Changes in the P2Y12 purinoceptor expression and function in monocyte-derived microglial like-cells from patients with Mesial Temporal Lobe Epilepsy with Hippocampal Sclerosis (MTLE-HS)

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Resumo

A epilepsia do lobo temporal mesial com esclerose hipocampal (MTLE-HS) é a síndrome epilética mais refratária, sendo que mais de 80% dos doentes apresentam uma resposta fraca aos medicamentos antiepiléticos convencionais. A elevada atividade neuronal durante as crises epiléticas resulta no aumento dos níveis extracelulares de ATP, que eventualmente é metabolizado em ADP, AMP e adenosina. O recetor $P2Y_{12}$, sensível ao ADP, é expresso de forma estável por células microgliais no SNC. Em modelos animais de epilepsia, foi observada uma diminuição transitória da expressão de $P2Y_{12}R$. Após essa breve diminuição, o estadio crónico da epilepsia carateriza-se por um aumento da expressão $P2Y_{12}R$ associado a uma extensa ativação microglial, sugerindo um papel duplo deste recetor no desenvolvimento da epilepsia e um potencial alvo terapêutico para o tratamento desta doença. Adicionalmente, a via de sinalização CX3CL1/CX3CR1 induz a libertação de IL-1β pela microglia, desempenhando um papel crucial na resposta inflamatória e perpetuando a neuroinflamação ao desencadear a libertação de ATP pelas dendrites neuronais.

O objetivo deste estudo foi o de avaliar os níveis do recetor purinérgico $P2Y_{12}$ na MTLE-HS humana, estudar o seu papel na neuro inflamação e como é que este recetor é modulado epigeneticamente. Para isso, (i) analisámos os níveis de mRNA codificante para o $P2Y_{12}R$ e CX3CR1 no tecido cerebral humano e (ii) desenvolvemos um modelo experimental que permite a cultura “in vitro” de células semelhantes à microglia derivadas de monócitos do sangue periférico de controlos e de doentes com MTLE-HS. Este modelo permitirá, num futuro próximo, analisar a atividade destes recetores, bem como a influência epigenética (e.g. microRNAs), durante a homeostasia e em situações inflamatórias. O objetivo final deste estudo será o de entender o impacto da neuroinflamação mediada por recetores purinérgicos na MTLE-HS humana, bem como na epileptogénese.

Neste trabalho, quantificaram-se os níveis de mRNA codificante para o $P2Y_{12}R$ e CX3CR1 no hipocampo e no neocórtex temporal de 19 doentes com MTLE-HS e em 10 controlos cadavéricos. Foram isolados células mononucleares do sangue periférico (PBMCs) do sangue periférico de 15 doentes com MTLE-HS e 5 dadores voluntários saudáveis, que foram subsequentemente diferenciadas (i) em células microglia-*like* derivadas de monócitos (MMG) utilizando fatores de diferenciação específicos para humanos e (ii) em macrófagos (grupo controlo). O mRNA isolado destas células foi

usado para avaliar o teor em transcritos codificantes para a seguintes proteínas: $P2Y_{12}R$, CX3CR1, $P2X_7R$ e IL-1β. Também se avaliaram os níveis de microRNAs, miR-146a, miR-22, miR-155, miR-21 e miR-223, presentes no meio de cultura.

Os doentes MTLE-HS possuíam um aumento significativo na expressão de $P2Y_{12}R$ e CX3CR1, tanto no hipocampo (1,78 e 1,96 vezes, respetivamente) quanto no neocórtex temporal (2,89 e 2,23 vezes, respetivamente), comparativamente com os controlos cadavéricos. As células MMG apresentaram uma morfologia microglial típica associada à expressão de $P2Y_{12}R$ e CX3CR1, ao contrário dos macrófagos que apresentavam uma morfologia ameboide e expressaram apenas CX3CR1. A estimulação das MMGs com LPS reduziu drasticamente a expressão de $P2Y_{12}R$ e aumentou os níveis de IL-1β; o aumento de IL-1β nas células estimuladas com LPS foi de apenas 2,76 vezes nos doentes portadores de MTLE-HS, enquanto se observou um aumento de 9,38 vezes nos indivíduos saudáveis. Na ausência de estimulação, as MMGs dos doentes com MTLE-HS possuíam níveis 3,46 vezes superiores de miR-146a comparativamente com os níveis medidos nos indivíduos saudáveis.

Estes resultados sugerem que os doentes com MTLE-HS apresentam uma desregulação da atividade microglial resultando numa possível propensão para uma ativação microglial prolongada. Esta alteração microglial associa-se frequentemente à formação de projeções neuronais aberrantes no hipocampo (e.g. giro denteado) resultando num ambiente propício ao desenvolvimento de crises convulsivas recorrentes. A variação da expressão dos recetores microgliais também no neocórtex temporal demonstra que esta região pode estar associada à epileptogénese. Este trabalho permitiu, ainda, demonstrar que é possível diferenciar monócitos do sangue periférico derivados de doentes MTLE-HS e de controlos saudáveis em células semelhantes à microglia (MMGs). A comprovação da funcionalidade destas células permite antecipar que a diferenciação de monócitos do sangue periférico em MMGs pode facilitar a realização de ensaios funcionais para caracterizar o papel de moduladores epigenéticos, recetores purinérgicos e neuroinflamação em indivíduos com vários estádios da doença, sem recorrer ao isolamento da microglia em amostras cerebrais de difícil obtenção apenas nas situações mais graves da doença.

Palavras-chave: Epilepsia; MTLE-HS; Sinalização purinérgica; $P2Y_{12}R$; CX3CR1; Células microgliais derivadas de monócitos; Neuroinflamação; Epileptogénese; MicroRNAs

Abstract

Mesial temporal lobe epilepsy with hippocampal sclerosis (MTLE-HS) is the most refractory epileptic syndrome, with more than 80% of patients showing a poor response to conventional antiepileptic drugs. The high neuronal activity during seizures results in elevated extracellular levels of ATP, which is eventually metabolized into ADP, AMP and adenosine. The $P2Y_{12}$ receptor, sensitive to ADP, is stably expressed by microglial cells in the CNS. In animal models of epilepsy, a transient decrease in $P2Y_{12}R$ expression has been observed. Following this brief reduction, the chronic stage of epilepsy is characterized by an increased expression of $P2Y_{12}R$ associated with extensive microglial activation, suggesting a dual role for this receptor in epilepsy development and a potential therapeutic target for its treatment. Additionally, under pathological conditions, the CX3CL1/CX3CR1 signaling pathway induces the release of IL-1β by microglia, playing a crucial role in the inflammatory response and perpetuating neuroinflammation by triggering ATP release from neuronal dendrites.

The aim of this study was to evaluate the levels of the purinergic receptor $P2Y_{12}$ in human MTLE-HS, investigate its role in neuroinflammation, and understand how this receptor is epigenetically modulated. For this, we (i) analysed mRNA levels coding for $P2Y_{12}R$ and CX3CR1 in human brain tissue and (ii) developed an experimental model that enables the in vitro culture of microglia-like cells derived from peripheral blood monocytes from both controls and MTLE-HS patients. This model will allow, in the near future, analysis of these receptors' activity as well as the influence of epigenetics (e.g., microRNAs) during homeostasis and in inflammatory situations. The ultimate goal of this study is to understand the impact of purinergic receptor-mediated neuroinflammation in human MTLE-HS as well as in epileptogenesis.

In this work, we quantified mRNA levels coding for $P2Y_{12}R$ and CX3CR1 in the hippocampus and temporal neocortex of 19 patients with MTLE-HS and in 10 cadaveric controls. Peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood of 15 MTLE-HS patients and 5 healthy volunteer donors. These were subsequently differentiated into (i) microglia-like cells derived from monocytes (MMG) using specific human differentiation factors and (ii) macrophages (control group). The mRNA isolated from these cells was used to evaluate transcript levels coding for the following proteins: $P2Y_{12}R$, CX3CR1, P2X7R, and IL-1β. Levels of microRNAs, miR-146a, miR-22, miR-155, miR-21, and miR-223 present in the culture medium were also assessed.

MTLE-HS patients showed a significant increase in $P2Y_{12}R$ and CX3CR1 expression, both in the hippocampus (1.78 and 1.96 times, respectively) and in the temporal neocortex (2.89 and 2.23 times, respectively), compared to cadaveric controls. MMG cells displayed a typical microglial morphology associated with the expression of $P2Y_{12}R$ and CX3CR1, unlike macrophages, which exhibited an amoeboid morphology and expressed only CX3CR1. Stimulation of MMGs with LPS drastically reduced $P2Y_{12}R$ expression and increased IL-1β levels; the increase in IL-1β in LPS-stimulated cells was only 2.76 times in MTLE-HS patients, while a 9.38-fold increase was observed in healthy individuals. In the absence of stimulation, MMGs from MTLE-HS patients showed levels of miR-146a that were 3.46 times higher than those measured in healthy individuals.

These results suggest that MTLE-HS patients exhibit a dysregulation of microglial activity, potentially leading to prolonged microglial activation. This microglial alteration is often associated with the formation of aberrant neuronal projections in the hippocampus (e.g., dentate gyrus), resulting in an environment conducive to the development of recurrent seizures. The variation in microglial receptor expression in the temporal neocortex also demonstrates that this region may be associated with epileptogenesis. This work further demonstrated that it is possible to differentiate peripheral blood monocytes derived from MTLE-HS patients and healthy controls into microglia-like cells. The validation of the functionality of these cells suggests that differentiating peripheral blood monocytes into MMGs may facilitate functional assays to characterize the role of epigenetic modulators, purinergic receptors, and neuroinflammation in individuals at various stages of the disease, without resorting to microglial isolation from brain samples, which is only feasible in the most severe cases of the disease.

Keywords: Epilepsy; MTLE-HS; Purinergic signalling; $P2Y_{12}R$; CX3CR1; Monocyte-derived microglia-like cells; Neuroinflammation; Epileptogenesis; MicroRNAs

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ADK	ADENOSINE KINASE
ADP	ADENOSINE 5'-DIPHOSPHATE
AKT	PROTEIN KINASE B
AMP	ADENOSINE MONOPHOSPHATE
AMPA	ALFA-AMINO-3-HYDROXY-5-METHYL-4-ISOXAZOLEPROPIONIC ACID
AR	ADENOSINE RECEPTOR
ASD	ANTISEIZURE DRUG
ATG16L1	AUTOPHAGY-RELATED 16-LIKE 1
ATP	ADENOSINE 5'-TRIPHOSPHATE
BBG	BRILLIANT BLUE G
CCL2	C-C MOTIF CHEMOKINE LIGAND 2
cAMP	CYCLIC ADENOSINE MONOPHOSPHATE
CA	*CORNU AMMONIS*
CHUDSA-ULSSA	NEUROSURGERY DEPARTMENT OF CENTRO HOSPITALAR E UNIVERSITÁRIO DE SANTO ANTÓNIO - UNIDADE LOCAL DE SAÚDE DE SANTO ANTÓNIO
CNS	CENTRAL NERVOUS SYSTEM
CT	CYCLE THRESHOLD
CX3CL1	C-X3-C MOTIF CHEMOKINE LIGAND
CX3CR1	C-X3-C MOTIF CHEMOKINE RECEPTOR 1
DAG	DIACYLGLYCEROL
DAMP	DANGER-ASSOCIATED MOLECULAR PATTERN
DNTP	DEOXYNUCLEOTIDE TRIPHOSPHATE
EEG	ELECTROENCEPHALOGRAPHY
FBS	FETAL BOVINE SERUM
FCT	FUNDAÇÃO PARA A CIÊNCIA E A TECNOLOGIA
FCUP	FACULTY OF SCIENCES OF THE UNIVERSITY OF PORTO
GABA	GAMMA-AMINOBUTYRIC ACID
GM-CSF	GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR
GPCR	G-PROTEIN COUPLED RECEPTOR
HS	HIPPOCAMPAL SCLEROSIS
ILAE	INTERNATIONAL LEAGUE AGAINST EPILEPSY
IFN	INTERFERON

IL	INTERLEUKIN
INMLCF-DN	INSTITUTO NACIONAL DE MEDICINA LEGAL E CIÊNCIAS FORENSES - DELEGAÇÃO DO NORTE
IP3	INOSITOL-3-PHOSPHATE
IRAK1/2	IL-1 RECEPTOR-ASSOCIATED KINASE-1/2
JAK	JANUS TYROSINE KINASE
KA	KAINIC ACID
LC3	MICROTUBULE-ASSOCIATED PROTEIN LIGHT CHAIN 3
LPCE	LIGA PORTUGUESA CONTRA EPILEPSIA
LPS	LIPOPOLYSACCHARIDE
MACS	MAGNETIC-ACTIVATED CELL SORTING
M-CSF	MACROPHAGE COLONY-STIMULATING FACTOR
MIR	MICRORNA
MMG	MONOCYTE-DERIVED MICROGLIA-LIKE
MTLE	MESIAL TEMPORAL LOBE EPILEPSY
MTOR	MAMMALIAN TARGET OF RAPAMYCIN
NF-KB	NUCLEAR FACTOR KAPPA-LIGHT-CHAIN-ENHANCER OF ACTIVATED B CELLS
NLRP3	NLR FAMILY PYRIN DOMAIN CONTAINING 3
NGF	NERVE GROWTH FACTOR
NMDA	N-METHYL-D-ASPARTATE
NTPDASE1 / CD39	TRIPHOSPHATE DIPHOSPHOHYDROLASE-1
NT5E / CD73	ECTO-5'-NUCLEOTIDASE
PBMC	PERIPHERAL BLOOD MONONUCLEAR CELL
PBS	PHOSPHATE-BUFFERED SALINE
PEG	POLYETHYLENE GLYCOL
PIP3	PHOSPHATIDYLINOSITOL (3,4,5)-TRISPHOSPHATE
PKC	PROTEIN KINASE C
PLC	PHOSPHOLIPASE C
PTEN	PHOSPHATASE AND TENSIN HOMOLOG
PTZ	PENTYLENETETRAZOLE
P2XR	P2X RECEPTOR
P2YR	P2Y RECEPTOR
RBCL	RED BLOOD CELL LYSIS
RT	REVERSE TRANSCRIPTION

RT-PCR	REAL TIME POLYMERASE CHAIN REACTION
SD	STANDARD DEVIATION
SE	STATUS EPILEPTICUS
SEM	STANDARD ERROR OF THE MEAN
SHIP1	SH2 DOMAIN-CONTAINING INOSITOL 5'-PHOSPHATASE1
SOCS1	SUPPRESSOR OF CYTOKINE SIGNALING
SPSS	STATISTICAL PACKAGE FOR THE SOCIAL SCIENCES
STAT	SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION
TGF	TRANSFORMING GROWTH FACTOR
TLR4	TOLL-LIKE RECEPTOR 4
TNF	TUMOUR NECROSIS FACTOR
TNFR	TUMOUR NECROSIS FACTOR RECEPTOR
TRAF6	TNF RECEPTOR-ASSOCIATED FACTOR 6
UDP	URIDINE 5'-DIPHOSPHATE
UBC	UBIQUITIN C
UP	UNIVERSITY OF PORTO
UTP	URIDINE 5'-TRIPHOSPHATE

Chapter 1: Introduction

1.1. Prevalence and classification of epilepsy

When the subject is neurological diseases, epilepsy is one of the most prevalent worldwide, impacting approximately 50 million individuals according to the World Health Organization [1, 2]. In Portugal, it affects around 50 thousand people according to the Liga Portuguesa Contra Epilepsia (LPCE) [3], with a major incidence in infants and people over the age of 50 and increasing with age [4-6].

Epilepsy is characterized by a predisposition to generate recurrent spontaneous seizures, which are a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain [7].

The disease can be classified at three different levels: seizure type, epilepsy type, and epilepsy syndrome. The International League Against Epilepsy (ILAE) classifies seizure types by the initial manifestations of the seizures, dividing these into three categories: a generalised onset, a focal onset or an unknown onset, in case of missed or obscured onset [6-8]. Seizures with a generalised onset affect both sides of the brain, whereas a focal onset affects a specific brain region (Figure 1).

Regardless of onset, seizures are further subdivided into motor or non-motor seizures and, in the case of focal seizures, can also be categorised by the level of awareness retained [7]. Focal seizures can evolve to generalised seizures and, as a consequence, some epilepsy syndromes can manifest both seizure types [8].

Focal epilepsy is frequently described by the area of the brain in which seizures originate. The most common focal epilepsy syndrome in adults, mesial temporal lobe epilepsy (MTLE), affects the medial or internal structures of the temporal lobe [9-12]. Seizures originate in an epileptogenic zone comprised by the hippocampus, parahippocampal gyrus and/or the amygdala [11, 13]. The excitatory stimuli in these regions can have long-term consequences, such as neuronal injury, neuronal death or even the modification of neuronal networks, depending on the type and duration of seizures. Thus, a common morphologic finding associated with MTLE is hippocampal sclerosis (HS), characterized by shrinkage of the hippocampus, caused by the selective and often profound loss of pyramidal neurons as well as scarring, as consequence of excessive proliferation and activation of glial cells (gliosis). This phenomenon is often associated with the reorganization of synaptic connections, where mossy fibres (granule

cells axons) sprout abnormally and form new, inappropriate neuronal connections, which has been argued to be a critical component in the development of recurrent seizures in patients with HS [14]. This pathological event can be bilateral and is associated with cognitive impairment such as the inability to form and retain new memories [9, 14-16].

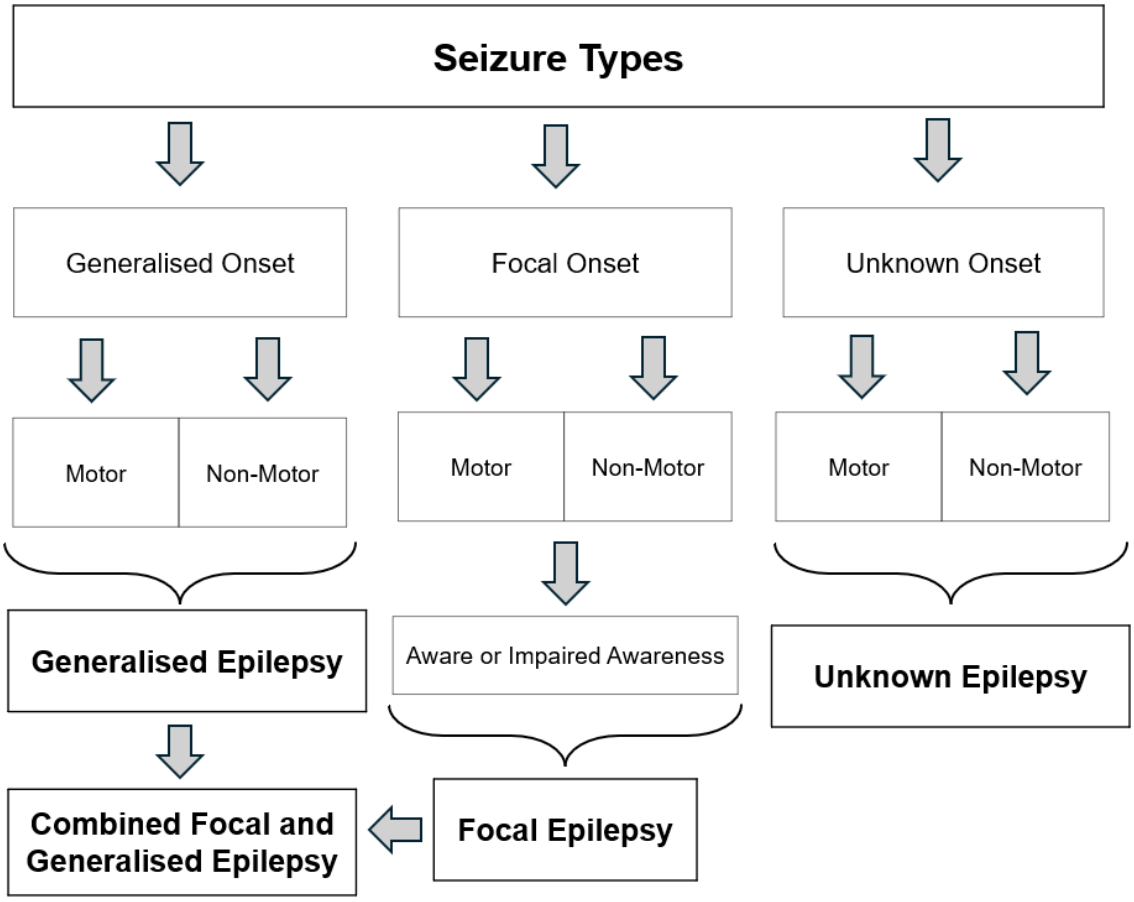

Figure 1 - Epilepsy and seizure types according to ILAE 2017 classification.

