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The role of circulating and exosomal miRNAs as biomarkers of drug-resistant epilepsy

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The role of circulating and exosomal miRNAs as biomarkers of drug-resistant epilepsy

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Dedication

"La scelta di un giovane dipende dalla sua inclinazione, ma anche dalla fortuna di incontrare un grande maestro."

(Rita Levi Montalcini)

"The choice of a youth depends on its inclination, but also from the chance to meet a great teacher."

(Rita Levi Montalcini)

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The role of circulating and exosomal miRNAs as biomarkers of drug-resistant epilepsy

Abstract

Epilepsy is estimated to affect about 65 million individuals worldwide, with up to 30 percent of drug-resistant patients who do not have remission despite appropriate therapy with antiepileptic drugs (AEDs). Therefore, it is important to distinguish drug-resistant epilepsy early in the course of disease to start a specific therapeutical approach as soon as possible.

Recently circulating miRNAs have been proposed as promising biomarkers for different neurodegenerative disorders, including epilepsy.

MiRNAs are a class of small non-coding RNA that regulate gene expression at a post-transcriptional level. The regulatory mechanisms controlling translation of mRNA transcripts represents to date a largely unexplored aspect of epilepsy. Several studies have demonstrated that miRNAs are differentially expressed in presence of drug-resistant epilepsy, with a specific expression pattern in brain regions connected to the epileptogenic activity.

Moreover miRNAs can be transmitted from one neuron to another across the synaptic cleft carried by exosomes. Because of the ability of exosomes to mediate drug efflux, it could be useful to study how they participate in the pathogenesis of drug-resistant epilepsy.

There is a need to establish the relationship between miRNA levels in lesional and non lesional drug-resistant epilepsy in order to use miRNAs as biomarkers of specific pathological condition such as intractable epilepsy.

We know that circulating miRNAs are stable in serum and their test in blood is broadly accessible, rapid and noninvasive. Previous studies already found a significant number of miRNAs differentially regulated in the epileptic state when compared to control animals, indicating a tight regulation of miRNAs associated with seizures in epilepsy models. Then our intent is also to understand the role of miRNAs in signaling pathways during corticogenesis, identifying differences in miRNA expression

between surgical and serum sample. This correlation would be a great goal for clinical practice, supporting the role of the miRNA as biomarkers in drug-resistant epilepsy associated to structural brain abnormalities. To date few studies have investigated the possible role of miRNAs in the pathogenesis of structural brain abnormalities, the heterogeneity of the abnormal cell population, in fact, is a critical limitation in this field of research.

Surely the observation of new specific miRNAs associated with drug-resistant epilepsy and structural brain abnormalities will broaden new horizons both for the clinical and the therapeutical approach of until now intractable epilepsy.

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List of abbreviations:

AEDs: antiepileptic drugs - miRNAs: microRNAs - BBB: blood-brain barrier
ILAE: International League Against Epilepsy - TLE:temporal lobe epilepsy -
HS:hippocampal sclerosis – SE: status epilepticus -
DNETs: dysembryoplasticneuroepithelioma - mTLE: mesial temporal lobe epilepsy lTLE: lateral lobe epilepsy - EEG: electroencephalogram – MRI: magnetic resonance imaging - GABA: aminobutyric acid - ASD: antiseizure drug – HSPs: heat-shock proteins – FCD: focal cortical dysplasia – TEM: Transmission Electron Microscopy – DLS: Dynamic Light Scattering – TCF: time autocorrelation functions – EC: enhanced chemiluminescence – qRT-PCR: Quantitative Real-Time Polymerase Chain Reaction - EIA: enzyme immuno-assay

CHAPTER 1: INTRODUCTION

Epilepsy is estimated to affect about 65 million individuals worldwide, with up to 30 percent of drug-resistant patients who do not have remission despite appropriate therapy with antiepileptic drugs (AEDs) *(Moshe S. et a;l 2014)*. Therefore, it is important to distinguish drug-resistant epilepsy early in the course of disease to start a specific therapeutical approach as soon as possible. *(Kwan P. et al; 2010)*

Recently circulating microRNAs (miRNAs) have been proposed as promising biomarkers for different neurodegenerative disorders, including epilepsy.

