (Kuang X et al. 2012; Bettoni I et al. 2008). Although some later work seems to contradict these results, showing that activation of nociceptive neurons by HMGB1 is maintained despite blocking TLR2 and TLR4 (Allette YM et al. 2014), the bulk of animal research supports the theory that the TLR4 pathway is essential in the development and chronification of neuropathic pain (Cao L et al. 2009). Studies on other receptors, such as TLR2 (Shi XQ et a. 2011) and TLR3 (Obata K et al. 2008), were subsequently added to research on TLR4. Hyperalgesia and allodynia associated with neuropathic damage were reduced in knock-out mice for TLR2 (Kim D et all. 2007) and the blockade of TLR3 in the spinal cord prevented the development of chronic pain and has even been proposed as a treatment (Carty M et al. 2011).

In models of neuropathic pain, the neurological damage leads to the release of endogenous ligands such as fibronectin, HSPs, hyaluronic acid, etc. Endogenous ligands can be released from necrotic or damaged cells, but also from cells simply activated or subjected to stress (Thakur KK et al. 2017). These ligands activate astrocytes, microglia, and other cells through TLRs, causing increased production of inflammatory mediators which in turn interact with

nociceptors. This interaction between TLRs and nociceptive neurons does not occur only indirectly after activation of the glia; the nerve terminals themselves express TLRs and respond directly to their ligands, without the need for interaction with the immune system. This is a mechanism of rapid response of neurons to pathogens. Therefore, nociceptive neurons not only perceive pain, temperature, or touch but through the expression of TLRs can recognize DAMPs, which can trigger avoidance behaviors and activate the innate and adaptive immune system (Ji RR et al. 2016). The expression of TLR4 in trigeminal neurons has been known since 2006 (Wadachi R et al. 2006) and its expression at the trigeminal nucleus level has been proven in several animal studies (Helley MP et al. 2015). Activation of neuronal TLR4 induces increased Ca^{+2} flow with vallinoid receptor type 1 sensitization (TRPV1) and increased CGRP secretion in rat trigeminal cell culture (Diogenes A et al. 2011). The activation of TLR4 by LPS of *Porphyromonas gingivalis* in the trigeminal nucleus sensitizes TRPV1 receptors and enhances the release of CGRP, so that by inducing pulpitis in the animal model, it increases the expression of TLR4, MyD88, TRIF, and NF-κB in neurons of the ipsilateral trigeminal nucleus to that gum; also, the blockade of TLR4 with Eritoran decreases the expression of TNF-α and IL-1β (Diogenes A et al. 2011). While the TPRV1-dependent pathway is important for the development of acute pain, in the case of chronic pain the MyD88 signaling pathway seems to be the most important one. Blocking this pathway results in decreased neuropathic pain in chemotherapy-induced pain models (Park CK et al. 2014). There are doubts about the composition of TLR4 expressed by sensory neurons; while in immune cells the receptor complex is composed of TLR4, CD14, and MD-2, neurons in the dorsal ganglia express CD14 and MD-2, but also MD-1 (Acosta C et al. 2008). The expression of MD-1, MD-2, MyD88, and TRAM shows that the two TLR4-dependent activation pathways are present in nociceptive neurons. The role of TLR4 in the development and maintenance of chronic pain has been demonstrated in a human model, through pre-activation with LPS and subsequent exposure to capsaicin (Hutchinson MR et al. 2013). TLR3, TLR7, and TLR9, are also expressed in trigeminal neurons and the dorsal ganglia (Qi J et al.

2011). The role of TLR2 and TLR3 in chronic pain models, at the moment, has only been demonstrated in animal models (Helley MP et al. 2015).

TLRs are also involved in central sensitization processes. Central sensitization involves changes in neurons and neuronal circuits. Repeated or continuous inflammation may contribute to these changes. DAMPs are expressed in the CNS in the absence of local damage, simply when there is damage to tissues or peripheral nerves. These DAMPs activate the TLRs and lead to the release of inflammatory mediators by the glia. The inflammatory mediators released by the microglia and astrocytes activate the pre- and post-synaptic terminals of nociceptive neurons (Kato J et al. 2016), increasing excitability and synaptic transmission. Besides, some pro-inflammatory factors such as IL1B, IL-6, prostaglandin E2 (PGE2), brain-derived neurotrophic factor (BDNF), and IFNϒ increase central sensitization by interfering with GABA or glycine dependent inhibitory circuits (Kawasaki Y et al. 2008). In TLR4 knock-out mice this transition to hypersensitivity or chronic allodynia does not occur (Christianson CA et al. 2011). TLR2 and TLR3 also appear to have a role in the central sensitization mechanisms. There is also evidence of expression of TLRs in pain-related brain areas, suggesting that they may have a role in the processing of pain signals at the central level (Nicotra L et al. 2012). The activation of TLRs in these locations releases pro-inflammatory mediators such as ROS, NO, IL-6, TNF-α and CGRP. The activation of TLR2 and TLR4 in microglia and astrocytes not only leads to an increase in the production of proinflammatory cytokines, but also to an increase in the self-expression of TLR2, TLR4 and TLR3 in those same cells, thus contributing to a positive feedback loop of neuroinflammation and chronification of pain (Marinelli C et al. 2015).

Finally, the relationship between TLRs and the physiopathology of pain is supported by the effects of certain drugs and hormonal variations on these receptors. At the pharmacological level, TLRs recognize xenobiotics, among other molecules with very different mechanisms of action, such as opioids (Hutchinson MR et al. 2007) or amitriptyline (Hutchinson MR et al. 2010), which suggests that they

play a role in the analgesic effect of these groups of drugs, as well as in opioid-induced paradoxical hyperalgesia (Shah M et al. 2017). From clinical experience and basic studies, we know the differences between the sexes in terms of chronic pain, inflammatory states, and pain threshold. There is an interaction between sex hormones and neuroimmunological signaling mediated by TLRs and purinergic receptors (Calippe B et al. 2010). The administration of intrathecal LPS causes allodynia in male but not female mice and depends on testosterone levels (Sorge RE et al. 2011). Regulation of TLR expression appears to be under hormonal influence in an age-dependent manner, so that estradiol increases mRNA-TLR4 expression in the microglia of female rats, but only when they are adults (Loram LC et al. 2012). Some studies suggest that sex differences in morphine response may be due to the interaction of TLRs with estradiol (Doyle HH et al. 2017).

1.3.4.4. TLRs and migraine

Several aspects of the physiopathology of migraine are closely related to the innate immune response. Although research on the role of TLRs in migraine is still scarce, the role of innate immune cells and PRRs in mechanisms such as sterile inflammation, nociceptor activation, CSD, peripheral sensitization, and central sensitization is better known.

The electrophysiological cortical phenomenon of CSD associates inflammation and activation of immediate immunity. The inflammatory cascade is initiated by the opening of the Pannexin I channel as a result of electrical changes and activation of caspase-I, which leads to the production of HMGB-1 by neurons that can then stimulate TLRs expressed by microglia and astrocytes (Yu S et al. 2016). CSD increases the expression of the immunomodulating enzyme Indoleamine 2,3-dioxygenase (IDO) and proinflammatory cytokines such as IL-6, IL-1β, and TNFα, which lead to activation of macrophages and mast cells, with the production of more cytokines. Animal studies have shown an increase in the expression of TLR3 and TLR4 in rats subjected to repeated CSD and a possible role of TLR3 in the regulation of the adaptive response in the CNS, protecting

neurons from CSD-derived damage (Ghaemi A et al. 2018). It has been suggested that astrocytes may regulate the threshold for CSD and that repeating CSD produces astrocytosis, although these mechanisms are not yet fully known nor is the importance of TLRs in them (Sukhotinsky I et al. 2011).

As previously mentioned, TLRs are involved in the processes of peripheral and central sensitization, which are essential in the chronification of migraine. The genetic defect of TLR4 or its pharmacological blockade by administration of the antagonist TAK-242 reduces light aversion (photophobia) in male mice and reduces activation of the trigeminal nucleus caudalis (Ramachandran R et al. 2019). This is the first experimental evidence of the involvement of TLR4 in initiating and maintaining migraine-associated behaviors, such as photophobia and allodynia, that translate into a process of central sensitization. With regard to peripheral sensitization, proinflammatory molecules such as IL1β, TNF, or PGE2 are very important in the process by stimulating the peripheral terminals of nociceptive neurons. When these receptors are activated post-translational changes happen and there is an increase in the expression of ion channels that contribute to the appearance of spontaneous pain, allodynia, and hyperalgesia. The constitutive absence of TLR reduces the production of inflammatory mediators and therefore the appearance of these effects (Christianson CA et al. 2011). But in addition to this indirect mechanism, TLRs contribute to sensitization directly by expressing themselves in the neurons of the peripheral nervous system (Wadachi R et al. 2006) and producing sensitization of the TRPV1 receptor when activated (Diogenes A et al. 2011).

Endothelial dysfunction is one of the key components of migraine and research on it is extensive. Alterations in the cells of the vascular endothelium are related to the disruption of BBB, also contributing to inflammation. In this regard, a recent study suggests that TLR4 stimulation in endothelial cells may interfere with the activation of *hedgehog* transduction pathways and alter barrier permeability (Moreau N et al. 2016).

The already mentioned relationship between steroid hormones and TLR expression is of special interest in the field of migraine,

considering that this is a pathology of greater incidence in women, mainly during the fertile period, and often related to the hormonal fluctuations characteristic of the menstrual cycle. In animal models, certain glucuronized metabolites of estrogens act as TLR4 ligands and produce increased pain in vivo in the form of allodynia, which ceases after blocking the TLR4 (Lewis SS et al. 2015).

The relationship between innate immunity and the physiopathology of migraine is not completely understood. TLRs, as the basic receptors of innate immunity, can be of great importance in the initiation or maintenance of the inflammatory mechanisms underlying the disorder. Moreover, evidence of their expression in neurons and endothelial cells suggests other ways of involvement in the physiopathology of the disorder, independent of inflammation. The exact role of TLRs in migraine is not known at present, and research on this subject has so far been anecdotal.

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2. JUSTIFICATION

Migraine is a very common neurological condition that affects about 1 billion people (Collaborators GBDH. 2018). Its importance lies in its high prevalence, the disability that it implies for those who suffer it, and its impact on the social and health level. Migraine tends to be trivialized as it does not entail vital risk or permanent sequelae, however, it constitutes the seventh cause of disability quantified as the number of YLDs (Vos T et al. 2012). CM is the most disabling form of chronic daily headache (Lanterini-Minet M et al. 2011), with a prevalence between 0.9 and 5.1%, in the general population, similar to epilepsy (Natoli JL et al. 2010).

Migraine has been known since ancient times, however, our understanding of its physiopathology is still partial. It is known that the TVS, the brainstem nuclei, the thalamus, and the hypothalamus are involved and that mechanisms such as CSD or sterile inflammation are triggered (De Simone R et al. 2013). The exact sequence of events or the primary cause of the onset of pain, as well as the reason behind the different individual susceptibilities, are still to be determined. Inflammation appears to play a central role (Pietrobon D et al. 2013), perhaps triggered by CSD (Bolay H et al. 2002), contributing to the maintenance of aberrant cycles of nociceptive activation essential in the chronification of pain (Xanthos DN et al. 2014).

Innate immunity is primarily responsible for inflammation. Glial cells, in particular microglia, are essential in the onset of pain and its chronification (Haight ES et al. 2019). Activation of microglia depends on the interaction between TLRs and exogenous or endogenous molecules. In the CNS, TLRs are expressed mainly in microglia, astrocytes, and oligodendrocytes, but also in the endothelium and neurons. Stimulation of the microglia by TLR agonists leads to an increase in the production of inflammatory

cytokines such as IFN-α, IFN-β, IL-1β, IL-6, IL-10, IL-12, IL-18, TNF-α, and NO (Trotta T et al. 2014).

TLRs are involved in multiple CNS pathologies, either infectious, such as bacterial or viral meningitis, or inflammatory and neurodegenerative. The role of TLRs has also been studied in cerebrovascular pathologies, such as ischemic stroke, in which it is related to the phenomenon of CSD. In animal models of chronic pain, there seems to be an increased expression of TLR2, TLR3, and TLR4 at the level of the brainstem and diencephalic nuclei and a reduction of pain after pharmacological blockade of TLR4 (Kuang X et al. 2012).

Despite the numerous shreds of evidence on the role of TLRs in inflammatory responses of the CNS, in the phenomenon of CSD and its implication in chronic neuropathic pain, studies about TLRs in EM and CM are scarce: the 4 896/G polymorphism of TLR4 has been associated with increased susceptibility to migraine in humans (Rafiei A et al. 2012); animal studies seem to indicate that the HMGB1-TLR2/4 axis plays a key role in glial activation after repeated CSD (Shibata M et al. 2017); the genetic defect of TLR4 or its pharmacological blockade with TAK-242 reduces photophobia and activation of the trigeminal nucleus caudalis in a murine model, being the first experimental evidence of the involvement of TLR4 in the initiation and maintenance of typical migraine behaviors (Ramachandran R et al. 2019); and finally, several drugs used for the acute treatment or prevention of migraine, such as botulinum toxin (Rojewska E et al. 2018), duloxetine (Zhou DM et al. 2018), or diclofenac (Barcelos RP et al. 2017) have been shown to have effects on glia and the TLRs themselves, although it is not known whether these mechanisms contribute to their effectiveness as a treatment for migraine.

In summary, to date, there is enough evidence the consider a potential role of TLRs in several of the mechanisms involved in migraine. This study aims to contribute to the understanding of the role of TLRs in neurogenic inflammation and the physiopathology of migraine chronification.

3. HYPOTHESIS

TLR2 and TLR4, as key receptors in the activation of microglia, could be relevant in the physiopathology of migraine. Migraine involves an inflammatory reaction with overexpression of endogenous ligands for TLRs. We propose that changes in TLR4 expression, either predetermined (genetic absence of TLR4) or provoked (pharmacological blockade of TLR4) produce a change in the response to stimulation in an animal model of CSD, as well as variations in the expression of inflammatory markers.

The production and release of DAMPs during migraine attacks and their interaction with TLRs may induce an increased expression of TLRs in cells of the monocyte-macrophage system in the context of migraine. We hypothesize, therefore, that expression of TLRs is increased in peripheral blood cells of subjects with CM, and that certain TLRs endogenous ligands may also be increased in plasma of CM patients.

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4. OBJECTIVES

The present work consists of an experimental study and a clinical study.

4.1. EXPERIMENTAL STUDY

4.1.1. Primary objective.

To demonstrate the participation of TLR4 receptors in the phenomenon of CSD in an animal model of migraine. For this purpose, response to CSD stimulation is evaluated in three experimental groups: wild-type mice (WT), knock-out mice for TLR4 (KO), and mice pre-treated with a selective TLR4 blocker in order to:

- Compare the response to CSD induction in normal and KO mice for TLR4.
- Determine if the response to CSD induction can be modified by pharmacological blockade of TLR4.

4.1.2. Secondary objectives.

- To determine the levels of systemic markers of inflammation in the three experimental groups.
- To evaluate the phenomenon of blood-brain barrier disruption in the three experimental groups.

4.2. CLINICAL STUDY

4.2.1. Primary objective.

To compare the expression of TLR2 and TLR4 in monocytes and neutrophils in peripheral blood in CM patients and healthy controls.

4.2.2.Secondary objectives.

- To study the relationship between levels of endogenous ligands in peripheral blood (HSP60 and cFN) and the expression of TLRs.
- To study the relationship between expression of TLRs in peripheral blood and the profile of markers of systemic inflammation (IL-6, IL-10, hs-CRP), neurogenic inflammation (CGRP), and endothelial dysfunction (PTX3, sTWEAK).

5. MATERIALS AND METHODS

5.1. GENERAL DESIGN AND ETHICAL ASPECTS

This work consists of an experimental study on animals and an observational cross-sectional clinical study.

The author (Clara Domínguez Vivero) has no conflict of interest to declare concerning this project.

The Ethics Committee of the University Hospital of Santiago de Compostela approved this study with the identification code 2016/085. The study meets the requirements specified by the World Medical Association in accordance with the Declaration of Helsinki.

Experimental protocols and animal handling were approved by the chief of the Servicio provincial de ganadería del departamento territorial da consellería de medio Rural e do Mar de la provincia de A Coruña being the main responsible Dr. Francisco Campos Pérez (15010/2019/004). The animal experiments were conducted by Paulo Àvila Gómez in accordance with the rules of the European Union Committee for Animal Studies (https://www.coe.int/en/web/conventions/full-list/-/conventions/treaty/123) and the regulations in force in Spain and EU (86/609/CEE, 2003/65/CE, 2010/63/EU, RD 1201/2005 AND RD 53/2013). The ARRIVE guidelines (Kilkenny C et al. 304) (Animal Research Reporting of in Vivo Experiments) were used as a guide in this experiment. All the procedures were carried out in the Health Research Institute of Santiago de Compostela (IDIS), with the registration number: ES1507802928[01].

5.2. EXPERIMENTAL STUDY

5.2.1. Experimental models of migraine

Current knowledge about migraine and its physiopathology derives mainly from animal models. These models reproduce some of the mechanisms of migraine, but none has been designed to reproduce

the whole process (Akerman S et al. 2013; Ayata C. 2013); therefore, all models have limitations. Given that they have demonstrated their translationality through pharmacological tests, they are regarded as acceptable approaches to the pathology.

Animal models of migraine can be classified according to several criteria: the physiopathological mechanism questioned, the method of stimulation or provocation, and the response recording system. Some of these techniques require the animal to be conscious, but most are performed on anaesthetized animals. The main existing models and the advantages and disadvantages of the one used in the present experimental design are discussed below. Existing models based on transgenic animals for migraine of monogenic inheritance will not be addressed given they are not related to our study design.

5.2.1.1. Animal models according to the underlying physiopathological mechanism

The existing models are eminently based on the activation of the TVS and the CSD phenomenon:

- Models based on TVS activation: given that the participation of trigeminal fibers in the origin of pain has been amply demonstrated in humans, many of the animal models are based on the activation of the nociceptive pathways of the TVS, their upward projections, and their regulation (Romero-Reyes M et al. 2014).
- Models based on CSD triggering: CSD is generally accepted as a model of migraine, despite the existing doubts regarding its physiopathological relevance in humans in cases of MwoA. The fact that several prophylactic migraine treatments suppress CSD supports its validity as an experimental model. There are several systems of provocation and registration of CSD in animals, but the physiopathological processes questioned by these models and their translational value have not been established with accuracy (Romero-Reyes M et al. 2014).

5.2.1.2. Animal models according to the stimulation method.

"Migraine" can be triggered in animals by stimulating the meninges or other structures of the trigeminal pathway, by inducing CSD, or by administering algogenic substances at a systemic level:

- Stimulation of trigeminal meningeal structures: intracranial dural stimulation models are based on the activation and sensitization of the TGV system using the nerve terminals present in the meninges. The two main methods of stimulation are the application of inflammatory mediators and electrical stimuli. The application of an "inflammatory soup" consists of instilling a series of chemical mediators on the dura, such as nitric oxide donors or CGRP. The main limitations of this model are that it compromises the integrity of BBB and that it corresponds more to a meningitis model than to a migraine model. Some models use direct electrical stimulation of the brainstem nuclei involved in the transmission or regulation of the nociceptive signal, involving a more aggressive injury to BBB.
- CSD triggering: CSD is triggered when the concentration of extracellular K^+ exceeds a certain threshold on a sufficient cortical surface. The stimulus capable of producing this critical change can be electrical, mechanical, or chemical. In electrical stimulation models, an electrical stimulus is applied at regular intervals until CSD is successfully triggered. The number and intensity of the required stimulus determine the threshold. This technique is highly variable due to irregularities in the junction between the electrode and the tissue. For this reason, chemical stimulation is often preferred, which offers greater intra- and inter-studio stability (Ayata C. 2013). For this purpose, a concentrated dilution of potassium chloride (KCl) (50 mM or higher), glutamate receptor agonists, Ca^{2+} channel agonists or Na^+ channel agonists are normally used. The concentration of

KCl or the volume applied is slowly increased to determine the threshold. An alternative method is to continuously apply a concentration directly above the threshold (1M) to trigger repeated CSD and determine their frequency. This approach has the limitation of depending on the refractory periods during which no new CSD can be triggered. Longer duration of CSD waves is associated with longer refractory periods and lower frequencies, which may be misinterpreted as low susceptibility to CSD. In such cases, the cumulative hourly CSD duration may be a more reliable parameter. In models based on KCl stimulation, the diameter of the cranial window and the preservation or absence of the dura mater cover are very important, so these conditions must be kept constant throughout the experiment, and ideally, between different experiments (Ayata C. 2013). Mechanical stimulation has also been used, but it is difficult to establish a threshold and the repetition of the stimulus is problematic. Besides, it seems to have a different pharmacological profile than chemical or electrical stimulation.

- Systemic stimulation: Intravenous administration of NO donors can produce hyperalgesia, thermal, and mechanical allodynia that revert with sumatriptan, as well as light aversion reactions (Storer RJ et al. 2015).

5.2.1.3. Animal models according to the registration method.

- Behavioral models: they require animals to be conscious in order to assess the presence of behaviors typically associated with pain. These models are usually based on the determination of the withdrawal threshold of a stimulus applied in the craniofacial region or the extremities (allodynia) or the evaluation of behaviors associated with pain, such as grimacing, changes in grooming behaviors, changes in the animal's usual exploratory behaviors, rest, inactivity...(Storer RJ et al. 2015). These models have been particularly useful in reproducing the chronification of the disorder, as it was found that after repeated administration of inflammatory substances

in the dura mater or injections of nitroglycerin (NTG), allodynia was maintained for several weeks without the need for further stimulation.

- CSD recording: This technique is performed on anaesthetized animals. The gold standard for the detection of CSD is the electrophysiological recording, which allows the observation of a slow negativization of membrane potential. This can be preceded by a neuronal hyperactivation. Susceptibility to the development of CSD, i.e. the electrical threshold for its provocation, is widely used as a model for migraine, but the mechanisms questioned by this model and their translational value have yet to be established. Measurement of CSD wave frequency during continuous KCl stimulation has shown complete agreement with electrical threshold determination and has the advantage of providing continuous data and being less affected by cortical surface irregularities, and is therefore considered a very consistent record. The relationship with the threshold is inverse, i.e. a higher frequency of CSDs in the continuous stimulation model represents a lower electrical threshold in the electrode stimulation model. It should be noted that a frequency ceiling exists (20-30 CSDs in one hour) regardless of the intensity of the stimulus. This depends on the refractory period (believed to be about 2-3 minutes). Other measures of the CSD wave, such as propagation speed, duration, or amplitude may also be relevant, but their relationship to CSD susceptibility is not well established. The determination of the propagation velocity seems to give the most consistent results. Various indirect measuring methods can be used as alternatives to the electrophysiological recording:
    - Diffusion-weighted magnetic resonance imaging (DW-MRI): This is a non-invasive technique, but the resolution is low.
    - Variations in cerebral blood flow (CBF): We know that activation of the TVS during migraine attacks releases vasoactive peptides that produce vasodilation of cerebral

and cranial vessels, and that the CSD phenomenon is accompanied by changes in dural and cortical blood flow. These changes are an indirect measure of both trigeminal activation and CSD, and are therefore considered an approximation to the electrophysiological recording of potential changes. In the pattern of changes in brain flow during CSD, four phases are distinguished: 1) initial brief hypoperfusion (5-30%) during the change in cortical voltage and lasting 5-30 seconds; 2) marked hyperemia (30-250% increase in flow) during repolarization and lasting a few minutes; 3) late, less marked hyperemia; 4) prolonged oligoemia of 10-40%. The measurement of variations in brain flow is an excellent predictor of the clinical efficacy of various treatments for migraine. Its main limitation is its susceptibility to changes in the animal's blood pressure and temperature under anesthetic conditions. There are two techniques for measuring variations in CBF: intravital microscopy and doppler laser flowmetry (Akerman S et al. 2013).

- Intravital microscopy: based on the visualization of the dural and pial vessels by microscopy and the measurement of the changes in their diameter. One of its main advantages is that it uses a closed cranial window in which the bone has simply been filed away, which reduces the effects of the surgical procedure on the condition of the vessels. This technique has shown that the administration of CGRP antagonists or triptans attenuates the vasodilation of the middle meningeal artery (Akerman S et al. 2013).
- Laser Doppler Flowmeter: records changes in the speed of meningeal blood flow as an indirect measure of the caliber of the vessels and, therefore, of the activation of the TVS. It can be used in any vascular bed. A low power laser beam from a fiber optic penetrates the tissue, is scattered by the blood

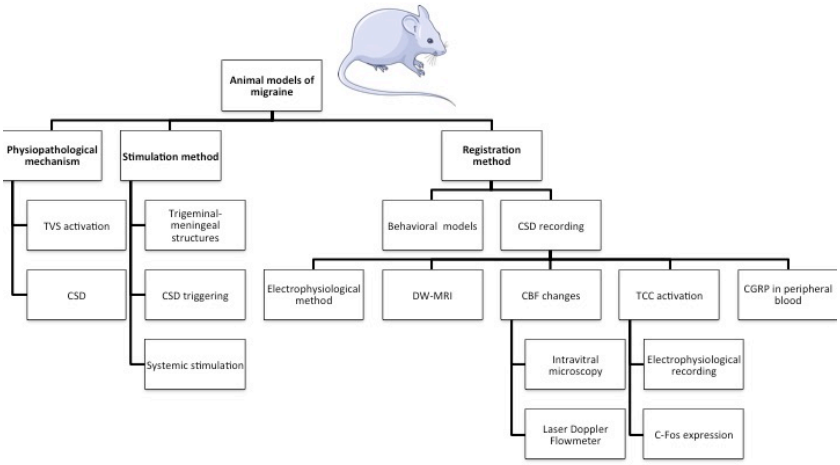
cells, and is returned to the detector. It has been proven in several studies that stimulation of dural vasculature causes reproducible increases in meningeal flow and that these changes are attenuated after administering 5HT1B/1D receptor agonists, CGRP antagonists, and nitric oxide synthase inhibitors (Akerman S et al. 2013). Unlike intravital microscopy, doppler flowmeter uses an open cranial window, with the consequent impact of the craniotomy procedure on the diameter of the cranial vessels. In contrast, the technique allows local application of substances to determine their effects on CBF without systemic side effects.

- Recording of TCC activation: The determination of changes in neuronal activity at this level is useful for the study of the physiopathology of migraine (Lewis SS et al. 2015). The activity of the TCC can be recorded in different ways:
    - Electrophysiological recording of TCC activity: allows a direct measurement of neuronal activation, in real-time and very sensitive, as it detects small-signal intensities through an electrode placed in the TCC. It has the disadvantage of the complex surgery required for the placement of the electrode in the TCC and requires very expensive equipment and intensive training to perform the technique reliably. This technique has also been tested with effective drugs for migraine (Akerman S et al. 2013).
    - Determination of c-Fos expression: c-Fos is the protein resulting from the transcription of c-fos, whose production increases with neuronal depolarization. Electrical, mechanical or chemical stimulation of dural structures increases the immunoreactivity of c-Fos in the nociceptive laminae of TCC, that decreases after administration of triptans and dihydroergotamine (Akerman S et al. 2013). The limitation of this method is that it is an indirect measurement, the expression of

c-Fos is non-specific and may be artifacted by surgical procedures.

- Quantification of CGRP in peripheral blood (Lewis SS et al. 2015): Stimulation of the TG results in an increase in the concentration of CGRP and substance P in the external jugular vein of the animals. This is an indirect and questionable method as the detection of the levels of these molecules in animals is complex due to their low concentrations and the small volume of plasma available for analysis.