The development of epilepsy cannot be prevented, yet recurrent seizures are avoided by the administration of antiseizure drugs (ASDs) that mainly target ion channels essential for synapse generation and action potential propagation, such as Na^+, Ca^{2+} or K^+ channels. Receptors involved in excitatory signalling pathways, such as the glutamate receptors, and inhibitory signalling pathways, such as the γ-aminobutyric acid (GABA) receptors, are also molecular targets of traditional ASDs [17].

MTLE has nearly 80% drug refractory rate and only 10% of patients presenting MTLE with HS become seizure free [18-20]. The unreliability of ASDs calls for additional treatments like neuromodulation devices, nerve stimulators, ketogenic diet and, as a last resource, amygdalohippocampectomy surgery, as a way to remove defective tissue and

avoid seizures [21]. Although surgery yields great short-term results, with 50 to 70% of patients becoming seizure free after a two-year follow up, there is an increased risk of seizure recurrence over time [22, 23]. Patients show a seizure recurrence rate of 45 to 50% and 60 to 70%, 10 years post temporal lobectomy and extratemporal resections, respectively [24].

People with epilepsy struggle to have a normal quality of life, as they may face societal stigma and lack of social support, resorting to social isolation in the fear of embarrassment and discrimination [25]. There are extra hurdles in education and higher rates of employment, leading to a lack of independence that imposes a great burden on the caregivers [8, 25].

Additionally, epilepsy patients seem to have a higher rate of psychological disorders, such as anxiety and depression, while also exhibiting a higher number of physiological comorbidities, some possibly exacerbated by the treatment.

Given the absence of a cure and the high intractability of MTLE-HS, this prompts the search for new and effective ASDs for seizure prevention and to control epileptogenesis.

1.2. Aetiology and epileptogenesis

The aetiology of the disease can be divided into six main categories: genetic, infectious, immune, structural, metabolic, and unknown. Although about 50% of patient cases do not have a known cause, common examples of epileptogenesis can be attributed to developmental brain abnormalities, infections, traumatic brain injury and stroke [6, 21].

This pathological process can usually be divided into three distinct phases: firstly, an initial brain insult. This injury, in turn, leads to a latent phase where there is a reorganization of the brain's neural connections, transforming the previously normal brain into an epileptic one. Over time, these changes can disrupt the delicate balance between inhibitory and excitatory signals in the limbic system, ultimately resulting in spontaneous seizures, corresponding to the third and final phase, chronic epilepsy [6, 21, 26].

The initial insult triggers an inflammatory response in order to repair the injured site. This otherwise beneficial process can aggravate epileptogenesis if the neuroinflammatory reaction persists for too long. A common example of the link between

neuroinflammation and epileptogenesis is observed in children, between 6 months and 5 years, where seizures are triggered by the rise in body temperature above 38.4 ºC in the absence of a CNS infection [27]. Viral or bacterial illness, certain vaccinations, and genetic predisposition are common risk factors for this general febrile response as a consequence of the release of several pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumour necrosis factor (TNF)-α [27]. Due to the increased expression of excitatory amino acid receptors, the increased cytokine release can trigger the febrile seizures, as the immature brain is hyperexcitable [28].

Prolonged febrile seizures in childhood are a precipitating event that seems to be associated with the developing of MTLE-HS, as 30 to 60% of MTLE patients reported a past history of prolonged febrile seizures [28]. Additionally, in rat pups, prolonged exposure to heated dry hair resulted in recurrent spontaneous seizures in 35% of adult animals [29]. Still, this relationship is somewhat controversial given the evidence that some patients have a pre-existing hippocampal malformation, ultimately playing a role in febrile seizure initiation and the development of hippocampal sclerosis [9, 12, 15, 30].

Although inflammation is an essential process to assure neuronal homeostasis and repair the injury site following a brain insult, the suggested correlation between prolonged febrile seizures in infants and increased MTLE-HS incidence points to a dual role of inflammation in epileptogenesis, as both preventive and favourable to this process.

1.3. Impact of neuroinflammation in epileptogenesis

To understand the impact of neuroinflammation in epileptogenesis, we must focus our attention on microglia, the resident immune cells of the central nervous system (CNS) that account for approximately 10% of brain cells. They are the primary effectors of the central inflammatory response to acute insults and chronic disorders [31, 32].

In homeostatic conditions, they survey their environment, searching for any signs of injury, infection, or abnormality by continuously extending and retracting their processes in all directions, physically interacting with other brain cells [33, 34]. Additionally, they secrete various neurotrophic factors and cytokines that support neuronal survival and function, while maintaining the integrity of the blood-brain barrier [35, 36].

However, following a brain insult, the damaged cells release danger-associated molecular patterns (DAMPs) that trigger the microglia activation and migration towards the lesion site using the chemoattractant gradient as a directional cue [37]. Once activated, microglia can adopt a variety of phenotypes, ranging from the classical pro-inflammatory (M1-like) state to the alternative anti-inflammatory (M2-like) state. The specific phenotype adopted is influenced by the nature of the stimulus, the time elapsed since exposure, and local environmental factors [38, 39].

The M1-like microglia are typically induced by exposure to bacterial products such as lipopolysaccharide (LPS), or proinflammatory cytokines, such as IL-1β, interferon (IFN)-γ and TNF-α [38, 39]. This activation leads to the production of high levels of pro-inflammatory cytokines and cytotoxic oxidative metabolites, ultimately causing inflammation and neuronal damage (Figure 2).

Regarding the M2-like microglia, there are three distinct subtypes described. The M2a phenotype, induced by anti-inflammatory cytokines, such as IL-4 and IL-13, contributes to tissue repair, regeneration and remodelling, generally functioning as an antagonist to the M1 pro-inflammatory responses. The M2b phenotype, induced by exposure to immune complexes and ligands of Toll-Like receptors, seems to have an immunoregulatory effect. Lastly, in response to specific anti-inflammatory factors, such as IL-10, glucocorticoids and growth factors, microglia adopt an immunosuppressive M2c phenotype, involved in matrix remodelling, immunoregulation and tissue remodelling [38, 39]. Microglia are capable of dynamically shifting between phenotypes, sometimes exhibiting different phenotypic markers, simultaneously expressing M1-like and M2-like markers. This prominently occurs in aging and pathological disease models [39].

Despite being an essential process for tissue repair, regeneration and remodelling, exacerbated activation of the pro-inflammatory microglial phenotype is a seizure-inducing process, as it promotes the prolonged release of pro-inflammatory cytokines that stimulate neuronal excitability by mediating synaptic neurotransmitter release.

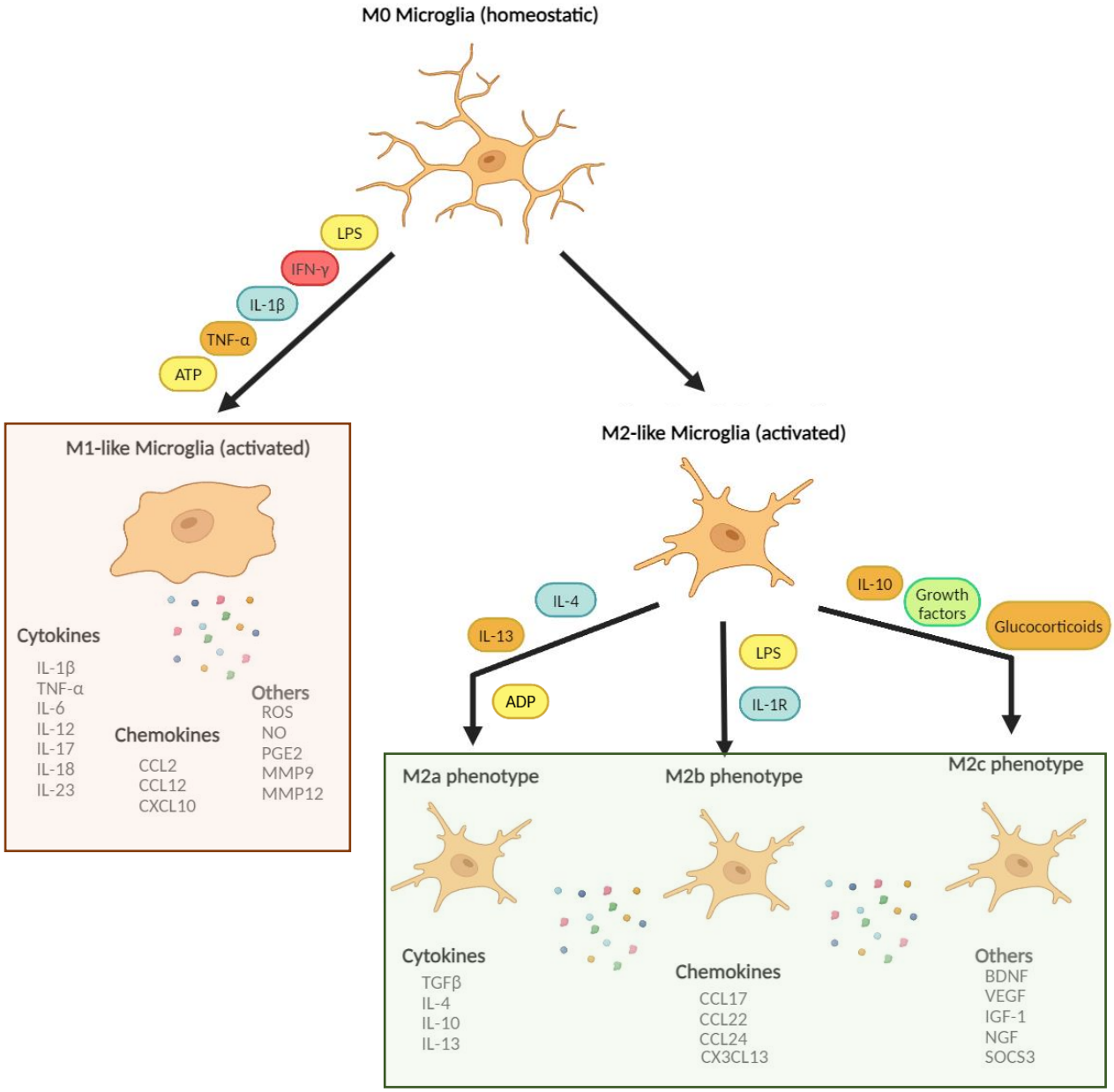

Figure 2 - Microglial activation phenotypes and released molecules. Initially in a resting, homeostatic state (M0), microglia can be activated by various signals into two main phenotypes: classically activated (M1-like) and alternatively activated (M2-like). This activation is triggered by interactions between inflammatory molecules and their specific receptors on the microglia surface. The M1 and M2 phenotypes are not just defined by their activation state, but also by the unique set of molecules they produce, such as chemokines, cytokines, and other signalling molecules. Figure created using BioRender.com, accessed on 15/09/2024.

For example, IL-1β enhances glutamate release and decreases its re-uptake, thereby increasing glutamate availability in neuronal synapses, while simultaneously decreasing GABA-mediated neurotransmission by up to 30% in TLE patients, promoting neuronal hyper-excitability and consequently inducing seizure generation [40, 41] (Figure 3). Additionally, IL-1β may contribute to glutamate-mediated neurodegeneration through the upregulation of N-methyl-D-aspartate (NMDA) receptors on post synaptic cells [42].

In the CNS, IL-6 is typically present in a low concentration prior to microglial stimulation and is upregulated by increased levels of other cytokines such as TNF-α, IL-1β, IFN-γ and IL-17 [43, 44]. This upregulation seems to be associated with decreased hippocampal neurogenesis and increased gliosis, which can contribute to epileptogenesis [44, 45].

TNF-α upregulates α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, exacerbating glutamatergic transmission, prompting an over-uptake of calcium, causing neurotoxicity [46], while simultaneously inducing GABA receptor endocytosis, reducing the inhibitory synaptic transmission essential to prevent neuronal excitability and seizure generation [47].

Exacerbated cytokine release following the initial insult dysregulates the synaptic neurotransmitter balance, inducing neuronal death, promoting neuronal hyper-excitability and seizure generation. The exacerbated ATP released from seizure-induced neuronal death further stimulates microglial activation, promoting a vicious cycle between neuroinflammation and seizure initiation.

However, it is still uncertain if in MTLE-HS seizures are initially generated as a consequence of an aggravated inflammatory reaction, or if the latter is a repercussion of the recurrent spontaneous seizures. To further delve into this question, we need to assess the specific role of microglia activation in MTLE, both during seizures and in the post- and inter-ictal phases, where the inflammatory reaction is accentuated. As microglial activation and subsequent cytokine release is mediated by different purinergic receptors, depending on the specific microglial activation state, we will focus our attention to the purinergic signalling role in epileptogenesis.

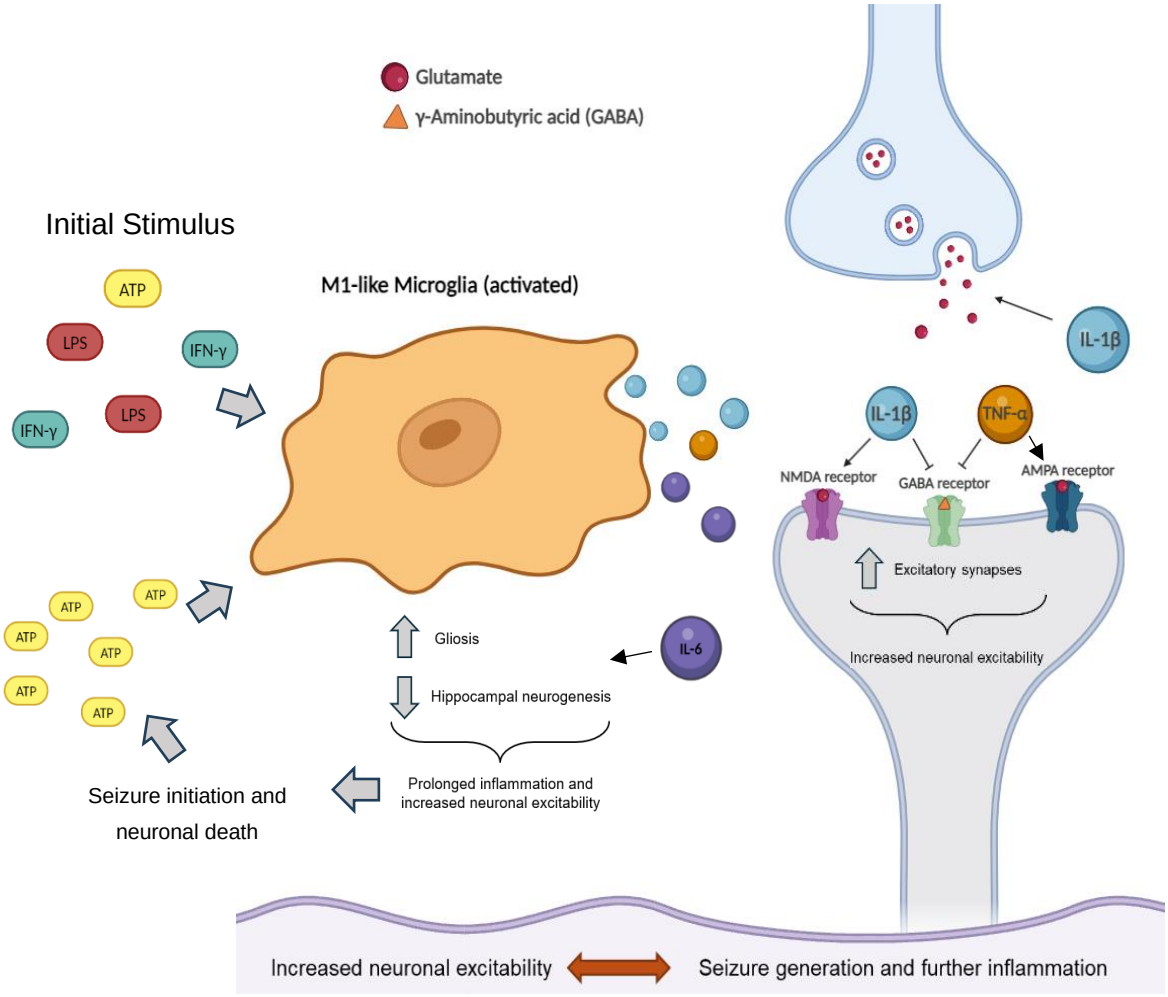

Figure 3 - Schematic representation of the vicious cycle between neuroinflammation and seizure initiation. An initial stimulus activates homeostatic microglia into the M1-like microglia phenotype. The activated microglia releases IL-1β, which enhances glutamate release and decreases its re-uptake, while simultaneously upregulating NMDA receptors and decreasing GABA-mediated neurotransmission. IL-6 decreases hippocampal neurogenesis and increases gliosis, stimulating an inflammatory environment. TNF-α upregulates AMPA receptors, while decreasing inhibitory GABAergic neurotransmission. If prolonged, the increased excitability triggers neuronal death by an over-uptake of calcium, lowering the seizure threshold and facilitating seizure generation. In turn, seizure releases abnormal amounts of ATP into the extracellular medium, further exacerbating the initial microglial activation. Figure created using BioRender.com, accessed on 15/09/2024.

1.4. Purinergic Signalling

Purinergic signalling is a fundamental communication pathway for the regulation of neuron-glia interactions, which has gained prominence in recent years. Evidence suggests that it is involved in the modulation of neuronal excitability pathways, with the ATP metabolite, adenosine, acting as an anticonvulsant and seizure terminator, observed in both *in vitro* and *in vivo* models of epilepsy [48, 49]. ATP presents a pro-convulsive and seizure initiating role, demonstrated by the increased extracellular ATP levels in seizure prone mice and increased generalised motor seizures caused by ATP

analogue microinjections in rodents [50]. It is increasingly obvious that, paired with the balance of glutamate/GABA levels, the ATP/adenosine balance is crucial to regulate brain hyperexcitability and microglia function [49].

The purinergic system is a ubiquitous signalling pathway where purine nucleotides and nucleosides are synthesized and released extracellularly, acting on pre- and post-junctional membrane receptors as modulators, transmitters, or gliotransmitters. It regulates synaptic transmission, both directly and/or through glia, responses to injury and inflammation, pain, myelination and neurogenesis [50].

Purinergic receptors are activated by purines, such as ATP and its breakdown products adenosine 5'-diphosphate (ADP) and adenosine [51, 52]. Some of these receptors also activate intracellular signalling pathways when bound to pyrimidines, for example uridine 5'-triphosphate (UTP), uridine 5'-diphosphate (UDP), and UDP-conjugated sugars, which prove to be more stable and more slowly degraded by ectonucleotide pyrophosphatases, consequently accumulating for longer periods of time [50-52].

Another intricate part of this system is the enzymatic machinery necessary to synthesize and eliminate such ligands from the extracellular space. Once released to the extracellular milieu, ATP can be broken down into ADP and AMP by different ectoenzymes, mainly the nucleoside triphosphate diphosphohydrolase-1 (NTPDase1/CD39), and AMP can be subsequently dephosphorylated into adenosine by the ecto-5'-nucleotidase (NT5E/CD73) [52, 53]. Adenosine is the substrate for adenosine deaminase to form inosine, and it can also be converted back into AMP inside cells via adenosine kinase (ADK) [52, 53].

1.4.1. P1 Receptors

The purinergic receptors can be divided into two major receptor families: P1 and P2. The P1 family is comprised by G-protein coupled receptors (GPCRs) sensitive to adenosine, the inhibitory modulator of the CNS. The adenosine receptors (ARs) can be divided into four subtypes: A_1, A_{2A}, A_{2B}, A_3.

1.4.2. P2 Receptors

Extracellular tri- and dinucleotides are endogenous ligands of the P2 receptor family [54], further subdivided into two distinct membrane receptor groups presenting

different mechanisms of action, pharmacology and sequence homology: the ionotropic P2X receptors (P2XRs) and the metabotropic P2Y receptors (P2YRs) [49].

1.4.2.1. P2X Receptors

P2X receptors are fast acting ATP-gated ion channels unable to be activated by dinucleotides or uracil nucleotides [49, 54]. P2X1-7 are the seven different mammalian subunits that have been characterised to date. These are assembled and form functional homo- or heterotrimeric channels permeable to small cations, including Na^+, K^+ and Ca^{2+} [49, 52, 54]. Independent of subunit arrangement, all P2XRs display a similar protein structure with two transmembrane domains, a large extracellular loop and an intracellular amino and carboxyl terminus [49].