MiRNAs are a class of small non-coding RNA that regulate gene expression at a post-transcriptional level. The regulatory mechanisms controlling translation of mRNA transcripts represents to date a largely unexplored aspect of epilepsy. Several studies have demonstrated that miRNAs are differentially expressed in presence of drug-resistant epilepsy, with a specific expression pattern in brain regions connected to the epileptogenic activity *(Bartel DP. et al 2009, Song Y.J. et al 2011; Sun Z. et al; 2013)* Moreover miRNAs can be transmitted from one neuron to another across the synaptic cleft carried by exosomes. These are small lipoprotein vesicles, derived from multivesicular bodies and the cellular endosome system, that cross the external plasma membrane to enter the perisynaptic space. Exosomes may cross the blood-brain barrier (BBB) and reach the circulation delivering their cargo (proteins, RNA) between specific cells. *(Gupta A. et al; 2014)* Because of the ability of exosomes to mediate drug efflux, it could be useful to study how they participate in the pathogenesis of drug-resistant epilepsy, a mechanism that is not completely clarified. Thus not only circulating miRNAs but also exosomal miRNAs show potential use as non-invasive biomarkers indicating disease states. There is a need to establish the relationship between miRNA levels in lesional and non lesional drug-resistant epilepsy in order to use miRNAs as biomarkers of specific pathological condition such as intractable epilepsy.

We know that circulating miRNAs are stable in serum and their test in blood is broadly accessible, rapid and noninvasive. Previous studies already found a significant number of miRNAs differentially regulated in the epileptic state when compared to control animals, indicating a tight regulation of miRNAs associated with seizures in epilepsy models. Thus, circulating miRNAs could be used as non invasive biomarkers to indicate drug-resistance.

This current growing evidence would change also both the pathophysiology and the therapeutical approach of epilepsy.

CHAPTER 2: EPILEPSY AND EPILEPTIC SYNDROMES

Epilepsy is one of the most common and serious neurological disorders worldwide, with a lifetime prevalence of 0.5%, affecting nearly 50 million people worldwide, resulting in a major socioeconomic burden.*(Chang B.S et al;2003)*

According to the International League Against Epilepsy (ILAE) we distinguish an *epileptic seizure* that is *"a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain"* from *epilepsy* that is a *"disorder of the brain characterized by an enduring predisposition to generate epileptic seizures and by the neurobiologic, cognitive, psychological, and social consequences of this condition" (Elger C.E. et al; 2008 - Fisher R.S. et al; 2005)*

Epilepsy is not one condition, but is a diverse family of disorders, having in common an alteration in the brain that increases the likelihood of future seizures. The phenotype of each seizure is determined by the point of origin and the degree of propagation of this pathological activity. Epilepsy is associated with increased psychiatric morbidity, cognitive impairment, scholastic difficulties, unemployment, lower rates of marriage, reduced leisure opportunities and greater social isolation and family dysfunction than those without the condition *(Baker GA.et al; 2000)*. Stigma and prejudice demarcate epilepsy from most other neurological conditions *(Jacoby A. et al; 2002)*

Furthermore epileptic patients have an increased risk of premature death that is two times higher than the general population, especially in the first few years after diagnosis. Common causes of death include seizure-related death (e.g. status epilepticus, Sudden Unexplained Death in Epilepsy Patients, SUDEP) and accidents (e.g. drowning or burns*) (Lhatoo SD. et al; 2001 – Pati S. et al; 2010).*

Typically, epileptogenesis is a self-promoted pathological process triggered through an initial insult. Increasing evidence has shown that epileptogenesis results in eventual neuronal death or dysfunction, ion channel dysfunction, mossy fiber sprouting, gliosis, neurogenesis and inflammation, etc. However, the underlying molecular mechanisms of this remain poorly understood. At the moment antiepileptic therapy is the primary treatments for epilepsy, and these therapies merely reduce or prevent the occurrence of seizures, ignoring the underlying pathophysiology of epilepsy.

Hence, there is an increasing need for the clarification of mechanisms of epileptogenesis and development of antiepileptogenic therapies. *(McNamara, J.O et al; 2006)*

2.1 Temporal lobe epilepsy (TLE)

Special attention has been given to temporal lobe epilepsy (TLE) which is one of the most common medically intractable neurological disorders, typically manifesting during childhood and is associated with hippocampal sclerosis (HS) and status epilepticus (SE).