Figure 5. 1. Animal models of migraine



5.2.2. Experimental design.

The selected animal model of migraine is based on the provocation of CSD in anaesthetized mice. We chose to stimulate continuously with KCl and record the response indirectly using laser flowmetry. We used 32 male C57BL/6 wild type (WT) mice and 14

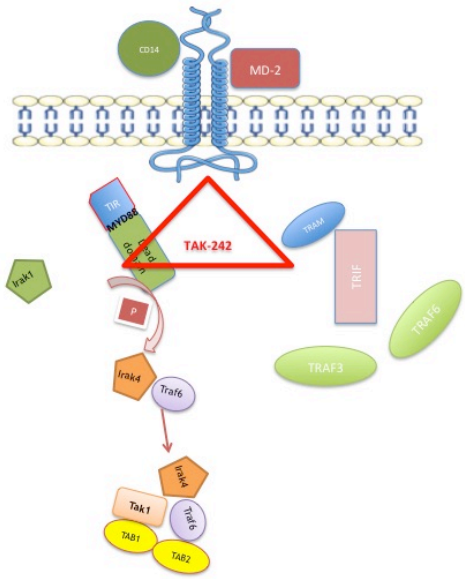
B6.B10ScN-Tlr4 knock-out (KO) mice weighing between 25 and 30 g (3-4 months). The mice were kept in stable environmental conditions, with a temperature of 23°C and relative humidity of 40%, submitted to a 12:12 hours light/dark cycle, and with continuous availability of water and food.

The design is divided into two phases:

- Phase 1: Determination of the effect of the absence of TLR4 on the morphology of the CSD wave measured by laser flowmetry by comparing a group of WT mice with a group of TLR4-KO mice.
- Phase 2: Determination of the influence of pharmacological manipulation of TLR4 on the morphology of the CSD wave. For this purpose, the characteristics of the CSD wave were determined after:
    - Stimulation with LPS: To verify that the changes observed in the CBF pattern are due to the blocking of TLR4 and not to other concurrent effects, animal models are stimulated with LPS (LPS, Sigma Aldrich™), the main ligand of TLR4.
    - Pharmacological blocking of TLR4 with TAK-242: A TLR4 antagonist, the cyclohexane derivative TAK-242 (Resatorvid®, Invivo Gen) is used. TAK-242 inhibits the TLR4 signaling pathway by binding to its intracellular TIR domain and preventing the signaling cascade. TAK-242 is injected into a vehicle of normal saline solution (NSS) and 1% dimethyl sulfoxide (DMSO).

As complementary studies to the laser flowmeter recording, we determined the levels of IL-6 in plasma in the different experimental groups before and after the procedure and the permeability of the BBB in the different experimental groups. The permeability of BBB was evaluated by performing a tissue stain with Evans Blue in vivo and later measuring its concentration in post-mortem brain tissue.

Figure 5.2. Effect of TAK-242 on TLR4 signaling pathway.



TAK-242 inhibits the TLR4 signaling pathway by binding to its intracellular TIR domain and preventing the signaling cascade.
Adapted from: Matsunaga N et al. 2011 with permission of ASPET.
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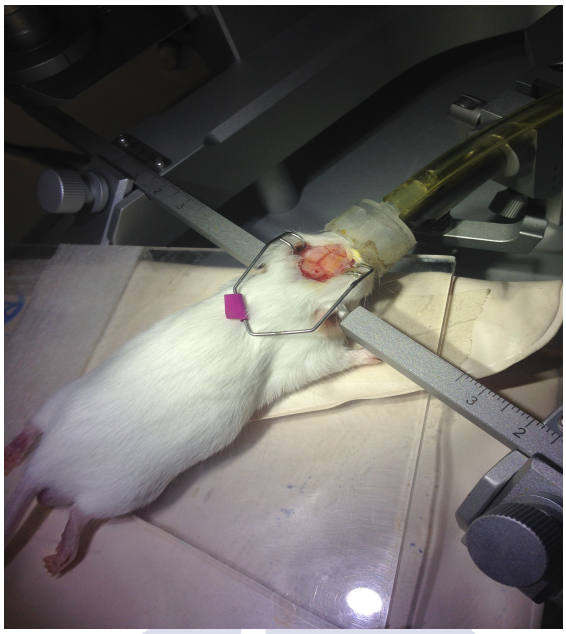
5.2.2.1. Anesthesia

Each animal was placed in an induction chamber where it was anaesthetized with sevoflurane (5%-6% for induction and 3%-4% for maintenance) evaporated in a mixture of oxygen and ambient air (30%:70%). Once anaesthetized, the temperature was controlled in all animals. The surgery was performed on a thermoregulated blanket controlled by an electronic thermostat, which was kept at around 37.0±0.5° throughout the procedure. The animal's head was fixed using a stereotactic frame (Stoelting Co. Wood Dale, IL, USA).

Figure 5.3. Setting of animals for surgery



5.2.2.2. Surgical procedure

After fixing and anesthetizing the animals, the surgery is started by following these steps:

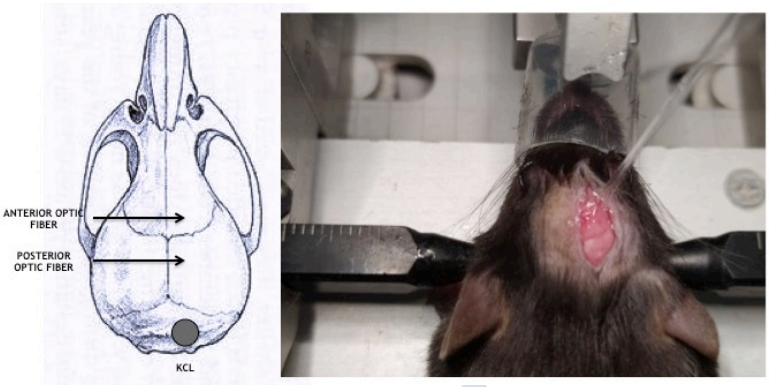
1) A 2 cm incision is made on the scalp, in the cranial midline. The subcutaneous tissue is set aside to expose the skull.

2) In order to locate the two optical fibers and monitor cerebral flow, two small erosions were made in the skull: the proximal 2 mm posterior to bregma and the distal 2 mm anterior to bregma. The optical fibers (MT B500-0 L120. Perimed) are fixed in these holes using a chemical adherent.

3) A 1 mm diameter hole is made in the occipital bone using a lathe. The dura mater is kept unharmed during this procedure. This is the usual procedure in mice, as the dura is thin enough to allow stimulation and is very difficult to remove in a non-

traumatic way (Akerman S et al. 2013). The area was kept hydrated with NSS.

Figure 5.4. Schematic representation of the cranial window for KCl administration and the arrangement of the recording electrodes



5.2.2.3. CSD induction

A small cotton ball soaked in KCl 300 mM is placed in the hole in the occipital bone to achieve continuous stimulation of the dural surface. Every 5 minutes solution is added to the cotton to keep it soaked and continue the stimulation.

5.2.2.4. Cerebral blood flow measurement

Variations in CBF are measured by doppler laser flowmeter using the two optical recording fibers, which are connected to a flowmeter measuring equipment (Periflux System 5000. Perimed) for recording. A basal recording is made for 10 minutes and CBF monitoring under KCL stimulation for 60 minutes. Changes in CBF are calculated for each pixel in relation to the basal recording.

5.2.2.5. Quantification of inflammatory markers in blood

Each animal was subjected to a baseline and post-procedure blood draw (1800µl). The samples were obtained by puncturing the jugular vein using a 30G needle. The samples were centrifuged at 1700 g and 700 µl of serum were extracted which was immediately transferred to 1.5 ml aliquots. Each aliquot was stored at -80°C until

the analysis was performed. ELISA (enzyme-linked immunosorbent assay) kits were used to measure IL-6 (Lifespan Biosciences) according to the manufacturer's instructions. Each sample was tested in duplicate and the mean concentrations were calculated and expressed as picograms of antigen per milliliter of protein (pg/mL). The minimum sensitivity of the IL-6 kit is <2.0 pg/ml with an intra-assay and inter-assay variability of <10% and 12%, respectively.

5.2.2.6. BBB permeability

In order to verify whether activation or blocking of TLR4 changes the patency of the BBB, a permeability test was performed using Evans Blue stain. This stain binds strongly to albumin, a large protein, which cannot pass through intact BBB.

The experimental groups were the same as in the previously described section. Additionally, a control group (n=6) was added, in which CSD stimulation was not performed and the cotton ball was impregnated in NS instead of KCl 300 mM. The purpose of this group was to rule out effects of the surgical procedure on barrier permeability.

To determine the BBB dysfunction, Evans Blue solution was injected into the jugular vein after the final blood draw. Subsequently, they were perfused with 20 ml of cold phosphate-buffered saline (PBS) and after slaughter, their brains were extracted and stored at -80°C. Both brain hemispheres were weighed and incubated separately in 1 mL of formamide for 24 hours at 55°C, to extract the Evans Blue. After centrifugation for 20 minutes at 10 000 rpm, the supernatant was extracted and the Evans Blue was quantified by measuring the optical density of the formamide extract at 610 nm. The absorbance was compared with a standard curve of 0.025 to 32 μg/mL. The extravasation was expressed as nanograms of Evans Blue per milligram of tissue.

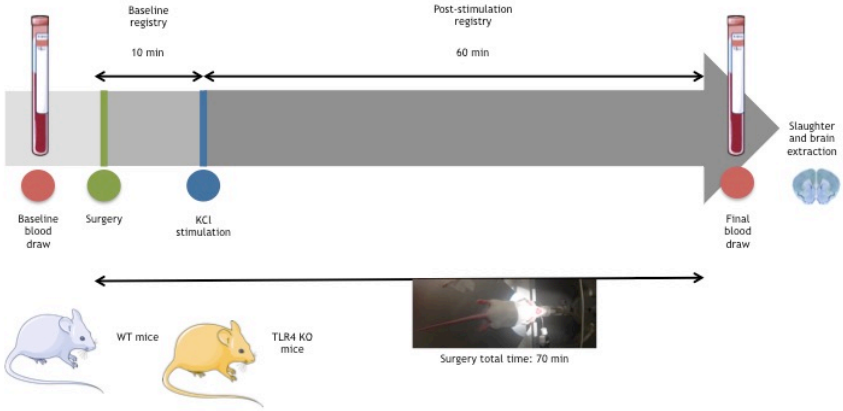
5.2.2.7. Slaughter

After the last blood draw or after perfusion of Evans Blue as appropriate and with the animal fully anesthetized, the slaughter was performed by intracardiac injection of 2 mL KCl.

5.2.2.8. Experimental groups

- Phase 1: The animals are anaesthetised, after the initial blood draw the surgical procedure is performed followed by a 10-minute baseline recording. Subsequently, induction of CSD is initiated and the record is kept for 60 minutes. Changes in CBF were calculated for each pixel in relation to basal CBF. This procedure is performed in two groups:
    - Group 0: WT mice (n=8)
    - Group 1: KO mice for TLR4 (n=8)

Figure 5.5. Procedures in Phase 1 of the experimental design



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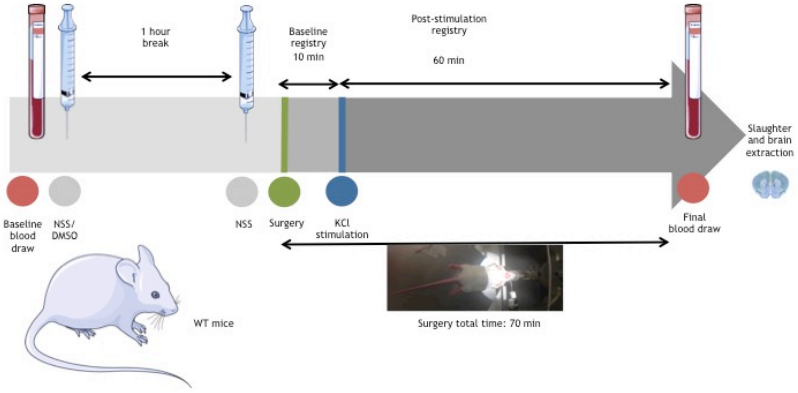
- Phase 2: After initial blood collection, the animals are injected intraperitoneally either with TAK-242 (100 µL; 3 mg/kg with its vehicle) or exclusively with its vehicle (100 µL DMSO 1% in NSS). The animals are returned to a warm box with access to food. One hour after the initial injection they are injected again intraperitoneally either with LPS (100 µL; 2.5 mg/kg) or its vehicle (NSS 100 µL) and the surgical procedure is started, performing again a basal registration and a 60 minutes registration during which the stimulation with KCl is maintained. The specimens are divided into the following groups:
    - Group 3: WT mice given vehicle (NSS/DMSO) and then NSS (n=6).

Figure 5.6. Proceedings in Group 3.



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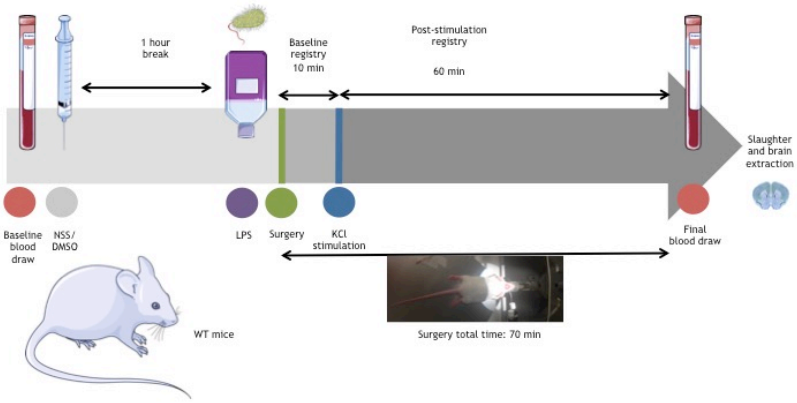
- Group 4: WT mice given vehicle (NSS/DMSO) and subsequently LPS (n=6)

Figure 5.7. Proceedings in Group 4.



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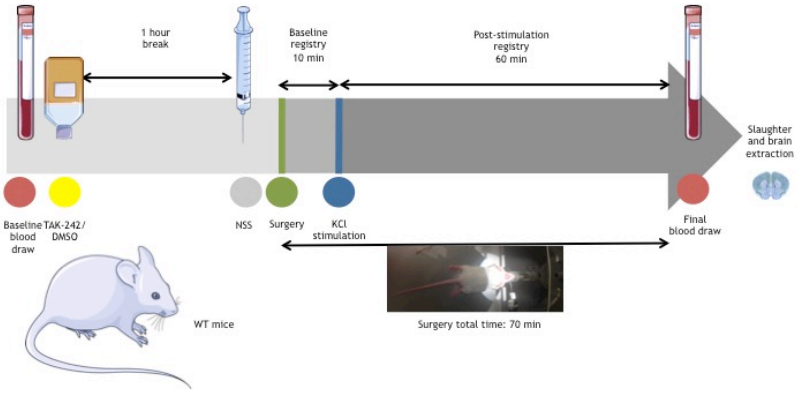
- Group 5: WT mice given TAK-242/DMSO and subsequently NSS (n=6)

Figure 5.8. Proceedings in Group 5.



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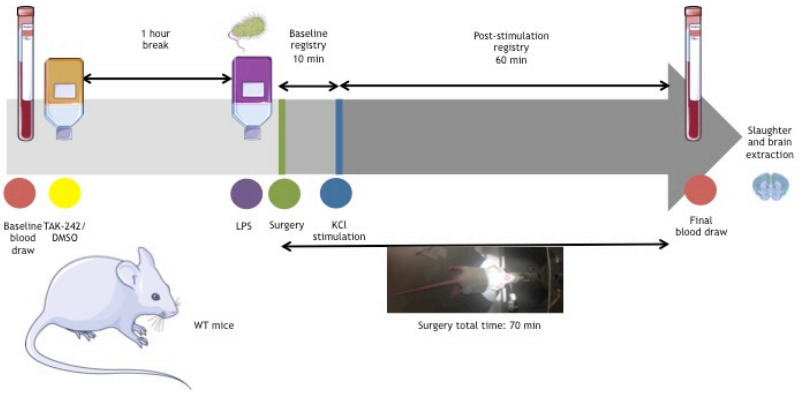
- Group 6: WT mice given TAK-242/DMSO and subsequently LPS (n=6)

Figure 5.9. Proceedings in Group 6.



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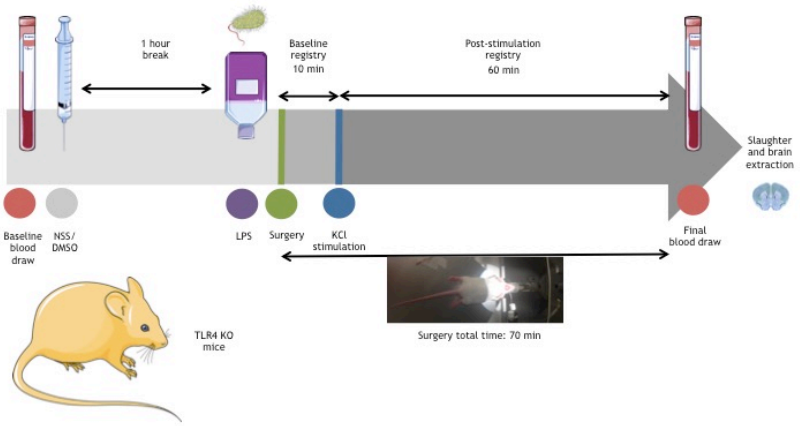
- Group 7: KO mice given vehicle (NSS/DMSO) and subsequently LPS (n=6)

Figure 5.10. Proceedings in Group 7.



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5.3. CLINICAL STUDY

The objective of the clinical study is to compare the levels of expression of TLR2 and TLR4 in monocytes and macrophages in peripheral blood in subjects with CM and healthy controls. Secondary objectives are to determine the levels of endogenous ligands of TLRs, such as celular fibronectin (cFN) and heat-shock protein 60 (HSP60); interleukins (IL6, IL10), high-sensitivity C-reactive protein (hs-CRP), markers of TVS activation including CGRP and soluble tumor necrosis factor (TNF)-like weak inducer of apoptosis (sTWEAK) and markers of endothelial dysfunction such as pentraxin 3 (PTX3). With this purpose, an observational cross-sectional study was designed and developed following the STROBE guidelines (Strengthening the Reporting of Observational Studies in Epidemiology). This study was developed in accordance with the Declaration of Helsinki of the World Medical Association (2008) and was approved by the Ethics Committee of the Galician Health Service (2016/085). Written informed consent was obtained from all participants.

5.3.1. Study population

5.3.1.1. CM subjects selection

The cases were recruited in the Headache Unit of the Hospital Clínico Universitario de Santiago de Compostela. They were offered to participate in the study during their scheduled visits to this Unit. All selected subjects met the following inclusion criteria:

- 18 years of age or older.
- Written informed consent.
- Meet diagnostic criteria for CM with or without aura according to ICHD 2013 (Headache Classification Committee of the International Headache Society. 2013) at the time of recruitment.

Exclusion criteria were as follows:

- Acute or chronic infectious pathology in the 4 weeks prior to the study.
- Acute or chronic inflammatory pathology in the 4 weeks prior to the study.

- Autoimmune pathology.
- Severe systemic pathology.
- Pregnancy or lactation.

5.3.1.2. Healthy controls selection.

Controls were recruited from among hospital workers and non-consanguineous companions of patients. The inclusion criteria for the recruitment of controls were as follows

- 18 years of age or older.
- Written informed consent.
- Not meet the diagnostic criteria for tension-type headache, migraine or other headache as set out in the ICHD 2013 (Headache Classification Committee of the International Headache Society, 2013).

The exclusion criteria for the selection of controls were the same as for cases:

- Acute or chronic infectious pathology in the 4 weeks prior to the study.
- Acute or chronic inflammatory pathology in the 4 weeks prior to the study.
- Autoimmune pathology.
- Severe systemic pathology.
- Pregnancy or lactation.

5.3.2.Clinical interview

After signing the informed consent and checking the inclusion and exclusion criteria, all subjects participated in a scheduled clinical interview with a neurologist (CD). During this interview, clinical records were reviewed and collected data were confirmed by the subjects.

- Age and sex.
- Socioeconomic level.
- Educational level.
- Toxic habits: tobacco consumption, daily units of alcohol, daily cups of coffee, consumption of other substances.

- Cardiovascular risk factors: diabetes mellitus, arterial hypertension, dyslipemia under dietary or pharmacological treatment.
- Previous or current diagnosis of pathologies comorbid with migraine, such as:
    - Anxiety-depressive syndrome.
    - Epilepsy.
    - Metabolic syndrome.
    - History of ischemic/hemorrhagic stroke.
    - Diagnosis of sleep apnea-hypopnea syndrome.
    - Diagnosis of asthma/respiratory allergy.
    - Diagnosis of fibromyalgia.
    - Personal history of (TBI).
    - Presence of sleep disorders requiring drug treatment.
- Characteristics of migraine:
    - Type of migraine (with or without aura)
    - Time of evolution since the beginning of the disorder
    - Other types of headache.
    - Intensity of attacks in the last 3 months: based on patient recall and measured by Visual Analog Scale (0-10).
    - Frequency of attacks in the last 3 months: recorded by calendar.
    - Duration of attacks in the last 3 months: based on patient recall.
    - Allodynia.
    - Review of current symptomatic treatments: non-steroidal anti-inflammatory drugs, triptans, ergotics.
    - Review of current preventive treatments: beta-blockers, neuromodulators, antihypertensives, calcium antagonists, antidepressants, botulinum toxin, others.
    - Review of concomitant treatments for other pathologies: hormonal contraceptives, antihypertensives, antidepressants, statins, corticoid-anti-inflammatory drugs, opioids.

5.3.3. Clinical examination

The height of the patients was determined using a handheld stadiometer (centimeters). The weight of the patients was measured with a digital scale (kilograms).

A brief neurological examination was performed.

Neuroimaging studies were available for those subjects who required it for clinical reasons.

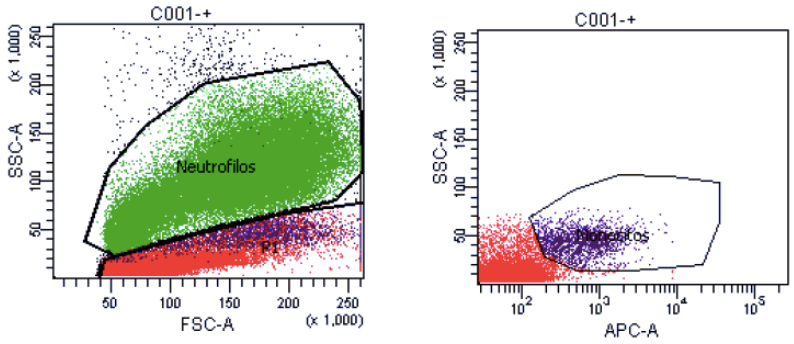
5.3.4. Blood collection and laboratory studies

Blood was drawn by venipuncture in the antecubital fossa. The extraction was performed on the same day of the interview if the patients had not presented headache in the previous 48 hours nor had they consumed anti-inflammatory drugs in this period. If this was not the case, they were asked to come 48 hours after the end of the crisis to have the sample taken. The samples were collected between 9 and 11 a.m. at the Neurology Service Day Hospital. Two different tubes were extracted in order to determine expression of TLRs (EDTA) and ligand levels in peripheral blood (tube with 3.2% sodium citrate).

5.3.4.1.Determination of TLR expression in peripheral blood

The expression of TLRs was determined in mononuclear cells (monocytes and neutrophils) in peripheral blood. Blood samples were collected in anticoagulated EDTA tubes and immediately processed at the Clinical Neurosciences Research Laboratory in Santiago de Compostela by flow cytometry. In this cytological analysis technique, the cells in suspension are passed one by one through a laser light beam. When the cells are hit, light is scattered and collected and measured by detectors (photodiodes and photomultipliers), which generate data that are analyzed by *ad hoc* software. The size of the cells is given by the front beam of light they emit (forward scattering signal, FSC) and the granularity of the cells by the side beam of light (side scattering signal, SSC). FSC and SSC are used to identify different cell lines in a mixed population based on their size and granularity.

Figure 5.11. Classification of cells according to FSC and SSC.



Cytometer differentiates neutrophils according to SSC and subsequently monocytes from other cell populations by APC staining.

Other cellular characteristics and the expression of different proteins and receptors can be determined if the samples are pre-stained with fluorophore labelled antibodies. Fluorophores absorb light and emit it at a certain wavelength, which is measured by different detectors. In addition to determining whether a cell type expresses a receptor or protein, measuring the intensity of the fluorescence signal allows the expression of these structures in each cell type to be quantified.

The samples were centrifuged at 400 g for 30 minutes at room temperature. The supernatant was aspirated and washed three times with NSS. From each sample 100 µl were extracted and distributed in two tubes (50 µl/tube). To remove the red blood cells, 500 µl of hypotonic solution (FACSLysing, fluorescent-activated cell sorting) were added to the samples and left to act until a translucent appearance was achieved. The samples were incubated for 15 minutes at room temperature and protected from light. They were then analysed in a FACSAria-II cytometer (BD Biosciences, 2 lasers, 7 channel configuration). Three stains were used:

- APC-marked anti-CD14 antibodies for binding to monocytes.
- Anti-CD282 antibodies marked with FITC for TLR2 binding.
- Anti-CD284 antibodies marked with PE-A for TLR4 binding.

Negative tubes, i.e. not marked with antibodies, were used as controls for each of the samples.

Figure 5.12. Control cytometry without fluorophore marking.

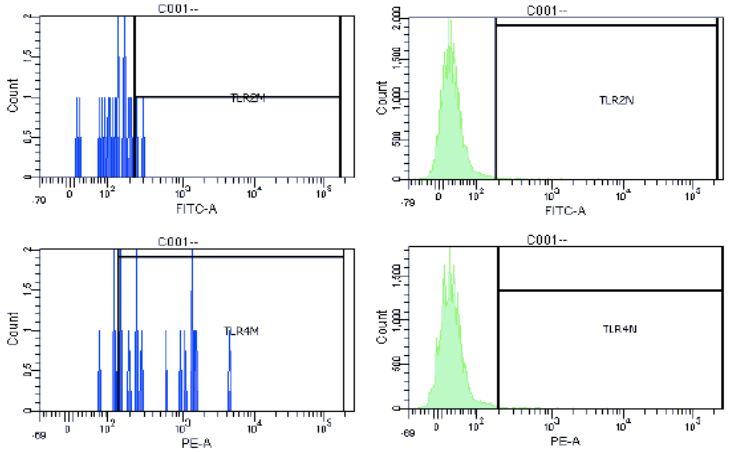
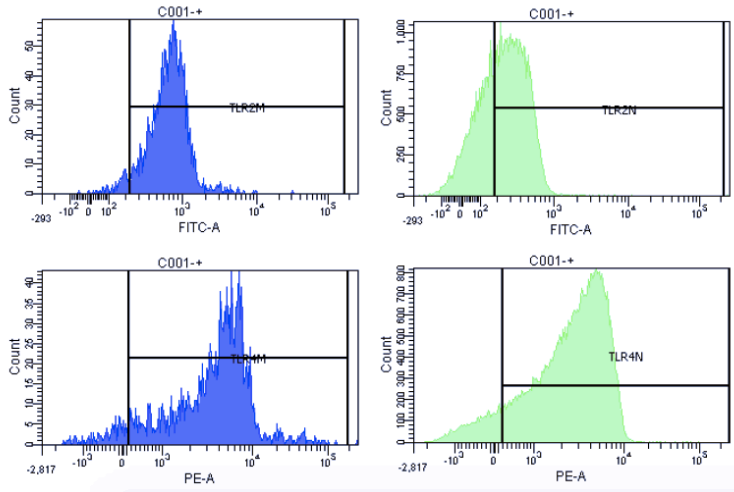


Figure 5.13. Cytometry in cells marked with fluorophores FITC-A and PE-A



The amplification parameters for FSC and SSC were used in linear mode and the fluorescence channels were used in logarithmic mode. Mononuclears and neutrophils were characterized by their complexity and size, according to their FSC and SSC values respectively.
We analyzed 50.000 events in the control tube (unmarked) and 100.000 events in the antibody-marked tube. The results are expressed as the mean of the corresponding population (neutrophils, monocytes) in Fluorescence Arbitrary Units (AU).