P2XRs are all expressed throughout the nervous system, yet each cell type expresses a specific set of subunits [49]. P2X4R and P2X7R expression is shared by neurons, microglia, and oligodendrocytes, whereas P2X2, P2X3, and P2X6 are neuron specific receptors. P2X7R is unambiguously expressed in oligodendrocytes and microglia, similar to virtually all other cell types associated with innate and adaptative immunity [54, 56]. In rodents, P2X7R expression in neurons and astrocytes is still controversial because of the poor selectivity of agonists and the failure of some antibodies to differentiate between the large number of active and inactive splice variants of the receptor [55]. Additionally, some recent works with improved methodologies did not find any expression of this ionotropic receptor in these cell types on rodents [56-58]. Despite this uncertainty in animal models, our group has consistently observed P2X7R expression in nerve terminals (synaptosomes) isolated from both human and rat cortical samples [2, 59].

As any other ligand-gated ion channels, P2XRs have the ability to contribute to fast synaptic transmission, but the excitatory post-synaptic currents produced as a result of receptor activation are small, rare and restrict to certain cells in the brain areas where this phenomenon occurs. As such, P2XRs function in neurons falls into the category of neuromodulatory rather than truly excitatory, indirectly regulating glutamatergic and GABAergic transmission [2, 54, 59].

Besides its implication in neurotransmitter release and synaptic transmission, the P2XR family is also involved in other cellular processes such as cell adhesion, migration and chemotaxis, proliferation, differentiation, maturation and survival [49].

Interestingly, P2X7R seems to be particularly relevant to the epileptogenic process as it is associated with microglial activation, cytokine production and secretion, as well as cell death in case of prolonged activation.

1.4.2.2. P2Y Receptors

The slower-acting P2Y receptors are GPCRs activated by adenine or uridine tri- and dinucleotides, unlike P2XRs [49, 52]. In mammals, the eight different metabotropic receptors of this family that have been characterised to date are: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$. P2YRs protein structure features the canonical seven transmembrane segments of GPCRs, an extracellular amino terminal and an intracellular carboxyl terminal [49, 52].

GPCRs are bound to intracellular heterotrimeric complexes consisting of three small G-proteins: one G_α, one G_β and one G_γ subunit [51]. Upon agonist binding, these G-proteins are responsible for initiating the signalling cascade, regulating metabolic enzymes, ion channels, transporter proteins, transcription, and other parts of the cell machinery.

G_α proteins can be divided into four families with distinct downstream targets and sequence homology: the G_s, G_i, $G_{q/11}$ and $G_{12/13}$ families [51]. G_α proteins of the G_s family stimulate adenylyl cyclase, consequently increasing the production of cyclic adenosine monophosphate (cAMP), a stimulator of protein kinase A (PKA). G_i has the opposite effect, decreasing intracellular cAMP concentration via adenylyl cyclase inactivation. $G_{q/11}$ stimulates phospholipase C (PLC) and leads to increased intracellular diacylglycerol (DAG) and inositol 3 phosphate (IP3) levels, resulting in the subsequent release of Ca^{2+} from intracellular reserves, subsequently activating protein kinase C (PKC). $G_{12/13}$ signals predominantly through the small G-protein Rho [17, 49]. G_β and G_γ subunits signal independently to the G_α protein, activating additional downstream targets.

Different subtypes of P2Y receptors are coupled with distinct G_α proteins. $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$Rs are associated with the $G_{q/11}$ protein family, while $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$Rs are coupled to G_i. $P2Y_{11}$R is an exception, with the capacity to bind either to the G_s or $G_{q/11}$ protein families [17, 49].

The endogenous ligands of these metabotropic receptors vary with each receptor subtype. $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ are sensitive to both ATP and ADP, with greater affinity for the dinucleotide ligand. $P2Y_2$ and $P2Y_4$ are both activated by UTP and ATP, with $P2Y_4$

displaying a higher affinity for the uridine nucleotide. $P2Y_6$ is activated by UDP, but also UTP and ADP with lesser sensitivity. $P2Y_{14}$ is the only receptor activated by a sugar-conjugated nucleotide, with UDP-Glucose as its endogenous ligand [17].

All neuronal cell types express functional P2YRs, but certain subtypes are predominant in specific cell types. Neurons express $P2Y_1R$, $P2Y_2R$, $P2Y_4R$, $P2Y_6R$, $P2Y_{13}R$ and $P2Y_{14}R$, microglia express $P2Y_1R$, $P2Y_2R$, $P2Y_4R$, $P2Y_6R$, $P2Y_{12}R$, $P2Y_{13}R$ and $P2Y_{14}R$, astrocytes express $P2Y_1R$, $P2Y_2R$, $P2Y_4R$, $P2Y_6R$ and $P2Y_{14}R$, oligodendrocytes express $P2Y_1R$, $P2Y_2R$, $P2Y_4R$, $P2Y_6R$, $P2Y_{11}R$ and $P2Y_{13}R$. The $P2Y_{12}R$ is exclusive to microglia.

This family of purinergic receptors, particularly $P2Y_{12}R$, is essential to the recognition of early stages of neuronal damage and the clearance of cellular debris, infected or damaged cells and tissue repair [60].

The neuroprotective role of these metabotropic purinoceptors is achieved by modulating microglial polarization, process extension and migration towards extracellular nucleotides [60, 61]. Ameboid/activated microglia also exert phagocytic and pinocytic activity depending on the subtype of P2Y receptor that is activated [39].

$P2Y_{12}R$ receptors also regulates seizure-induced neurogenesis, immature neuronal projections, synaptic plasticity, blood-brain barrier repair, neuroinflammation, cell survival and microglial-neuronal physical interactions [39, 62].

1.5. Purinergic signalling in Epilepsy

1.5.1. Mechanisms of ATP release in epilepsy

Physiologically, extracellular ATP concentrations are extremely low (around 10nM) but can increase dramatically into the millimolar range during pathological conditions [63]. Neuronal hyperexcitation, such as prolonged or repeated seizures, inflammation and cell death provoke the release of ATP at millimolar concentrations to the neuronal extracellular medium, where it mediates the release of gliotransmitters, and influences synaptic structure and neuroinflammatory cascades [17].

Once in the extracellular medium, ATP will promptly be metabolized into ADP and AMP by CD39. In the brain, this ectoenzyme is exclusively expressed on microglia, and on endothelial and smooth muscle cells of the vasculature [39]. Despite the obvious

role in purinergic signalling, by regulating ATP levels, CD39 could also indirectly influence microglial polarization towards M1- or M2-like phenotypes, regulating neuroinflammatory pathways.

1.5.2. $P2Y_{12}R$ function in epilepsy

Surprisingly, the ADP-sensitive $P2Y_{12}R$, a specific microglial marker in the CNS, has not received much attention despite being extremely important to the microglia function in pathological conditions.

In earlier studies, Avignone *et al*. found a significant increase in hippocampal $P2Y_{12}R$ mRNA expression 24 and 48h following kainic acid (KA)-induced epilepsy mice models [64]. Similarly, nerve injury induces an increase in mRNA and protein expression of microglial $P2Y_{12}R$ in the spinal cord [65].

More recently, Alves *et al*. found no significant changes in the $P2Y_{12}R$ mRNA expression in the hippocampus of TLE patients and in experimental epilepsy animal models. In contrast to Avignone *et al*., shortly after status epilepticus (SE), both mRNA expression and protein levels were downregulated in KA-induced epilepsy mice models [17]. However, they later observed a significant increase in $P2Y_{12}R$ protein levels, two weeks post-SE.

This variation in $P2Y_{12}R$ expression and protein levels suggests a possible dual role in epileptogenesis, initially involved in tissue repair and seizure termination, while later promoting a seizure-inducing environment.

Recently, a new model of microglia-neuron interaction in epilepsy was proposed, suggesting that neuronal excitation and excessive glutamate release activates neuronal NMDA receptors, evoking the release of the C-X3-C motif chemokine ligand (CX3CL1) from neurons, consequently activating the microglial C-X3-C motif chemokine receptor 1 (CX3CR1) [66]. When activated, CX3CR1 induces microglial chemotaxis to the site of inflammation, promoting the release of IL-1β by the microglia, which consequently triggers ATP release by neuronal dendrites [66]. As previously mentioned, a molecular stimulus, such as increased extracellular ATP concentrations, will trigger the shift in polarization states of microglia, a dynamic heavily modulated by purinergic receptors. The chemoattractant ATP is metabolized into ADP by CD39, subsequently stimulating the $P2Y_{12}R$ and initiating the first step in microglial activation [67, 68]. This metabotropic receptor contributes to maintain the ramified microglial morphology and, when activated,

mediates the extension of microglial processes towards the site of injury, in cooperation with the A3 receptor. Both these receptors are upregulated during this stage of microglial activation [39].

Subsequently, $P2Y_{12}R$ downregulation and $A_{2A}R$ upregulation induce the retraction of these microglial processes. Simultaneously, the upregulation of the P2X4R mediates the microglial migratory activity towards the source of the ATP released. Following the total retraction of the microglial processes, microglia display an amoeboid morphology and exert phagocytosis ($P2Y_6R$), pinocytosis ($P2Y_4R$) or secretory activity (P2X4R and P2X7R) depending on the purinergic receptor involved [39].

Additionally, the detrimental effects prompted by blood-brain barrier (BBB) breakdown are attenuated by $P2Y_{12}R$ activation, inducing the rapid closure of the damaged region by forming a dense aggregate at the site of injury [69].

The functional contribution of the $P2Y_{12}R$ during epilepsy was analysed in two different epilepsy mouse models: one induced by systemic administration of KA and the other induced by intra-cerebroventricular injection of KA [70]. Eyo *et al.* also demonstrated that $P2Y_{12}R$-deficient mice exhibited reduced seizure-induced increases in microglial process numbers and more severe KA-induced seizure behaviours, indicating that $P2Y_{12}R$ might have an initial neuroprotective role [70].

Supporting this, $P2Y_{12}R$ also appears to have a more definite inhibitory role in the synaptic transmission. The activation of $G_{i/o}$-coupled P2Y receptors mediates the inhibition of Ca^{2+} channels and, consequently, decreases the voltage-dependent influx of Ca^{2+} [71, 72]. This subsequently affects the Ca^{2+}-dependent vesicular exocytosis, limiting neurotransmitter secretion and synaptic transmission [71, 72].

These $G_{i/o}$-coupled receptors also decrease cAMP production and PKA activation. This is in agreement with the inhibitory role of these receptors, as cAMP analogues have been shown to enhance epileptiform activity [73], while PKA inhibition is correlated with a significant decrease in number of seizures, seizure duration and neuronal hyperexcitability in the cortex and hippocampus of rat and mice seizure models [74-77]. This is supported by the fact that $P2Y_{12}R$ activation limits the release of noradrenaline [78, 79].

Overall, the involvement of $P2Y_{12}R$ in the microglial migratory activity paired with neurotransmitter regulation is essential for the clearance of cellular debris, infected or damaged cells and for tissue repair, preventing neuronal excitability and future seizures.

However, $P2Y_{12}R$ also mediates seizure-induced neurogenesis and the sprouting of immature neuronal projections subsequent to KA application in mice models. This is thought to create aberrant neuronal networks that pave a landscape for spontaneous recurrent seizures to occur [62]. If the delicate process of microglia activation is dysregulated, a prolonged stimulation might promote epileptogenesis, as the increase in ATP levels in the brain can generate or exacerbate seizures, supporting the involvement of ATP in brain hyperexcitability [80].

1.5.3. P2X7R function in epilepsy

The ATP-sensitive P2X7R, essential for microglial proliferation and cytokine release. Some of the initial studies concerning P2X7R expression in the brain showed an up-regulation of the purinergic receptor following kainic acid (KA) and pilocarpine-induced seizures in rat hippocampus, seemingly restricted to microglia [81, 82]. Subsequent studies consistently reported an increase in the mRNA and protein levels of the P2X7R in the hippocampus and temporal neocortex of animal epilepsy models and pharmacoresistant TLE patients. [64, 80, 83, 84].

The upregulation of P2X7R observed in TLE patients goes in accordance with the receptor's pharmacological characteristics. Due to its low affinity for ATP ($EC_{50} \geq 100\,\mu M$), P2X7R activation typically occurs under pathological conditions when extracellular ATP reaches higher concentrations [51, 52], as is the case during a SE. It plays a crucial role in mediating the release of inflammatory cytokines, particularly IL-1β and IL-18, as the application of antagonists or the genetic knockout of this receptor impairs the release of these inflammatory mediators [85-89].

Mechanistically, the release of IL-1β and IL-18 is a two-signal process. Firstly, an inflammatory signal binds to Toll-like receptor 4 (TLR4), initiating a signalling pathway that culminates in the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factor in the nucleus, consequently promoting the upregulation of the pro-inflammatory cytokines, pro-IL-1β and pro-IL-18, and the NLRP3 protein [90]. Secondly, ATP binds to the P2X7R, inducing the efflux of K^+ needed for the activation of the NLR family pyrin domain containing 3 (NLRP3) inflammasome without compromising global cellular K^+ homeostasis [88]. The NLRP3 inflammasome triggers the activation of caspase-1, which cleaves pro-IL-1β and pro-IL-18 for subsequent release of IL-1β and IL-18 [91].

This IL-1β/ NF-κB signal-induced inflammation leads to neurogenesis, sprouting, neuronal damage and death, eventually causing neuronal excitotoxicity that leads to SE [92].

Although P2X7R stimulation induces microglia proliferation, prolonged P2X7R activation might promote cell death via pyroptosis, a form of inflammatory programmed cell death where the permeability of the cell membrane increases, inducing swelling and subsequent cell death [93].

Additionally, P2X7R activation can also stimulate the production of other inflammatory mediators such as IL-6 and TNF-α [94, 95]. TNF-α can initiate another classical NF-κB signalling pathway via the activation of the TNF receptor (TNFR), promoting the activation of the NF-κB transcription factor and the release of pro-inflammatory cytokines, consequently perpetuating the inflammatory conditions in the brain [96].

While previous studies primarily identified P2X7R expression on microglia, some authors also reported a strong increase in neuronal P2X7R expression in both animal epilepsy models and TLE patients [80, 83, 84], suggesting a role in synaptic transmission. P2X7R activation seems to mediate ATP-evoked efflux of excitatory amino acids, glutamate and D-aspartate, as well as the release of the inhibitory neurotransmitter, GABA [97-101]. Additionally, prolonged P2X7R activation induces ATP release via pannexin hemichannels, boosting inflammation [102].

Although the P2X7R predominantly plays an excitatory role, promoting synaptic transmission, its activation can also prevent overexcitation when activated pre-synaptically, suggesting a context and location-specific role in neuroexcitation and synaptic transmission [103, 104].

To substantiate this excitatory role, it was observed that the genetic deletion of the P2X7R or the use of pharmacological antagonists, such as A438079 and Brilliant Blue G (BBG), displayed notable anticonvulsive properties, reducing seizure severity and duration, while restoring motor and cognitive deficits [80, 84, 105-107]. Additionally, there was a significant reduction (up to 61%) in seizure-induced neuronal death, in the hippocampus and neocortex of intra-amygdala KA model of SE in mice and rat pups, and in the pentylenetetrazole (PTZ) rat kindling model [80, 84, 105]. Conversely, pre-treatment with BzATP, a P2X7R agonist, prior to seizure-induction resulted in a significant increase in seizure duration, supporting the involvement of the ionotropic receptor in seizure exacerbation [80].

In contrast to data from KA and PTZ kindling models, Kang *et al*. found that P2X7R inhibition resulted in increased hippocampal neuronal death and exacerbated seizures. However, the same group found no changes in seizure severity following P2X7R inhibition in systemic KA or picrotoxin seizure models.

The conflicting outcomes might be a product of differences in the process of seizure induction in these models. The intra-amygdala KA-induced SE mouse model exhibits significant hippocampal neuronal damage [108], whereas the cell death is minimal in the pilocarpine-induced SE mouse model [109]. Increased cell death is accompanied by a considerable increase in extracellular ATP, essential for P2X7R activation.

A recent study further elaborated on this, demonstrating that P2X7R regulation of brain excitability seems to be cell-type dependent. While deletion of the P2X7R from microglia displayed an anti-convulsive and anti-epileptogenic effect, loss of this receptor in neurons was correlated with an increase in brain excitability, indicating a possible pro-epileptogenic role in neurons [110]. To complement this, they also demonstrated that overexpression of the P2X7R in GABAergic interneurons reduces seizures in epileptic models [110]. In alignment with these observations, our group has previously reported that P2X7R upregulation inhibits GABA reuptake by neocortex nerve terminals in human drug-resistant epilepsy [2], while also increasing GABA over glutamate release in cerebral cortex nerve terminals when external Ca^{2+} is available [59]. This suggests that neuronal P2X7Rs, under high-frequency firing, increase the bioavailability of inhibitory neurotransmitters in the brain environment, supressing a seizure-inducing environment, thereby unbalancing glutamatergic neuroexcitation.

Different P2X7R antagonist doses and delivery routes, paired with the cell type-dependent divergent effects of P2X7R activation, may account for the varying seizure-modifying actions observed.

1.6. Epigenetic modulation

As aforementioned significant changes in the expression of $P2Y_{12}R$ and P2X7R in an epilepsy setting may occur, which can be justified by a dysregulation in specific epigenetic modulators. Epigenetics is the study of how environmental factors and behaviours can cause changes in gene expression without altering the underlying DNA

sequence. Epigenetic modifications include DNA methylation, histone modification and non-coding RNAs.

MicroRNAs (miRs) are 18-25 nucleotides long, small, noncoding RNAs which regulate gene expression at the posttranscriptional level by binding to 3'-untranslated regions of the target mRNAs and promoting transcript degradation or inhibition of translation, thereby fine-tuning their function [111].

They are essential to maintain cellular homeostasis in healthy conditions and have a significant impact in pathologies in all tissues [112]. The aberrant expression of miRs has been implicated in numerous pathologies, including epilepsy.

Circulating cell-free miRNAs are associated to protein complexes and encapsulated in vesicles, preventing them from being degraded by extracellular RNases. Their freeze resistant properties, in addition to being stable in a vast array of pH levels, makes them ideal biomarkers for pathological conditions [112].

1.6.1. Impact of miR-146a in epilepsy

One of the most impactful miRNAs in the epilepsy development seems to be miR-146a, an anti-inflammatory miRNA that influences the TLR signalling pathway, regulating the expression of IL-1 receptor-associated kinase-1/2 (IRAK1/2), TNF receptor-associated factor 6 (TRAF6) (Figure 4), and typical inflammatory modulators, like NF-κB, TNF-α, IL-1β, and IL-6) [113]. Additionally, other signalling pathways targeted by miR-146a regulate biological processes aside from inflammation, including intracellular Ca^{2+} changes, apoptosis, oxidative stress and neurodegeneration [113].

Pharmacologically, the administration of intra-nasal miR-146a mimetic drugs before the injection of pilocarpine decreased seizure severity, extended the latency to generalized convulsions, and increased the percentage of animals with no induced seizures [114]. Altogether, this diminished the hippocampal damage associated with SE.

Similar results were obtained by the intra-cerebroventricular injection of a miR-146a mimetic drug in a pilocarpine-induced SE immature rat model [113, 115]. The injection of anti-miR-146a had opposite outcomes, thus shortening the latency phase, aggravating the degree and duration of seizures, and significantly increasing the levels of IL-1β, TNF-α, TLR4, and NF- κB [115].

However, Huang *et al*. observed contradictory results in the pilocarpine-induced SE rat model. The silencing of miRNA-146a significantly ameliorated the seizure-induced hippocampal damage and neuronal death and was accompanied by a decrease in IL-1β, IL-6, and IL-18 levels [113, 116].

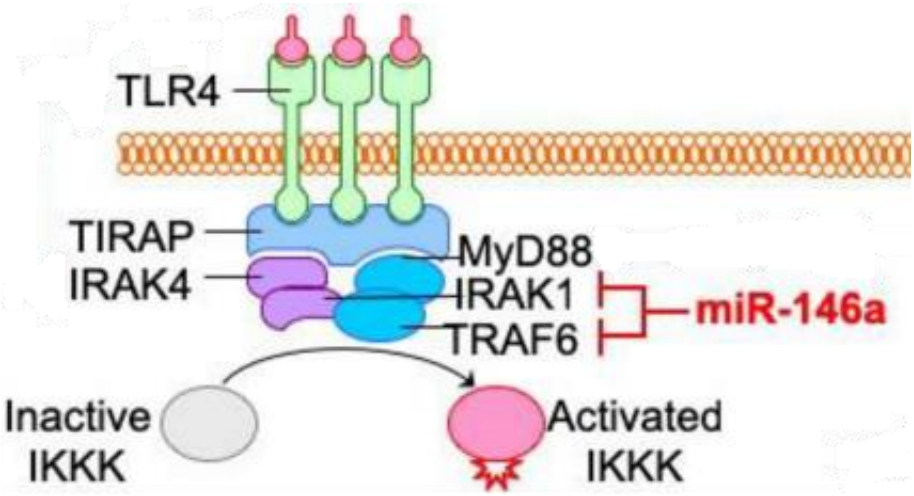

Figure 4 - Schematic representation of miR-146a mechanism of action. Adapted from Guo Y et al. Frontiers in Molecular Neuroscience. 2019;12:125. DOI: 10.3389/fnmol.2019.00125.

1.6.2. Impact of miR-22 in epilepsy

MicroRNAs can also regulate the expression of purinergic receptors. There is an inverse correlation between miR-22 serum levels and P2X7R expression in the hippocampus and neocortex of MTLE-HS patients [10]. These patients displayed a significant decrease in miR-22 serum levels.

Engel *et al*. further stated that miR-22 transcription and, consequently, P2X7R downregulation, is calcium sensitive. The miR-22 transcription is hindered if seizure activity is sufficient to elicit neuronal death, meaning that miR-22 transcription is blocked by extremely elevated Ca^{2+} levels in a KA-induced SE mouse model [117].

The intra-cerebroventricular injection of anti-miR-22 increased P2X7R expression and cytokine levels in the hippocampus, following intra-amygdala KA-induced SE in mice models. The epileptic phenotype was also aggravated as spontaneous seizures became more frequent, cognitive performance was impaired and astrogliosis was exacerbated [118]. The major pro-inflammatory and hyperexcitability effects of miR-22 silencing were prevented by treatment with a specific P2X7R

antagonist or in P2X7R knockout mice [118]. The injection of a miR-22 mimetic drug had opposite effects, supressing spontaneous seizures, further demonstrating the *in vivo* anti-seizure effect of a miR-22 [118].

The genetic deletion of miR-22 also aggravated the epilepsy phenotype following intra-amygdala KA-induced SE, whereby spontaneous seizures occurred sooner, more frequently and were longer [119]. Aberrant SE-induced neurogenesis is also supressed by miR-22, as its inhibition exacerbates aberrant migration of newly formed neurons post SE [120].

1.6.3. Impact of miR-155 in epilepsy

MicroRNAs can also promote the inflammatory reaction, as it is the case of miR-155, which is strongly associated with the microglia activation and its pro-inflammatory signaling [121, 122].

It exerts its pro-inflammatory effects by targeting anti-inflammatory regulators, such as: Suppressor of Cytokine Signaling (SOCS1) (Figure 5), a negative regulator of cytokine signalling, and SH2 Domain-Containing Inositol 5'-Phosphatase1 (SHIP1), a suppressor of TNF-α and IL-6 production [122].

SOCS1 is part of a group of proteins inhibiting the cytokine signalling pathways, particularly the JAK-STAT pathway. By inhibiting Janus tyrosine kinases (JAKs), SOCS1 prevents the phosphorylation and subsequent activation of signal transducer and activator of transcription (STAT), a promoter of the transcription and release of pro-inflammatory cytokines like IL-1β and IL-6 [122].

The miR-155 expression in TLE patients was significantly higher than healthy controls [123]. Children with chronic TLE also presented an increase in miR-155 levels, accompanied by a higher TNF-α expression [124].

KA and pilocarpine-induced SE mice models displayed significantly increased levels of miR-155 following SE [124, 125]. The antagonism or genetic silencing of miR-155 alleviated the seizure severity and reduced the seizure duration and frequency, concomitantly lowering seizure-induced mortality and reducing hippocampal apoptosis. Additionally, there was a decrease in microglia activation and cytokine release, with lower IL-1β and IL-6 protein levels [124-127].

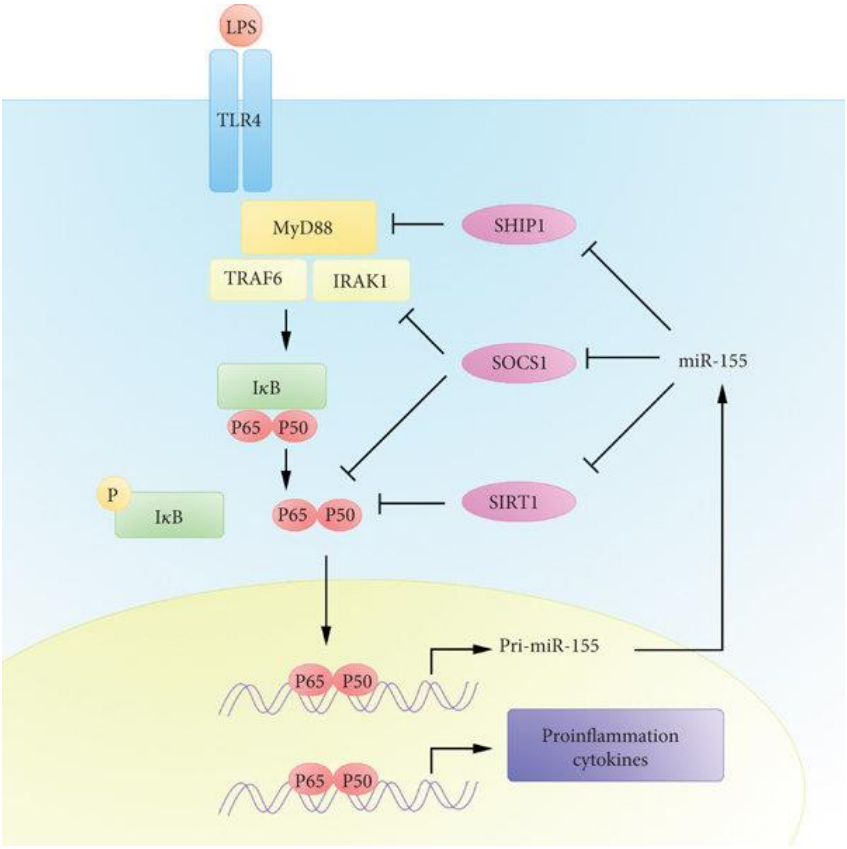

Figure 5 - Schematic representation of miR-155 mechanism of action. Adapted from Chen et al. Mediators of Inflammation. 2021(9);1-10. DOI: 10.1155/2021/8874854

1.6.4. Impact of miR-21 in epilepsy

By targeting and inhibiting pathways responsible for pro-inflammatory cytokine release and apoptosis, miR-21 seems to have a significant role in epileptogenesis.

Tang *et al*. demonstrated KA-induced seizures in rats resulted in an increase in that both miR-21 and mammalian target of rapamycin (mTOR) expression, whereas phosphatase and tensin homolog (PTEN) was downregulated during acute, latent, and chronic stages of epileptogenesis. This demonstrates that PTEN-mTOR is a target of miR-21 in a KA model of epilepsy [128].

PTEN dephosphorylates phosphatidylinositol (3,4,5)-trisphosphate (PIP3), thereby inhibiting the activation of Protein Kinase B (AKT). By downregulating PTEN, miR-21 indirectly activates the PI3K/AKT signaling pathway (Figure 6). AKT, once activated, can then phosphorylate and activate mTOR, promoting cellular growth, proliferation, and survival.

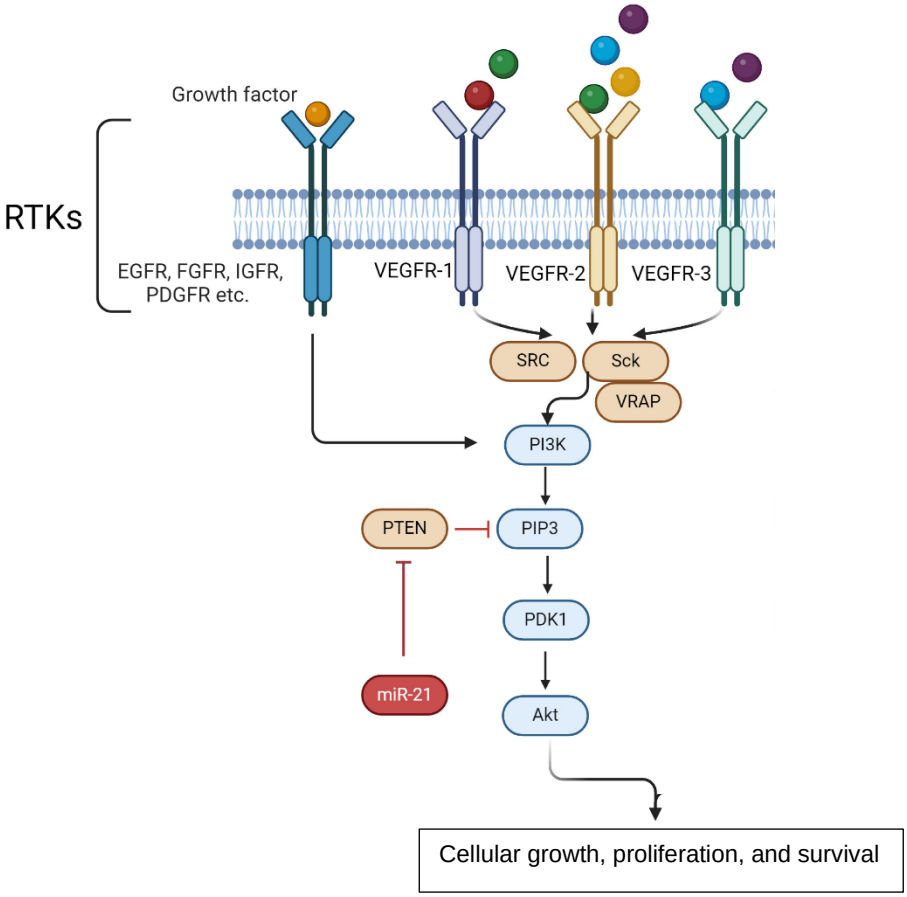

Figure 6 - Schematic representation of miR-21 mechanism of action. Adapted from Chen et al. Cells 2024, 13, 948. https://doi.org/10.3390/cells13110948

The genetic silencing of miR-21 24 hours prior to SE reduced seizure severity and neuronal death, while improving the cognitive and memory impairment [128]. Considering this, miR-21 seems to aggravate the severity of the pathology in the KA-induced epilepsy rat model.

Further elaborating on the role of miR-21, Zhang *et al*. described a possible interaction between miR-21 and STAT3. Contrary to what was previously found, the group observed a decrease in the expression level of miR-21 following pilocarpine-induced SE in the hippocampus of rat models [129]. This was associated with an increase in STAT3, IL-6 expression levels and loss of hippocampal neurons [129].

The inhibition of miR-21 5 hours post-SE further increased the expression level of IL-6, promoting neuroinflammation and aggravating the hippocampal neuronal death associated with SE, suggesting that miR-21 might have a neuroprotective role via the STAT3 signalling pathway [129].

However, other studies have disparagingly reported an increase in miR-21 expression following pilocarpine-induced SE in rat models [130, 131]. Despite this, it

seems that miR-21 role in epilepsy might vary pre- and post-SE by regulating distinct signalling pathway.

1.6.5. Impact of miR-223 in epilepsy

Even though not much is known about the role of miR-223 in epilepsy and neuroinflammation, evidence suggests that it might be a new prospect for the treatment of drug-resistant MTLE-HS.

The experimental validation of the predicted binding site for miR-223 supports the notion that $P2Y_{12}R$ mRNA seems to be a possible target, revealing a possible influence in the microglial function [132, 133].

Accordingly, miR-223 levels were elevated in the serum of epileptic children, in the temporal neocortex of TLE patients and in KA-induced SE mouse models, suggesting a possible involvement of this microRNA in epileptogenesis [134, 135].

In hippocampal microglia of TLE patients and the murine KA model of TLE, sequestosome 1 (P62) expression was remarkably increased in association with miR-223, whereas autophagy-related 16-like 1 (ATG16L1) and microtubule-associated protein light chain 3 (LC3) levels were significantly decreased [134]. ATG16L1 was determined as a direct target of miR 223 using a dual-luciferase reporter assay [134] (Figure 7).

ATG16L1 forms a crucial complex essential for the elongation of the phagophore, which eventually matures into the autophagosome. This complex facilitates the eventual lipidation of LC3, a pivotal step in autophagosome membrane expansion and cargo recognition. A decrease in ATG16L1 levels would mean that autophagy and cellular homeostasis was compromised.

Treatment with an antagonist of miR-223 alleviated epilepsy, prevented abnormalities in electroencephalography (EEG) recordings and increased the ATG16L1 and LC3 levels in KA-treated mice [134].

These findings show that miR-223 affects microglial autophagy via ATG16L1 in the KA model of TLE, possibly aggravating seizure severity.

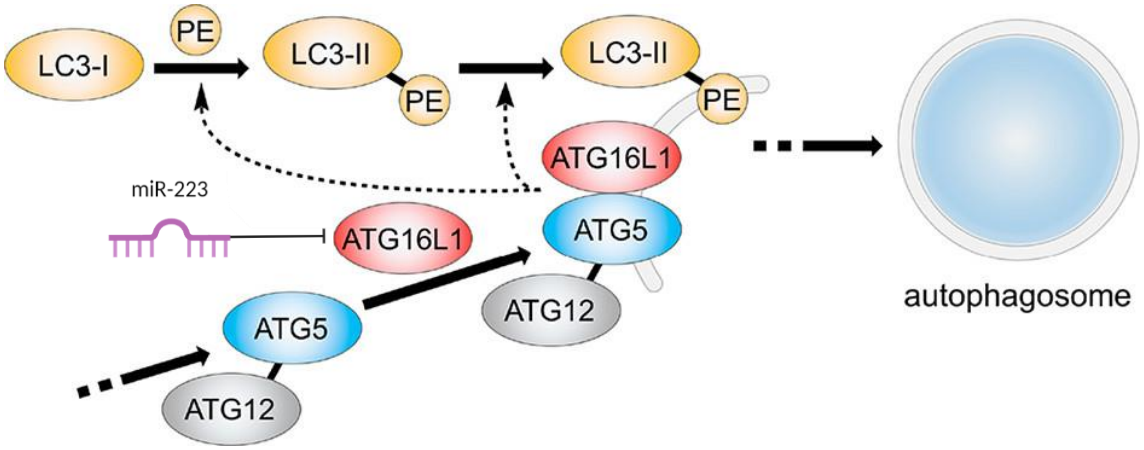

Figure 7 - Schematic representation of miR-223 mechanism of action. Adapted from Cui et al. Journal of the American Chemical Society 2022 144 (38), 17671-17679 DOI: 10.1021/jacs.2c07648

Chapter 2: Aim

MTLE-HS is a highly drug-resistant form of epilepsy and it typically follows a three-phase progression. An initial brain insult, which triggers a cascade of pathological processes, followed by a latent phase marked by the reorganization of neural circuits, which transforms normal brain networks into epileptic ones. Finally, a chronic phase characterized by recurrent spontaneous seizures [6, 21, 26]. This progression is tied closely to an imbalance between inhibitory and excitatory signals in the brain.

Neuroinflammation was identified as a key factor in driving these epileptic changes, known as epileptogenesis. The inflammatory response, while crucial for tissue repair after the initial brain injury, can exacerbate epileptogenesis if it becomes chronic. Prolonged febrile seizures in childhood are cited as a potential precipitating event, with 30–60% of MTLE-HS patients reporting a history of such seizures [28]. This suggests that inflammation may play a dual role in MTLE-HS: while it is necessary for neuronal homeostasis and injury repair, excessive or prolonged inflammation can facilitate the onset and progression of epilepsy.

The role of microglia, the resident immune cells of the CNS, is central to this neuroinflammatory process, as they are the primary responders to both acute brain insults and chronic neurological disorders [31, 32]. Pro-inflammatory activation of microglia has significant consequences in epileptogenesis. When excessively activated, microglia release pro-inflammatory cytokines, which increase neuronal excitability by influencing synaptic neurotransmitter release, exacerbating seizure severity. However, there remains uncertainty regarding whether this exaggerated inflammatory response is a cause of seizures in MTLE-HS patients or a consequence of the recurrent seizures themselves. This question is particularly relevant for understanding the role of microglial activation in different seizure phases (ictal, postictal, and interictal), where inflammation is most pronounced.

Given that microglial activation is strongly regulated by purinergic receptors, we set out to study how purinergic signalling influences microglial activation and function, with a particular emphasis on the microglia-specific ADP-sensitive $P2Y_{12}R$, which is exclusively expressed by these cells in the CNS. During epileptic seizures, elevated synaptic activity and neuronal death leads to increased levels of extracellular ATP, which is broken down into ADP and adenosine. In experimental models, SE induces a temporary decrease in $P2Y_{12}R$ levels. However, during the chronic phase of epilepsy, $P2Y_{12}R$ becomes overexpressed, corresponding with sustained microglial activation.

This suggests that $P2Y_{12}R$ plays a dual role, both in the early stages of seizures and in chronic epilepsy and may represent a target for therapeutic interventions.

A new model of microglia-neuron interaction has recently been proposed, adding further complexity to our understanding of neuroinflammation in epilepsy. This model suggests that neuronal excitation and the excessive release of glutamate during seizures activate neuronal NMDA receptors, which in turn trigger the release of CX3CL1 from neurons. CX3CL1 then binds to and activates the CX3CR1 on microglia [66]. Once activated, CX3CR1 induces the release of IL-1β by microglia, which plays a critical role in the inflammatory response. IL-1β release further triggers the release of ATP from neuronal dendrites, perpetuating the cycle of neuroinflammation. This model highlights a feedback mechanism between neurons and microglia where neuronal hyperactivity directly stimulates microglial activation via chemokine signaling, driving further cytokine release and ATP-mediated microglial activation. This cascade could exacerbate both neuronal excitability and the inflammatory response, contributing to seizure generation and progression in MTLE-HS.

As seizures progress, the neuronal death caused by these events releases further ATP, which in turn stimulates continued microglial activation. This creates a vicious cycle where neuroinflammation and seizure initiation reinforce one another: increased ATP levels due to neuronal death sustain microglial activation, worsening inflammation and promoting further seizures.

To explore this process, our study focused on the role of purinergic signalling, specifically the $P2Y_{12}R$ receptor, in human MTLE-HS and how it is modulated epigenetically. To achieve this, we analysed the $P2Y_{12}R$ and CX3CR1 mRNA expression in human MTLE-HS brain tissue and develop a monocyte-derived microglia-like cell culture model. This model will be used to assess $P2Y_{12}R$ functions in both homeostatic and inflammatory conditions, and how it is regulated by other purinoceptors and epigenetic factors, such as microRNAs. The ultimate goal was to clarify the impact of neuroinflammation, driven by purinergic signalling, on MTLE-HS and its progression, hoping at identifying novel targets for epilepsy treatment.

Chapter 3: Materials and Methods

3.1. Brain samples from epileptic patients and cadaveric controls

Resected fresh human brain samples were obtained from 19 MTLE-HS patients (11F / 8M, 43.51 ± 9.96 years old) (Table 1) who underwent epilepsy surgical treatment (selective amygdalohippocampectomy or anterior temporal lobectomy) at the Neurosurgery Department of Centro Hospitalar e Universitário de Santo António - Unidade Local de Saúde de Santo António (CHUdSA-ULSSA). The decision for surgery was taken by the multidisciplinary epilepsy team incorporating neurologists, neurosurgeons, neuroradiologists, neurophysiologists, and neuropsychologists [10, 136].

Surgical specimens of the hippocampus and anterior temporal lobe (neocortex) were collected. A complete coronal slice of 0.5 cm thickness was removed 3 cm posterior to the tip of the temporal pole. Samples were recovered in ice-cold artificial cerebrospinal fluid (CSF: 10 mM glucose, 124 mM NaCl, 3 mM KCl, 1 mM MgCl2, 1.2 mM NaH_2PO_4, 26 mM $NaHCO_3$, 2 mM $CaCl_2$, pH = 7.40) and immediately cryopreserved in liquid nitrogen. The amount of tissue removed did not differ from the strictly necessary for successful surgical practice.

Control brain samples were obtained using an identical procedure on 10 human cadavers (8M / 2F, 67.10 ± 10.93 years old) with no previous history of neurologic disease, which were submitted to forensic autopsies performed within 4–7 h after post-mortem, the tissue viability window for functional assays [10, 136].

Brain samples were discarded if post-collection assessment revealed active infectious or neoplastic diseases, chronic inflammatory conditions, neurologic poisoning, and/or past history of alcoholism and drug addiction. Brain samples were made available by the Instituto Nacional de Medicina Legal e Ciências Forenses - Delegação do Norte (INMLCF-DN), according to Decree-Law 274/99, of 22 July, published in Diário da República - 1st SERIE A, No. 169, of 22-07-1999, Page 4522, regarding the regulation on the ethical use of human cadaveric tissue for research.

Table 1 - Clinical and demographic data from MTLE-HS patients submitted to gene expression quantification.