5.3.4.2. Determination of endogenous ligands and biomarkers of inflammation, trigeminal activation and endothelial dysfunction

The samples were collected in a tube with 1.3% sodium citrate, and were allowed to coagulate at room temperature for 1 hour. Subsequently, the serum was separated from the blood by centrifugation (15 minutes at 3000 g), and 0.5 ml of serum was extracted and transferred to aliquots. Each aliquot was stored at -80°C until the time of analysis. The levels of all biomarkers were measured by ELISA according to the manufacturer's instructions.

Table 5.1. ELISA kits characteristics

	Manufacturer	Range	Sensitivity	Intra coefficient of variation	Extra coefficient of variation
IL-6	Biolegend, San Diego, California, USA.	7.8-500 pg/ml	1.6 pg/ml	<8%	<9%
IL-10	Biolegend, San Diego, California, USA.	3.9-250 pg/ml	2pg/ml	<6%	<8%
cFN	Abbexa, Cambridge, UK	12.5-800 ng/ml	<7.5ng/ml	<8%	<10%
PTX-3	Rockland antibodies&assays, Limerick PA, USA	312-2000 pg/ml	<10 pg/ml	<6%	<7%
sTWEAK	ThermoScientific, Massachusetts, USA	46.88-3000 pg/ml	40 pg/ml	<10%	<12%

HSP60	AssayPro, StCharles, USA	2.5-80 ng/ml	1.9 ng/ml	<5.3%	<10%
CGRP	Cloud-clone, Texas, USA	12.35-1000 pg/ml	<5.35 pg/ml	<10%	<12%
Hs-CRP	Cusabio, Texas, USA	0.625-40 ng/ml	0.156 ng/ml	<8%	<10%

5.4. SAMPLE SIZE CALCULATION

5.4.1. Experimental study

Sample size for in vivo studies was calculated using EPIDAT software (http://www.sergas.es/Saude-publica/EPIDAT-4-2) based on previous studies and with a power (1-β) of 0.8 and an $\alpha = 0.05$. Statistical significance was set at $p<0.05$. Animals were randomly assigned to treatment groups, and researchers were blinded to treatment administration and result analysis.

5.4.2. Clinical study

Based on the results of an exploratory study carried out in our laboratory on TLR4 in monocytes, a sample size of 162 subjects was calculated (81 migraine patients and 81 healthy controls), assuming a risk $\alpha= 0.05$ and a risk $\beta= 0.1$, for a statistical power of 80%. The sample size was calculated using EPIDAT software (http://www.sergas.es/MostrarContidos_N3_T01.aspx?IdPaxina=62714).

5.5. STATISTICAL ANALYSIS

5.5.1. Experimental study.

Results were expressed as mean ± Standard Deviation (SD). Data was first examined to assess distribution using the D'Agostino and Pearson omnibus normality test. Statistical analyses were performed using a t-student test or one-way analysis of variance (ANOVA) followed by a Bonferroni posthoc analysis. All analyses were

conducted using Graphpad Prism 6 for Mac (GraphPad Software, La Jolla California USA).

5.5.2.Clinical study

All data analyses were performed with IBM SPSS Statistics 20.0 software for Mac (SPSS Inc., Chicago, IL, USA). Continuous normally distributed variables analysed with Kolmogorov-Smirnov test were reported as mean ± standard deviation, whereas continuous non-normally distributed variables were expressed as median [P_{25}, P_{75}]. All biochemical variables were log transformed if normality assumptions were not met.

Categorical variables were reported as percentages. Differences between two groups were assessed by independent t test (continuous normally distributed variables), Mann- Whitney test (continuous non-normally distributed variables) and X^2 test (categorical variables). Spearman´s correlation coefficient (r) was used to correlate TLR expression with significant ligands and biomarkers.

Binary logistic regression models were performed to test potential associations between migraine status and TLR expression.

To determine the best discriminant cut-off point of TLR expression to identify CM patients, a receiver operating characteristic (ROC) analysis was carried out.

All tests were performed at a significance level of $\alpha = 0.05$.

6. RESULTS

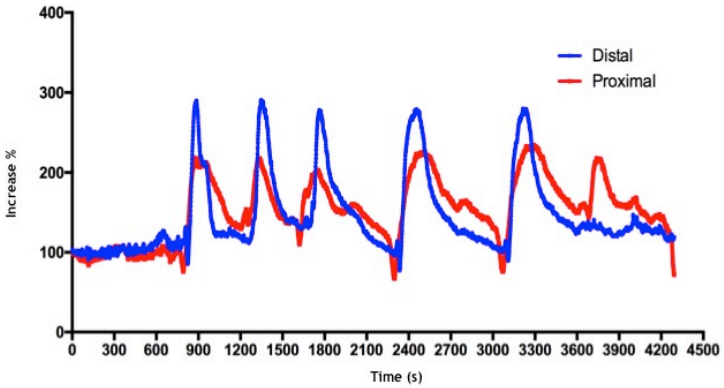
6.1 EXPERIMENTAL MODEL

6.1.1. Initial test. Wild type mice versus TLR4-KO mice.

In our model we observed a wave-shaped propagation of vascular changes secondary to the appearance of CSD, similar to what previous studies have reported. Each animal was monitored for 70 minutes (10 minutes of basal recording followed by 60 minutes of recording with KCl esposure as the trigger of CSD). The same wave pattern was recorded on the proximal recording fibre as on the distal fibre with an average delay of 20 seconds between the two.

The increase in brain flow was calculated as a percentage of basal flow. In each animal, the wave duration in seconds (s) and the number of waves during the recording period were measured.

Figure 6.1. CBF changes record.

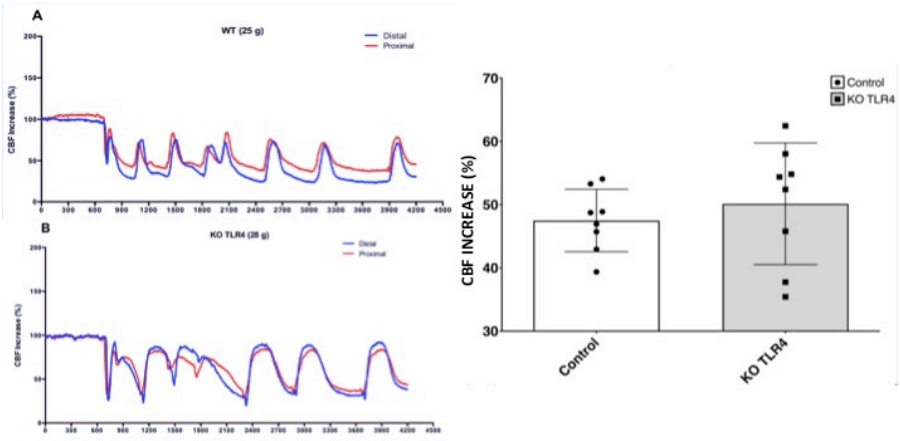


Waves of CBF variation in both registration points: proximal (red) and distal (blue). On this record the average increase over the basal level (%), the duration of the waves in seconds (s) and the number of waves recorded per 60 min period are calculated for each animal.

6.1.1.1. Changes in CBF

The increase in CBF (%) was slightly higher in the KO mice group compared to the WT group, although these differences did not reach statistical significance (47.49±4.932 and 50.14±9.620, respectively; p=0.5004).

Figure 6.2. CBF changes in KO and WT animals

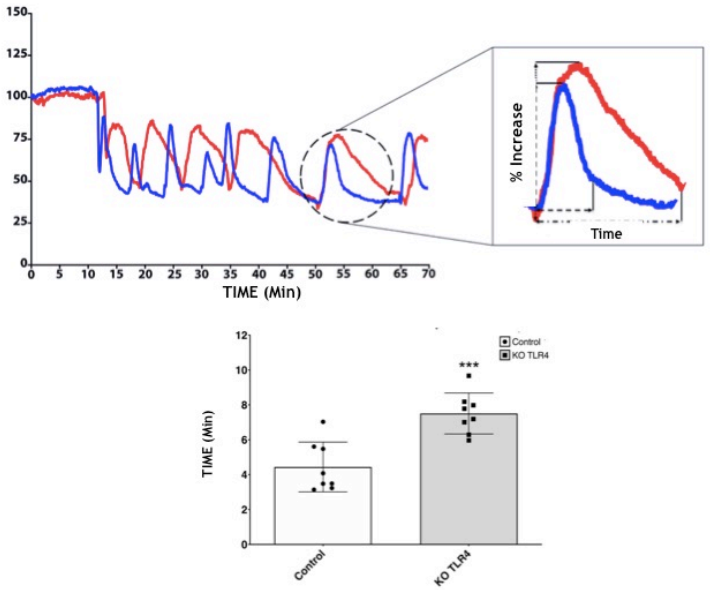


(1) WT (A) and KO (B) records after continuous KCl stimulation, where a pattern is observed in the proximal (red) and distal (blue) recording fibre. (2) Percentage increase in CBF from baseline (%).*p<0.05; **p<0.01; ***p<0.001.

6.1.1.2. CSD wave duration

The duration of the wave was defined as the time between the start of the hyperemia and the start of the next wave, measured in minutes. KO animals for TLR4 had a longer mean duration than wild animals (7.509 ± 1.172 and 4.443 ± 1.428, respectively) (p<0.001).

Figure 6.3. CSD wave length in WT and KO for TLR4.



The image shows a longer duration of the CSD wave in group 1 (KO), in red, in relation to group O (WT), in blue. B. Difference between the average wave duration of group 0 and group 1 measured in minutes. *p<0.05; **p<0.01; ***p<0.001.

6.1.1.3.Number of events

No significant differences were found in the number of CSD events during the registration period between the two groups.

6.1.2.Pharmacological manipulation: activation and blockade of TLR4 receptors.

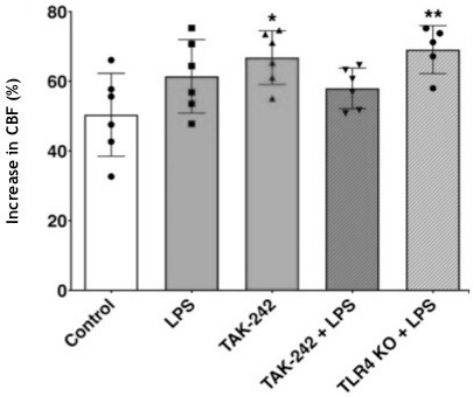
As a complement to previous results, the effect of pharmacological manipulation of TLR4s on the characteristics of the CSD wave was determined.

6.1.2.1. Increase in CBF

CBF was measured in each animal during 70 minutes (initial 10 minutes to have a basal record followed by 60 minutes after beginning stimulation). The wave pattern was similar in both recording fibres. CBF levels were slightly higher in all groups than in

the control group. However, only the group treated with TAK-242 (66.82±7.714%; $p<0.05$) and the KO animals treated with LPS (69.09±6.897%; $p<0.01$) showed statistically significant increases in CBF.

Figure 6.4. Increase in CBF.

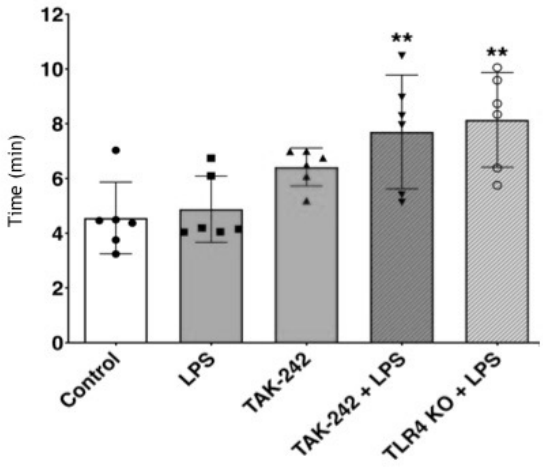


Increase of CBF over baseline (%) in the control group (white), group 4 (WT with DMSO and LPS administration), group 5 (WT with TAK242 and NSS administration), group 6 (WT with TAK242 and LPS administration) and group 7 (KO with DMSO and LPS administration)
***p<0.05; **p<0.01; ***p<0.001**

6.1.2.2. CSD wave duration.

The average wave duration in the control group was 4.557±1.308 min. Administration of LPS (2.5 mg/kg) 1 hour before CSD induction did not prolong the wave duration (4.877±1.211 min). Administration of TAK-242 (3 mg/kg) 1 hour prior to surgical procedure and NSS administration increased wave duration, but the difference was not statistically significant (6.417±0.6933 min). However, WT mice pretreated with TAK-242 that received LPS showed a significantly longer wave of CBF changes (7.700±2.082 min; $p<0.01$). Similarly, when KO mice for TLR4 were stimulated with LPS (2.5 mg/kg) the wave duration was significantly prolonged (8.140±1.729 min; $p<0.001$).

Figure 6.5. CSD wave duration.

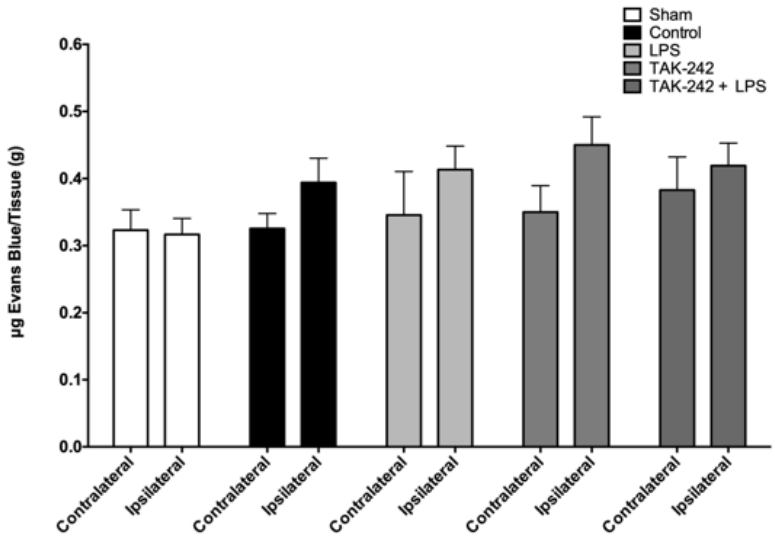


A prolongation of the CBF wave measured in minutes is observed in the KO groups for TLR4 or in those where TLR4 has been blocked with TAK242, mainly when stimulation with LPS occurs.
***p<0.05; **p<0.01; ***p<0.001**

6.1.3. BBB permeability

In our experimental conditions the staining filtration of Evans Blue was greater in the hemisphere where the CSD was induced with respect to the contralateral. No differences were found in terms of barrier permeability between control, KO or TAK-242-blocked animals.

Figure 6.6. BBB disruption in the different experimental groups

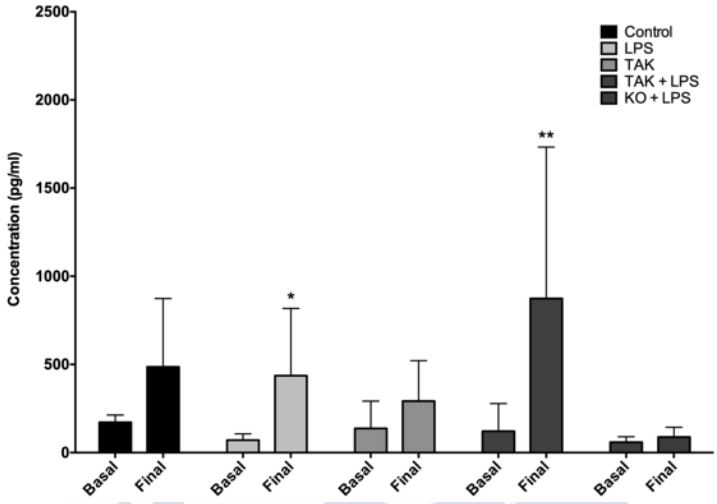


Ratio between µg Evans Blue per g of tissue in both hemispheres (ipsilateral to CSD and contralateral) in the different groups. In all groups there was a greater filtration of Evans Blue in the ipsilateral hemisphere, demonstrating the effect of the repeated CSD on BBB permeability, but we did not find differences among experimental groups. *p<0.05; **p<0.01; ***p<0.001

6.1.4. IL-6 levels

In all experimental groups, IL-6 levels were higher after the procedure than before. KO animals for TLR4 showed a very discrete increase in IL-6 levels after CSD induction, but the differences were not significant. Animals exposed to TAK-242 and LPS showed higher levels of IL6 in post-procedure determination.

Figure 6.7. Pre- and post-procedure IL-6 levels (pg/ml) in the different experimental groups.

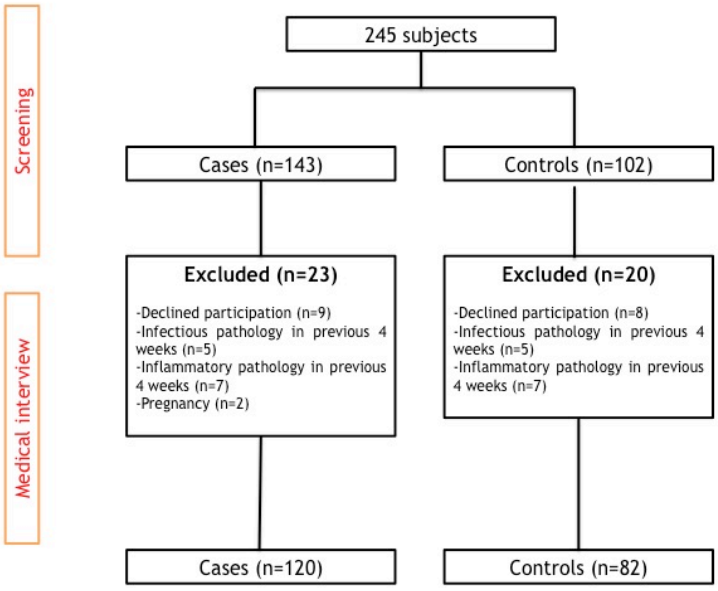


After the procedure, IL-6 levels were higher in the groups exposed to LPS and TAK-242 + LPS but no statistically significant differences were found. IL-6 levels were lower in KO animals for TLR4 than in the control group.
***p<0.05; **p<0.01; ***p<0.001**

6.2. CLINICAL STUDY

A total of 245 subjects were asked to participate in the study. Of these, 20 controls and 23 cases were excluded for several reasons (Figure 6.1). The final study sample for the clinical study consisted of 120 subjects with a diagnosis of CM and 82 healthy controls.

Figure 6.8. Flow chart of the clinical study



6.2.1 Socio-demographic characteristics

The socio-demographic characteristics of the sample are summarised in the table below.

Table 6.1. Socio-demographic characteristics of the sample

		CONTROLS (n=82)	CM (n=120)	p
Age (years)		48.44 ± 10.52	48.58 ± 11.37	0.932
Sex (% women)		97.6%	97.5%	1.000
Education level	Basic	1.2%	51.7%	**0.000**
	Secondary	32.9%	29.2%	
	Degree	65.9%	19.2%	
Income	TSI 001	0%	6.7%	**0.000**
	TSI 002	1.7%	20.8%	
	TSI 003	39.7%	53.3%	
	TSI 004	55.2%	19.2%	
	TSI 005	3.4%	0%	

TSI 001: retired, with non-contributive pension
TSI 002: retired, pension less than 100 000 euros/year
TSI 003: active, income less than 18 000 euros/year
TSI 004: active, income between 18 000 and 100 000 euros/year
TSI 005: active, income over 100 000 euros/year

There are no significant differences between groups in terms of sex as in both groups most of the subjects are women (97.5% cases vs. 97.6% controls; p=1.000).

The mean age of cases is 48.58 years (±11.37) and 48.44 years (±10.52) in the control group, with no difference between both groups.

In contrast, educational level and socioeconomic status are different in both groups. A lower level of education predominates among patients with CM; while 65.9% of the controls have completed higher education, only 19.2% of the cases have accessed university studies. 51.7% of cases have completed only basic education. Income is determined on the basis of the "Tarjeta Sanitaria Individual" code (TSI). Among cases, most of the subjects were in group 003 (53.3%) or 002 (20.8%) while in the control group the income was slightly higher with 55.2% of subjects with TSI 004 and 39.7% with TSI 003. These differences are significant.

6.2.2 Clinical characteristics

The clinical characteristics of controls and subjects with CM are summarized in Table 6.2.

Table 6.2. Clinical characteristics of the sample

	CONTROLS (n=82)	CM (n=120)	p
BMI (kg/m^2)	23.83 ± 4.22	26.95 ± 4.40	0.000
Toxic habits			
Smoking (%)	23.5%	14.2%	0.092
Alcohol abuse[1]	1.2%	2.5%	0.649
Caffeine abuse[2]	23.2%	22.5%	0.911
Other drugs	1.2%	0.8%	1.000
Cardiovascular risk factors			
HBP	2.4%	13.3%	0.010
DLP	7.3%	19.2%	0.018
DM	2.4%	2.5%	1.000
Migraine comorbidities			
Anxiety/depression	9.8%	43.3%	0.000
Epilepsy	0.0%	0.8%	1.000
Metabolic syndrome[3]	0.0%	1.7%	0.515
Cardiovascular disease	0.0%	0.8%	1.000
PFO	0%	0.8%	1.000
OSA	1.2%	1.7%	1.000
Allergy	7.3%	14.2%	0.176
Fibromyalgia	0%	10.8%	0.001
TBI	4.9%	9.2%	0.289
Sleep disorders	18.3%	55.8%	0.000
Medical treatments			
Hormone contraceptives	12.1%	15.8%	0.505
Anti-hypertensives	3.4%	15.8%	0.023
Antidepressants	8.6%	31.7%	0.001
Statins	8.6%	16.7%	0.148
Opioids	0.0%	14.2%	0.002

BMI: Body mass index, HBP: high blood pressure, DLP: dyslipidemia, DM: diabetes mellitus, PFO: patent foramen ovale, OSA: obstructive sleep apnea syndrome, TBI: thraumatic brain injury. [1]Defined as > 3 Standard Beverage Units (SBUs) per day for men and 2 for women. [2]Defined as >3 cups of coffee per day. [3]Defined as increased blood pressure, high blood sugar levels, excess body fat around the waist, and abnormal cholesterol or triglyceridees levels

Patients with CM had a higher BMI (26.95±4.40) than controls (23.83±4.22) (p=0.000). In terms of toxic habits, we found more

smokers in the control group (23.5% vs. 14.2%), although the difference was not significant. Excessive alcohol consumption, defined as >4 units per day for men and >2 for women, was slightly more frequent among subjects with CM (2.5% vs. 1.2%), but in any case, anecdotal. Excessive caffeine consumption (>3 drinks per day) was around 20% in both groups. Only 1 subject in each group admitted to abuse other substances.

Subjects with CM presented a higher prevalence of high blood pressure (13.3% vs. 2.4%; p=0.010) and dyslipidemia (19.2% vs. 7.3%; p=0.018), while the prevalence of diabetes mellitus was almost identical in both groups (2.5% vs. 2.4%; p=0.674).

A review of migraine comorbidities was conducted both by interview and by review of medical chart. The prevalence of anxiety-depressive syndrome was much higher in subjects with CM than in controls (43.3% vs. 9.8%, p=0.000), as was the prevalence of allergy (14.2% vs. 7.3%, p=0.176), fibromyalgia (10.8% vs. 0%, p=0.001), sleep disorders (55.8% vs. 18.3%, p=0.000) and history of traumatic brain injury (9.2% vs.4.9%, p=0.289). In contrast, the prevalence of epilepsy, metabolic syndrome, stroke, patent foramen ovale (PFO) and obstructive sleep apnoea (OSA) syndrome was similar between both groups.

In accordance with these findings, patients with CM had a significantly higher use of lowering blood pressure drugs (15.8% vs 3.4%, p=0.023), antidepressants (31.7% vs 8.6%, p=0.001) and opioids (14.2% vs 0%, p=0.002).

6.2.3. Characteristics of CM patients

Characteristics of CM patients in our sample are reflected in the following table. The median time of evolution since the first migraine diagnosis was over 26 years, with high intensity headache (VAS 8) and a median of 20 days of pain per month. Most crisis (>68%) last longer than 24 hours and occur without aura. Almost half of patients do not have another accompanying headache. Painkiller abuse was present in 20% of CM patients in our sample. Regarding treatments, the most used preventative was OnabotulinumtoxinA and during

migraine attacks, the most frequent symptomatic treatment was NSAIDs, followed by triptans.

Table 6.3. Clinical characteristics of CM subjects

		Median [percentiles] / %
Time of evolution (years)		26.50 [15.00,42.75]
VAS score		8 [7,10]
Days of pain per month		20 [15,25]
Duration of crisis	<12 h	14.2%
	12-24h	17.5%
	>24h	68.3%
Migraine Charateristics	With aura	7.5%
	Without aura	63.3%
	With/without aura	29.2%
Allodynia		60%
Other headaches	No	49.2%
	Tension headache	46.7%
	Neuralgia	1.7%
	Other	1.7%
Painkiller abuse		20%
Preventative treatments		
OnabotulinumtoxinA		71.7%
Beta-blockers		40%
Neuromodulators		28.3%
Antihypertensives		0%
Calcium antagonists		15%
Antidepressants		36.7%
Others (Tizanidine, Ciclobenzaprine)		18.3%
Symptomatic treatment		
NSAIDs		87.5%
Triptans		72.5%
Ergotics		0%

6.2.4. Expression of TLRs

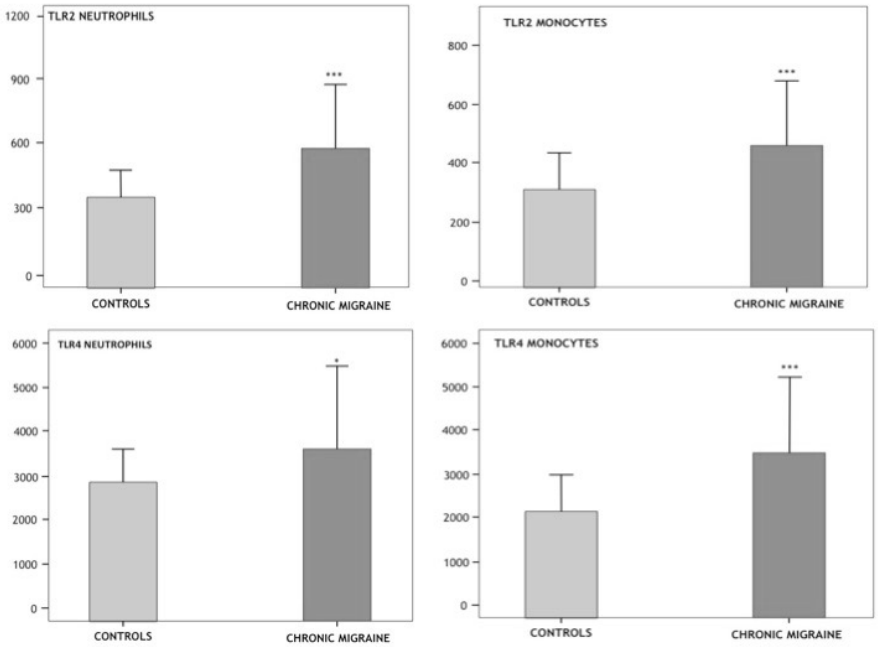
Subjects with CM showed increased expression of TLR2 and TLR4 in neutrophils and monocytes in peripheral blood. This difference was statistically significant.

Table 6.4. Expression of TLR2 and TLR4 in monocytes (M) and neutrophils (N)

	CONTROLS (n=82)	CM (n=120)	p
TLR2 N (AU)	367,30±94,80	546,23±316,56	0.000
TLR2 M (AU)	339,50±133,09	469,22±204,50	0.000
TLR4 N (AU)	2997,74±749.33	3597,45±1865,25	0.037
TLR4 M (AU)	2110,80±865,86	3436,83±1751,03	0.000

Results expressed as mean (standard deviation) Comparison of means calculated using the T-student test for the variables transformed by Log10.

Figure 6.9. Expression of TLRs in controls and subjects with CM.



Expression of TLR2 in monocytes (A), TLR2 in neutrophils (B), TLR4 in monocytes (C) and TLR4 in neutrophils (D).
***p<0.05; **p<0.01; ***p<0.001**

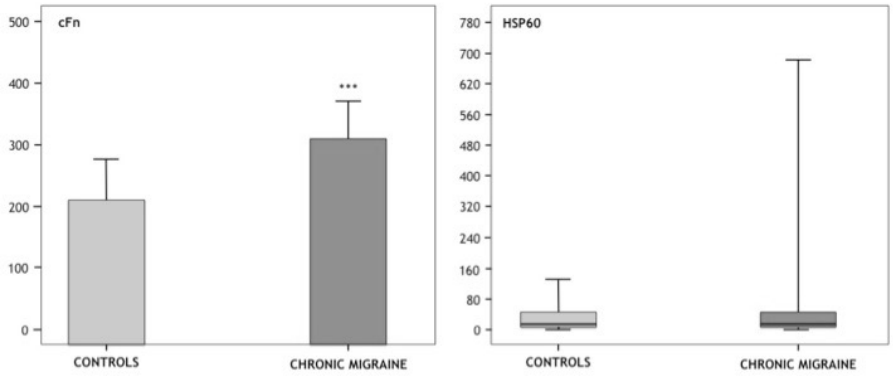
6.2.5. Ligands of TLRs

The levels of two ligands of TLRs in peripheral blood were determined: cFN and HSP60. Significantly higher levels of cFN were found in subjects with migraine. HSP60 levels are higher in subjects with migraine, but differences do not reach statistical significance.

Table 6.5. Ligands of TLRs in controls and subjects with CM.

	CONTROLS (n=82)	CM(n=120)	p
cFN (ng/ml)	211.79 ± 70.91	306.51 ± 59.68	0.000
HSP60 (ng/ml)	12.31 [2.47,43.33]	15.79 [3.81,42.97]	0.187

Figure 6.10. Levels of cFN (A) and HSP60 (B)



***p<0.05; **p<0.01; ***p<0.001**

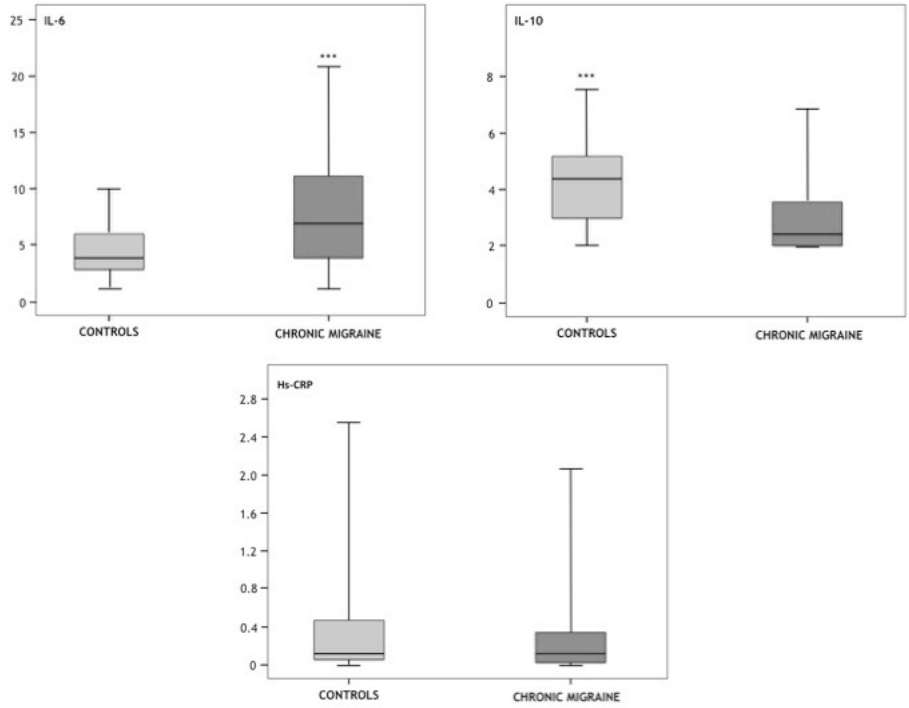
6.2.6. Inflammatory cytokines: IL-6, IL-10 and hs-CRP.

Analysis of the levels of several cytokines related to neuroinflammation revealed a significant elevation of IL-6 levels in subjects with CM. IL-6 is one of the main products of the activation of the signaling pathways of TLR4. In contrast, levels of IL-10, a cytokine with anti-inflammatory effects, were significantly lower in subjects with CM. Levels of hs-CRP, a non-specific marker of systemic inflammation, were not different between groups.

Table 6.6. Cytokine levels in controls and CM

	CONTROLS (n=82)	CM (n=120)	p
IL-10 (pg/ml)	4.45 [3.07,5.12]	2.37 [2.00,3.50]	0.000
IL-6 (ng/ml)	3.75 [2.77,5.92]	7.00 [3.87,11.58]	0.000
Hs-CRP (mg/dl)	0.07 [0.02,0.44]	0.11 [0.03,0.36]	0.501

Figure 6.11. Levels of inflammatory cytokines



Box plots showing IL-6(A), IL-10 (B) and hs-CRP levels in controls and subjects with CM.
*p<0.05; **p<0.01; ***p<0.001

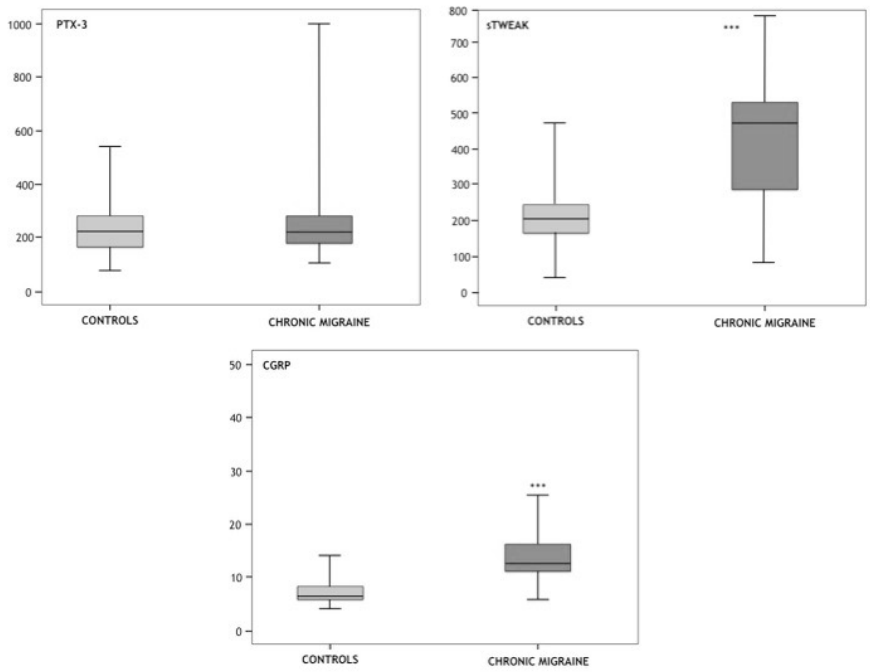
6.2.7. Trigeminal-vascular activation and endothelial dysfunction biomarkers

Neuropeptide levels related to endothelial dysfunction (sTWEAK, PTX3) and trigeminal-vascular activation (CGRP) were determined. Subjects with CM presented higher levels of sTWEAK and CGRP. Levels of PTX3 were higher in this sample, although differences did not reach statistical significance.

Table 6.7. Markers of trigeminal-vascular activation and endothelial dysfunction

	CONTROLS (n=82)	CM (n=120)	p
PTX-3 (pg/ml)	204.50 [176.87,270.93]	217.50 [185.77,289.61]	0.065
sTWEAK (pg/ml)	201.17 [170.05,241.38]	483.54 [297.64,527.77]	0.000
CGRP (pg/ml)	6.36 [5.35,8.36]	12.74 [11.54,15.51]	0.000

Figure 6.12. Box plots showing PTX-3(A), sTWEAK (B) and CGRP levels in controls and subjects with CM.



Box plots showing PTX-3(A), sTWEAK (B) and CGRP levels in controls and subjects with CM.
***p<0.05; **p<0.01; ***p<0.001**

6.2.8. Correlation analysis

6.2.8.1.Correlation between TLR expression and levels of TLR ligands

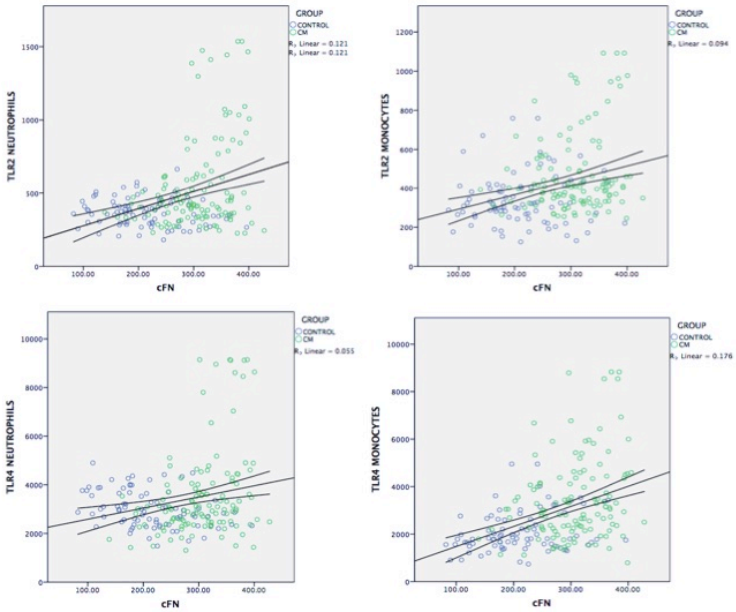
In the total sample, our data showed a significant correlation between levels of cFN and expression of TLR2 in neutrophils and monocytes, as well as expression of TLR4 in monocytes. When examining healthy controls and migraineurs together, levels of HSP60 are correlated with expression of TLR2 in neutrophils and monocytes, as well as expression of TLR4 in neutrophils.

Among migraineurs, cFN levels are correlated with expression of TLR2 in neutrophils and TLR4 in neutrophils and monocytes. When examining only migraineurs, HSP60 levels are correlated with expression of all TLRs.

Table 6.8. Correlations between TLRs and endogenous ligand levels.

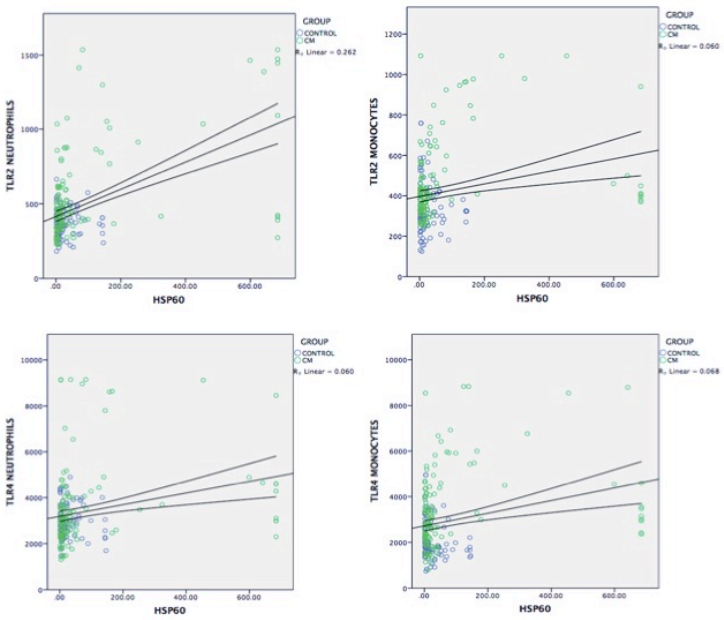
		cFN	HSP60
TLR2N	r	0.213	0.321
	p	0.002	<0.001
TLR2M	r	0.302	0.241
	p	<0.001	0.001
TLR4N	r	0.125	0.253
	p	0.077	0.000
TLR4M	r	0.428	0.101
	p	<0.001	0.154

Figure 6.13. Correlation between TLR2 and TLR4 expression in neutrophils and monocytes and cFN.



Scatter plots showing the correlation between cFN and TLR2 in neutrophils (A), TLR2 in monocytes (B), TLR4 in monocytes (C) and TLR4 in neutrophils (D).

Figure 6.14. Correlation between TLR2 and TLR4 expression in neutrophils and monocytes and HSP60.



Scatter plots showing the correlation between HSP60 and TLR2 in neutrophils (A), TLR2 in monocytes (B), TLR4 in monocytes (C) and TLR4 in neutrophils (D).

6.2.8.2. Correlation between TLR expression and levels of inflammatory biomarkers.

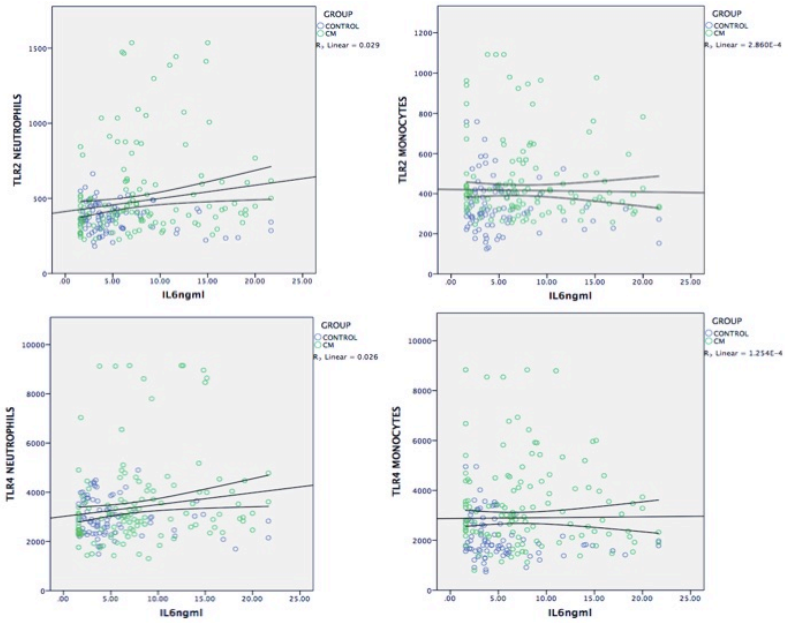
Among all subjects, IL6 levels were correlated with the expression of TLR2 and TLR4 in neutrophils, and this relation was maintained when examining only migraineurs. IL10 levels were correlated negatively with the expression of TLR2 and TLR4 in monocytes in the whole sample, although this correlation was lost when examining only the group of migraineurs.

We found no correlation among levels of hs-CRP and expression of TLRs.

Table 6.9. Correlations between TLRs and cytokine levels

		IL6	IL10	Hs-CRP
TLR2N	r	0.202	-0.114	0.048
	p	0.004	0.105	0.497
TLR2M	r	0.17	-0.243	-0.22
	p	0.808	<0.001	0.192
TLR4N	r	0.166	-0.003	0.054
	p	0.018	0.963	0.447
TLR4M	r	0.019	-0.336	-0.159
	p	0.790	<0.001	0.130

Figure 6.15. Correlation between TLR2 and TLR4 expression in neutrophils and monocytes and IL-6.



Scatter plots showing the correlation between IL6 and TLR2 in neutrophils (A), TLR2 in monocytes (B), TLR4 in monocytes (C), and TLR4 in neutrophils (D).

6.2.8.3. Correlation between TLR expression and biomarkers of trigeminal-vascular activation and endothelial dysfunction.

In our sample we found a relationship between PTX3 levels and TLR2 expression in neutrophils, that existed also in migraineurs.

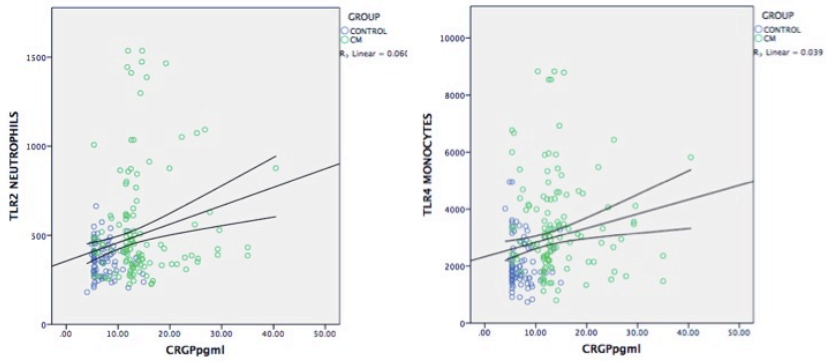
The levels of sTWEAK are correlated with the expression of TLR2 in neutrophils, TLR4 in monocytes, and TLR4 in neutrophils in the whole sample, but when examining only migraineurs, the correlation persists only with TLR2 in monocytes and is negative.

CGRP levels are correlated with the expression of TLR2 in neutrophils and TLR4 in monocytes in the whole sample. We found no correlation between CGRP and expression of TLRs in migraineurs.

Table 6.10. Correlations between TLRs and trigeminovascular activation and endothelial dysfunction biomarker levels.

		PTX3	sTWEAK	CGRP
TLR2N	r	0.155	0.212	0.185
	p	0.028	0.002	0.008
TLR2M	r	0.002	0.086	0.135
	p	0.978	0.224	0.055
TLR4N	r	0.091	0.123	0.055
	p	0.197	0.080	0.437
TLR4M	r	0.022	0.184	0.282
	p	0.758	0.009	<0.001

Figure 6.16. Scatter plots showing the correlation between TLR2 expression in neutrophils and monocytes and TLR4 expression in monocytes and CGRP.



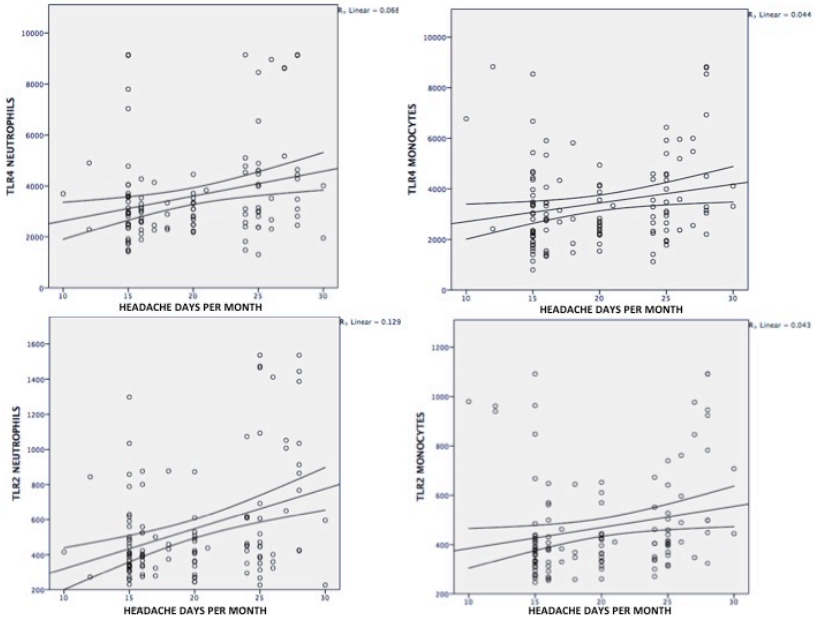
6.2.8.4 Correlation between TLR expression and migraine characteristics

In our sample, we found a correlation between the frequency of migraine attacks, measured as days with pain per month, and the expression of TLR2 in neutrophils and monocytes. Additionally, we found a correlation between the expression of TLR in neutrophils and time from diagnosis, and a specific correlation between the expression of TLR2 in neutrophils and intensity of pain measured using the VAS scale.

Table 6.11. Relationship between the expression of TLRs and characteristics of migraine

		Time from diagnosis (years)	Intensity (VAS)	Frequency (days with pain per month)
TLR2N	r	0.354	0.332	0.280
	p	<0.001	<0.001	0.002
TLR2M	r	0.172	0.147	0.280
	p	0.060	0.109	0.002
TLR4N	r	0.240	0.174	0.224
	p	0.008	0.057	0.014
TLR4M	r	0.143	0.134	0.234
	p	0.119	0.145	0.010

Figure 6.17. Scatter plots showing the correlation frequency of migraine attacks and TLRs.



Scatter plots showing the correlation between frequency of migraine attacks (measured in days of pain per month) and TLR2 in neutrophils (A), TLR2 in monocytes (B), TLR4 in monocytes (C), and TLR4 in neutrophils (D).

6.2.9. Association between the expression of TLRs and CM

6.2.9.1 Model I: Expression of TLRs and CM diagnosis

There is an association between the diagnosis of CM and the expression of TLR2 in neutrophils and monocytes, as well as between the diagnosis of CM and expression of TLR4 in neutrophils and monocytes. Results are shown in Table 6.12

Table 6.12. Model I

	OR	CI	P
TLR2N	1.005	1.003-1.008	0.000
TLR2M	1.005	1.003-1.008	0.000
TLR4N	1.000	1.000-1.001	0.010
TLR4M	1.001	1.001-1.001	0.000

6.2.9.2. Model II: Expression of TLRs and CM diagnosis adjusted by clinical variables

Taking into account the clinical variables in which there are differences between subjects with CM and healthy controls (socioeconomic level, BMI, HBP, DLP, anxiety-depressive syndrome, fibromyalgia, and sleep disorders), an association is maintained between the diagnosis of CM and the expression of TLR2 in neutrophils (OR=1. 005, 95%CI:1.002-1.009; p=0.002), TLR2 in monocytes (OR=1.005, 95%CI: 1.002-1.009; p=0.001), TLR4 in neutrophils (OR=1.000, 95%CI:1.000-1.001; p=0.032) and TLR4 in monocytes (OR=1-001, 95%CI:1.000-1.0001; p=0.001).

Table 6.13. Model II

	OR	CI	P
TLR2N	**1.005**	**1.002-1.009**	**0.002**
Socioeconomical level	0.272	0.136-0.545	0.000
BMI	1.121	1.014-1.240	0.026
HBP	0.977	0.126-7.604	0.982
DLP	0.530	0.119-2.361	0.405
Anxiety/depression	3.585	1.190-10.798	0.998
Sleep disorders	2.010	0.853-4.734	0.110
Fibromyalgia	0.000	0.000	0.999

	OR	CI	P
TLR2M	**1.005**	**1.002-1.009**	**0.001**
Socioeconomical level	0.287	0.149-0.556	0.000
BMI	1.104	1.003-1.217	0.044
HBP	1.540	0.196-12.069	0.681
DLP	0.407	0.092-1.789	0.234
Anxiety/depression	2.766	0.942-8.116	0.064
Sleep disorders	2.554	1.086-6.006	0.032
Fibromyalgia	0.000	0.000	0.999

	OR	CI	P
TLR4N	**1.001**	**1.000-1.001**	**0.034**
Socioeconomical level	0.267	0.138-0.518	0.000
BMI	1.099	0.997-1.210	0.056
HBP	1.153	0.162-8.183	0.887
DLP	0.560	0.128-2.445	0.440
Anxiety/depression	3.475	1.206-10.008	0.021
Sleep disorders	1.988	0.873-4.525	0.102
Fibromyalgia	0.000	0.000	0.999

	OR	CI	P
TLR4M	**1.001**	**1.000-1.001**	**0.001**
Socioeconomical level	0.255	0.133-0.489	0.000
BMI	1.100	0.998-1.214	0.056
HBP	1.903	0.263-13.776	0.524
DLP	0.385	0.085-1.739	0.215
Anxiety/depression	3.212	1.071-9.638	0.037
Sleep disorders	2.454	1.038-5.803	0.041
Fibromyalgia	0.000	0.000	0.999

6.2.9.3. Model III: Expression of TLRs and CM diagnosis adjusted by ligands

Considering blood levels of cFN, a weak association is maintained between the diagnosis of CM and the expression of TLR2 in neutrophils (OR=1. 005, 95%CI: 1.002-1.009;p=0.002), TLR2 in monocytes (OR=1.005, 95% CI: 1.002-1.007; p=0.001) and TLR4 in monocytes (OR=1.001, 95% CI: 1.000-1.001; p=0.001). However, adjusting by cFN, association between the expression of TLR4 in neutrophils and CM is no longer significant.

Table 6.14. Model III

	OR	CI	P
TLR2N (UA)	**1.005**	**1.002-1.009**	**0.002**
cFN (ng/ml)	1.020	1.014-1.026	0.000

	OR	CI	P
TLR2M (UA)	**1.005**	**1.002-1.007**	**0.001**
cFN (ng/ml)	1.020	1.014-1.027	0.000

	OR	CI	P
TLR4N (UA)	**1.000**	**1.000-1.001**	**0.219**
cFN (ng/ml)	1.021	1.015-1.027	0.000

	OR	CI	P
TLR4M (UA)	**1.001**	**1.000-1.001**	**0.001**
cFN (ng/ml)	1.019	1.013-1.025	0.000

6.2.9.4. Model IV: Expression of TLRs and CM diagnosis adjusted by biomarkers

After controlling the association between TLRs and migraine by the levels of interleukins (IL-6 and IL-10) and biomarkers of neuroinflammation (CGRP and sTWEAK), the association between MC and expression of TLR2 in neutrophils and monocytes is maintained (OR=1. 006, 95%CI:1.002-1.011, p=0.010 and OR=1.010, 95%CI:1.006-1.014, p=0.000), as well as the association between expression of TLR4 in monocytes and CM (OR=1.001, 95%CI:1.001-1.002, p=0.000)

Table 6.15. Model IV

	OR	CI	P
TLR2N (UA)	**1.006**	**1.002-1.011**	**0.010**
IL-6 (ng/ml)	1.037	0.933-1.152	0.501
IL-10 (pg/ml)	0.448	0.297-0.677	0.000
CGRP (pg/ml)	1.329	1.138-1.552	0.000
sTWEAK (pg/ml)	1.010	1.005-1.014	0.000

	OR	CI	P
TLR2M (UA)	**1.010**	**1.006-1.014**	**0.000**
IL-6 (ng/ml)	1.036	0.921-1.164	0.557
IL-10 (pg/ml)	0.529	0.345-0.809	0.003
CGRP (pg/ml)	1.526	1.234-1.888	0.000
sTWEAK (pg/ml)	1.012	1.007-1.018	0.000

	OR	CI	P
TLR4N (UA)	1.000	1.000-1.001	0.204
IL-6 (ng/ml)	1.028	0.930-1.138	0.587
IL-10 (pg/ml)	0.447	0.300-0.677	0.000
CGRP (pg/ml)	1.307	1.126-1.518	0.000
sTWEAK (pg/ml)	1.010	1.005-1.015	0.000

	OR	CI	P
TLR4M (UA)	**1.001**	**1.001-1.002**	**0.000**
IL-6 (ng/ml)	1.074	0.960-1.202	0.213
IL-10 (pg/ml)	0.561	0.368-0.853	0.007
CGRP (pg/ml)	1.384	1.152-1.663	0.001
sTWEAK (pg/ml)	1.012	1.007-1.018	0.000

6.2.10. Expression of TLRs as a predictor of CM diagnosis

Expression of TLR2 in monocytes and neutrophils, as well as expression of TLR4 in monocytes are associated with the diagnosis of CM, and this association persists after adjustment for other clinical features and biomarkers that have shown a relationship with the diagnosis.