Clinical/demographic data	***MTLE-HS patients***
F / M	11 / 8
Age ± SD, years (range)	43.51 ± 9.96 (29-63)
Age of onset ± SD, years (range)	10.95 ± 7.55 (1-28)
Disease mean duration ± SD, years (range)	32.56 ± 9.07 (18-53)
Hippocampal Sclerosis (Left / Right)	12 / 7
Febrile Seizures antecedents (Yes / No)	11 / 8

3.2. RNA extraction from brain tissue samples

The RNA was isolated using the commercially available RNeasy Lipid Tissue Mini Kit® (Qiagen, Germany), according to the manufacturer's instructions. Briefly, tissue samples were removed from -80 ºC storage, avoiding thawing during handling and homogenised with 1 mL QIAzol Lysis Reagent (Qiagen, Germany)

The homogenate was further mixed by pipetting up and down and posteriorly incubated at room temperature for 5 minutes, ensuring cell lysis and the dissociation of nucleoprotein complexes. Post incubation, 200 µL of chloroform were added to the homogenate and thoroughly mixed by vortexing, followed by an incubation at room temperature for 3 minutes. Afterwards, the samples were centrifuged for 15 minutes at 12000 g at 4ºC in a Sigma 2-16K (Sigma Zentrifugen, Germany) centrifuge. The upper aqueous phase, containing RNA, was transferred to a new collection tube containing 600 µL volume of ethanol 70% and it was thoroughly mixed by vortexing. The transfer of the interphase and organic phase, containing DNA and proteins, respectively, was avoided. The samples were then pipetted into a RNeasy MinElute spin column and centrifuged at 12000 g for 30 seconds at room temperature in a Sigma 1-14 MicroCentrifuge (Sigma Zentrifugen, Germany). The flow-through was discarded and the column was washed at 12000 g for 30 seconds at room temperature with 700 µL of RW1 and 500 µL of RPE buffer, supplied with the kit. 50 µL of RNase-free water were directly added to the centre of the column membrane. To elute the mRNA >200 nucleotides, the column was centrifuged at 16000 g for 1 minute and 20 seconds at room temperature. This elution

step was repeated using 30 µL of RNase-free water, providing a second RNA sample with less concentration. RNA samples were stored at -80 ºC until further use.

3.3. RNA quantification

RNA quantification and purity were evaluated using a NanoDrop 1000 Spectrophotometer v3.6.0 (Thermo Fisher Scientific, USA). The absorbance measurement at 260 nm allows for the nucleic acid quantification, whereas at 230 nm and 280 nm it determines the concentration of organic salts and proteins in the sample, respectively. Samples with a 260/280 nm and 260/230 nm absorbance ratio between 1.5 and 2.0 were considered acceptable. RNA degradation was not assessed but according to previous reports, it remains stable for at least 36 h post-mortem in the case of human brain samples, and storage at -80 ºC for up to 23 years yields high-quality RNA [137].

3.4. cDNA synthesis from human brain tissue samples RNA

RNA was reverse-transcribed to cDNA using the commercially available NZY First-Strand cDNA Synthesis Kit (NZYTech, Portugal), according to the manufacturer's instructions. The total volume of the reaction solution was 20 µL per sample. Each solution was comprised by the RNA sample, NZYRT Master Mix and NZYRT Enzyme Mix. The volumes and final concentrations of each component are described in Table 2. The NZYRT Master Mix includes oligo(dT)18 primers, random hexamers, Mg^{2+} (reverse transcriptase co-factor), deoxynucleotide triphosphates (dNTPs) and an optimized reverse transcription (RT) buffer containing enhancers and stabilizer additives. The NZYRT Enzyme Mix includes both NZY Reverse Transcriptase (RNase H minus) and a powerful NZY Ribonuclease Inhibitor to protect RNA against degradation due to ribonuclease contamination. cDNA synthesis was performed using a MiniAmp Plus Thermal Cycler (Applied Biosystems by Thermo Fisher Scientific, USA), employing the thermic profile described in Table 3. Upon conclusion of the first-strand cDNA synthesis, 1 µL of RNase H (from *E. coli*) was added to the samples, with the purpose of degrading the RNA template in cDNA-RNA hybrids. A second and final reaction is then performed employing the thermic profile described in Table 4.

Table 2 - Volume and concentration of each solution used in the reverse transcription of brain tissue mRNA into cDNA.

Solutions	Volume (µL)	Initial Concentration	Final Concentration
NZYRT 2X Master Mix	10	2 X	1 X
NZYRT Enzyme Mix	2	200 U/µL	20 U/µL
RNA	8	20ng/µL	8ng/µL

Table 3 - Thermic profile used in the reverse transcription of mRNA into cDNA.

Temperature	Time
25ºC	10 minutes
50ºC	30 minutes
85ºC	5 minutes
4ºC	Hold

Table 4 - Thermic profile used for the RNase reaction.

Temperature	Time
37ºC	20 minutes
85ºC	5 minutes
4ºC	Hold

3.5. Quantification of gene expression in human brain tissue samples

The quantification of the genetic expression of the $P2Y_{12}R$, CX3CR1 and the reference gene Ubiquitin C (UBC) in brain tissue was performed by TaqMan Real Time PCR. The total volume of the reaction solution was 8 µL per sample. Each solution was comprised by the cDNA sample, a specific primer and probe solution (TaqMan Gene

Expression Assays, Applied Biosystems, USA) and a NZYSpeedy qPCR mastermix (NZYTech, Portugal). The specific primers and probes used are detailed in Table 5, while the volumes and final concentrations of each component of the solution are described in Table 6. The reaction was performed using a Rotor-Gene 6000 Real Time Thermocycler machine (Corbett Research, UK), employing the thermic profile described in Table 7. Each reaction was performed in triplicate and the average cycle threshold (Ct) value was used in the analysis. The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. ΔCt is a reference gene normalization in which ΔCt = Ct target gene – Ct reference gene. The ΔΔCt is calculated with the formula ΔCt patients - ΔCt controls.

Table 5 - List of primers and probes used in the TaqMan Real Time-PCR, for both brain tissue and cell mRNA quantification.

Primer and probe solution	Assay ID
$P2Y_{12}R$	hs00224470_m1
P2X7R	hs0017521_m1
CX3CR1	hs00365842_m1
IL-1β	hs01555410_m1
UBC	hs05002522_g1

Table 6 - Reaction conditions for the quantitative TaqMan Real Time-PCR in the qRT-PCR.

Solutions	Volume (µL)	Initial Concentration	Final Concentration
NZYSpeedy qPCR mastermix	4	2 X	1 X
TaqMan probes	0.4	20 X	1 X
cDNA	1	---	
ddH_2O	2.6	---	

Table 7 - Thermic profile used in gene expression assays.

Temperature	Time	Number of cycles
95ºC	10 minutes	1
92ºC	15 seconds	50
58ºC	40 seconds	
72ºC	20 seconds	

3.6. Data presentation and statistical analysis from brain tissue sample

$P2Y_{12}R$ and CX3CR1 mRNA levels were expressed as fold change of control individuals. Results are expressed as mean ± standard error of the mean (SEM) and the ‘‘n’’ shown in graphs represents the total number of individuals. Normal distribution was evaluated with Kolmogorov-Smirnov test. Spearman’s correlation coefficients were used to test interactions between age, age at onset, disease duration, and expression levels. Differences in ΔCt were evaluated using the unpaired Student’s t-test and the Mann-Whitney test, accordingly. Statistical analyses were performed with SPSS (Statistical Package for the Social Sciences) v29 and $p < 0.05$ values were considered statistically significant.

3.7. Blood samples from epileptic patients and healthy controls

Serum samples were obtained from peripheral blood of 15 MTLE-HS patients (1M / 14F, 51.4 ± 13.8 years) (25 – 70) and 5 age-matched controls (1M / 4F, 51.2 ± 9.9 years) (40 – 65) without known neurological diseases (Table 8). Patients were followed up at the Epilepsy Outpatient Clinic of the CHUdSA-ULSSA. Control individuals were voluntarily recruited among blood donors, ethnically matched, from the same geographic region.

Table 8 - Clinical and demographic data from MTLE-HS patients and healthy controls submitted to microRNA expression quantification.

Clinical/demographic data	***MTLE-HS patients***	***Healthy Controls***
F / M	14 / 1	4 / 1
Age ± SD, years (range)	51.4 ± 13.8 (25-70)	51.2 ± 9.9 years (40 – 65)

3.8. *In vitro* differentiation of monocytes into microglia-like cells and macrophages

The protocol for monocyte-derived microglia-like (MMG) cells differentiation was adapted from previously described methods [138, 139]. Whole blood was collected using BD Vacutainer® CPT™ tubes (BD Biosciences, USA), containing a Ficoll™ Hypaque™ solution, which allows for the separation of peripheral blood mononuclear cells (PBMCs) by centrifugation. The centrifugation was performed at 1690 g for 30 minutes at room temperature in a Heraeus Megafuge 16R centrifuge (Thermo Fisher Scientific, USA). The PBMCs (in the upper layer) were then transferred onto a 15 mL Falcon tube and, then, separated from the plasma by centrifugation at 600 g for 10 minutes at room temperature. Subsequently, they were re-suspended in 1 X phosphate-buffered saline (PBS) (Gibco, Thermo Fisher Scientific, USA) and centrifuged once more at 600 g for 10 minutes at room temperature, as a washing step.

PBMCs were plated, at a density of 2 million cells per well, in 6-well plates (Sarstedt, Portugal) with a RPMI Medium 1640 + GlutaMAX™ (Gibco, Thermo Fisher Scientific, USA) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin. Cell cultures were incubated at 37 ºC, 5% CO_2 and saturating humidity. After 24h, the medium was removed, together with all the non-adhering cells. Adherent monocytes were culture in RPMI Medium 1640 + GlutaMAX™ containing 100 U/mL penicillin, 100 µg/mL streptomycin, and supplemented with 10 ng/mL human M-CSF, 10 ng/mL human GM-CSF, 10 ng/mL human β-NGF, 20 ng/mL human TGF-β2, 100 ng/mL human IL34 and 100 ng/mL human CCL2 (PeproTech, Thermo Fisher Scientific, USA) for MMG differentiation. As controls, monocyte-derived macrophages were obtained from adherent monocytes cultured in RPMI Medium 1640 + GlutaMAX™ with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and supplemented with 25

ng/mL human M-CSF (Figure 1). Media changes were performed at day three and five maintaining the differentiation conditions (Figure 1).

As an inflammatory stimulus, 100 ng/mL of LPS was added to culture medium at day 5. Cell collection was done at day seven. Cells were washed once with PBS, and Versenne, a non-enzymatic dissociation buffer, was added for cell detachment. After the addition of RPMI Medium 1640 + GlutaMAX™ containing 100 U/mL penicillin, 100 µg/mL streptomycin and 10 % FBS to the wells, cells were gently scrapped and collected. The cell suspension was centrifuged at 800 g for 5 minutes at 4 ºC and the supernatant was discarded. 350 µL of RLT buffer, a lysis buffer, were added to the cells and thoroughly mixed. The cell lysate stored at -80 ºC until RNA extraction.

Cell culture media was collected, separated in multiple aliquots and stored at -80 ºC until further use.

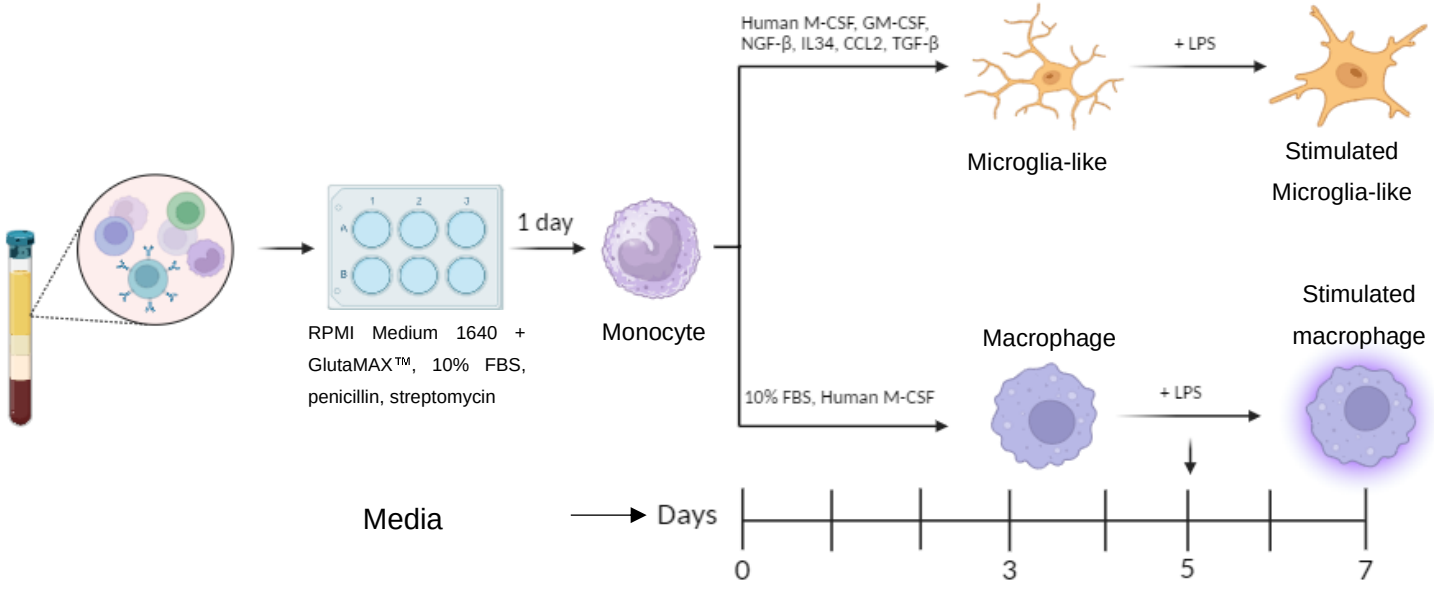

Figure 8 - Schematic representation of the in vitro differentiation of monocytes into microglia-like cells and macrophages. Media changes were performed at day 3 and 5, maintaining differentiation conditions. LPS stimulation (100 ng/mL) was done at day 5. The cells and media were collected at day 7.

3.9. Monocyte extraction from blood samples

Peripheral blood collected using 5% VACUETTE® EDTA tubes (Greiner Bio-One, Austria) was centrifuged at 1500 g for 15 minutes at room temperature in a Rotofix 32A centrifuge (Hettich, Germany). The plasma and buffy coat were extracted, together with some erythrocytes. Red blood cell lysis (RBCL) buffer (Tris- HCl 1mM pH 7.2, NaCl 5 M

and $MgCl_2 \cdot 6H_2O$ 1 M) was added to lyse the erythrocytes, and the solution was centrifuged at 828 g for 10 minutes at 4 ºC in a Heraeus Megafuge 16R centrifuge (Thermo Fisher Scientific, USA). The supernatant was discarded, the leukocytes were re-suspended in RBCL and the solution was once again centrifuged. The pellet (PBMCs) was stored at -20 ºC until the monocyte extraction.

For monocyte extraction, the PBMCs were thawed and re-suspended in PBS (Gibco, Thermo Fisher Scientific, USA) and subsequently centrifuged at 600 g for 10 minutes at room temperature. The supernatant was discarded, and the pellet was washed with magnetic-activated cell sorting (MACS) buffer (Miltenyi Biotec, Germany). Following a centrifugation with the previously described conditions, the pellet was yet again re-suspended in MACS buffer and 150 µL of $CD14^+$ MicroBeads (Miltenyi Biotec, Germany) were added to the suspension. The beads are 50 nm superparamagnetic particles that are conjugated to highly specific antibodies against the CD14 cell surface antigen. Following a 20-minute incubation at 4 ºC, the cells were transferred to LS Columns (Miltenyi Biotec, Germany) in a MACS® MultiStand (Miltenyi Biotec, Germany) and were extracted using positive selection with the $CD14^+$ MicroBeads. The columns were washed 3 times with MACS buffer before eluting. The $CD14^+$ monocyte suspension was centrifuged at 600 g for 5 minutes at 4 ºC and the supernatant was discarded. 350 µL of RLT buffer, were added to the cells and thoroughly mixed by pipetting up and down and vortexing. The cell lysate was transferred to a 1,5 mL Eppendorf and stored at -80 ºC until RNA extraction.

3.10. Analysis of gene expression of monocytes, macrophages and microglia-like cells

The RNA was isolated from the cells using the commercially available AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Germany), according to the manufacturer's instructions. Briefly, the homogenized lysate was thawed and transferred to an AllPrep DNA column and centrifuged at 16000 g for 3 minutes at room temperature in a Sigma 1-14 MicroCentrifuge (Sigma Zentrifugen, Germany). The column was used for DNA extraction and stored at 4 ºC for further use. The flow-through was used for RNA extraction. 600 µL of ethanol 70% was added to the flow-through and thoroughly mixed by pipetting up and down and vortexing. The sample was transferred to a RNeasy column, centrifuged at 16000 g for 3 minutes at room temperature and the flow-through was discarded. The flow-through was discarded and the column was washed at 16000

g for 3 minutes at room temperature with 700 µL of RW1 and 500µL of RPE buffer, supplied with the kit. RNA was eluted with the addition of 15 µL of RNase-free water to the column membrane and centrifugation at 16000 g for 5 minutes at room temperature. This elution step was repeated using another 15 µL of RNase-free water. Obtained RNA was reverse-transcribed to cDNA using the commercially available NZY First-Strand cDNA Synthesis Kit (NZYTech, Portugal), according to the manufacturer's instructions as described in in section 1.4.

The quantification of the mRNA levels of the $P2Y_{12}R$, P2X7R, IL-1β and UBC genes was performed by TaqMan Real Time PCR. The experimental protocol was identical to the one described in section 1.5. (See above). The specific primers and probes used are detailed in Table 5. Each reaction was performed in triplicate and the average Ct value was used in the analysis. The relative expression was calculated using the $2^{-\Delta\Delta Ct}$, as previously described.

3.11. cDNA synthesis from the culture media microRNA

The microRNA was reverse-transcribed to cDNA using the commercially available TaqMan™ MicroRNA Reverse Transcription kit (Applied Biosystems by Thermo Fisher Scientific, USA), according to the manufacturer's instructions. The total volume of the reaction solution was 7.5 µL per sample. Each solution was comprised by the microRNA sample, a specific primer, dNTPs, Reverse Transcriptase (MultiScribe™), Reverse Transcription Buffer, RNase Inhibitor and nuclease-free water. The specific primers used are detailed in Table 9, while the volumes and final concentrations of each component of the solution are described in Table 10. After gently mixing the solution, the cDNA synthesis was performed using a MiniAmp Plus Thermal Cycler (Applied Biosystems by Thermo Fisher Scientific, USA), employing the thermic profile described in Table 11.

Table 9 - List of primers used in the reverse transcription of microRNA into cDNA.

Primers	Assay ID
miR-146a	000468
miR-22	000398
miR-155	002623
RNU48	001006
miR-21	000397
miR-223	002295

Table 10 - Volume and concentration of each solution used in the reverse transcription of microRNA into cDNA.

Solutions	Volume (µL)	Initial Concentration	Final Concentration
dNTPs	0.08	100 mM	1 mM
Reverse Transcriptase	0.5	50 U/µL	3,33 U/µL
Reverse Transcription Buffer	0.75	10 X	1 X
RNase Inhibitor	0.1	20 U/µL	0,27 U/µL
DdH_2O	2.08	-----	
Primer	1.5	5 X	1 X
microRNA	2.5	2 ng/µL	0,67 ng/µL

Table 11 - Thermic profile used in the reverse transcription of microRNA into cDNA.

Temperature	Time
16ºC	30 minutes
42ºC	30 minutes
85ºC	5 minutes
4ºC	Hold

3.12. MicroRNA extraction from the culture media of microglia-like cells and macrophages

The microRNA was extracted using the miRNeasy Serum/Plasma Kit® (Qiagen, Germany), according to the manufacturer's instructions.

After thawing, 1mL of QIAzol Lysis Reagent (Qiagen, Germany) was added to 200 mL of sample, mixed by vortexing, and posteriorly incubated at room temperature for 5 minutes. Chloroform of an equal volume to the starting sample was added to the homogenate and thoroughly mixed by vortexing, followed by an incubation at room temperature for 3 minutes. Post incubation, the samples were centrifuged for 15 minutes at 12000 g at 4 ºC in a Sigma 2-16K (Sigma Zentrifugen, Germany) centrifuge. The upper aqueous phase, containing RNA, was transferred to a new collection tube containing 900 µL volumes of ethanol 100% and it was thoroughly mixed by pipetting. The samples were then pipetted into a RNeasy MinElute spin column and centrifuged at 12000 g for 30 seconds at room temperature in a Sigma 1-14 MicroCentrifuge (Sigma Zentrifugen, Germany). The flow-through was discarded and the column was washed at 12000 g for 30 seconds at room temperature with 700 µL of RWT and 500 µL of RPE buffer, supplied with the kit. It was posteriorly washed with 80% ethanol, at 12000 g for 2 minutes and 45 seconds at room temperature. The RNeasy MinElute spin column was then placed in a new collection tube, with the lid open, and centrifuged at 16000 g for 5 minutes and 45 seconds at room temperature to dry the membrane. 14 µL of RNase-free water were directly added to the centre of the column membrane. Lastly, to elute the microRNA, the column was centrifuged at 16000 g for 1 minute and 20 seconds at room temperature. RNA samples were stored at -80 ºC until further use.