ROC curves were calculated for TLR2 in neutrophils and monocytes and TLR4 in monocytes with the following results.

6.2.10.1. Expression of TLR2 in neutrophils

The ROC analysis showed an area under the curve (AUC of 0.681 (95% CI:0.609-0.753, P<0.000), suggesting that an expression of TLR2 in neutrophils ≥389 UI is associated with CM with a sensitivity of 63.3% and a specificity of 61%. After categorizing the expression of TLR2 in neutrophils (<389 IU and ≥389 IU) this biomarker was associated with the diagnosis of CM (OR=2.699, 95%CI:1.513-4.813; p=0.001). After adjustment for clinical variables, ligands, and biomarkers of inflammation that showed an association with CM diagnosis (socioeconomic level, anxiety-depression disorder, sleep disorders, cFN, IL-10, sTWEAK, and CGRP), expression of TLR2 remained associated with CM diagnosis (OR=7.397, CI:1.091-50.166; p=0.40)

Figure 6.18. COR curve for TLR2 expression in neutrophils and CM.

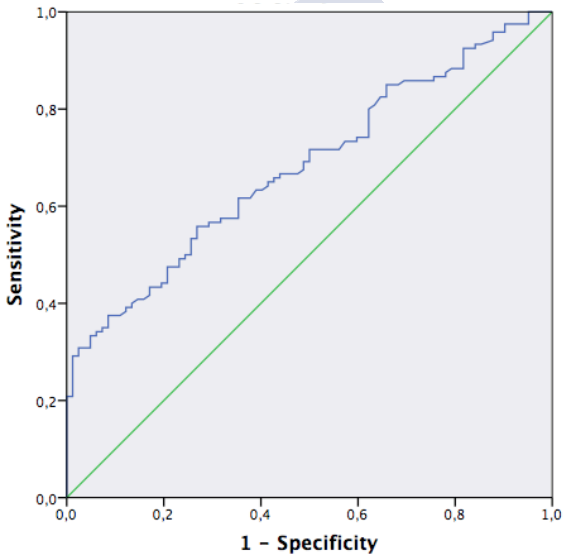


Table 6.16. Association between TLR2 expression in neutrophils higher than ≥389 and CM status. Model I (unadjusted) and model II (adjusted by clinical characteristics and biochemical parameters)

	OR	CI	P
TLR2N (UA)	**2.699**	**1.513-4.813**	**0.001**

	OR	CI	P
TLR2N ≥389 UA	**2.646**	**1.212-5.780**	**0.015**
Socioeconomical level	0.101	0.019-0.540	0.007
Anxiety/depression	32.008	3.148-325.460	0.003
Sleep disorders	1.608	0.256-10.090	0.612
cFN (ng/ml)	1.028	1.028-1.008	0.005
IL-10 (pg/ml)	0.345	0.171-0.695	0.003
CGRP (pg/ml)	1.344	1.038-1.740	0.025
sTWEAK (pg/ml)	1.015	1.005-1.025	0.003

6.2.10.2. Expression of TLR2 in monocytes

The ROC analysis showed an AUC of 0.721 (95% CI:0.648-0.794, p<0.000), indicating that an expression of TLR2 in monocytes ≥350 UI is associated with CM with a sensitivity of 69% and a specificity of 64%. After categorizing the expression of TLR2 (<350 IU and ≥350 IU) it was associated with the diagnosis of CM adjusted by clinical variables, ligand level, and biomarkers (OR=16.585, 95%CI:2.101-130.903; p=0.008).

Figure 6.19. COR curve for TLR2 expression in monocytes and CM.

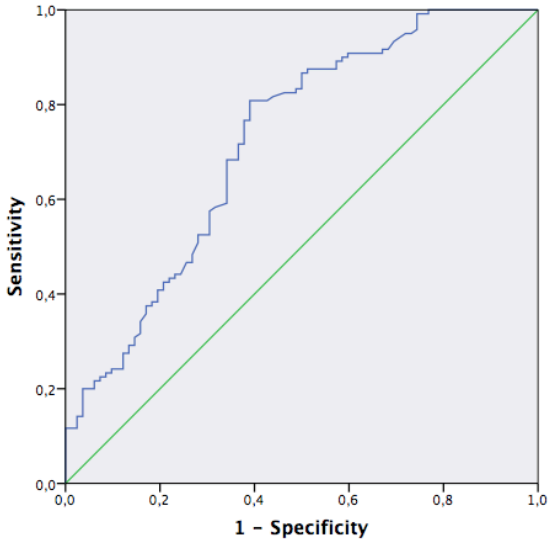


Table 6.17. Association between TLR2 expression in monocytes higher than ≥350 and CM status. Model I (unadjusted) and model II (adjusted by clinical characteristics and biochemical parameters)

	OR	CI	P
TLR2M ≥350 (UA)	**4.044**	**2.230-7.335**	**0.000**

	OR	CI	P
TLR2M ≥350 UA	**16.585**	**2.101-130.903**	**0.008**
Socioeconomical level	0.065	0.012-0.351	0.001
BMI	1.141	0.929-1.401	0.210
Sleep disorders	1.608	0.256-10.090	0.612
cFN (ng/ml)	1.028	1.008-1.049	0.007
IL-10 (pg/ml)	0.370	0.186-0.737	0.005
CGRP (pg/ml)	1.246	0.990-1.569	0.060
sTWEAK (pg/ml)	1.018	1.008-1.028	0.001

6.2.10.3. Expression of TLR4 in monocytes

The ROC analysis showed an AUC of 0.763 (95% CI:0.697-0.829, P=0.000), indicating that an expression of TLR4 in monocytes ≥2232 UI is associated with chronic migraine with a sensitivity of 75% and a specificity of 71%. After categorizing the expression of TLR4 (<2232 IU and ≥2232 IU) this biomarker was associated with the diagnosis of CM after adjusting the analysis by clinical variables, ligands, and biomarkers (OR=6.207, 95%CI: 1.124-34.271; p=0.036).

Figure 6.20. COR curve for TLR4 expression in monocytes and MC.

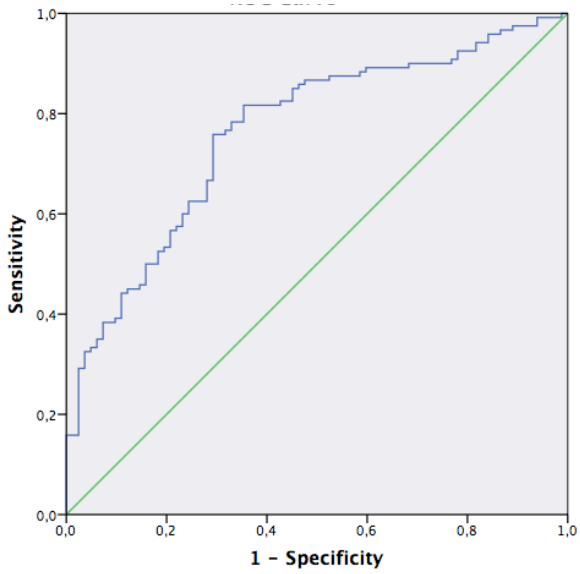


Table 6.18. Association between TLR4 expression in monocytes higher than ≥2232 and CM status. Model I (unadjusted) and model II (adjusted by clinical characteristics and biochemical parameters)

	OR	CI	P
TLR4M ≥2232 (UA)	**7.154**	**3.814-13.421**	**0.000**

	OR	CI	P
TLR4M ≥2232 UA	**6.207**	**1.124-34.271**	**0.036**
Socioeconomical level	0.059	0.008-0.452	0.006
Anxiety-depression	28.079	3.107-253.788	0.003
BMI	1.085	0.892-1.319	0.417
cFN (ng/ml)	1.030	1.007-1.053	0.009
IL-10 (pg/ml)	0.348	0.168-0.721	0.005
CGRP (pg/ml)	1.268	0.992-1.622	0.058
sTWEAK (pg/ml)	1.018	1.007-1.029	0.001

7.DISCUSSION

7.1. EXPERIMENTAL STUDY

7.1.1. Summary of results

Our experimental model shows that KO animals for TLR4 present changes in the CSD pattern recorded by laser flowmetry after KCl stimulation, consisting of a time prolongation of the depolarization wave. These changes are reproduced in WT mice if a TLR4 antagonist (TAK-242) and LPS are administered before inducing CSD by KCl stimulation. As said, changes observed in the CBF wave are of a temporal profile, affecting the duration of the waveform, while other characteristics such as its amplitude or morphology do not seem to be affected by manipulation of TLR4. In KO animals for TLR4 treated with LPS and in WT animals pre-treated with TAK-242 there was also a significant increase in CBF compared to other groups. Pharmacologically blockade of TLR4 (TAK-242 and LPS) entailed as well a significant increase in post-procedure IL-6 levels. Finally, no differences were found in the permeability of BBB in those specimens with genetically determined or pharmacological absence of TLR4, but there were differences between the stimulated hemisphere and the contralateral, demonstrating that repeated CSD alters the barrier permeability.

7.1.2. Choice of experimental model

Our animal design is a murine model subjected to CSD provocation through meningeal stimulation with KCl and recording of CBF changes by laser flowmetry.

Since there is no electrophysiological evidence of the existence of CSD in humans with migraine, particularly in MwoA, the translational value of animal models of CSD has been questioned. Nevertheless, the recording of CSD is among the best defined and most studied animal models of migraine. It has served to demonstrate

the efficacy of several preventive and symptomatic treatments, and is, therefore, a generally accepted model and one of the most used in previous studies (Akerman S et al. 2013). Our group has extensive experience in CSD models and CBF variations in cerebral ischemic pathology, which facilitated the development of this experimental model.

The chosen methodology for CSD provocation - continuous application of KCl to the meninges through a cranial window - has some advantages: it is a specific, localized, direct, and easily controlled method compared with, for example, the administration of substances on a systemic level. The effects of KCl on a local level can be accurately measured and compared, as the number of factors involved in the response is reduced. Compared to direct electrical meningeal stimulation, the continuous application of KCl is more stable and reproducible. However, this methodology does not allow the exact determination of the threshold for the appearance of CSD, as the changes recorded focus on the duration and frequency of CSD waves. The measurement of these parameters provides continuous data and is not affected by cortical surface irregularities, and is therefore considered statistically more powerful than the determination of the electrical threshold, with which it has also demonstrated total correlation (Ayata C. 2013).

As for the response recording system, doppler flowmetry was chosen. The gold standard for recording CSD is electrophysiological recording, but doppler flowmetry has shown a high correlation with the determination of the electrical threshold by electrophysiological recording and is an excellent predictor of the effectiveness of various migraine treatments (Ayata C et al.2015). Interest in models based on vascular changes is increasing, as these changes have been observed in humans during the aura of migraine, unlike electrophysiological changes, increasing the translationality of the model (Cutrer FM et al. 2000).

The use of KO mice for TLR4 together with pharmacologically manipulated WT mice allows us to compare the effects of the constitutive absence of receptors with their blockade. The TLR4 antagonist used in our experimental model is TAK-242 (Resatorvid),

the only known molecule that directly antagonizes TLR4. TAK-242 is the most widely used TLR inhibitor in other animal models of neurological pathologies, such as stroke (Hua F et al. 2015). TAK242 binds to the Cys747 residue of the intracellular domain of the receptor and completely inactivates it. The other antagonist most used and known in inflammation models, E 5564 (Eritoran), is a synthetic molecule containing an N-acetylglucosamine residue similar to LPS and which acts as an MD2 ligand. Eritoran binds to a location too far away from MD2, however, to be considered a direct antagonist of TLR4. There are other compounds that are currently in preclinical development, such as CRX-526, 1A6, a monoclonal antibody against the TLR4/MD2 complex, and OPN-401, a protein of viral origin that inhibits the TLR4-dependent signaling pathway, but its use is still in the experimental phase (Zaffaroni et al. 2018). Both TAK-242 and E5564 (Eritoran) have been tested in human clinical trials in pathologies such as pain (Bruno K et al. 2018) and sepsis (Rice TW et al. 2010) with encouraging results that also demonstrate a good safety profile and acceptable tolerability (Opal SM et al. 2013), increasing the potential of our findings in terms of migraine therapy.

The experimental model is complemented by the determination of one of the end products of the TLR-4 signaling pathway, IL-6, and the measurement of changes in BBB permeability. The determination of IL-6 makes it possible to approximate the influence of the manipulation of TLRs on inflammation and to prove that changes evidenced in CBF correlate with inflammatory mechanisms. Similarly, the study of BBB permeability allows us to determine whether there is a relationship between the absence or blockage of TLRs and BBB dysfunction, which is another mechanism involved in neurogenic inflammation.

7.1.3. Changes in CSD wave

Our findings show that the constitutive absence of TLR4 produces a prolongation in the duration of CBF changes associated with CSD (measured by laser flowmetry). The interpretation of these findings is complex and must be made in relation to previous animal models of CSD. These studies show that the increase in the duration

of the CSD wave corresponds to a higher threshold for CSD and therefore, lower susceptibility to stimulation.

In animal models of migraine of monogenic origin, like the existing models of FHM1 and FHM2, lower stimulation thresholds for the generation of CSD have been registered. In the case of the FHM1 model (van den Maagdenberg AM et al. 2004) an increase in the speed of propagation of the electrophysiological phenomenon was also found, while in the case of FHM2 (Leo L et al. 2011) there is no change in the speed of propagation or duration of depolarization waves.

Pharmacological manipulation models of CSD show similar results to our findings. Hosseini-Zare and collaborators (Hosseini-Zare MS et al. 2017) studied the phenomenon of CSD after pharmacologically blocking the astrocyte glutamate transporters (GLT-1 and GLAST) with TFB-TBOA (2S,3S-3-3-4-trifluoromethyl benzoylamino benzyloxy aspartate), increasing the glutamate concentration in the cellular environment. The electrophysiological recording showed a reduction in cell excitability and amplitude of CSD waves, with shorter inter-CSD periods, as well as a delay in the onset of CSD and a reduction in its rate of propagation. The effect of TFB-BOA was to reduce the cortical excitability and the duration of CSD, reducing the propagation speed. The authors concluded that blocking glutamate transporters was a potential therapeutic target for diseases in which CSD has a role, such as migraine.

The paradigmatic study by Ayata and collaborators on the effect of different preventive treatments (topiramate, valproate, propranolol, and amitriptyline) on CSD showed how these drugs were capable of suppressing CSD and that, even when it was triggered, the speed of propagation was much slower, without differences in the amplitude of the voltage changes (Ayata et al. 2006). In the same way, pregabalin does not lower the threshold of CSD, but it does cause it to have a slower propagation both ex vivo and in vivo (Cain SM et al. 2017). Studies with topiramate, on the other hand, have not shown differences in the speed of propagation, but have shown its efficacy to avoid CSD triggering (Akerman et al. 2005).

Our results, therefore, support the findings of previous studies carried out with CSD inhibitor drugs and point towards a relationship between activation of TLR4 and susceptibility to CSD.

7.1.3.1. TLRs and CSD susceptibility

CSD is associated with activation and hypertrophy of microglia. A single episode of CSD does not produce relevant changes in microglia, but the induction of several CSD episodes can induce an increase in microglial size. This glial activation is believed to be at least partially mediated by TLRs (Bruno K et al. 2018). Ghaemi and co-workers induced repeated CSDs in brain tissue and astrocyte cultures over 4 weeks and were able to observe an increase in the number and volume of astrocytes, without changes in the number of neurons, and an increase in glial expression of TLR3 and TLR4, as well as in the expression of molecules derived from the TLR activation pathway (IL-6, IL-1β, and TNF-α) (Ghaemi A et al. 2018). The HMGB1-TLR2/4 axis could be fundamental in glial activation after repeated CSD (Shibata M et al. 2017). CSD activates Pannexin 1 channels and induces the release of HMGB1 by neurons. HMGB1 is normally located in the cell nucleus, but upon repeated CSD, its transcription increases, and it is released into the extracellular space. HMGB1 then binds to TLR2 and TLR4 and initiates signaling cascades that produce inflammatory mediators such as ROS in the microglia, IL-1β, and TNFα. In KO mice for TLR4/TLR2 or after application of an anti-HMGB1 antibody to the cortical surface these morphological changes in the glia do not occur (Tian X et al. 2017). Other TLRs may have a regulatory role. Stimulation with Poly I:C, a TLR3 agonist, prior to the induction of repeated CSD, reduces the expression of TNF-α and IFN-γ in the brain, the proliferation of dark neurons, and the production of TNF-α and IL-4 in the spleen (Ghaemi A et al. 2016). The results of this study suggest that systemic administration of a TLR3 agonist modulates inflammation and neuronal damage mediated by CSD, not only at the CNS level but also systemically.

In summary, it seems clear that CSD induces an increase in the expression of TLRs in the glia and activation of their signaling

pathways. On the other hand, studies in animal tissue and in vivo have shown that repeated CSD lowers the threshold for CSD (Grinberg YY et al. 2017). The proliferation of inflammatory factors and the increase in the expression of TLRs could be responsible for the perpetuation of positive feedback mechanisms that lead to increased susceptibility to CSD. The exact role of TLRs in these mechanisms remains to be determined, but our results suggest that in animals with a constitutive deficit of TLRs or pharmacological blockade of TLRs susceptibility to CSD is lower.

7.1.4. Role of TLRs in animal models of migraine

Numerous preclinical studies have demonstrated the importance of the innate immune system in chronic pain, through inflammatory mechanisms (Lacagnina MJ et al. 2018). Work carried out specifically on migraine models is scarce.

There is a very recent study on the role of TLR4s in migraine using a murine model of aversion to light (photophobia) to which the compound 48/80 is administered. This compound produces mast cell degranulation. Animals treated with the compound have an aversion to light for 2 hours, which disappears if they receive Sumatriptan. The work shows that the genetic defect of TLR4 or its pharmacological blockade by administration of the antagonist TAK-242 reduces the aversion to light in male mice, but not in females. These results are complemented by the study of trigeminal nucleus caudalis activation, which increases when administering compound 48/80 and decreases after treatment with sumatriptan or TAK-242 (Ramachandran R et al. 2019). This is the first animal model that involves TLR4s in the initiation and maintenance of typical migraine-related behaviors. It is striking that this effect is produced in the study only in male mice, and perhaps this is related to the activation that the glucuronized metabolites of estrogens produce on TLR4s (Lewis SS et al. 2015).

Pretreatment with TAK-242 also reduces hyperalgesia in an animal model (rats) after administration of inflammatory soup in the meninges (Su M et al. 2018). This effect is mediated by a reduction in glial activation and release of neurotrophic factors. A previous animal model of facial allodynia induced by the application of inflammatory

soup (histamine, bradykinin, serotonin, and prostaglandin E2) showed how the occurrence of allodynia is prevented by pre-treating the animals with the TLR4 antagonist naltrexone (Wieseler J et al. 2017).

Our results complement the findings of these studies by showing that congenital depletion of TLR4 or its pharmacological blockade produces changes in the pattern of CSD. This offers a physiopathological explanation for the reduction of certain clinical manifestations accepted as a correlate of migraine in animal models, such as photophobia and allodynia.

7.1.5. Role of TLRs in animal models of pain

Previous works show an increase in the expression of TLRs and the inflammatory molecules derived from their signaling pathways in other types of chronic pain. The effects of inhibition of TLR2 and TLR4 in animal models of pain and inflammation support a possible role of these receptors in migraine, a pathology in which inflammation and nociceptive transmission at the trigeminal nerve is essential.

Most animal models of neuropathic pain produce pain by ligation of the spinal nerve L5 and then measure the response by evaluating behavioral changes in animals. These studies have found not only an increase in the expression of TLR2 and TLR4 in rats with neuropathic damage (Jin G et al. 2018) but a reduction in hyperalgesia and associated glial activation in KO animals for TLR2 and TLR4 (Jurga AM et al. 2016). The CD14-TLR4 dimer is essential for the activation of TLRs in this context, but it is still unknown with which ligand it interacts, either HSPs, HMGB1, or β-defensins. The absence of TLR4 in a model of spinal cord injury in mice attenuates microglial activation, pro-inflammatory cytokine release, and mechanical hypersensitivity associated with the injury (Tanga FY et al. 2005). In KO mice for TLR2, after the section of a spinal nerve, thermal and mechanical hypersensitivity, as well as microglial activation and expression of pro-inflammatory factors, are reduced (Kim D et al. 2007).

In the case of pain of inflammatory origin it has been shown that the induction of pulpitis in an animal model produces an increase in the expression of TLR4, an increase in the production of MyD88,

TRIF, NF-κB, TNF-α, and IL-1β in the ipsilateral trigeminal nucleus, and a reduction in the pain threshold in its innervation area (Lin JJ et al. 2015). The increase in TLR4 expression in this model occurred mainly in medium and small caliber nerve fibers, which express TRPV1 and CGRP. Blocking TLR4 with Eritoran reverses all these effects, demonstrating the role of the receptor in this model of facial pain. The results of this study suggest that blocking TLR4 may inhibit inflammation and pain, and therefore is a potential therapeutic target in orofacial pain. Specimens with a constitutive deficit of TLRs did not show hypersensitivity to mechanical stimuli in a microglial induction model using ds-HMGB1 (Agalave NM et al. 2014). Similarly, a model of joint inflammation in mice showed how KO specimens for TLR4 were protected from hypersensitivity once the inflammatory phase was overcome (Raghavendra V et al. 2004).

Regarding pharmacological manipulation of TLRs, numerous animal studies have shown that blocking TLR2 and TLR4 decreases or inhibits pain of varied origin (Hutchinson MR et al. 2008): daily intrathecal administration of an antisense oligodeoxynucleotide for TLR4 attenuates mechanical allodynia and thermal hyperalgesia after C5 root injury in an animal model (Kuang X et al. 2012); intrathecal administration of RNA for TLR4 interference decreases the hypersensitivity associated with sciatic nerve compression damage in another model (Wu FX et al. 2010); sparstolosin B, a substance isolated from Sparganium Stoloniferum and used in Chinese medicine, acts as an inhibitor of TLR2 and TLR4 and reduces allodynia in experimental animals (Jin G et al. 2018). In an animal model of bone cancer, intrathecal administration of a TLR4 antagonist attenuated microglial activation and expression of IL-1β and TNF-α, as well as mechanical allodynia, pointing to the role of microglial TLR4 in chronic pain of oncological origin (Lan LS et al. 2010). Finally, several studies have demonstrated the effects of different TLR4 inhibitory molecules, such as naloxone (Lewis SS et al. 2012) or FP-1 (a synthetic TLR4 antagonist), in animal models of chronic neuropathic pain (Bettoni I et al. 2008).

There are no studies on the relationship between pain and the expression of TLRs specifically in neurons. The creation of a KO

animal model for the expression of TLRs specifically in neurons is one of the keys to finding out if the PRRs expressed in these cells have a role in the transmission of nociceptive impulses (Kato J et al. 2016) or if the effect of the absence/blocking of TLRs on pain is mediated by inflammatory mechanisms and depends on microglial activation.

7.1.6. IL-6 levels

Our results show an increase in IL-6 levels in all specimens after the surgical procedure, which is lower in KO animals for TLR4, but without differences reaching statistical significance. In animals treated with TAK-242 and subsequently stimulated with LPS, post-procedure IL-6 levels are significantly higher.

IL-6 is an inflammatory cytokine whose levels are increased in patients with migraine in both ictal and interictal periods (Wang F et al. 2015). Its levels correlate with those of IL-1β, which is another of the final products of the Myd88-dependent pathway (Moresco EM et al. 2011), and with CGRP levels (Han D. 2019). IL-6 increases excitability of dural trigeminal afferents and contributes to allodynia in animal models of migraine; it inhibits ERK1/2 at the trigeminal receptors, a protein that has been implicated in the induction and maintenance of several painful states through its effects on the voltage-dependent sodium channel Nav1.7 (Yan J et al. 2012). IL-6 is also able to activate meningeal nociceptors contributing to pain in migraine and other chronic pain syndromes (Zhang X et al. 2012). In vitro studies show that effective substances for the treatment of migraine inhibit the translocation of NF-(κB) to the nucleus, reducing glial activation and the production of IL-6 (Magni P et al. 2012).

CSD induction in astrocyte cultures increases the expression of IL-6, IL-1β, and TNFα, as well as TLR3 and TLR4 (Ghaemi A et al. 2018). Inflammatory cytokine levels in animal models of CSD can be altered by the surgical procedure itself, but this is not the only reason: non-invasive induction of CSD by optogenetics also increases cytokine levels, mainly of IL-1β and TNF-α, and much more modestly of IL-6 (Takizawa T et al. 2020). According to these results, IL-6 levels would be less specific. It is possible that in our model IL-6

elevation is mainly due to the surgical procedure and not so much to the effects of CSD, explaining the absence of significant differences between groups. Stimulation with LPS may have increased IL-6 levels perhaps via other pathways, independent of TLRs. However, the small number of animals in the experimental design, only 6 per group, should be taken into account, and our results may be interpreted carefully.

7.1.7. BBB permeability

The results of our animal model show that induction of repeated CSD alters the permeability of the BBB in the stimulated brain hemisphere. We have not found differences in BBB permeability between WT and KO specimens or those where TLR4 was pharmacologically blocked.

Migraine attacks can be considered a test for the homeostatic systems of the brain. To accommodate the increased blood flow resulting from vasodilation of the brain vessels, the BBB expands, aided by the expression of matrix metalloproteinase 9 (MMP-9) which widens the narrow intercellular junctions of the vascular endothelium (Gursoy-Ozdemir Y et al. 2004). The disruption of BBB depends on caveolin-1 and the timing of this disruption remains controversial. While some studies point out that it begins between 3-6 hours after CSD and is maintained for 24 hours (Sadeghian H et al. 2018), other works using KCl stimulation show that changes in the BBB begin even 30 minutes after stimulation, which is more congruent with our results (Cottier KE et al. 2018). On the contrary, in one of the most complete studies on the disruption of BBB in an animal model of migraine, it was observed that the disruption of BBB occurs only after chronic stimulation and exclusively in the area of the TCC (Fried NT et al. 2018).

In all aforementioned studies, changes in the BBB were accompanied by activation of astrocytes and microglia, suggesting that TLR-mediated glial activation influences the phenomenon of barrier disruption. Brain pericytes, wall cells located in the brain capillaries that are key to the maintenance of barrier permeability, express TLR2 and TLR4, and are affected by neuroinflammation

(Nyul-Tóth Á et al. 2017). The stimulation of animal models with TLR2 agonists initiates neuroinflammatory processes and produces a deterioration of certain components of BBB, an effect that is enhanced by the co-stimulation of TLR4 with LPS (Mayerhofer R et al. 2017). However, studies on the effect of TLR activation on BBB are scarce, and none of them has been carried out exclusively on migraine. In MS, the TLR-Myd88 pathway is involved in increasing the permeability of BBB (Zheng C et al. 2020); and studies in sepsis indicate that the entry of LPS into the brain and its inflammatory effects first require the binding of LPS to the TLR4 receptors of endothelial cells, improving permeability (Singh AK et al. 2004).

Our results seem to confirm the effects of CSD over BBB, since permeability was increased in the stimulated cerebral hemisphere. However, we have not found significant differences in barrier permeability between WT and KO mice for TLR4 or those blocked with TAK-242. The relationship between the activation of TLR4 and BBB permeability is just one of the many processes involved and these changes depend on other molecular pathways not related to TLRs.

7.1.8. Limitations of the experimental model

The animal model used in our design has several limitations that derive from both the choice of mice as experimental animals and the stimulation and recording proceedings.

The choice of a murine model has the advantage of allowing comparison with previous studies, mostly carried out on mice. However, it entails a basic limitation in the translationality of the model: mice have a lysencephalic brain, which is more susceptible to CSD than the human, gyrencephalic brain, added to the fact that the larger the brain the less susceptibility to CSD. The determinants of these interspecies differences are not fully known but could be due to a different ratio of astrocytes to neurons (Akerman S et al. 2013). While the use of KO mice for TLR is useful and widely developed in various pathologies, we should take into consideration that TLRs play an important role in neurological development and their absence could have an impact on several responses in the adult mouse or cause the

development of unknown compensatory mechanisms, non-existent in the wild animal (Trotta T et al. 2014). Another important limitation of the use of murine models in TLR research is that the pattern of TLR expression differs from that of humans; human astrocytes only express TLR3 while murine astrocytes express TLR2, 4, 5, and 9 (Kielian T. 2009). For this reason, the use of animal models should be complemented with models based on human tissues and human cell cultures.

As in many previous studies in the field of pain, we only used male mice. This is an important limitation in the study of a pathology such as migraine, with great inter-sexual variability. The differences in susceptibility to CSD induction as well as in the activation of TLRs between both sexes are well known. Intrathecal injection of LPS induces allodynia in male mice, but not in females, suggesting that in females hyperalgesia may depend on a glia-independent pathway (Sorge RE et al. 2011). These differences may also be mediated by modulation of TLR4 by sex hormones, probably estradiol metabolites (Lewis SS et al. 2015).

Regarding the experimental design itself, our study is limited in the control of physiological parameters during surgery (blood pressure, CO2 blood levels, or glycemia levels). In any model with anesthetized animals, it is key to perform a strict control of both the depth of anesthesia and the animal physiology (Ayata C. 2013). In the particular case of CSD studies, changes in blood pressure can modify vessel caliber and brain flow. The duration of CSD is inversely proportional to blood pressure and hypotension prolongs its duration, in turn lengthening the refractory period. If the animal's BP is low, the frequency of CSD during the experiment will be reduced. We did not control BP during surgery, so undetected fluctuations in this parameter may have affected our findings. Nevertheless, as mice tend to have hypotension when undergoing prolonged anesthesia and intubation, we have tried to minimize this effect by maintaining mask ventilation. Gas concentration and pH values also affect CSD, although the exact meaning of their influence is unknown. The temperature was kept under control with the use of a thermoregulated blanket, but continuous monitoring of the animal temperature was not

possible. Plasma glucose levels also affect CSD, hyperglycemia reduces susceptibility and hypoglycemia prolongs its duration, so ideally it should have been controlled. Finally, anesthetics can also have effects on cranial vasculature or susceptibility to CSD; isofluorane, together with nitrous oxide, reduces susceptibility to CSD induction and hinders its spread. Besides, isoflurane and sevoflurane attenuate TLR4-dependent signaling by binding to critical areas of the TLR4-MD-2 complex (Okuno T et al. 2019). Barbiturates, urethane, or α-chloralose have a minor influence on these parameters, but they induce hypotension and respiratory depression more frequently so that animal control is more complex. Technical limitations for the control of the physiological state in our experiment led us to opt for sevoflurane despite its effects on TLR4 signaling.

As for the recording of CSD, laser flowmeter is a subrogated technique to the gold standard, (electrophysiological recording). However, laser flowmetry has been widely used and is considered a validated method. This method requires an open cranial window, which induces irritation of the trigeminal terminals and dilation of intracranial vessels, making it essential to control the provocation of CSD during surgery, which can modify the speed and duration of the following CSD waves (Akerman S et al. 2013). The occurrence of these CSDs secondary to the surgical procedure should be monitored during preparation and therefore it is advised not to initiate stimulation immediately, as was done in our experimental model, by performing a baseline recording between the start of surgery and KCl stimulation. Bleeding can also irritate the meningeal terminals but in our case we have tried to reduce this effect by applying wax to the skull and moisturizing the cranial window with warm mineral oil. On the other hand, the advantage of this technique with an open bone window is that it allows inflammatory substances to be applied directly to the dura, producing a local effect similar to the one we assume underlies migraine pathophysiology.

7.2. CLINICAL STUDY

7.2.1. Summary of results

In our study, we analyzed data from 120 subjects with CM and 82 healthy controls. In both groups, there was a majority of women. No significant differences in age were found between groups. Educational level is lower in the group of subjects with CM, as well as socioeconomic status. In terms of clinical characteristics, subjects with CM have a higher BMI and a higher prevalence of HBP, DLP, anxiety/depression, fibromyalgia, and sleep disorders. In the group of subjects with CM, most of the patients present MwoA, with attacks of >24 duration and without criteria of drug abuse.

Regarding the main goal of the study, the expression of TLR4 and TLR2 in monocytes and macrophages is significantly increased in patients with CM. We determined the levels of two endogenous TLR ligands (DAMPS): HSP60 and cFN. Subjects with CM show elevated levels of both molecules compared to controls, although only differences in cFN have reached statistical significance.

Subsequently, we determined the levels of two cytokines whose production is related to the activation of TLR-dependent pathways: IL-6 and IL-10, as well as a non-specific systemic inflammation marker, hs-CRP. In our sample levels of IL-6 are significantly increased in patients with CM, while levels of IL-10, an anti-inflammatory cytokine, are lower than in controls. No differences in hs-CRP levels were found.

In order to approximate the effect of TLR activation on other mechanisms involved in the physiopathology of migraine, we determined several neuropeptides whose levels are high in CM: CGRP, related to vascular trigeminal activation and PTX3 and sTWEAK, related to endothelial dysfunction. In our sample, subjects with CM show significantly higher levels of sTWEAK and CGRP, consistent with the results of previous studies. Differences in PTX3 levels, which were higher in CM patients, were not significant.

The expression of TLRs in peripheral blood mononuclear cells was correlated to the levels of cFN, IL6, CGRP, and sTWEAK. IL10 levels were inversely correlated with the expression of TLRs.

After adjusting our findings for clinical and molecular variables that were significantly different in patients with CM, the expression of TLR2 in neutrophils and monocytes, and the expression of TLR4 in monocytes were associated with CM diagnosis. We could determine that the expression of TLR2 in neutrophils ≥389 AU, TLR2 in monocytes ≥350 AU, and TLR4 in monocytes ≥2232 AU can predict CM status.

7.2.2. Characteristics of the sample

7.2.2.1. Socio-demographic characteristics

The demographic characteristics of our group of patients with CM are similar to those of other previously published cohorts (Burch RC et al. 2015). The sample is composed of a majority of women in their 40s. A high percentage of subjects with CM in our sample have not extended their education beyond basic level (52.1%), a percentage that is higher than previously published findings in series of migraine patients in Spain (García-Cabo Fernandez C et al. 2016), and also higher than the percentage of people with basic education in the Galician population in general, 26.23% according to the Galician Institute of Statistics (https://www.ige.eu/web/mostrar_actividade_estatistica.jsp?idioma=es&codigo=0203002). Regarding the socioeconomic level, most CM subjects belong to the TSI groups 001 (6%), 002 (21.4%), 003 (53.8%), following data provided by most previously published epidemiological studies. CM has been associated with low socioeconomic level and there is debate as to whether this is a risk factor or rather a consequence of the difficulties that the disorder entails in carrying out normal academic and work-related activities (Schwedt TJ. 2014).

7.2.2.2. Clinical characteristics

Subjects with CM have a BMI significantly higher than healthy controls, with an average value of 26.95, which corresponds to the overweight category. These results are consistent with those published in previous studies (Lipton RB. 2011). Obesity is a risk factor for the chronification of migraine, possibly mediated by a prolonged state of inflammation and in which certain cytokines released by adipose tissue may play a role (Dominguez C et al. 2018).

In terms of toxic habits, CM patients smoke less than controls (14.2% versus 23.5%), have a slightly higher percentage of alcohol abuse, although low (2.5% versus 1.2%) and a similar consumption of caffeine, with 22.5% of subjects admitting to take more than 3 cups of coffee or caffeinated drinks a day. Only one patient in each study group acknowledged consuming drugs of abuse. Caffeine consumption is recognized as a chronification factor according to several previous studies (Schwedt TJ. 2014), but there are no data regarding the influence of tobacco or alcohol consumption in migraine chronification.

Subjects with CM in our sample had a higher frequency of high blood pressure (13.3% vs. 2.4%) and dyslipidemia (19.2% vs. 7.3%), with similar incidences of diabetes mellitus (2.5% vs. 2.4%) and metabolic syndrome. Only one subject had a history of ischemic stroke and had been diagnosed with a PFO. Studies linking cardiovascular risk to migraine are numerous and in particular, HBP and a history of stroke have been found more frequently in patients with CM (Lipton RB. 2011). In our study we found no differences in the incidence of cerebrovascular disease probably due to the small number of subjects and their average age. Both metabolic syndrome (Buse DC et al. 2010) and PFO (Hildick-Smith D et al. 2017) have been associated with migraine and its chronification. In our sample, we did not perform an active search for PFO, and the only case was diagnosed during hospital admission after a stroke. As regards the relationship between lipid metabolism disorders and migraine, previous studies are scarce, but it is interesting how our results seem to support findings from other groups that correlate total cholesterol

and LDL cholesterol levels with the frequency and intensity of migraine (Tana C et al. 2015).

Our data on the different comorbidities of migraine, in line with previous studies, reveal a higher prevalence of anxiety-depressive syndrome (43.3% vs. 9.8%), fibromyalgia (10.8% vs. 0%), and sleep disorders (55.8% vs. 18.3%) among chronic migraineurs. All these disorders have been previously associated with migraine and are recognized as risk factors for chronification.

Concerning the clinical characteristics of subjects with CM, 36.7% of the subjects present episodes of aura. The time elapsed since the diagnosis of migraine was 26.50 years on average and the monthly headache frequency was 20 days, similar to the frequencies recorded in previous studies such as the CaMEO or the AMPP (Lipton RB et al. 2016). Fifty-nine percent of patients did not present allodynia, which is a low incidence of this central sensitization syndrome compared to previous studies (Chen PK et al. 2018).

7.2.3. Expression of TLRs in peripheral blood

In our sample, subjects with CM showed an increased expression of TLR2 and TLR4 in peripheral blood mononuclear cells when compared to healthy controls. This increase may reflect changes in the expression of these receptors throughout the phagocytic-mononuclear system, including the CNS myeloid cells (microglia) and suggests an overactivation of TLR signaling pathways. At the time of printing, this is the first study dedicated to analyzing the expression of TLRs in patients with migraine and one of the few studies focusing on the relationship between TLRs expression and migraine in humans. The interpretation of our results, therefore, must be made with caution, given the lack of previous studies to compare with.

7.2.3.1. Relationship between the expression of TLRs in microglia and the monocyte-macrophage system in peripheral blood

Current theories unanimously accept that neuroinflammation is an essential part of the physiopathology of migraine. There is not much data on the specific role of innate immunity in the disorder, given that its study *in situ* in the CNS is complex. Although most of

the work on inflammation in migraine focuses on the function of mast cells, there are two studies on monocytes and macrophages at a peripheral level in subjects with migraine which have shown deficits in the mechanisms of phagocytosis (Covelli V et al. 1990; Gallai V et al. 1993). This suggests that changes of the phagocytic-mononuclear system occur at a systemic level in migraine subjects. It remains unknown if the dysfunction of the peripheral mononuclear cells is related to changes in TLRs signaling pathways.

Most of the studies on TLR expression in CNS cells have been performed in animals. For obvious reasons, work in vivo in humans is limited to measuring the expression of TLRs in peripheral blood, using them as an approximation of their expression in other tissues. Although there are no studies on migraine, the expression of TLRs at the peripheral level has been analyzed in other neurological pathologies that share physiopathological mechanisms, such as CSD - this is the case of ischemic stroke (Deng L et al. 2017) - or inflammation - as is the case with chronic pain, Alzheimer's disease (Zhang W et al. 2012), acute demyelinating polyneuropathy (Wang YZ et al. 2012) or MS (Hasheminia SJ et al. 2014). A correlation between local and peripheral expression of TLR2 and TLR4 has also been found in non-neurological pathologies such as keratoconus (Malfeito M et al. 2019; Sobrino T et al. 2017). Many studies suggest that there is an association between the expression of TLRs in the CNS and peripheral blood (Brea D et al. 2011; Drouin-Ouellet J et al. 2014; Cassiani-Ingoni R et al. 2006). In conditions of neuroinflammation, the peripheral immune system and microglia communicate directly, but in addition, after the onset of inflammation, the release of pro-inflammatory cytokines leads to BBB disruption, which facilitates the migration and activation of monocytes/macrophages, neutrophils, and T cells (Downes CE et al. 2010). According to this evidence, we can argue that the expression of TLRs in peripheral immune cells is related to their glial expression and that our findings in peripheral blood may reflect the over-expression of TLRs in the CNS of CM patients.

Our findings in peripheral monocytes and neutrophils suggest an association between CM and the expression of TLRs, and a role for

these receptors in the physiopathology of the disorder, whatever the exact mechanism. The existing evidence on the role of TLRs in some migraine-related pathologies, such as stroke or chronic pain, helps us to elaborate different hypotheses to explain our results.

7.2.3.2. Hypothesis 1: over-expression of TLRs is genetically determined and predisposes to CM.

Overexpression of TLRs in CM patients may be genetically predetermined and condition, at least partially, the individual predisposition to develop the disorder. Overexpression of these receptors on endothelial cells, neurons, and microglia would facilitate the emergence of CSD and the release of inflammatory mediators.

The only human study to date concerning the role of TLRs in patients with migraine is a genetic study that analyses the frequency of 4 896/G polymorphism of TLR4 and its relationship with migraine diagnosis in a group of 170 patients and 170 controls (Rafiei A et al. 2012). Its results associate the expression of the G allele with the diagnosis of migraine. However, neither the transcription of this gene at the protein level nor the expression of TLRs in the two study groups was evaluated. This suggests that TLRs could be part of the set of factors that determine predisposition to migraine and point to a potential causal role. However, with the information available at this moment, we cannot determine if the genetic variations translate into a greater or lesser expression of the receptor, or into some conformational modification that changes its activity.

A greater expression of TLRs may lead to a greater susceptibility to CSD, both in ischemic pathology and in migraine. Several genetic studies associate different polymorphisms of TLR2 and TLR4 with the risk of ischemic stroke and the evolutionary course after it: rs5743708, rs1927911, rs4986790, and TLR4-C119A (Tajalli-Nezhad S et al. 2019). The presence of a greater number of receptors in the microglia and neurons could magnify the inflammatory response and produce a more intense activation of the meningeal pain receptors. The endothelial cells of the brain and meningeal vessels can be activated by exogenous and endogenous TLR ligands and initiate the production of inflammatory mediators, so if in a subject these cells

express more TLRs, the inflammatory response to CSD may be greater than expected. The expression of TLRs would also offer a new physiopathological mechanism to explain the epidemiological correlation between migraine and ischemic stroke.

7.2.3.3. Hypothesis 2: TLRs overexpression is related to repeated CSD episodes.

There are no studies on the relationship between TLRs and CSD in humans. A recent work that used 11C-PBR28, a radioligand marker of glial activity, in patients with MwA, showed accumulation of the radioligand in the areas involved in the aura as well as in the areas related to pain processing (Albrecht DS et al. 2019). This study demonstrated that, indeed, activation of glial cells occurs during aura in migraine. Whether or not TLRs are involved in this glial activation is unknown.

Numerous studies in cerebral vascular pathology have helped to understand the relationship between TLRs and the phenomenon of CSD. The spread of CSD in models of cerebral ischemia produces local neurogenic inflammation and activation of macrophages and mast cells that release different inflammatory mediators (Gehrmann J et al. 1993). These mediators, mostly cytokines, and the substances released by astrocytes (cytokines, prostanoids, and nitric oxide derivatives) alter the sensitivity of intracranial meningeal nociceptors and generate pain. In addition, CSD alters the activation of different inflammatory pathways: it increases the expression of TNF-α and IL-1 and reduces the expression of IL-2, IL-10, IL-12, and C4 complement. It also produces activation of caspase-1 and release of HMGB1, as well as IL-1β from neurons and NF-kB from astrocytes. HMGB1 is one of the main endogenous ligands of TLRs and TNF-α and IL-1 are the main products of the TLR4 activation pathway. At the time of publication of this work, there are no studies on the expression of HMGB1 in subjects with migraine.

The expression of TLRs is modulated by many of these inflammatory mediators. The expression of TLR2 in monocytes increases with IL-1, IL-10 and decreases with TNF, IL-4, and IFN-g; as for TLR4, its expression in the microglia increases after stimulation

with IL-1β (Tajalli-Nezhad S et al. 2019). About our findings, the release of endogenous ligands as a result of repeated CSD over time may lead to an overexpression of TLRs. Several studies in brain ischemia models have shown that TLR4 is overexpressed in microglia and astrocytes after CNS inflammation (Caso JR et al. 2007) and CSD may produce a similar effect (Gehrmann J et al. 1993). In turn, activation of TLRs may modify susceptibility to CSD: increased production of cytokines and activation of microglia with the release of different mediators, such as BDNF, may affect brain bioelectrical activity and contribute to the transformation of EM into CM (Kraig RP et al.2010).

7.2.3.4. Hypothesis 3: Overexpression of TLRs is a result of sterile inflammation.

Increased expression of TLRs at the peripheral level may be a consequence of a pro-inflammatory environment. According to this hypothesis, the constant presence of pro-inflammatory factors in subjects with CM or the repetition of migraine attacks during which DAMPs are released, such as cFN, HMGB1, or HSPs, favor the activation of TLRs, setting a positive feedback loop.

Migraine entails sterile inflammation, with activation of mast cells and T cells, leading to increased expression of cytokine coding genes, TLR2, and TLR4 (Conti P et al. 2019). According to these findings, the increased expression of TLRs would be a consequence of repeated or prolonged inflammation and not a causal phenomenon. This activation, in turn, increases the production of inflammatory cytokines and contributes to the initiation of the adaptive immune response and the prolongation of the inflammatory state. The increased expression of TLRs would be part of the pro-inflammatory environment that facilitates the appearance of new attacks, contributing to the vicious circle of chronification.

The contribution of TLRs to the maintenance of deleterious mechanisms of inflammation has not been studied in migraine, but it has been studied in closely related pathologies, such as stroke and chronic pain. Studies in animals have shown that TLR2 and TLR4 participate in the damage produced by ischemia and reperfusion.

Expression of TLR2 and TLR4 in brain tissue increases at the onset of reperfusion, is sustained over time, and results in increased cytokine production (Wang Y et al. 2013). In humans, it has also been shown that there is a parallel increase in the expression of TLRs in peripheral blood neutrophils immediately after stroke (Brea D et al. 2011) and this increase is associated with higher levels of cytokines (Yang QW et al. 2008). Manipulation of TLRs seems to affect the evolution after ischemic injury: pre-conditioning with TLR4 and TLR2 agonists is neuroprotective in animal models, as is treatment with TLR4 antagonists such as TAK-242, Eritoran, or naloxone. KO animals for TLR2 and TLR4 develop smaller ischemic lesions (Anttila JE et al. 2017) and blocking of one of the main TLR agonists, HMGB1, is being investigated as a treatment for stroke (Tian X et al. 2017).

As for chronic pain, immunity is known to be relevant in its pathogenesis. The perpetuation of pain requires the activation of the cells of the immune system in the CNS and continuous communication with nociceptors. Several works have studied the peripheral expression of TLRs in chronic pain. Kwok et al. analyzed both their expression and their activation in peripheral mononuclear cells in patients with chronic neuropathic pain and their results show an increased response to TLR2, TLR4, and TLR7 ligands (Kwok YH et al. 2012). Macrophages produce inflammatory mediators (chemokines and cytokines) that favor the release of glutamate, reactive species of oxygen or nitric oxide by the glia, molecules that activate nociceptors, inducing the onset and maintenance of pain(Watkins LR et al. 2001). All these substances have a protective role in conditions of infection, as they help to destroy pathogens, but in the case of sterile neuroinflammation, they are deleterious and perpetuate pain. In turn, substances released by nociceptors in response to this stimulation, such as various neuropeptides and chemokines, stimulate macrophages via TLR-dependent pathways, so that inflammation and pain are enhanced and perpetuated (Chen O et al. 2019). In this sense, the concept of "TLR Radical Cycle Pathway" is of great interest. According to this theory, the release of PAMPs and DAMPs in response to neuroinflammatory and oxidative mechanisms can produce an activation of the TLR complex with self-

amplification of the response and activation of a positive feedback loop (Lucas K et al. 2015) that contributes to the chronification of pain. Certain inflammatory cytokines dependent on the activation of TLRs, such as IFN-α, IL-1β, and IL-6 do not appear in acute pain conditions, but their expression does increase in those cases where pain becomes chronic, suggesting a role for TLRs in the appearance of feedback loops and perpetuation of inflammation. CM can be framed as a type of pathological pain involving these mechanisms and in which neurogenic inflammation of the meninges is key to the stimulation and sensitization of trigeminal fibers (Fernández de las Penas C et al. 2007).

Finally, the participation of TLRs in migraine allows us to hypothesize that non-identified molecules, either TLR ligands or regulators of their signaling pathways, are responsible for starting the inflammatory cascade that leads to pain. The expression of TLRs is not static but is rapidly modulated by contact with ligands, cytokine levels, and environmental stressors, in the same way that migraine is characterized by its cyclical course and dependence on environmental factors and triggers (Vidya MK et al. 2018). In this sense, the interaction between steroid hormones and TLRs is particularly suggestive. Estrogens are known to affect the expression of TLR2 and TLR4. An acute increase in estrogen load reduces the expression of TLRs, while chronic estrogen exposure increases resistance to infection by promoting the expression of TLR4 and CD14, as well as TLR2 (Vidya MK et al. 2018). Progesterone and its metabolites reduce the expression of TLR2/TLR4 and regulate the activation of TLR4 and the NF-κB pathway in subarachnoid hemorrhage, brain trauma, and brain ischemia (Tajalli-Nezhad S et al. 2019). Androgens, on the other hand, reduce immunity to bacterial endotoxin (Vidya MK et al. 2018). These interactions between sex hormones and TLRs offer an attractive hypothesis about the possible role of TLR in a pathology such as migraine, in which the influence of the menstrual cycle and sex hormones is evident but has not yet been fully explained.

7.2.3.5. Pharmacological evidence on the role of TLRs in pain

Several pharmacological studies relate TLRs to the mechanisms of action of some of the preventive and symptomatic treatments commonly used in migraine.

One of the main pieces of evidence on the role of TLR4 in pain and its chronification derives from studies with morphine and naltrexone. These studies show that opioid agonists activate the TLR4 pathway and induce the production of inflammatory mediators, which produce a paradoxical increase in pain. In contrast, naltrexone, an opioid antagonist, blocks this same pathway and reduces inflammation (Hutchinson MR et al. 2008). As a result of these findings, naltrexone has been studied as a possible treatment for fibromyalgia, one of the main comorbidities of CM (Younger J et al. 2009). These results could explain the well-known clinical relationship between the consumption of opioid analgesics and the chronification of migraine, in addition to their limited effect in the treatment of pain (Shah M et al. 2017).

Some of the commonly used preventive treatments for migraine influence TLR signaling pathways. Onabotulinum toxin A (OnabotA), the only preventive treatment approved exclusively for CM, exerts part of its effect through microglia: OnabotA acts on the microglia through the SNAP23 protein and reduces the phosphorylation of NF-κB, p38, and ERK1/2. This means it has a direct effect on the TLR2 and TLR4 signaling pathways, inhibiting the production of nociceptive factors (Rojewska E et al. 2018). Tricyclic antidepressants have also an antagonistic effect on TLR4 (Li J et al. 2016), specifically amitriptyline, imipramine, myanserin, cyclobenzaprine, ketotifen and desipramine. Other preventive drugs such as duloxetine (Zhou DM et al. 2018) or symptomatic drugs such as diclofenac (Barcelos RP et al. 2017) interact with the CNS TLR pathway.

At the experimental level, drugs such as Rifampin (Wang X et al. 2013), which bind to MD-2, the main TLR4 co-receptor and inhibit the activation of the signaling pathway, have already been tested in animal models of migraine and have been proved to reduce allodynia.

AV411 (ibudilast) is a TLR4 antagonist that inhibits the production of cytokines in glial cells and increases the production of IL-10 and neurotrophic factors. It has shown great efficacy in models of neuropathic pain and opioid withdrawal, although it is in the clinical research phase (Thakur KK et al. 2017). A double-blind clinical trial has recently been published in patients with CM who were administered Ibudilast, without observing changes in the frequency or intensity of the headache (Kwok YH et al. 2016). Other natural compounds such as curcumin, resveratrol, and fisetin appear to be able to have an effect on neuropathic pain through TLRs (Thakur KK et al. 2017). The possible role of other TLRs, other than TLR4, in chronic pain has been poorly studied. Modulating molecules of TLR5 have been tested in animals for the treatment of allodynia and neuropathic pain, for their role in Aδ and C fibers, and as mediators of the entry of molecules with an analgesic effect (QX-314) (Peirs C et al. 2015).

We are far from understanding by what specific physiopathological mechanisms TLRs interfere with the appearance of migraine. The large number of neurological processes that involve TLRs says much about their therapeutic potential, but also about the caution with which treatments targeting these receptors should be considered.

7.2.4. Expression of TLR ligands

In our study, we determined the levels of two specific ligands of TLR4 and TLR2: HSP60 and cFN. Subjects with CM present significantly higher levels of cFN, while no significant differences were found regarding the levels of HSP60.

HSP60 is a chaperone of the heat shock protein family. It is released into the extracellular space in situations of cellular stress and acts as a signal to macrophages and dendritic cells via activation of TLR2 and TLR4 (de Graaf R et al. 2006). Its effects on the immune system appear to depend on its concentration (Zininga T et al. 2018). There are no previous studies regarding the levels of HSP60 in patients with migraine, but the role of this molecule in other neuroinflammatory processes is known: it contributes to

neurodegeneration and chronification by activating the TLR4 pathway (Lehnardt S et al. 2008). In our sample, the subjects with CM present higher levels of HSP60, although differences do not reach statistical significance. This may be due to the fact that the results obtained are very disparate, in part because of the great variability in the expression of HSP60 and its susceptibility to other mediators, such as exposure to bacterial LPS or hormonal variations (Heiserman JP et al. 2015). Previous studies in cerebral ischemic pathology did not find significant differences in HSP60 levels in blood either, while the interaction between HSP60 and TLRs was proved in vitro (Brea D et al. 2011).