3.13. Quantification of microRNA expression in the cell culture media

The quantification of the expression of miR-146a, miR-22, miR-155, miR-21, miR-223 and, the RNU48 used for normalisation, was performed by TaqMan Real Time PCR. The experimental protocol was identical to the one described in section 1.5. (See above). The specific primers and probes used are detailed in Table 9, Each reaction was performed in triplicate and the average Ct value was used in the analysis. The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method, as previously described.

3.14. Data presentation and statistical analysis from cell mRNA and microRNA expression

For the MMG cell culture model characterization, $P2Y_{12}R$ and CX3CR1 expression was displayed as a percentage of cell cultures expressing the receptors. For the functional characterization of the model, $P2Y_{12}R$, P2X7R and IL-1β mRNA levels were expressed as fold change of control individuals and as fold change of non-stimulated cells. Medium miR-146a, miR-22, miR-155, miR-21 and miR-223 levels were expressed as -ΔCt. Results are expressed as mean ± SEM and the ‘‘n’’ shown in graphs represents the total number of individuals. Differences in ΔCt were evaluated using the paired or unpaired Mann-Whitney test, accordingly. Statistical analyses were performed with SPSS v29 and $p < 0.05$ values were considered statistically significant.

Chapter 4: Results

4.1. Quantification of $P2Y_{12}R$ and CX3CR1 gene expression in human brain tissue samples

When compared to control individuals, MTLE-HS patients overexpress $P2Y_{12}R$ and CX3CR1 mRNA transcripts in the hippocampus and temporal neocortex. $P2Y_{12}R$ mRNA transcripts were 1.78 ± 0.24-fold (p = 0.004) and 2.89 ± 0.48-fold (p < 0.001) higher in the hippocampus (Figure 9A) and temporal neocortex (Figure 9B) of MTLE-HS patients, respectively. CX3CR1 mRNA transcripts were 1.96 ± 0.27-fold (p = 0.004) and 2.23 ± 0.36-fold (p = 0.001) higher in the hippocampus (Figure 9C) and temporal neocortex (Figure 9D) of MTLE-HS patients compared to the cadaveric controls, respectively.

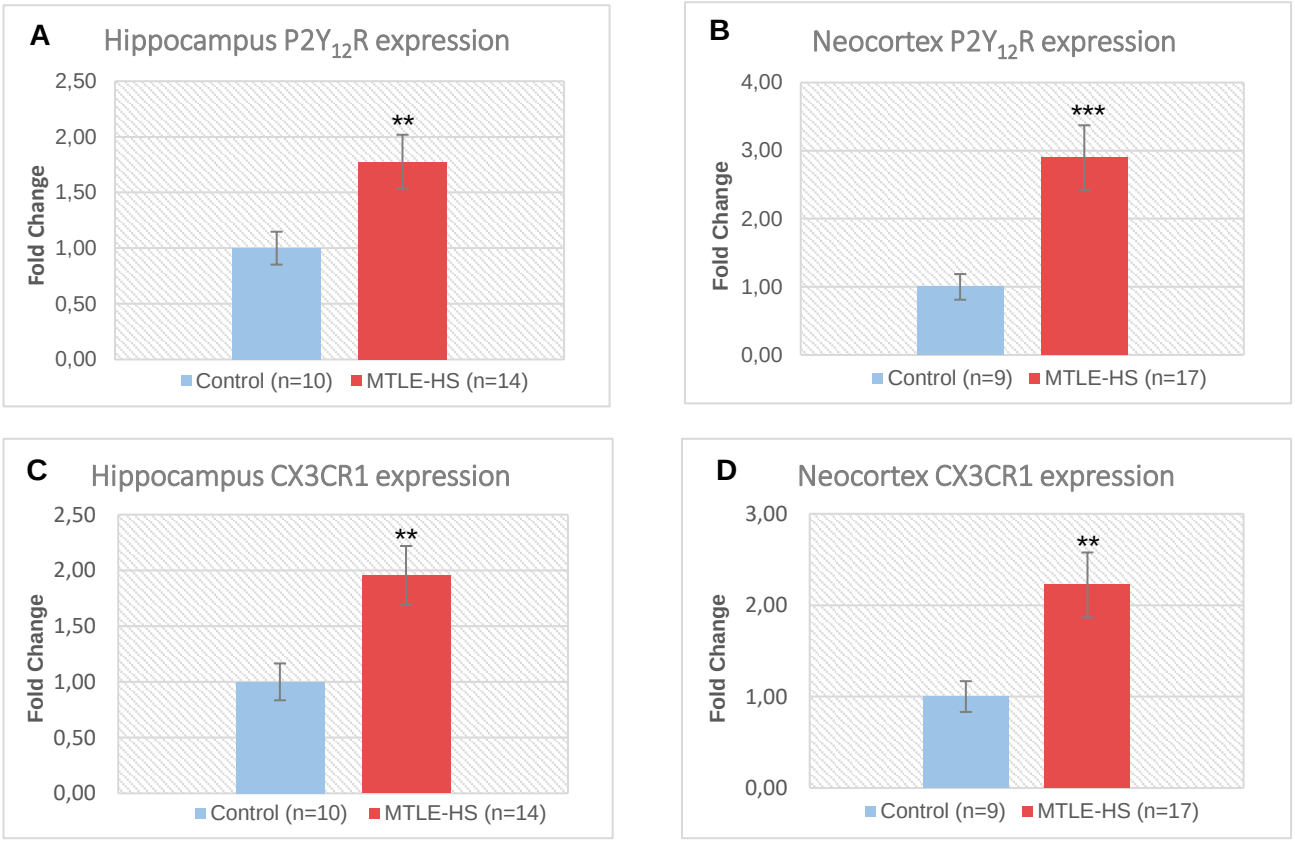

Figure 9 - The $P2Y_{12}R$ and CX3CR1 mRNA expression in the hippocampus and temporal neocortex of drug-refractory MTLE-HS patients is significantly increased when compared to non-epileptic cadaveric controls. $P2Y_{12}R$ mRNA expression in the hippocampus (**A**) and in the temporal neocortex (**B**) of MTLE-HS patients, compared to cadaveric controls. CX3CR1 mRNA expression in the hippocampus (**C**) and in the temporal neocortex (**D**) of MTLE-HS patients, compared to cadaveric controls. Fold change (compared to cadaveric controls) is expressed as mean ± SEM; "n" represents the number of individuals among 10 controls and 19 MTLE-HS patients in which quality assessment of retrieved RNA samples was suitable for quantification. Differences in expression were evaluated using the unpaired Student's t-test and the Mann-Whitney test, accordingly. Representing statistically significant differences between epilepsy patients and control individuals: * p < 0.05, ** p < 0.01, *** p < 0.001.

Considering that epileptic patients submitted to surgery were significantly younger than the cadaveric controls (43.51 ± 9.96 years old *vs* 67.10 ± 10.93 years old, respectively, $p < 0.0001$), and since aging can affect microglial $P2Y_{12}R$ and CX3CR1 mRNA expression [140-142], we examined if our results could be biased by the age difference. We observed no significant correlation ($p > 0.05$) between the receptor's expressions and age of cadaveric controls and MTLE-HS patients, both in the hippocampus and temporal neocortex (Figure 10 and 11, respectively). Additionally, $P2Y_{12}R$ and CX3CR1 expression in the hippocampus and temporal neocortex were not correlated ($p > 0.05$) with age of epilepsy onset, nor the duration of the disease condition (Figure 11).

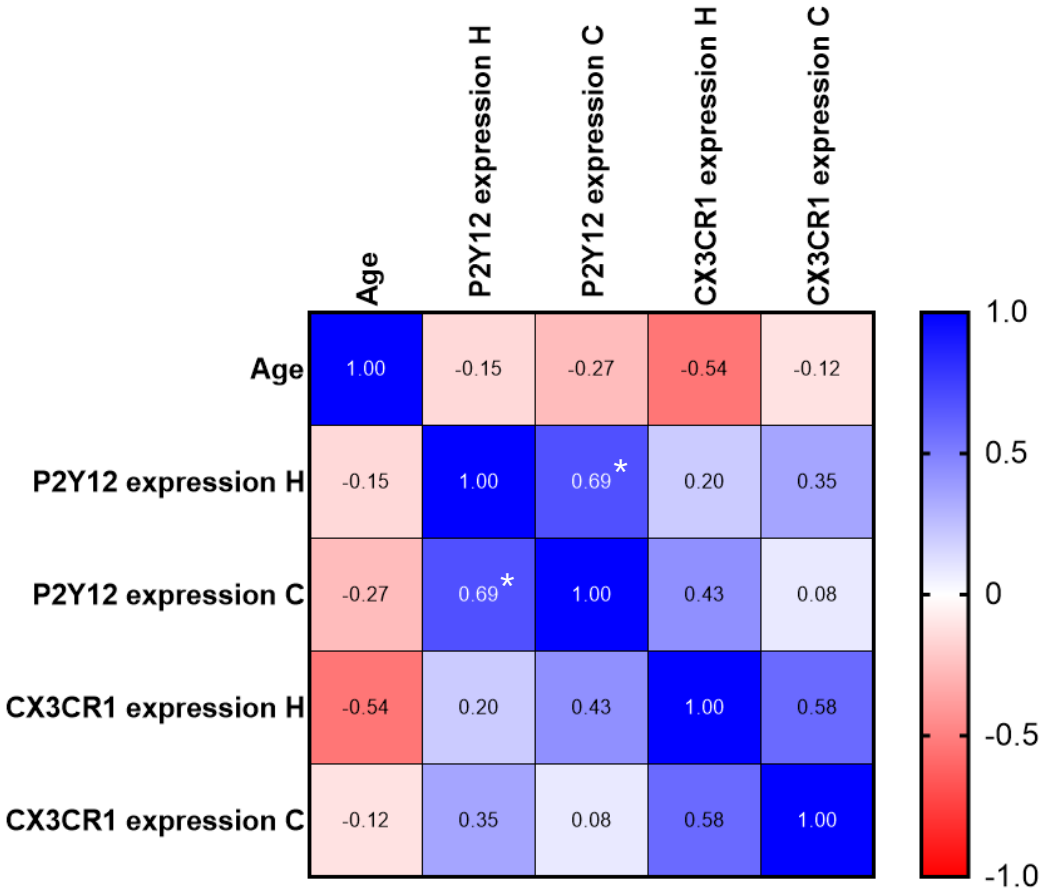

Figure 10 - Square matrix showing the correlation between $P2Y_{12}R$ and CX3CR1 expression and age of cadaveric controls, both in the hippocampus (H) and in the temporal neocortex (C). Age is expressed in years and receptor expression is expressed in ΔCt (arbitrary units). The values in the matrix represent Spearman's rho. Values with * represent a significant correlation at the 0.05 level (2-tailed).

Of note, in cadaveric controls the $P2Y_{12}R$ expression in the hippocampus was positively correlated with $P2Y_{12}R$ expression in the temporal neocortex with a correlation coefficient of 0.69 ($p = 0.041$, Figure 10). In addition, in MTLE-HS patients, CX3CR1

expression in the hippocampus was positively correlated with CX3CR1 expression in the temporal neocortex with a correlation coefficient of 0.71 (p = 0.010, Figure 11).

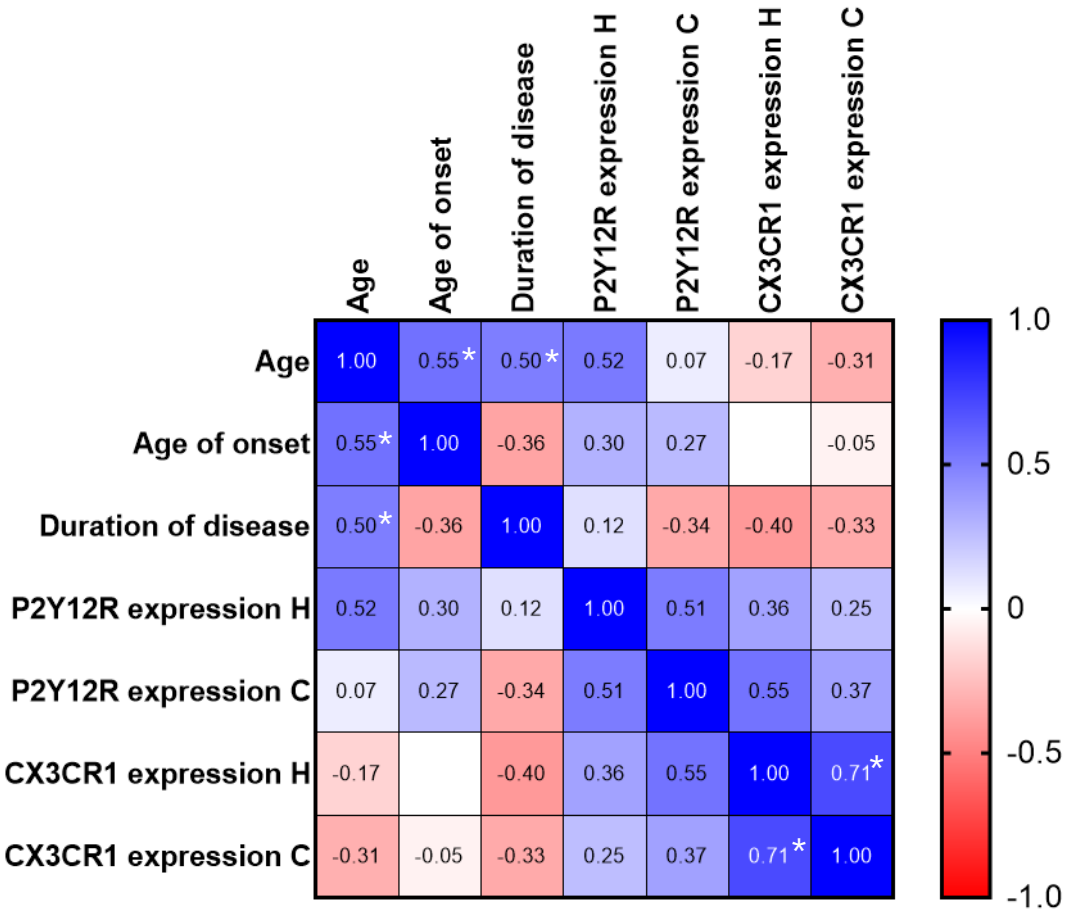

Figure 11 - Square matrix showing the correlation between $P2Y_{12}R$ and CX3CR1 expression, age, age of onset and duration of the disease in MTLE-HS patients, both in the hippocampus (H) and in the temporal neocortex (C). Age, age of onset and duration of the disease are expressed in years and receptor expression is expressed in ΔCt (arbitrary units). The values in the matrix represent Spearman's rho. Values with * represent a significant correlation at the 0.05 level (2-tailed).

4.2. Monocyte-derived microglia-like cell model optimization

We hypothesized that variations in gene transcription of $P2Y_{12}R$ and CX3CR1 microglial receptors could indicate dysregulation of microglial activity in MTLE-HS patients. Thus, we developed a cell culture model of monocyte-derived microglia-like (MMG) cells to examine this hypothesis using more accessible blood samples.

Cultured MMG cells exhibited elongated processes at culture day 5 (Figure 12A). These cells developed process ramifications at culture day 7, which is considered a signature of microglial cells morphology (Figure 12B). Monocyte-derived macrophage

cultures were used as controls; these cells exhibited an ameboid morphology that is characteristic of macrophages at day 7 of culture (Figure 12C).

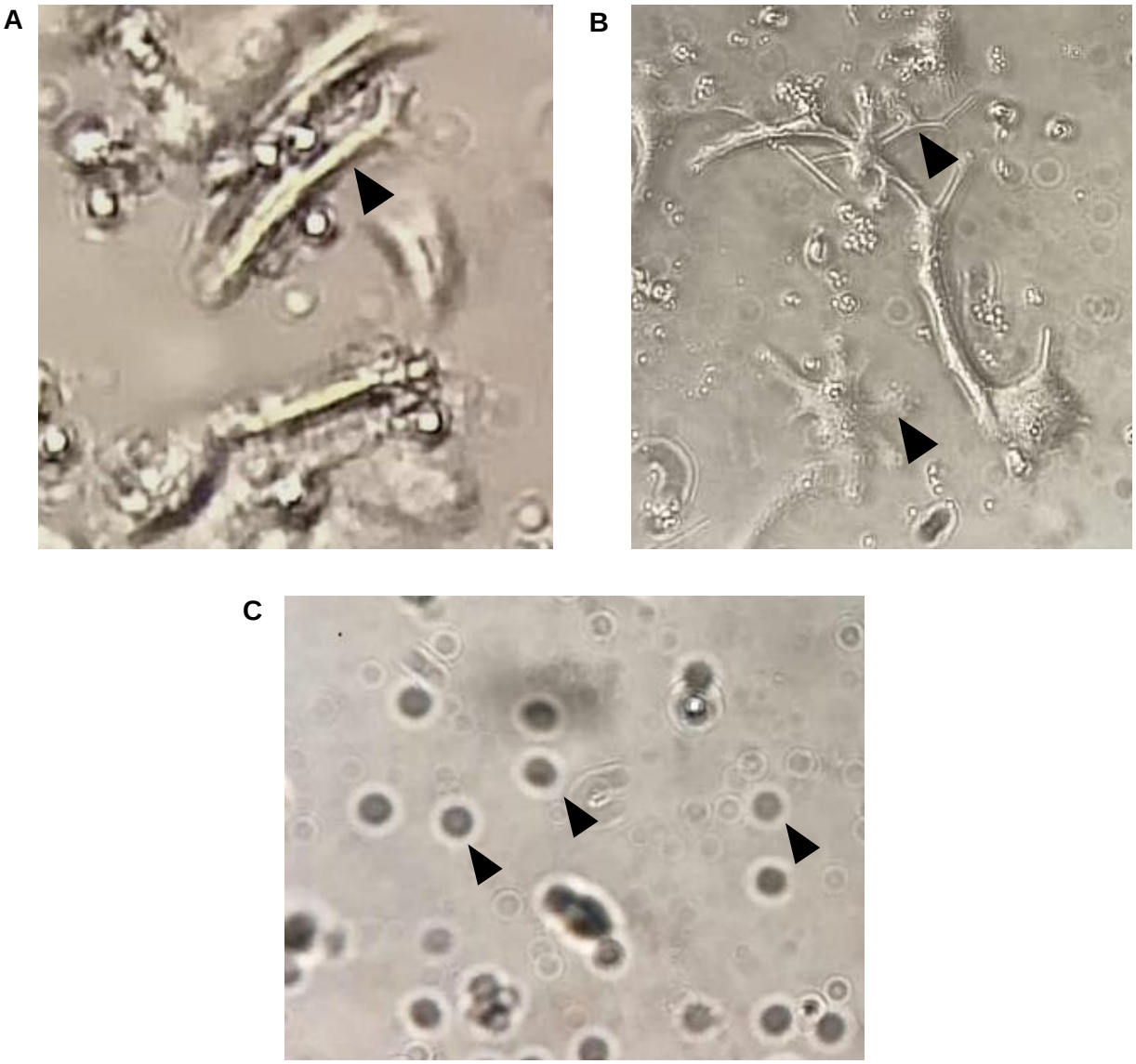

Figure 12 - Images obtained by optical microscopy with a 10X objective. (**A**) Monocyte-derived microglia-like (MMG) cells presenting elongated processes at day 5 of cell cultures. (**B**) MMG cells exhibiting process ramification at day 7 of cell cultures. (**C**) Monocyte-derived macrophages with an ameboid morphology at day 7 of cell cultures. The black arrows point to the (**A**) elongated processes, (**B**) process ramifications and (**C**) ameboid macrophages.

The microglia-like morphology of MMG cells prompted us to perform a molecular characterization of these cells both in homeostatic and stimulated conditions. To this end, we measured the transcriptional levels of the $P2Y_{12}R$, a molecular marker that is characteristic of microglia cells present in the CNS. For comparison reasons, we also

quantified the expression of CX3CR1, a non-selective biomarker of myeloid cells, including microglia, monocytes and macrophages.

We detected $P2Y_{12}R$ mRNA gene transcripts in MMG cells of 14 out of the 20 cultured individuals (70% of experiments, Figure 13A), but this microglial biomarker was absent in isolated RNA samples from monocyte and macrophage-derivative cultures (Figure 13A). Conversely, we detected CX3CR1 mRNA in 2 out of 12 monocytes isolated from the peripheral blood, and in 2 out of 20 MMG-derivatives and 1 out of 20 macrophage cultures (Figure 13B).