Fibronectin is a multi-domain glycoprotein found in the extracellular matrix (ECM), in different body fluids and on the surface of cells. It can be found in two forms; soluble, which is an inactive form secreted by hepatocytes, or insoluble, also called cellular (cFN), which is produced locally. The cellular form of fibronectin (cFN) is mainly synthesized by fibroblasts and endothelial cells (Speziale P et al. 2019). In addition to cell differentiation, growth, and migration, fibronectin is involved in the mechanisms of inflammation, specifically in chemotaxis and leukocyte function. The EDA domain of cFN promotes chronic inflammation and is produced mainly by smooth muscle in blood vessels. Fibronectin is an endogenous ligand of TLR4 and TLR2 (Lemanska-Perek A et al. 2019). There are few studies on the expression of fibronectin in migraine. In two of them, patients with CM showed higher levels of cFN and a correlation between them and the presence of iron deposits in the periaqueductal grey substance (Dominguez C et al. 2019). Our group found elevated cFN levels in ischemic stroke and a correlation with the expression of TLR2 and TLR4 in peripheral blood monocytes. In the parallel *in vitro* study, it was further demonstrated that cFN binds to TLRs (Brea D et al. 2011). Levels of cFN are also high in other migraine comorbid pathologies, such as fibromyalgia and obesity (Pay S et al. 2000) as well as in disorders that involve a disruption of the integrity of the BBB, such as intracranial hemorrhages. In acute inflammatory pathologies, like sepsis, fibronectin levels decrease. Plasma fibronectin extravasated from plasma stimulates the microglia,

accentuating inflammation. In models of brain damage, fibronectin is an early marker of BBB disruption and is also produced later by activated macrophages and astrocytes (Howe MD et al. 2018).

In our study, the levels of HSP60 and cFN are correlated with the expression of TLR2 and TLR4 in mononuclear cells in peripheral blood. Adjusted regression models show a possible association between cFN levels and TLR expression. These results suggest that this molecule, previously studied as a possible biomarker of migraine and its chronification, could promote a state of chronic neuroinflammation by activating TLR-dependent pathways. The findings regarding HSP60 are less striking, in line with the absence of previous studies on the role of this molecule in the physiopathology of migraine.

7.2.5. Interleukin levels.

In our work, subjects with CM present significantly higher levels of IL-6 and lower levels of IL-10. We observed no differences in hs-CRP levels.

IL-6, in addition to its acute inflammatory functions, participates in the physiopathology of chronic pain, allodynia, and hyperalgesia. Whether IL-6 levels are increased in migraine is still controversial, as some studies found no differences between subjects with migraine and healthy controls, while others found higher levels in migraineurs, as in our sample (Perini F et al. 2005). IL-6 effects over pain have been strongly remarked by the finding that central or peripheral administration of IL-6 or TNF-α produces hyperalgesia (Koçer A et al. 2009). In our sample, IL-6 levels are correlated with TLR2 and TLR4 expression in neutrophils, although the association with TLRs peripheral expression does not persist after adjustment for other molecules related to neurogenic inflammation (CGRP and sTWEAK). Activation of TLR4 through the MyD88 pathway induces the expression of several pro-inflammatory cytokines and IL-6 is among them (Moresco EM et al. 2001), therefore, if there is a persistent inflammatory state mediated by TLRs in CM, we would expect that the activation of its signaling pathways condition higher levels of IL-6. Our results, however, do not allow us to establish a direct

relationship between the expression of TLRs and IL6, maybe because this relation is mediated by other molecules such as CGRP (Cuesta MC et al. 2002).

In our sample of CM subjects, IL-10 levels are lower than in controls, and we found a correlation between the expression of TLRs in monocytes and IL-10 levels. IL-10 is a cytokine inhibitor factor or an anti-inflammatory cytokine that inhibits immune mediator release, antigen expression, and phagocytosis (Kany S et al. 2019). IL-10 is produced by microglia after activation by TLRs and has been studied as a possible therapy in pathologies where persistent neuroinflammation leads to neurodegeneration, as well as in pain (Vanderwall AG et al. 2019). Previous findings of IL-10 levels in subjects with migraine are disparate; while some studies have found reduced levels of IL-10 in patients with migraine during interictal periods (Domínguez C et al. 2018), others have found them increased (Boćkowski L et al. 2010). The profile of its levels has even been studied throughout the migraine cycle so that it is known to rise just before and during attacks, and to be lower than in controls during inter-critical periods (Perini F et al. 2005). There are no studies on the relationship between the expression of TLRs and IL-10 levels specifically in migraine, but their interactions in other neurological pathologies have been studied. Studies carried out in MS show that IL-10 production depends mainly on TLR3 activation and IFN-β mediation, but also on TLR-2 and TLR-4 and IFN-ϒ activation (Lobo-Silva D et al. 2017). Regulation of TLR-2 and TLR-4 by melanocyte-stimulating hormone favors the development of an immunoregulatory phenotype of monocytes and macrophages with increased expression of IL-10 (Carniglia L et al. 2016). These studies undoubtedly show that the regulation of IL-10 production is very complex and many of the known factors influencing it have not been analyzed in our sample.

Concerning hs-CRP values, we found no differences between subjects with CM and controls, nor any relationship between hs-CRP values and the expression of TLRs. Hs-CRP is a polypeptide molecule of the pentraxin family, from the subgroup of short-chain pentraxins. Hs-CRP is mainly synthesized in the liver in response to certain

inflammatory cytokines and plays an essential role in innate immunity, complement activation and immunoglobulin binding to its receptors (Moutachakkir M et al. 2017). Hs-CRP levels are elevated during infections but also during chronic and acute inflammatory processes, such as rheumatoid arthritis or cardiovascular pathologies. Hs-CRP production is induced by IL-6, in a complex process that involves many other molecular mediators. Hs-CRP levels may also be altered by factors such as age, weight, lipid levels, or blood pressure (Sproston NR et al. 2018), which may have influenced our results. Hs-CRP interacts with monocytes and macrophages by inducing cytokine release and phagocytosis, as well as with endothelial cells, where it favors the mechanisms of cell adhesion (Salazar J et al. 2014). In fact, it has been shown that cells of the monocyte-macrophage system synthesize and release hs-CRP after being stimulated through TLR4, either by DAMPs or PAMPs (Haider DG et al. 2006). CRP values in migraine have been studied extensively, but with contradictory results; some studies report high values and others have found no differences between subjects with migraine and healthy controls (Lippi G et al 2014). This variability in the results is probably due to its non-specific nature and to the presence of two CRP isoforms, with differentiated effects over the mechanisms of inflammation. The relationship between CRP levels and the expression of TLRs in migraine has not been studied to date and our exploratory results point to an absence of association between them.

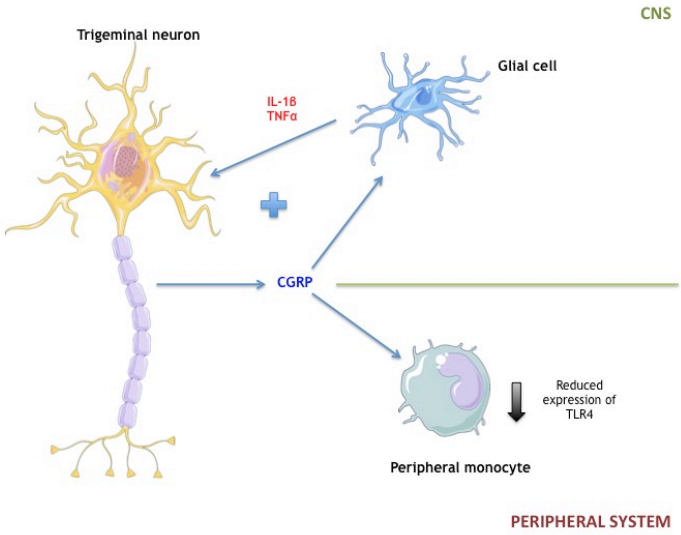
7.2.6 Biomarkers of TVS activation and endothelial dysfunction

Subjects with CM in our sample have higher plasma levels of CGRP and sTWEAK, in line with previous studies (Santos-Lasaosa S et al. 2019; Leyra Y et al. 2020). PTX3 levels are higher in subjects with CM, but these results are not statistically significant. We found a relationship between elevated levels of sTWEAK and CGRP and the expression of TLRs in mononuclear cells.

CGRP is the main biomarker in migraine. There is plenty of evidence regarding its importance in the physiopathology of migraine: its levels are increased during attacks and interictal periods, its

exogenous administration causes headache of similar characteristics to migraine in predisposed patients and its blockade by monoclonal antibodies reduces headache (Santos-Lasaosa S et al. 2019). Trigeminal neurons produce CGRP that stimulates adjacent glial cells resulting in the release of IL-1β and TNF-α, end products of the TLR signaling pathway (Iyengar S et al. 2019). These inflammatory molecules, in turn, act on trigeminal neurons by increasing the production and release of CGRP (Bowen EJ et al. 2006), in a positive feedback loop. Besides, CGRP increases the production of cytokines not only by glial cells but also by the neurons themselves, maintaining a hypersensitive state and increasing pain (Thalakoti S et al. 2007; Durham PL. 2016). Studies in sepsis models have shown that CGRP inhibits cells from innate immunity and adaptive immunity at the systemic level and reduces the expression of TLR4 at the transcriptional level (Fox FE et al. 1997). This regulation is mediated by the production of ICER (inducible CAMP early repressor), which reduces the transcription of TLR4 and its expression in the cell membrane and inhibits the translocation of NF-κB to the nucleus (Li W et al. 2006).

Figure 7.1. Interaction between CGRP, microglia, and peripheral monocytes



Activation of glial cells increases the production of inflammatory cytokines that stimulate the release of CGRP by neurons. This, in turn, increases the release of inflammatory molecules by microglia and reduces expression of TLR4 at the peripheral level.

Our results seem to contradict previous findings, by suggesting a positive correlation between CGRP levels and the expression of both TLR4 and TLR2 in peripheral blood. The inhibitory effect of CGRP in TLR expression may need higher levels of the molecule, like those found in sepsis, while the CGRP levels reached in migraine are not sufficient to produce this downregulation. We do not have enough data to venture the meaning of this association, but we can affirm that our findings support the existence of an interaction between CGRP and innate immunity, highlighting the potential role of TLRs in neuroinflammation and migraine.

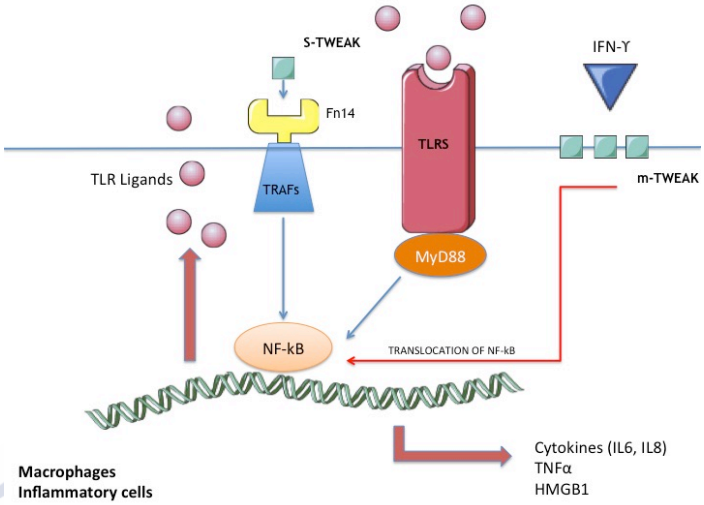
TWEAK is a member of the TNF superfamily of cytokines. It is synthesized as transmembrane protein type II (mTWEAK) from

which a soluble fragment with biological activity (sTWEAK) is released (Yepes M et al. 2013). Plasma levels of sTWEAK have been studied as biomarkers of cardiovascular pathology and endothelial dysfunction (Blanco-Colio LM et al. 2007). TWEAK, both in its soluble form (sTWEAK) and in its membrane-anchored form (mTWEAK) binds to Fn14, a membrane receptor that when activated interacts with the TLR-dependent NF-κβ pathway (Burkly LC et al. 2007) and induces different types of response depending on the cell type. sTWEAK is expressed in many tissues, including the CNS, where it is released or expressed on the membrane of monocytes, macrophages, astrocytes, and microglia and can stimulate endothelial cells, astrocytes and neurons (Yepes M. 2007). Although sTWEAK exerts its main effect on endothelial cells, it also induces the expression of cytokines such as IL-6, IL-8, and ICAM-1 by inflammatory cells (Saas P et al. 2000). This increase in inflammatory cytokine expression is produced by direct interaction of sTWEAK and innate immunity cells or indirectly by increasing expression of TLR ligands (Novoyatleva T et al. 2014). For example, sTWEAK cooperates with the TLR2 ligand Pam3CysSK4 in the stimulation of IL-8 synthesis by endothelial cells (Hans ES et al. 2010) and can stimulate the secretion of HMGB1 (Moreno JA et al. 2013). sTWEAK also acts as a ligand for TLRs and an inducer of necroptosis during brain development (Thornton C et al. 2015). In addition to these pro-inflammatory effects, TWEAK also has an anti-inflammatory function: it regulates and inhibits the transition from innate to adaptive immunity and it induces the association of the nuclear factor p65 κβ with histone deacetylase 1, suppressing cytokine production (Maecker H et al. 2005). TWEAK (the insoluble form of the molecule) is expressed in circulating monocytes and its production is increased after stimulation with IFN-γ (Nakayama M et al. 2000), its activation promotes the nuclear translocation of NF-kB. TWEAK, therefore, acts by directly modulating innate immunity, increasing the secretion of IL-10, and reducing the secretion of pro-inflammatory cytokines (IFN-ϒ and IL-12).

The levels of sTWEAK are elevated in several neurological pathologies such as stroke, MS, or TBI (Tang B et al. 2019), and also

in migraine (Domínguez-Vivero C et al. 2020). There are no specific studies about the relationship between TLRs and sTWEAK in neurological pathology, nor in migraine, but what we know about their interaction in other diseases suggests that the simultaneous elevation of TLRs and sTWEAK in subjects with CM could reflect a maintained pro-inflammatory state. Although the role of TWEAK in the regulation of inflammatory processes is complex and depends on the expression of many other mediators and regulatory proteins, our findings suggest that its effect on inflammation in migraine may depend, at least partially, on TLRs.

Figure 7.2. Interaction between TWEAK and TLRs



sTWEAK induces the expression of cytokines such as IL-6, IL-8, and ICAM-1 by inflammatory cells. This increase in inflammatory cytokine expression is produced by direct interaction of sTWEAK and innate immunity cells via FN14 or indirectly by increasing expression of TLR ligands. mTWEAK (the insoluble form of the molecule) is expressed in circulating monocytes and its production is increased after stimulation with IFN-γ, its activation promotes the nuclear translocation of NF-kB, inhibiting the production of cytokines.

PTX3 is a molecule related to endothelial dysfunction and inflammation. Its levels rise or fall in parallel with those of sTWEAK in various infectious and inflammatory diseases (Fan WC et al. 2017). Pentraxins are a family of pattern recognition molecules (PRMs), essential components of humoral immunity that promote the activation of complement and the processes of opsonization and agglutination. PTX3 is the prototype of long pentraxin (as opposed to short pentraxins, such as hs-CRP). It is secreted by immune cells and somatic cells in response to pro-inflammatory stimuli following activation of TLRs (Mantovani et al. 2013). PTX3 is considered an optimal marker of local inflammation since unlike other PTXs produced in the liver, such as CRP, it is secreted at the precise site of inflammation by endothelial cells and macrophages after stimulation of these cells by inflammatory cytokines (IL-1β, TNF-α), TLR agonists and microbial components. In the CNS, PTX3 production occurs in several cell types including microglia, dendritic cells, fibroblasts, and endothelial cells and is mediated by activation of TLRs (Ummenthum K et al. 2016). It is believed that PTX3 may regulate inflammatory pathways in the CNS, although this function is not fully known. The production of PTX3 by glial cells depends mainly on the activation of TLR3 in infectious pathologies and on the stimulation of TLR2 by HspB5 in inflammatory contexts in which it also exerts anti-inflammatory effects. The production of PTX3 by endothelial cells, however, does not seem to depend on the activation of TLRs (Ummenthum K et al. 2016). In other pathologies the biological effects of PTX3 are different; for example, in melanoma, it modifies the cell migration and this effect depends on its interaction with TLR4 (Rathore M et al. 2019). PTX3 also exerts effects on the TLRs themselves and may act by inhibiting the function of TLR4s (Bozza S et al. 2014). PTX3 levels are elevated in various neurological diseases such as stroke, MS and optic neuritis (Ummenthum K et al. 2016); in the case of migraine, the role of PTX3 as a potential biomarker has recently begun to be studied based on its relationship with both the mechanisms of neurogenic inflammation and endothelial dysfunction. While in the works published so far

PTX3 levels are higher in subjects with CM or during migraine attacks (Domínguez-Vivero C et al. 2020; Ceylan M et al. 2016), in our sample we have not found increases in PTX3 among subjects with CM. The reasons for the discrepancies between our findings in this sample and the findings in previous studies are unknown, although perhaps it is due to the great variability of the levels in our sample, which has not allowed to reach statistical significance. These contradictory results require further research on the role of PTX3 in migraine. There are no studies to date on the relationship between PTX3 levels and expression of TLRs in neurological pathology and we did not find a correlation between expression of TLRs and levels of PTX3.

7.2.7. Limitations

Our clinical study has several limitations. Some of the most frequent clinical features in the group of subjects with CM, such as HBP, fibromyalgia or anxiety-depressive syndrome may influence levels of inflammatory markers, the immune system state, and TLRs performance (García Bueno B et al. 2016). Their effect was controlled by adjusting the analysis, however ideally these potential cofactors should be absent or at least equally distributed among groups. Similarly, the presence of other types of headache and preventive or symptomatic treatments for migraine could have influenced our results. Given that the role of TLRs in migraine has hardly been explored previously, the comparison between healthy controls and CM subjects seemed to be the most appropriate choice to assess potential differences; however, it would have been interesting to include a group of subjects with EM. This would have allowed us to determine if there is a continuum in inflammatory mechanisms in migraine or if changes in innate immunity only appear after the chronification process. Exclusion criteria prevented all those subjects with infectious/inflammatory pathologies or with known autoimmune conditions to participate in the study, but TLRs have indeed been implicated in a large number of pathologies, such as cardiovascular disease (Semlali A et al. 2019), which we have not registered or assessed, and therefore may have influenced our findings. A major

limitation of this work is that levels of sex hormones were not taken into account. It has been shown that estrogens and progesterone are relevant to TLR expression and function in previous studies (Calippe B et al. 2010). Our sample included pre-, peri- and post-menopausal women, some were using hormonal contraceptives and blood samples were collected at different moments of the menstrual cycle. These factors may have had an influence in the expression of TLRs or in the activation of their signaling pathways and, therefore, in the expression of inflammatory cytokines (Lewis SS et al. 2015). Opioid use may also have influenced the expression of TLRs or their activity; patients with frequent opioid use should perhaps have been excluded, although this would have left out a very common profile among CM (Lewis SS et al. 2012).

Regarding the techniques used for the determination of TLRs in peripheral blood, we opted for flow cytometry and antibody marking of TLRs. Flow cytometry requires immediate processing of the samples, but offers a more reliable and dynamic picture of the expression of TLRs at the time of the study, allows the identification of cellular subpopulations, and allows the distinction between cytoplasmic and surface expression of TLRs. On the other hand, the results of this technique are more variable and also depend on the fluorophore used. The alternative method (gene transcription of TLRs and their associated molecules, MyD88 and CD14), would have allowed a more complete characterization of the signaling pathway. However, gene transcription analysis is more appropriate when looking for changes in gene regulation, and not in the expression of a particular molecule or receptor.

Levels of ligands and biomarkers were analyzed using ELISA. The levels of cFN had been previously studied by our group, finding an elevation of cFN in CM, but those of HSP60 had not been previously determined. The values of HSP60 found in our sample are very variable and this may be due to the fact that the techniques of determination of heat shock proteins by ELISA still offer some problems. It is controversial if HSP can be measured using the standard detergents included in most immunoassay kits and the results are very different depending on the fluid in which these levels are

determined. Differences between serum and plasma may alter the determinations in different biological samples (Pockley AG et al. 2018). On the other hand, the determination of HMGB1 would have been of great interest. HMGB1 levels would have given us more specific information regarding TLRs signaling pathways in migraine, given that HMGB1 is one of the TLR ligands that has been shown to be elevated in the condition and which is released after activation of trigeminal neurons (Ramachandran R et al. 2019).

The determination of inflammatory cytokines is common in clinical practice and therefore the analytical technique has been contrasted in various pathologies. The determination of IL6 and IL10 levels gives us an overview of the production of pro- and anti-inflammatory cytokines and their relationship with the expression of TLRs. It would also have been informative to determine the levels of other cytokines involved in TLR signaling pathways such as IL1, IFN-α, and IFN-β. Some of these mediators have also been directly involved in the physiopathology of migraine and are related to the expression of CGRP (Neeb L et al. 2016).

There is also an important variability when comparing the levels of biomarkers found in this study and in previous ones. Regarding CGRP, this variability may stem from the laboratory methodology: CGRP has a short half-life, of minutes, so immediately after the sample is taken it must be cooled in a tube with EDTA and protease and peptidase inhibitors. Samples should be kept at -80° until final processing. Another limitation is that the concentration of CGRP is relatively small and is very variable between individuals, especially if the samples are extracted at a distance (radial vein) where they are more diluted. It is not known at what point in the migraine attack the concentrations increase in the peripheral circulation and how long these increases last (Akerman S et al. 2013). These limitations explain the variability in CGRP levels between studies and the differences in results found by our group in previous work. The same occurs with sTWEAK and PTX3, in which the results found in this study differ markedly from previous ones.

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8. CONCLUSIONS

The results of this work contribute to the existing knowledge about inflammation and innate immunity in the physiopathology of migraine.

Our findings allow us to conclude that:

- Subjects with CM show increased expression of TLR2 and TLR4 in neutrophils and monocytes in peripheral blood.
- Subjects with CM present higher levels of ligands of TLRs (cFN), inflammation biomarkers (IL6 and sTWEAK) and trigeminal vascular activation markers (CGRP). Healthy controls show higher levels of IL10, an anti-inflammatory interleukin.
- Expression of TLR2 and TLR4 in peripheral blood mononuclear cells is correlated with levels of TLRs ligands, inflammation and trigeminal-vascular activation biomarkers.
- Expression of TLR2 in peripheral blood monocytes and neutrophils and expression of TLR4 in peripheral blood monocytes is independently associated with CM status (after adjusting for clinical variables and levels of biomarkers of inflammation and trigemino-vascular activation).
- Levels of expression of TLR2 in neutrophils and monocytes and levels of expression of TLR4 in monocytes may have a predictive role in CM diagnosis.
- We can confirm the role of TLRs on CSD based in an animal model of migraine.
- Either absence of TLRs or their pharmacological blockade can modulate vascular response to CSD stimulation, with no significant changes in inflammatory response.

These findings suggest a key role of TLRs and innate immunity in migraine pathophysiology, as factors enabling both initiation and chronification of the condition. The possibility of pharmacological

blockade of TLRs opens new potential therapeutic pathways for migraine.

9. FUTURE RESEARCH

Our work shows for the first time an association between the expression of TLRs in peripheral blood mononuclear cells and CM. Further studies are needed to elucidate the nature of this association; whether overexpression of TLRs is a contributing factor to the origin or maintenance of pain or whether it is a consequence of a persistent state of inflammation has not been addressed by this work. Study designs including larger samples and measuring the expression of TLRs in healthy controls, EM subjects, and CM subjects are required to define this association more accurately. Follow-up studies assessing the expression of TLRs in treatment-responsive and non-responsive patients may also contribute to elucidate the role of TLR expression in response to adequate therapy.

Some particularly interesting aspects related to the signaling pathways of TLRs have not been analyzed in our work, remarkably HMGB1 levels, the most studied endogenous ligand in other types of chronic pain (Allete YM et al. 2014). New models are needed to determine HMGB1 levels and their relationship with the expression of TLRs specifically in CM.

TLRs are potentially interesting therapeutic targets in chronic pain due to their central position in the signaling cascades of inflammation. Unlike other molecules such as TNFα, treatments directed against TLR4 would allow simultaneous regulation of many immune-activation pathways with greater effects on the inflammatory response and on pain (Mayerhofer R et al. 2017). Several compounds directed against TLR2, TLR3, TLR4 and TLR9 are currently undergoing clinical and preclinical research (Li J et al. 2016). They have been tested in other pathologies, such as sepsis, without adverse effects (Rice TW et al. 2010). Research with these compounds in animal models of migraine would be of great interest to define new therapeutic targets.

The results of our experimental model support the relationship between CSD and glial activation, although the nature of this relationship is far from being fully understood. Studies are needed to determine whether microglia has a role in susceptibility to initiation of CSD, whether microglial activation is an adaptive or harmful mechanism, and if its local activation in the cortex can influence sistemic immunity.

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11. APPENDIX

11.1. RESUMO EN GALEGO

Alteración da inmunidade innata na migraña crónica. Papel dos receptores Toll-like 2 e 4 como elementos chave na cronificacion da migraña e potenciais dianas terapéuticas.

INTRODUCIÓN

A migraña, unha cefalea primaria, é un dos trastornos neurolóxicos máis comúns. As súas repercusións a nivel individual e social son enormes; segundo a Enquisa Global Burden of Disease publicada en 2018 (Colaboradores de GBDH. 2016), uns 1.000 millóns de personas sofren de migraña cunha prevalencia xeral do 14.4% e do 18.9% en mulleres. A súa elevada prevalencia e o perfil demográfico das persoas que a padecen condicionan un maior impacto social, familiar e laboral, facendo da migraña a sétima causa de discapacidade medida en anos vividos con discapacidade. A migraña implica tamén un maior risco de desenvolver outras comorbilidades físicas e psiquiátricas. As consecuencias persoais e sociais son aínda maiores na migraña crónica (MC), na que as crises de dor aparecen 15 ou máis días ó mes. A MC adoita resultar dun lento aumento na frecuencia das dores de cabeza durante meses ou anos. Cada ano, arredor dun 2.5% dos pacientes diagnosticados de migraña episódica (ME) pasan a cumprir criterios diagnósticos de MC.

Malia o seu impacto na saúde global, os mecanismos patofisiolóxicos que desencadenan e perpetúan unha crise de migraña e conducen á cronificación aínda non se comprenden completamente. Acéptase en xeral que a migraña é un trastorno multifactorial que afecta á excitabilidade cerebral e á regulación sensorial. A susceptibilidade xenética individual, xunto con diversos factores epixenéticos, conduce á activación dunha serie de mecanismos, entre eles o fenómeno de despolarización cortical propagada (DCP), a inflamación estéril, a

estimulación do sistema trixémino-vascular (STV) e as interaccións reguladoras co tronco do encéfalo e os núcleos diencefálicos que participan na transmisión da dor.

A dor, o sintoma esencial da migraña, depende da activación do STV. O mecanismo polo cal se estimulan as fibras nociceptivas na migraña aínda está por determinar, pero implica a activación de determinadas áreas da corteza, a inflamación neuróxena e a liberación de neuropéptidos. A activación da corteza pode estar relacionada coa DCP, que non se ten rexistrado en humanos pero que se caracterizou perfectamente en modelos animais de migraña. A DCP activa ó STV facendo que as terminais nerviosas liberen neuropéptidos vasoactivos coma o péptido intestinal vasoactivo (VIP) e o péptido relacionado co xen da calcitonina (CGRP) nas terminais parasimpáticas perivasculares. Estes neuropéptidos producen vasodilatación dos vasos sanguíneos, aumento do fluxo sanguíneo, extravasación de plasma e degranulación das células plasmáticas da duramadre, unha inflamación estéril que estimula ós nociceptores menínxeos (Levy D et al.2010) nun ciclo de retroalimentación positiva.

A contribución da inflamación ás enfermedades neurolóxicas foi ignorada durante décadas, xa que durante a meirande parte do século XX o SNC considerouse un santuario inmunolóxico. Hoxe en día sabemos que a activación glial é esencial en moitas enfermidades do SNC, incluida a dor crónica (Buchanan MM et al.2010); nembargantes, as investigacións sobre o papel dos mecanismos inmunolóxicos no caso específico da migraña son aínda escasas. A resposta inmunolóxica, sexa innata ou adaptativa, depende da capacidade de diferenciar o propio do alleo. Este recoñecemento exércese mediante unha serie de receptores dirixidos contra moléculas moi conservadas, chamados receptores de recoñecemento de patróns (RRP). A familia de receptores Toll-like (TLR) representa o sistema máis importante de recoñecemento de patóxenos nos mamíferos. Describíronse dez TLRs en seres humanos (Liu Y et al. 2014); os tipos 1, 2, 4, 5, 6 e 10 localízanse na superficie da célula, mentres os tipos 3, 7, 8 e 9 atópanse no interior da célula e recoñecen ácidos nucleicos (Akira S. 2006). Ademáis dos patróns moleculares asociados a patóxenos (PAMPs), os TLR poden recoñecer unha

variedade de moléculas endóxenas, que normalmente están illadas do contacto coa resposta inmunolóxica e se denominan patróns moleculares asociados a perigo (DAMPs). A activación dos TLRs polos DAMPs xera unha resposta inflamatoria estéril que é chave nas enfermidades inflamatorias non infecciosas.

A resposta inmunolóxica innata no sistema nervioso consiste principalmente na activación da microglía seguida dun aumento na produción de citoquinas proinflamatorias e astrocitosis reactiva (Olson JK et al. 2004). A microglía expresa un amplo repertorio de TLRs (1-9), pero os TLR2, TLR3 e TLR4 son, con diferencia, os máis numerosos (Bsibsi M et al. 2002). Os niveis de expresión dos TLR na microglia in vivo parecen ser baixos en condicións fisiolóxicas, pero atopouse un aumento de súa expresión en modelos animais de diversas patoloxías neurolóxicas. Os ligandos endóxenos máis relevantes a nivel do SNC son as proteínas de choque térmico, o HMGB1 e a fibronectina. En situacións de estrés ou dano fíltranse ó espacio extracelular e activan a resposta inflamatoria despois de unirse a TLR2 e TLR4 (Yu L et al. 2010).

Os primeiros estudos sobre o papel dos TLR en patoloxía do SNC centráronse en enfermidades infecciosas. Non se tardou en descubrir que os TLR, en particular o TLR2 e o TLR4, participaban en patoloxías inflamatorias do SNC ó unirse a ligandos endóxenos. Numerosos estudos en modelos animais demostraron a contribución dos TLR á xénese e ó mantemento da dor neuropática. Os danos nos nervios conducen á liberación de ligandos endóxenos como a fibronectina, as proteínas de choque térmico, o ácido hialurónico, etc. Estes ligandos activan ós astrocitos, á microglia e a outras células a través dos TLRs, provocando un aumento da produción de mediadores inflamatorios que, ó tempo, interactúan cos nociceptores (Thakur KK e outros 2017). Os procesos centrais de sensibilización tamén implican a activación dos TLRs. Os DAMPs activan ós TLRs e provocan a liberación de mediadores inflamatorios pola glía, que interactúan con receptores situados nas neuronas aumentando a excitabilidade e a transmisión sináptica, ademáis de alterar a transmisión GABAérxica, que é inhibitoria. Tamén hai probas da expresión dos TLRs nas zonas da cortiza relacionadas coa dor, o que

suxire que poden ter un papel no procesamento dos sinais de dor a nivel cortical (Nicotra L e outros 2012).
Algúns aspectos da fisiopatoloxía da migraña, como a inflamación estéril, a activación dos nociceptores, a DCP, a sensibilización periférica e a sensibilización central, están estreitamente vencellados coa resposta inmunolóxica innata e a activación dos TLRs. Non obstante, aínda son limitadas as investigacións sobre o papel destes receptores na migraña. Como receptores básicos da inmunidade innata, os TLRs poden ter unha grande importancia no inicio e o mantemento dos mecanismos inflamatorios da migraña; ademáis, o feito de que se expresen en neuronas e células endoteliais abre novas vías potenciais de participación na fisiopatoloxía do trastorno, independentes da inflamación.

XUSTIFICACIÓN E OBXETIVOS

Descoñecemos o papel exacto dos TLR na migraña, e as investigacións sobre este tema son, ata o de agora, anecdóticas. Este traballo pretende contribuir á comprensión do papel dos TLR na fisiopatoloxía e os mecanismos de cronificación da migraña. Os obxectivos básicos deste estudo son:

- Demostrar a participación dos receptores TLR4 no fenómeno de despolarización cortical propagada a través dun modelo animal de migraña.
- Comparar a expresión de TLR2 e TLR4 en células mononucleares de sangue periférico de pacientes con MC e controis sans e relacionala cos niveis de ligandos, citoquinas e biomarcadores de activación trixéminovascular e disfunción endotelial.

MATERIAIS E MÉTODOS

Este traballo inclúe un modelo experimental e un estudo clínico.

Modelo experimental

O modelo animal de migraña seleccionado baséase na provocación de DCP en ratos anestesiados usando estimulación continua con KCl. A resposta evalúase a través dos cambios no fluxo sanguíneo cerebral (FSC) utilizando *laser flowmetry*. Usáronse 32

ratos macho C57BL/6 tipo salvaxe (WT) e 14 ratos B6.B10ScN-Tlr4 knock-out (KO). O deseño divídese en dúas fases:

- Fase 1: Comparación da morfoloxía dos cambios en FSC despois da provocación da CSD nun grupo de ratos WT e un grupo de TLR4-KO.
- Fase 2: Comparación da morfoloxía dos cambios de FSC despois da provocación de DCP en ratos WT con e sen bloqueo farmacolóxico de TLR4 usando TAK-242.
- Como estudios complementarios ó rexistro do fluxómetro láser, determinamos os niveis plasmáticos de IL-6 no plasma dos diferentes grupos experimentais antes e despois do procedemento, así como a permeabilidade da barreira hematoencefálica (BHE) por tinción tisular con Evans-Blue *in vivo*.

Estudio clínico

Diseñouse un estudo transversal incluíndo suxeitos diagnosticados de MC e controis sans, dacordo coas directrices do STROBE (Strengthening the Reporting of Observational Studies in Epidemiology).

- Determináronse os niveis de expresión de TLR2 e TLR4 en monocitos e macrófagos en ambos grupos mediante citometría de fluxo.
- Determináronse en ámbolos dous grupos mediante ELISA os niveis de ligandos endóxenos de TLR, como a fibronectina celular (cFn) e a proteína de choque térmico 60 (HSP60); interleuquinas (IL6, IL10), proteína C reactiva de alta sensibilidade (hs-CRP), marcadores de activación do STV, incluido o CGRP e o *soluble tumor necrosis factor (TNF)-like weak inducer of apoptosis* (sTWEAK) e niveis de marcadores de disfunción endotelial, como a pentraxina 3 (PTX3).

RESULTADOS

Modelo experimental

O noso modelo experimental mostra que os animais KO para TLR4 presentan cambios no patrón de DCP rexistrado por fluxometría láser tras estimulación con KCl, consistentes nunha prolongación da onda de despolarización. Estes cambios tamén se observan nos ratos WT si se administra un antagonista de TLR4 (TAK-242) antes de inducir a DCP. Non se atoparon diferencias entre os grupos experimentais na expresión de IL-6 nin na permeabilidade da BHE.

Estudo clínico

Recrutamos 120 suxeitos con MC e 82 controis sans. A expresión de TLR4 e TLR2 en monocitos e macrófagos foi significativamente maior nos pacientes con MC. Os suxeitos con MC mostraron niveis significativamente máis altos de cFn, IL-6, sTWEAK e CGRP. Atopáronse niveis máis altos de IL-10 en controis sans. Non houbo diferencias entre os grupos de estudio nos niveis de HSP60, hs-CRP e PTX3.

A expresión de TLR nas células mononucleares de sangue periférico correlacionáronse cos niveis de cFn, IL6, CGRP e STWEAK. Os niveis de IL10 correlacionáronse inversamente coa expresión de TLRs.

Despois de axustar os resultados polas variables clínicas e moleculares que foron significativamente diferentes nos pacientes con MC, tanto a expresión de TLR2 en neutrófilos e monocitos como a expresión de TLR4 en monocitos mantiveron a asociación co diagnóstico de MC. Utilizando curvas ROC puidemos determinar que a expresión de TLR2 en neutrófilos ≥389 IU, TLR2 en monocitos ≥350 UI e TLR4 en monocitos ≥2232 UI poden predicir o diagnóstico de MC.

DISCUSIÓN

Modelo experimental

A interpretación dos nosos achados é complexa e debe facerse en relación con anteriores modelos animais de DCP. Estudos anteriores

que usaron rexistros electrofisiolóxicos e fluxometría láser demostraron que o aumento na duración da onda de DCP se correlacionou con unha menor susceptibilidade á estimulación. Polo tanto, os nosos resultados suxiren que a ausencia ou o bloqueo de TLR4 poden dificultar o inicio da DCP.

Estes achados son congruentes con outros estudos en tecido animal e *in vivo* que mostran como a DCP repetida induce un aumento na expresión dos TLR na glía e unha activación das súas vías de sinalización, disminuíndo o umbral para a DCP (Grinberg YY et al. 2017). A proliferación de factores inflamatorios e o aumento da expresión de TLR poderían ser responsables da perpetuación dos mecanismos de retroalimentación positiva que conducen a unha maior susceptibilidade á DCP. Polo tanto, a ausencia e o bloqueo farmacolóxico de TLR4 podería reducir a susceptibilidade a DCP.

Esta relación entre a expresión de TLR, a inflamación e a DCP explicaría achados anteriores en modelos de comportamento animal en migraña, nos que o defecto xenético de TLR4 ou o seu bloqueo farmacolóxico pola administración do antagonista TAK-242 reduce a aversión á luz e a activación do núcleo trixeminal caudal (Ramachandran R et al. 2019). O pretratamento con TAK-242 tamén reduce a hiperalxesia nun modelo de rata despois da administración de sopa inflamatoria nas meninxes (Su M et al. 2018).

En modelos animais doutros tipos de dor crónica hai un aumento da expresión de TLR e das moléculas inflamatorias derivadas das súas vías de señalización. Estes estudos atoparon non un aumento na expresión de TLR2 e TLR4 en ratas con dano neuropático (Jin G et al. 2018), se non unha redución da hiperalxesia e a activación glial asociada en animais KO para TLR2 e TLR4 (Jurga AM et al. 2016). O bloqueo de TLR2 e TLR4 disminúe ou inhibe a dor de distinta orixe (Hutchinson MR et al. 2008).

Curiosamente, non atopamos diferencias nos niveis de IL-6 entre os grupos de estudio. Estes resultados deben interpretarse con cautela, xa que os niveis de IL-6 son menos específicos para a activación da vía de TLR4 que outros biomarcadores inflamatorios e poderían verse influídos polo procedemento quirúrxico. No que respecta ós resultados de permeabilidade da BHE, a relación entre a inflamación e

a permeabilidade de barreira é máis complexa e depende doutras vías e procesos moleculares, non só os relacionados cos TLR.
En resumo, os nosos resultados suxiren unha implicación dos TLR4 no fenómeno da DCP. Estes achados son coherentes cos datos anteriores sobre a relación entre o aumento da expresión dos TLR e a disminución do umbral de susceptibilidade á DCP ou a relación entre a repetición das DCP e o aumento da expresión dos TLR gliais.

Estudo clínico

As características demográficas e clínicas da nosa mostra son similares ás de outras mostras de MC publicadas con anterioridade (García-Cabo Fernández C et al. 2016). Os suxeitos con MC mostraron unha maior expresión de TLR2 e TLR4 nas células mononucleares de sangue periférico cando se compararon con controis sans e estas diferencias sobreviviron ó axuste por varios factores clínicos e biomarcadores. A expresión de TLR2 e TLR4 podería incluso predecir o diagnóstico de MC na nosa mostra. Estes achados reflicten un aumento na expresión dos TLR en todo o sistema fagocítico-mononuclear, incluíndo as células mieloides do SNC (microglia) e suxiren unha sobreactivación das vías de sinalización dos TLR. No momento de imprimir este traballo, é o primeiro estudo adicado a analizar a expresión dos TLR en pacientes con migraña. Polo tanto, a interpretación dos nosos resultados debe facerse con precaución, dada a falta de estudos previos cos que comparalos.
A sobreexpresión de TLR en pacientes con MC pode estar xenéticamente predeterminada e condicionar, alomenos parcialmente, a predisposición destes individuos a desenvolver o trastorno. O único estudo en humanos realizado ata a data sobre o papel dos TLR en pacientes con migraña é un estudo xenético que analiza a frecuencia do polimorfismo 4 896/G de TLR4 e a súa relación coa presencia de migraña. Nembargantes, varios estudos xenéticos asocian diferentes polimorfismos de TLR2 e TLR4 co risco de eventos cerebrovasculares isquémicos e o impacto da DCP e a inflamación no contexto da isquemia. Tamén é posible que a liberación de ligandos endóxenos como resultado da DCP repetidas conduza a unha sobreexpresión dos TLRs. Varios estudos en modelos de isquemia

cerebral demostraron que o TLR4 se sobreexpresa na microglia e os astrocitos despois da inflamación do SNC (Caso JR et al. 2007) e a DCP pode producir un efecto similar (Gehrmann J et al. 1993). Ó tempo, a activación dos TLR pode modificar a susceptibilidade á DCP: o aumento da produción de citoquinas e a activación da microglia con liberación de distintos mediadores, coma o factor neurotrófico derivado do cerebro, pode afectar á actividade bioeléctrica do cerebro e contribuir á transformación da ME en MC (Kraig RP et al.2010). Por último, o aumento da expresión de TLR a nivel periférico podería ser consecuencia dun entorno proinflamatorio. Segundo esta hipótese, a presencia constante de factores proinflamatorios en suxeitos con MC ou a repetición de ataques de migraña durante os cuais se liberan DAMPs, como cFn, HMGB1 ou HSP, favorecen a activación dos TLR, establecendo un mecanismo de retroalimentación positiva. Neste sentido, o concepto de "Ciclo Radical de TLR" é de grande interese; segundo esta teoría a liberación de PAMPs e DAMPs en resposta a mecanismos neuroinflamatorios e oxidativos pode producir unha activación do complexo TLR con autoamplificación da resposta e retroalimentación positiva (Lucas K et al. 2015) que contribúe á cronificación da dor.
Atopamos niveis máis altos de IL6, CGRP e sTWEAK en pacientes con MC, e unha correlación coa expresión de TLR. A cFn é un ligando de TLR 2 e 4 coñecido e a IL-6 é un dos produtos finais da súa vía de sinalización. Estes achados reforzan a noción de que os TLR están sobreactivados na MC. O CGRP é ata hoxe o péptido mellor estudiado na fisiopatoloxía da migraña; os nosos achados apoian a existencia dunha interación entre o CGRP e a inmunidade innata, destacando o papel potencial dos TLRs na neuroinflamación. Aínda que o papel de TWEAK na regulación dos procesos inflamatorios é complexo e depende da expresión doutros moitos mediadores e proteínas reguladoras, os nosos achados suxiren que o seu efecto sobre a inflamación na migraña pode depender parcialmente dos TLR.

Limitacións

Tanto o noso modelo animal coma o noso estudio clínico teñen varias limitacións. Hai diferencias relevantes entre humanos e ratos nos mecanismos de DCP e a expresión glial de TLR, que poderían afectar á traslacionalidade dos nosos resultados. Só usamos ratos machos, un erro común nos estudos sobre migraña, considerando que é unha patoloxía con claras diferencias de sexo e na que o estróxeno e os seus metabolitos teñen unha grande influencia. Durante a cirurxía, o control dos parámetros fisiolóxicos nos animales non foi óptimo, xa que debimos ter controlado estrictamente a presión sanguínea e os niveis de osixenación. Finalmente, aínda que xa foi amplamente validada, a fluxometría láser é unha técnica subrogada ó patrón ouro, que é o rexistro electrofisiolóxico.

No noso estudo clínico, aínda que os resultados se axustaron polas diferencias entre grupos, moitas comorbilidades de migraña, a presencia doutros tipos de cefalea e o uso de tratamentos preventivos poden ter influido nos nosos resultados. Tería sido interesante incluir suxeitos con EM para evaluar a posible expresión ascendente dos TLRs a medida que a enfermidade progresa. A principal limitación é sen dúbida a falta de control das variacións hormonais, considerando a probada influencia do estróxeno e a proxesterona na expresión e a función dos TLR (Calippe B et al. 2010). A variabilidade de estudios previos en relación coas análises ELISA de HSP60, PTX3 ou CGRP tamén poden ter influido nos nosos achados e disminuido a súa fiabilidade.

CONCLUSIONS

Os suxeitos con MC mostran unha maior expresión de TLR2 e TLR4 en neutrófilos e monocitos en sangue periférico. A expresión de TLR2 en monocitos e neutrófilos en sangue periférico e a expresión de TLR4 en monocitos en sangue periférico asóciase de forma independente coa MC e pode ter un papel predictivo no seu diagnóstico.

A ausencia ou o bloqueo de TLR4 cambia o patrón da onda de DCP e modula a resposta vascular nun modelo animal de migraña.

Estes achados suxiren un papel clave dos TLR e a inmunidade innata na fisiopatoloxía da migraña, como factores implicados tanto no seu

inicio como na súa cronificación. A posibilidade de bloquear farmacolóxicamente os TLR abre potenciais novas vías terapéuticas para a migraña.

11.2. COPIA DO INFORME FAVORABLE DO COMITE DE ETICA PARA REALIZACION DO ESTUDIO

XUNTA DE GALICIA
CONSELLERÍA DE SANIDADE
Secretaría Xeral Técnica

Edificio Administrativo San Lázaro
15703 SANTIAGO DE COMPOSTELA
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galic

DICTAMEN DEL COMITÉ AUTONÓMICO DE ÉTICA DE LA INVESTIGACIÓN DE GALICIA

Paula M. López Vázquez, Secretaria del Comité Autonómico de Ética de la Investigación de Galicia

CERTIFICA:

Que este Comité evaluó en su reunión del día 10/05/2016:

> **Título:**Alteración de la inmunidad innata en la migraña. Papel de los receptores TLR-2 y TLR-4 como elementos clave en la cronificación de la migraña y posibles dianas terapéuticas
> **Promotor***:* Rogelio Leira Muiño
> **Código de Registro:** 2016/079

Y, tomando en consideración las siguientes cuestiones:

- La pertinencia del estudio, teniendo en cuenta el conocimiento disponible, así como los requisitos legales aplicables, y en particular la Ley 14/2007, de investigación biomédica, el Real Decreto 1716/2011, de 18 de noviembre, por el que se establecen los requisitos básicos de autorización y funcionamiento de los biobancos con fines de investigación biomédica y del tratamiento de las muestras biológicas de origen humano, y se regula el funcionamiento y organización del Registro Nacional de Biobancos para investigación biomédica, la ORDEN SAS/3470/2009, de 16 de diciembre, por la que se publican las Directrices sobre estudios Posautorización de Tipo Observacional para medicamentos de uso humano, y la Circular nº 07 / 2004, investigaciones clínicas con productos sanitarios.
- La idoneidad del protocolo en relación con los objetivos del estudio, justificación de los riesgos y molestias previsibles para el sujeto, así como los beneficios esperados.
- Los principios éticos de la Declaración de Helsinki vigente.
- Los Procedimientos Normalizados de Trabajo del CEIC de Galicia

Emite un **INFORME FAVORABLE** para la realización del estudio por el/la investigador/a del centro:

Centros	Investigadores Principales
C.H. Universitario de Santiago	Rogelio Leira Muiño

*NOTA: El diseño transversal que se ha elegido no permitirá demostrar asociación tal como se plantea en algun objetivo secundario. Para ello, es preciso un diseño de seguimiento prospectivo.

Y HACE CONSTAR QUE:

1 El CAEIG cumple los requisitos legales vigentes (R.D 1090/2015 y la Ley 14/2007)
2 El CAEIG tanto en su composición como en sus PNTs cumple las Normasde Buena Práctica Clínica (CPMP/ICH/135/95).
3 La composición actual del CAEIG es:

Manuel Portela Romero. (Presidente). Médico Especialista en Medicina Familiar y Comunitaria.
Irene Zarra Ferro. (Vicepresidenta). Farmacéutica de Atención Especializada.
Paula Mª López Vázquez, (Secretaria). Médico Especialista en Farmacología Clínica.
Juan Vázquez Lago (Secretario Suplente). Médico Especialista en Medicina Preventiva y Salud Pública.
Jesús Alberdi Sudupe. Médico especialista en Psiquiatría.
Rosendo Bugarín González. Médico Especialista en Medicina Familiar y Comunitaria.
Juan Casariego Rosón. Médico Especialista en Cardiología.
Xoán X. Casas Rodríguez. Médico Especialista en Medicina Familiar y Comunitaria.
Juana Mª Cruz del Río. Trabajadora Social.
Juan Fernando Cueva Bañuelos. Médico Especialista en Oncología Médica.
José Álvaro Fernández Rial. Médico Especialista en Medicina Interna.
José Luis Fernández Trisac. Médico Especialista en Pediatría.
Mª José Ferreira Díaz. Diplomada Universitaria de Enfermería
Pablo Nimo Ríos. Licenciado en Derecho. Miembro externo
Pilar Gayoso Diz. Médico Especialista en Medicina Familiar y Comunitaria.
Agustín Pía Morandeira. Farmacéutico de Atención Primaria
Salvador Pita Fernández. Médico Especialista en Medicina Familiar y Comunitaria.
Carmen Rodríguez-Tenreiro Sánchez. Licenciada en Farmacia.
Susana María Romero Yuste. Médico Especialista en Reumatología.
Mª Asunción Verdejo González. Médico Especialista en Farmacología Clínica.

En Santiago de Compostela, a 11 de mayo de 2016

XUNTA DE GALICIA
SECRETARÍA XERAL TÉCNICA
CONSELLERÍA DE SANIDADE
COMITÉ AUTONÓMICO DE ÉTICA DA INVESTIGACIÓN

Firmado digitalmente por LOPEZ VAZQUEZ PAULA MARIA - DNI 46900339G
Nombre de reconocimiento (DN): c=ES, o=XUNTA DE GALICIA, ou=certificado electrónico de empleado público, serialNumber=46900339G, sn=LOPEZ VAZQUEZ, givenName=PAULA MARIA, cn=LOPEZ VAZQUEZ PAULA MARIA - DNI 46900339G
Fecha: 2016.05.11 12:06:51 +02'00'

11.3 RESOLUCION DE AUTORIZACION DE PROXECTOS DE EXPERIMENTACION ANIMAL

XUNTA DE GALICIA
CONSELLERÍA DO MEDIO RURAL

Departamento Territorial
Edificio Administrativo de Servizos Múltiples
Vicente Ferrer, 2
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RESOLUCIÓN DE AUTORIZACIÓN DE PROXECTOS DE EXPERIMENTACIÓN ANIMAL

Expediente núm.: 15010/2019/004 **Data de inicio:** 05.02.2019
Persoa interesada: Francisco Campos Pérez **Procedemento:** resolución de autorización
Forma de inicio: solicitude da persoa interesada

ANTECEDENTES

A persoa interesada, como representante do centro CIMUS (Universidade de Santiago de Compostela), presentou con data 05.02.2019 unha solicitude para a realización do proxecto de experimentación animal (entrada no Rexistro Electrónico da Xunta de Galicia 2019/246024), cuxos datos se detallan a continuación:

Denominación do proxecto: *Nanopartículas biomiméticas para a administración dirixida de nanomedicinas*
Nome do centro usuario: Animalario do CIMUS
Persoa responsable do proxecto: Francisco Campos Pérez
Establecemento onde se realizarán os procedementos do proxecto (ou lugar xeográfico no caso de traballos de campo): Animalario do CIMUS
Clasificación do proxecto : Tipo I ☐ Tipo II ☐ Tipo III ☒

CONSIDERACIÓNS LEGAIS E TÉCNICAS

1 O Real decreto 53/2013, de 1 de febreiro (BOE 34, do 8 de febreiro), polo que se establecen as normas básicas aplicables para a protección dos animais utilizados en experimentación e outros fins científicos, incluíndo a docencia, establece no seu artigo 33 as condicións de autorizacións dos proxectos con animais de experimentación.

2 O artigo 88 da Lei 39/2015, de 1 de outubro, do procedemento administrativo común das administracións públicas (BOE 236, do 2 de outubro de 2015) establece que a resolución que poña fin o procedemento decidirá todas as cuestións expostas polos interesados e aquelas outras derivadas deste.

3 O Servizo de Gandaría da Coruña revisou a documentación achegada na solicitude e o resultado favorable da avaliación do proxecto, realizada polo órgano habilitado da Sección de Experimentación Animal do Comité de Bioética da Universidade de Santiago de Compostela.

Esta xefatura territorial é competente para ditar unha resolución, de conformidade co Decreto 149/2018, do 5 de decembro, polo que se establece a estrutura orgánica da

Departamento Territorial
Edificio Administrativo de Servizos Múltiples
Vicente Ferrer, 2
15071 – A Coruña

Consellería do Medio Rural e se modifica parcialmente o Decreto 177/2016, do 15 de decembro, polo que se fixa a estrutura orgánica da Vicepresidencia e das consellerías da Xunta de Galicia (DOG 235, do 11 de novembro).

De acordo con todo o indicado, RESOLVO:

1. Autorizar o proxecto solicitado.
2. O mencionado proxecto precisa someterse a unha avaliación retrospectiva tras finalizar a súa autorización.
3. A autorización deste proxecto terá unha duración de dous anos e unha vez transcorrido este tempo deberá ser autorizado de novo.

A citada autorización é unicamente válida nas condicións que figuran no expediente. Ante calquera cambio significativo no proxecto que poida ter efectos negativos sobre o benestar dos animais, deberá solicitar a confirmación da autorización ao Servizo Provincial de Gandaría.

Esta autorización poderá ser suspendida, no caso de que o proxecto non se leve a cabo de acordo coas condicións de autorización e retirala, previo expediente tramitado ao que se lle dará audiencia.

Contra a presente resolución, que non lle pon fin á vía administrativa, poderá interpoñer un recurso de alzada ante o conselleiro de Medio Rural. O prazo comezará a contar dende o día seguinte ao da recepción desta resolución. Todo isto, segundo o disposto nos artigos 121 e 122 da citada Lei 39/2015.

Mediante este escrito notificaselle ao CIMUS da USC esta resolución segundo o esixido no artigo 40.1 da antedita Lei 39/2015.

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