Despite the expression of the non-selective myeloid cell biomarker, CX3CR1, being less evident in tested cell cultures, we obtained a substantial enrichment of the $P2Y_{12}R$ gene transcription in MMG cells. The overexpression of this selective microglial biomarker indicates that MMG cells derived from PBMCs display enough morphological and molecular similarities for using this procedure as a more manageable microglial model for “in vitro” assays.

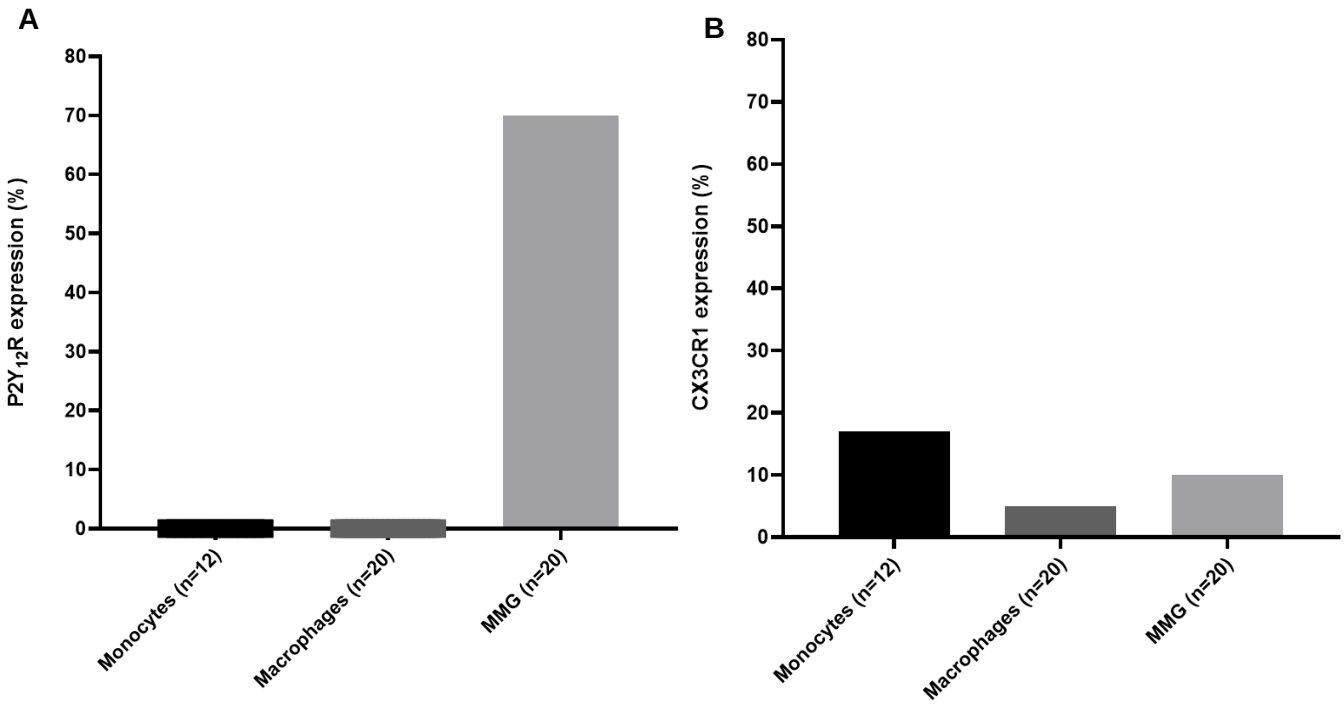

Figure 13 - Percentage of experiments in which $P2Y_{12}R$ (**A**) and CX3CR1 (**B**) mRNA transcripts were detected in blood monocytes, monocyte-derived macrophage and MMG cells. “n” represents the number of cell cultures among 5 controls and 15 MTLE-HS patients in which quality assessment of retrieved RNA samples was suitable for quantification.

4.3. Characterization of the monocyte-derived microglia-like (MMG) cells for “in vitro” functional assays

Considering that microglia are the resident immune cells of the brain and play a major role in the inflammatory pathways, we examined the ability of isolated MMG cells to respond to LPS as a proxy of inflammation-induced microglial cells activation.

Although the number of viable RNA samples did not allow significant ($p > 0.05$) conclusions, data show that MMG cells stimulated with LPS display a reduction in the $P2Y_{12}R$ gene transcription compared to non-stimulated cultures, independently on whether the cells were obtained from healthy volunteers (82.05-fold) or MTLE-HS patients (74.45-fold) (Figure 14A). We observed no changes concerning the P2X7R mRNA in these cells under similar experimental conditions (Figure 14B). On the other hand, exposure of MMG cells to LPS increased the mRNA levels of the pro-inflammatory cytokine, IL-1β, by 9.38-fold ($p = 0.068$) and 2.76-fold ($p = 0.005$) when the cells were isolated from healthy volunteers and MTLE-HS patients, respectively (Figure 14C).

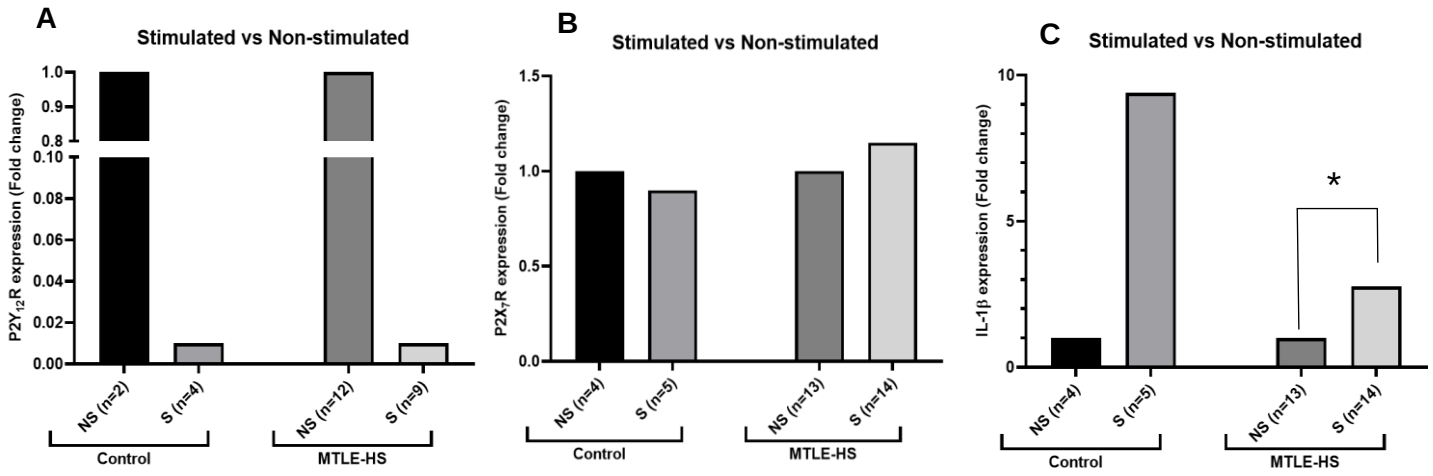

Figure 14 - Levels of $P2Y_{12}R$, P2X7R and IL-1β mRNA expression in stimulated (S) and non-stimulated (NS) monocyte-derived microglia-like cell (MMG) cultures, of both refractory MTLE-HS patients and healthy controls. Ordinates represent the fold change of $P2Y_{12}R$ (**A**), P2X7R (**B**) and IL-1β (**C**) expression levels in stimulated MMG cultures compared to non-stimulated, of both healthy controls and MTLE-HS patients. “n” represents the number of individuals among 5 controls and 15 MTLE-HS patients in which quality assessment of retrieved RNA samples was suitable for quantification. Quantification of each sample was compared to the corresponding non-stimulated sample, which was set to 1.0. Differences in ΔCt were evaluated using the paired Mann-Whitney test. SEM was not represented due to the low number of individuals from both healthy controls and MTLE-HS patients, as this is yet a preliminary study. Representing statistically significant differences between the stimulated and non-stimulated cultures: ** $p < 0.01$.

Both the decrease in $P2Y_{12}R$ mRNA expression and the increase in IL-1β levels are expected in microglial cells upon stimulation with LPS. Likewise, one should not expect changes in the P2X7R at the transcriptional level after LPS stimulation, as anticipated in previous studies [143, 144]. Thus, besides the morphology and molecular characteristics, MMG cells differentiated from monocytes of the peripheral blood are also functionally compatible with microglia in what concerns to the response to inflammation activators, like LPS.

4.4. Are there functional differences between MMG cells originated from MTLE-HS patients and healthy volunteers?

The aforementioned results prompted us to evaluate the functional differences between MMG cells obtained from the peripheral blood of MTLE-HS patients and healthy volunteers, concerning the transcriptional expression of $P2Y_{12}R$, P2X7R and IL-1β under homeostatic conditions and after stimulation with LPS.

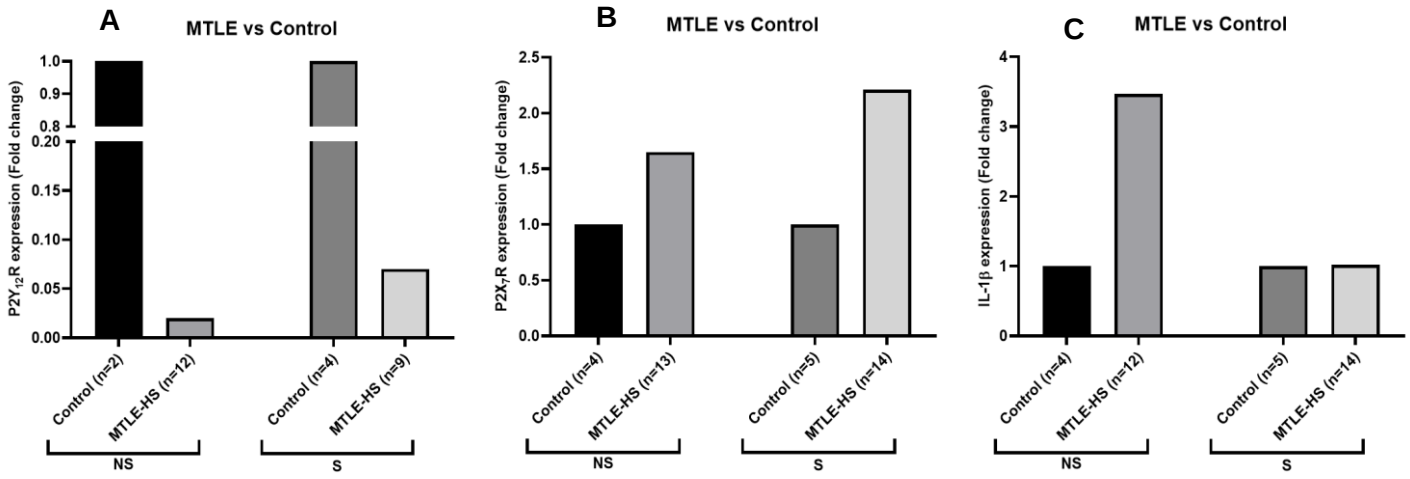

Figure 15 - Levels of $P2Y_{12}R$, P2X7R and IL-1β mRNA expression in monocyte-derived microglia-like cell (MMG) cultures derived from refractory MTLE-HS patients and healthy controls, in both stimulated (S) and non-stimulated (NS) conditions. Ordinates represent the fold change of $P2Y_{12}R$ (**A**), P2X7R (**B**) and IL-1β (**C**) expression levels in MTLE-HS patients compared to healthy controls, both in stimulated MMG cultures and non-stimulated. "n" represents the number of individuals among 5 controls and 15 MTLE-HS patients in which quality assessment of retrieved RNA samples was suitable for quantification. Quantification of each sample was compared to the control group which was set to 1.0. Differences in ΔCt were evaluated using the unpaired Mann-Whitney test. SEM was not represented due to the low number of individuals from both healthy controls and MTLE-HS patients, as this is yet a preliminary study.

Under homeostatic conditions, MMG cells isolated from MTLE-HS patients exhibited lower $P2Y_{12}R$ mRNA amounts (40.77-fold; Figure 15A) and overexpressed

both P2X7R (1.6-fold; Figure 15B) and IL-1β (3.5-fold; Figure 15B) at gene transcriptional level mRNA compared to healthy volunteers.

A similar profile was obtained concerning $P2Y_{12}$ (15.25-fold reduction; Figure 15A) and P2X7 (2.21-fold increase; Figure 15B) receptors upon stimulation of MMG cells with LPS. However, no differences were observed regarding the IL-1β gene transcription after LPS treatment of the cells among MTLE-HS patients and healthy controls.

4.5. Quantification of microRNA levels in MMG cells culture medium

Epigenetic modulators can regulate purinergic-mediated inflammatory pathways involved in the initiation/propagation of seizures. Therefore, we analysed the expression of important microRNAs controlling the inflammatory response in the culture medium of MMG cells.

Under homeostatic conditions, we detected all five microRNAs, miR-146a, miR-22, miR-155, miR-21 and miR-223, investigated in the culture medium of MMG cells originated from both MTLE-HS patients and healthy volunteers in the context of this work (Figure 16 A-E). On average, the culture medium of MMG cells from MTLE-HS patients had higher microRNAs amounts compared to those obtained from healthy controls, but this difference only reached statistical significance regarding miR-146a (3.46-fold increase; $p = 0.029$). Non-significant increases ($p < 0.05$) were observed among the two groups concerning miR-22 (2.77-fold), miR-155 (3.08-fold), miR-21 (3.57-fold) and miR-223 (1.83-fold).

LPS failed to induce changes ($p > 0.05$) in the expression of interest microRNAs in the culture medium of MMG cells (Figure 16 A-E). However, there was a trend for the up-regulation of miR-146a (2.24-fold; Figure 16A) and miR-22 (1.65-fold; Figure 16A) in the culture medium of MMG cells originated from healthy volunteers following LPS stimulation. Conversely, miR-22 (1.5-fold; Figure 16B), miR-21 (2.2-fold; Figure 16D) and miR-223 (1.6-fold; Figure 16E) levels decreased ($p > 0.05$) in the culture medium of MMG cells from MTLE-HS patients following LPS stimulation.

No significant differences were observed in the expression of microRNA comparing stimulated MMG cells derived from MTLE-HS patients and healthy controls (Figure 16 A-E). A trend for the upregulation of miR-155 (2.13-fold; Figure 16C) and miR-21 (1.92-fold; Figure 16CD) was found in the culture medium of MTLE-HS patients.

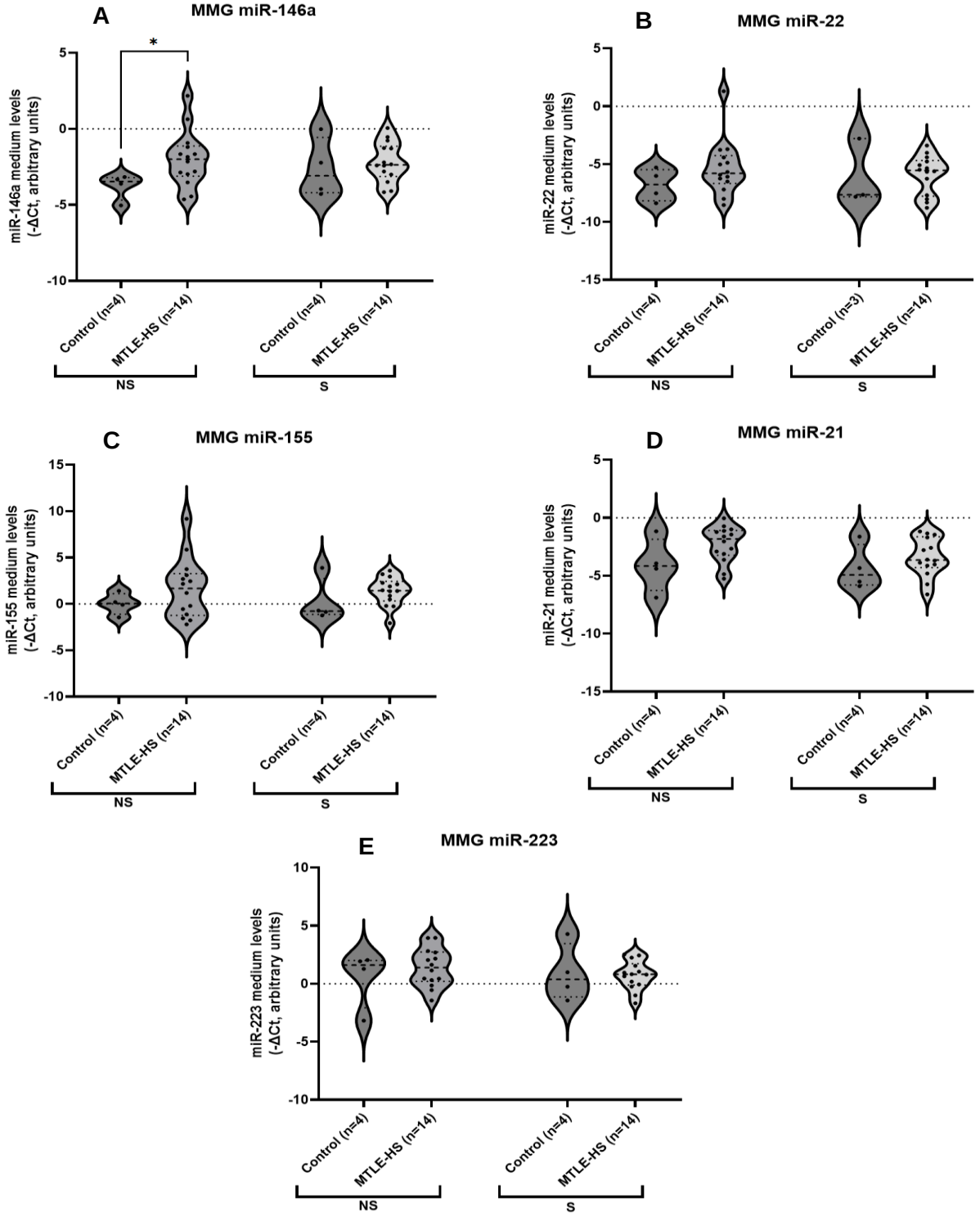

Figure 16 - Levels of miR-146a, miR-22, miR-155, miR-21 and miR-223 expression in the medium of stimulated (S) and non-stimulated (NS) monocyte-derived microglia-like cell (MMG) cultures, of both refractory MTLE-HS patients and healthy controls. Ordinates represent -ΔCt variation of miR-146a (**A**), miR-22 (**B**), miR-155 (**C**), miR-21 (**D**) and miR-223 (**E**) expression levels in the medium of non-stimulated and stimulated MMG cultures of healthy controls and MTLE-HS patients. "n" represents the number of individuals among 5 controls and 16 MTLE-HS patients in which quality assessment of retrieved RNA samples was suitable for quantification. Differences in expression were evaluated using the unpaired Mann-Whitney test. Representing statistically significant differences between the MTLE-HS patients and healthy controls: * $p < 0.05$.

Chapter 5: Discussion and Conclusions

Microglia, the resident immune cells of the CNS, are responsible for maintaining cerebral homeostasis while in their resting state, by constantly surveying the environment surrounding them. However, in response to various stimuli, such as brain injury, infection, or even the excessive neuronal activity associated with seizures, microglia become activated, dynamically shifting between phenotypes. Initially, they remove cellular debris and contribute to tissue repair, regeneration and remodelling of neuronal networks. However, a prolonged microglial activation and sustained inflammatory reaction seems to increase neuronal excitability, lower seizure thresholds, and promote seizure generation. The microglia activation process is profoundly mediated by purinergic signalling. Therefore, in this work we intended to focus on the $P2Y_{12}$ purinoceptor expression and function, since this receptor is a selective molecular biomarker of microglia in the brain.

We show here that the hippocampus and temporal neocortex of MTLE-HS patients overexpress the $P2Y_{12}R$ compared to cadaveric controls. These observations are in line with the studies of Avignone *et al*. who found a significant increase in hippocampal $P2Y_{12}R$ mRNA expression 24 and 48h following kainic acid (KA)-induced status epilepticus (SE) in mice [64]. Conversely, Alves *et al*. demonstrated that the $P2Y_{12}R$ is downregulated shortly after SE at both mRNA and protein levels [17]. This situation changed 14 days after the SE, when the protein levels of the $P2Y_{12}R$ increase without any changes at the mRNA transcription level [17]. Likewise, the hippocampus of TLE patients also displayed no significant changes in $P2Y_{12}R$ mRNA transcripts compared to healthy controls [17].

These results suggest that the $P2Y_{12}R$ may have different roles depending on the epilepsy stage. Initially, the increased neuronal activity paired with neuronal death foster the release of high ATP amounts, which may be rapidly dephosphorylated to ADP by microglial CD39. The adenine dinucleotide activates $P2Y_{12}R$ triggering the extension of microglial processes towards the site of injury, thus initiating the first step of microglial activation [67, 68]. Subsequently, there is a downregulation of the $P2Y_{12}R$ and an upregulation of other purinergic receptors, causing the retraction of the microglial processes and promoting the microglial migratory activity towards the source of the ATP [39]. Additionally, the seizure-induced ATP release will activate the P2X7R, which further drives microglial activation and proliferation, while simultaneously inducing the production and release of inflammatory mediators, such as IL-1β, IL-6, IL-18 and TNF-α

[85-89, 94, 95, 145]. The microglial activation, via P2X7R and $P2Y_{12}R$, seems to be critical to clear cellular debris and repair damaged tissue, actively displaying an anti-epileptic role [39]. Supporting this notion, studies have shown that the deletion of the $P2Y_{12}R$ proved to worsen seizure severity and increase seizure frequency in epilepsy models. [70, 146]. Even though an initial microglial activation might be essential to repair the damaged brain region and to avoid recurrent seizures, if dysregulated, prolonged inflammation and aberrant neuronal sprouting might promote excessive brain excitability, eventually perpetuating the seizure-inducing process.

Besides its role in injury detection and repair, the $P2Y_{12}R$ has an essential role in the modulation of neuron-microglia interactions. In the days following the epileptogenic insult, microglial $P2Y_{12}R$ adopts a new role, as it becomes crucial in the seizure-induced neurogenesis and formation of immature neuronal projections [62]. An increased expression of $P2Y_{12}R$ suggests a greater extent of immature neuronal sprouting, resulting in atypical excitatory synaptic connections that accentuate a seizure-inducing environment. This could possibly answer the question of how a region with such dramatic depletion of neurons, like the hippocampus, is capable of still generating spontaneous seizure activity.

Despite HS being the major histopathological phenomenon of MTLE, which characterized by significant neuronal loss and extensive hippocampal gliosis, multiple studies also showed an atrophy, of lesser extent, of extrahippocampal regions, suggesting a possible correlation of these regions with epileptogenesis [147-150]. Supporting this theory, our group has previously observed that altered DNA methylation patterns encompassing multiple pathways known to be involved in epileptogenesis in the temporal neocortex, whereas the hippocampus exhibited less prominent changes [151]. This suggests that the temporal neocortex of MTLE-HS patients might also be involved in epileptogenesis, perhaps in a different phase of this process, given the differential anatomical damage and epigenetic modulation. Our observations of a significant increase in both $P2Y_{12}R$ and CX3CR1 confirms a dysregulation of the microglia cells in this region, which could possibly be associated with an exacerbated inflammatory response and involvement in epileptogenesis. The abnormal ATP release as consequence of hippocampal neuronal death, might be the molecular stimulus triggering this initial microglial activation and $P2Y_{12}R$ upregulation in the temporal neocortex.

Additionally, the upregulation of CX3CR1 observed in the hippocampus and temporal neocortex of MTLE-HS patients is in line with other studies showing an increase in CX3CR1 expression in chronic epilepsy animal models, as well as in the hippocampus

of epilepsy patients [152-154]. A current model of neuron-glia interaction proposes that neuronal excitation and excessive glutamate release characteristic of seizures [155], evoke the release of CX3CL1 from neurons, consequently activating the microglial CX3CR1 and inducing the chemotaxis of microglia in order to reach the site of inflammation. Under homeostatic conditions, the CX3CL1/CX3CR1 signalling axis induces microglia phagocytosis, enhancing its ability to clean up brain cell debris, thereby promoting tissue repair, while also inhibiting the release of inflammatory factors and excitatory glutamatergic synaptic transmission [156]. Endogenous CX3CL1 is therefore essential to maintain microglia in a homeostatic state, exerting an overall neuroprotective effect characteristic of a M2-like anti-inflammatory microglial phenotype. However, under pathological conditions, the CX3CL1/CX3CR1 pathway may display a different functionality, promoting the microglial activation and the release of inflammatory factors, essentially shifting the microglial phenotype to a M1-like pro-inflammatory state and exacerbating the neuronal excitability associated with epilepsy [157]. In keeping with this, Yeo *et al*. found that CX3CL1 infusion accelerated SE-induced microglial activation [158], as fractalkine induces actin rearrangement and shape changes that promote microglial chemotaxis [159]. The impairment of CX3CR1 activation also results in a reduction of microglia activation, neurodegeneration and neuroblast production, possibly alleviating the neuronal hyperexcitability characteristic of MTLE-HS [152].

Overexpression of CX3CR1 as we observed here in the hippocampus and temporal neocortex of MTLE-HS patients may occur as a compensatory mechanism. This is hypothesized since the increased release of inflammatory mediators may cause neurons and microglia to upregulate the expression of CX3CL1/CX3CR1 in an attempt to control the excessive microglial activation and to maintain the dynamic equilibrium between M1- and M2-type microglia [156]. However, given the dysregulation of this pathway's function observed under pathological conditions, specifically in epilepsy, we assume that overexpression of CX3CR1 results in additional M1-like microglial activation, fostering cytokine release and exacerbating excitatory synaptic transmission, which promotes seizure generation. Additionally, CX3CR1 has a regulatory role in modulating basal hippocampal neurogenesis [160]. The increased basal neurogenesis as consequence of CX3CR1 upregulation can make MTLE-HS patients more susceptible to aberrant neuronal sprouting, thus promoting excessive brain excitability as mentioned above.

Overexpression of both $P2Y_{12}R$ and CX3CR1 in the hippocampus and temporal neocortex of MTLE-HS patients suggests that these individuals are more prone to

microglial activation, chemotaxis, initiation of the inflammatory pathway and release of ATP, which are common features aggravating the severity of seizures by promoting the vicious cycle relating inflammation and seizure initiation. To support this notion of a pro-inflammatory and seizure-inducing environment in the hippocampus and temporal neocortex of MTLE-HS patients, our group had previously shown an increase in IL-1β and P2X7R mRNA levels [10, 136]. However, data also showed that MTLE-HS patients had increased levels of IL-10 in these brain regions, possibly as a compensatory mechanism to supress the aggravated inflammatory response [136]. Given that IL-10 promotes the shift of microglia into a M2-like anti-inflammatory phenotype, this would suggest a stimulation of the clearance of cell debris, tissue repair, regeneration and remodelling, displaying a neuroprotective role and preventing seizure generation [38, 39]. This shift to a M2-like phenotype could contribute to promote the upregulation of the $P2Y_{12}R$ transcriptional level observed, as the anti-inflammatory phenotype typically exhibits $P2Y_{12}R$ overexpression [38, 39].

The immunostaining of protein surface markers of M1-like pro-inflammatory microglia (CD16 and CD86) and M2-like anti-inflammatory microglia (CD163 and CD206) would allow us to evaluate if different microglial subpopulations are restricted to specific brain regions of MTLE-HS patients [38]. As such, we could appraise if the increase in $P2Y_{12}R$ and CX3CR1 mRNA expression is associated with a pro-inflammatory M1-like or anti-inflammatory M2-like microglia phenotype. Additionally, as we merely analysed brain tissue homogenates, co-localization experiments focused on $P2Y_{12}R$, P2X7R, CX3CR1 and the microglia activation marker, Iba-1, using immunohistochemistry could be informative to determine if mRNA expression changes observed in the brain of MTLE-HS patients is region-specific. In any case, the upregulation of these receptors in both brain regions suggests a dysregulation in the microglial cell function and an exacerbated immune response. As consequence, this would suggest that regulatory mechanisms, such as epigenetic modulation via microRNAs silencing or DNA methylation, were also altered or not.

The $P2Y_{12}R$ mRNA expression disparity between previous experimental data and our observations may also stem from using different housekeeping genes. Although commonly used in epilepsy research, β-actin and GAPDH may be less stable than UBC in neocortical tissue [161]. Similarly, in the hippocampus of chronic epilepsy animal models, β-actin is considered one of the least stable reference genes, as its expression seems to vary, depending on brain region and epilepsy phase (SE, latent and chronic phases), while HPRT and UBC are considered more stable options [162, 163].

Much of our knowledge about microglia biology was obtained in laboratory settings using rodent cell cultures. However, understanding the role of microglia in human pathologies proves to be a harder task, as obtaining primary microglia for research poses significant challenges. Various models have been used to investigate microglial roles in neurological diseases and explore potential treatments. These include primary cultures of microglia isolated from post-mortem brains or surgical resections and microglia derived from induced pluripotent stem cells (iPSCs) [164].

Human microglia research has been significantly hampered by the low reproducibility of *in vivo* traits in cell culture models. While post-mortem and *ex vivo* microglia offer direct insights into the human physiology, they are limited by ethical concerns and technical difficulties. Additionally, the yield of cells isolation is often insufficient for extensive research, and these cells can rapidly change their phenotype and transcriptomic signature when transferred to a laboratory environment. The transition to an *in vitro* tissue culture environment rapidly decreases the expression of microglial signature genes, such as CX3CR1 and $P2Y_{12}R$, while activating genes associated with acute inflammatory responses, thus suggesting that the culture model cannot effectively mimic the homeostatic conditions [165].

To circumvent this problem, the use of microglia-like models gained popularity. A common concern regarding the use of microglia-like models is their distinct ontogeny compared to *in vivo* microglia, as these originate from the yolk sack erythromyeloid progenitor cells. As such, to emulate the microglial ontogeny, iPSC-derived microglia can be used to study human microglia. However, the differentiation protocols take over 25 days to obtain and, for large cohort screenings, would require significant automation, cost and time resources [164]. Despite the ability to differentiate microglia into every microglial activation state, the lack of a standardized protocol can affect the phenotypic, molecular and functional characteristics of the cell models.

In light of these limitations, we decided for the use of the *in vitro* model of MMG cells, which has gained prominence in recent years due to the quick and easy collection (blood samples) and differentiation process. We followed the MMG cell differentiation protocol developed by Ryan *et al*., with some minor adjustments. Whereas they isolated monocytes by magnetic separation using CD14 microbeads, which impair LPS sensitivity and response [164], we opted for an isolation by plastic adhesion considering the low quantity of initial PBMCs collected. In addition to the growth factors used by their group,

we supplemented our cultures with 20 ng/mL human TGF-β2, as it is helpful in the *in vitro* development of adult microglia and aids microglia-like cells to maintain their identity [166, 167]. Perhaps because of these changes, we were able to detect $P2Y_{12}R$ mRNA expression in MMG cells, while observing no expression of this receptor in monocytes and monocyte-derived macrophages, whereas other authors found no difference in $P2Y_{12}R$ mRNA expression between the different cell types [138, 168]. Although we did not observe significant CX3CR1 mRNA expression differences between MMG cells, monocytes and monocyte-derived macrophages, other groups have reported similar results [169]. Additionally, Quek *et al*. previously observed that the $P2Y_{12}R$ mRNA expression was significantly increased in MMG cells at culture day 14 compared to day 7 [170]. Considering this, adopting a protocol with additional days of the cells in culture could help us increase the expression of the microglia-specific receptors in our MMG cells.

In our hands, we proved that MMG cells functionally mimic brain microglial cells, since we detected significant increases in IL-1β mRNA expression in MMG cells isolated from MTLE-HS patients following stimulation with LPS. This expected situation may result because LPS activates the TLR4/NLRP3 pathway, inducing the production and release of IL-1β, among other cytokines [171]. The levels of miR-146a in non-stimulated MMG cells from MTLE-HS patients were also significantly increased. It is known that pro-inflammatory molecules stimulate miR-146a expression, which will act on a negative feedback loop targeting the TLR4 signalling pathway, inhibiting NF-κB activation, and, therefore, diminishing IL-1β release. Although not statistically significant, we observed that non-stimulated MMG cells from MTLE-HS patients also displayed varying levels of miR-22, miR-155, miR-21 and miR-223 in the culture media, when compared to control-derived cells. In a similar fashion, MTLE-HS-derived MMG cells stimulated with LPS displayed non-significant variations in several microRNAs compared to cells from healthy controls. Despite not reaching statistical significance, possibly due to the low sample size, the observed variations point to a dysregulation of the compensatory mechanisms involved in the microglial inflammatory response in MTLE-HS.

As previously stated, microglia activation initially induces $P2Y_{12}R$ upregulation but, at a later time point, $P2Y_{12}R$ expression is decreased (Figure 1A). On the other hand, CX3CR1 mRNA expression is significantly decreased (Figure 1B) after LPS stimulation in microglia [142, 172], but cells display a higher surface expression of this receptor [142]. The MMG cell model set-up in this study needs a further characterization of the different stages of $P2Y_{12}R$ and CX3CR1 mRNA expression following LPS stimulation, as

one could not confirm following the 2-day LPS exposure the microglial activation phase the MMG cells are. It could also be of interest to stimulate MMG cell cultures with IL-10 instead of LPS, while complemented with the aforementioned growth factors to determine if these cultures can differentiate into an anti-inflammatory M2-like microglia phenotype. If such differentiation is possible, we could then assess the differences between M2-like MMG cells from MTLE-HS patients in comparison to the ones derived from healthy controls.

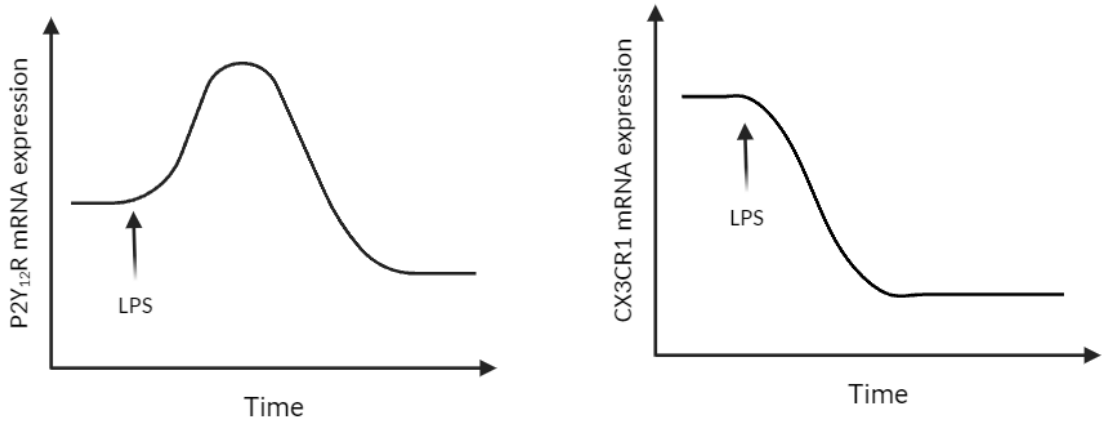

Figure 17 Schematic representation of the (**A**) $P2Y_{12}R$ and (**B**) CX3CR1 mRNA expression variation following microglial activation, based on the observations of Inoue et al. PloS one vol. 16,5 e0252118. 21 May. 2021, doi:10.1371/journal.pone.0252118 and Morillas et al. International journal of molecular sciences vol. 22,4 1636. 6 Feb. 2021, doi:10.3390/ijms22041636.

As ATP breakdown into ADP is essential for $P2Y_{12}R$ and microglia activation, it is essential to analyse the kinetics of the extracellular catabolism of ATP via CD39 ectoenzyme in MMG cells from MTLE-HS patients compared to healthy controls. Furthermore, we intend to use our MMG cell model to evaluate the impact of purinergic signalling in MTLE-HS patient's by stimulating MMG cells with ATP and ATP+LPS, while monitoring Ca^{2+} oscillations by time-lapse confocal microscopy to study the mechanisms underlying microglial activation. Additionally, we will test the therapeutic effect of $P2Y_{12}R$ antagonism following LPS stimulation in MTLE-HS-derived MMG cells. Furthermore, our model is ideal to study the nuances of epigenetic modulation of purinergic signalling and neuroinflammation in MTLE-HS patients compared to healthy controls.

Notwithstanding, this model has its limitations, as the impact of the in vitro tissue culture environment is unknown due to the lack interaction with other brain cell populations and a three-dimensional spatial arrangement. A straightforward method for

investigating the crosstalk between microglia and neurons or astrocytes involves either transferring conditioned medium from these cell types cultured individually or employing Transwell systems, which enable bidirectional exchange of secreted factors between different cell types.

Overall, our observations indicate that MTLE-HS patients might have a microglial dysregulation and be more susceptible to prolonged microglial activation that has deleterious effects on the neuronal network, together with aberrant neuronal projections that promote a seizure-inducing environment. This effect might be mostly noticeable in the hippocampus as histopathological changes make this region more susceptible to inflammation and activation of the $P2Y_{12}R$ and CX3CR1. Nonetheless, the increase in $P2Y_{12}R$ and CX3CR1 mRNA expression in the temporal neocortex supports the hypothesis that this region might also be a seizure-inducing environment, perhaps in the earlier stage of epileptogenesis. Our cell model proved to be functional and, therefore, it is of great value to study the interactions between different epigenetic modulators, purinergic signalling and neuroinflammation mediators.

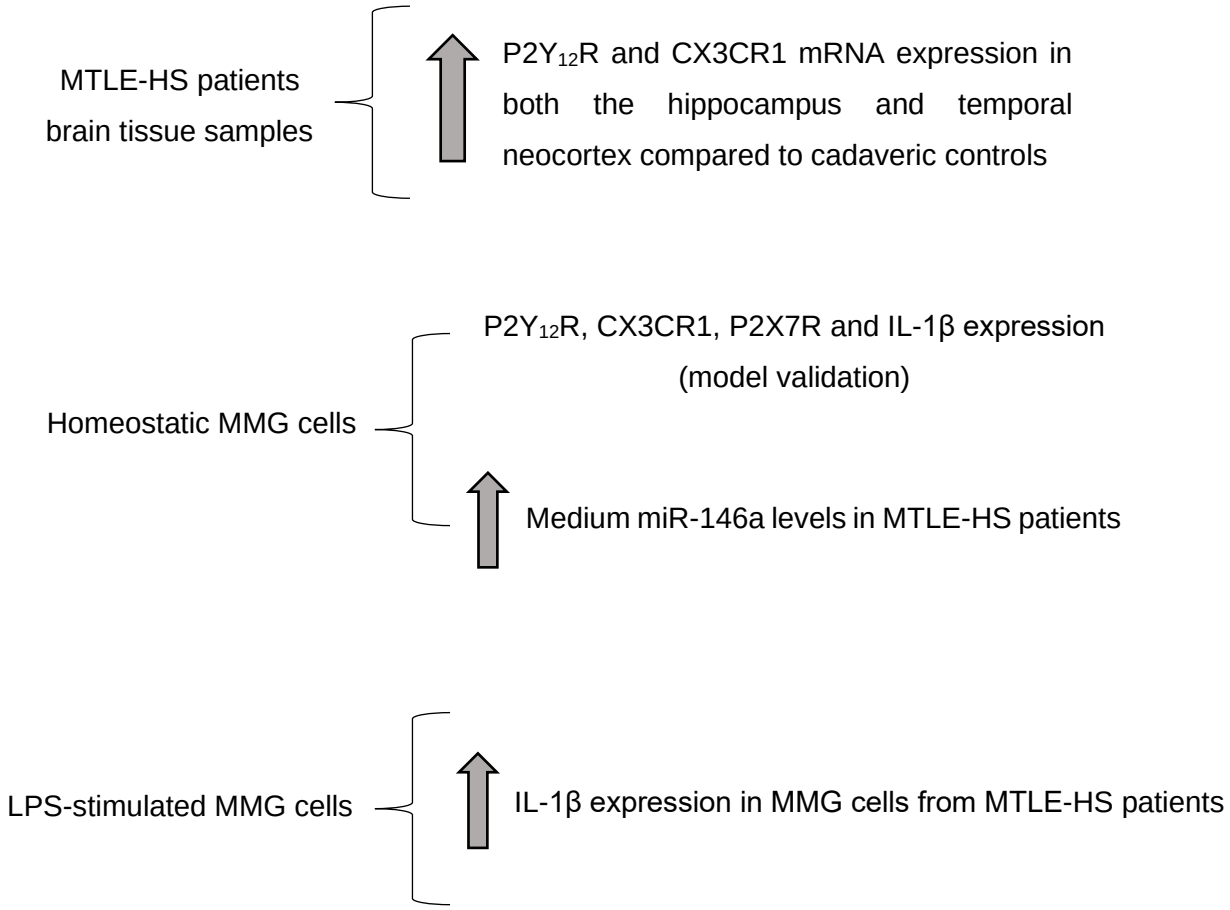

Figure 18 - Brief review of the statistically significant observations in (**A**) MTLE-HS patients brain tissue samples, (**B**) Homeostatic MMG cells, and (**C**) LPS-stimulated MMG cells.

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