Temporal lobe epilepsy (TLE) is the most common form of focal epilepsy in adults, accounting for 60% of cases (*Shorvon S. et al; 2010*). A number of sub-classifications exist regarding the neuroanatomical origin of the seizures, with the distinction between mesial and lateral temporal seizure onsets being one of the most widely used.

Mesial temporal lobe epilepsy (MTLE) arises in the hippocampus, parahippocampal gyrus and amygdale, *lateral temporal lobe epilepsy* (LTLE) arises in the neocortex on the outer surface of the temporal lobe of the brain.
Seizures may involve only one or both lobes, giving rise to focal seizures (without loss of consciousness) or secondarily generalized seizures (associated with loss of consciousness).
About 40% to 80% of people with TLE also perform repetitive, automatic movements (called automatisms), such as lip smacking and rubbing the hands together. As seizures usually involve areas of the limbic system which control emotions and memory, some individuals may have problems with memory, especially if seizures have occurred for more than five years. However these memory problems are almost never severe.
More than 90% of patients with mesial temporal lobe epilepsy (mTLE) report a visceral aura, most commonly an epigastric sensation that has a rising character. Other auras maybe characterized by an abnormal sense of taste, an aversive smell, déjà vu or a dreamy sensation. Fear is the most reported affective symptom, although other complex emotional symptoms may also occur. Autonomic symptoms include changes in skin colour, blood pressure, heart rate and piloerection.
The latter is characterized by prominent behavioral changes, often a motionless stare. Speech usually ceases or is severely disrupted if the seizure involves the language dominant temporal lobe (normally the left). If the seizure onset is in the non-language dominant hemisphere, speech may be retained throughout the seizure, but is generally marked by meaningless repetitive vocalisations. Behavioral automatisms which demarcate a seizure focus as originating in mesial temporal lobe structures are usually oroalimentary (e.g. lip-smacking, chewing, swallowing) or gestural (e.g. fumbling, fidgeting, repetitive motor action, undressing, walking, running or

sexually directed actions), and are often prolonged. Limb automatisms are usually ipsilateral to the epileptogenic focus, with contralateral dystonic posturing. Following a temporal lobe complex partial seizure, confusion and headache are common. Post-ictal nose-rubbing may occur and most frequently occurs ipsilateral to the epileptogenic zone.

EEG correlates of mTLE often show anterior and mid-temporal spikes. Further changes include intermittent or persisting slow activity over the temporal lobes, which can be unilateral or bilateral. With advances in magnetic resonance imaging (MRI), structural abnormalities are often identified in mesial temporal brain structures *(Elger CE. et al; 2008 - Shorvon S. et al; 2010).*

Seizures occur after an initial insult like an infection, stroke or trauma, vascular malformation or prolonged febrile seizures; a genetic cause is less frequent. Between the initial insult and the onset of the crisis, a so called latent period characterized by the absence of seizures, occurs. During this period, changes in structure and physiology of the brain tissue ("epileptogenesis") happen.

The commonest pathology underlying mesial temporal lobe epilepsy is *hippocampal sclerosis*.

It is characterized by severe loss of the principal neurones associated with widening of the granule cell layer of the dentate gyrus, termed granule cell dispersion, which is observed in about 40-50% of surgical temporal lobe specimens. *(el Bahh B. et al; 1998 - Blümcke I. et al; 2002)* This condition is often associated with a history of febrile seizures in infancy.

Other aetiologies include dysembryoplasticneuroepithelioma (DNETs) and other benign tumours, cavernous angiomas, glioma, malformations of cortical development, or gliosis as a result of encephalitis or meningitis.

Mesial temporal lobe epilepsy, especially with pathologically or radiologically proven mesial temporal sclerosis is considered a highly refractory and drug-resistant type of epilepsy.

A recent paper reported that U.S. prevalence of drug-resistant TLE-HS is 0.51-0.66 cases per 1000 people, and the estimated U.S. incidence is 3.1-3.4 cases per 100,000 people per year.

Based on a U.S. population of 324 million, it is estimated that as many as 143,000-191,000 U.S. patients still suffer from drug-resistant TLE -HS and are in need of surgery or other therapeutic options. *(Lewis DV.et al; 2014)*

Temporal lobe epilepsy associated with HS is often progressive with worsening of seizures, impairment of cognitive function, psychiatric disorders and is accompanied by significant morbidity and mortality *(De Oliveira et al; 2010 – Asadi-Pooya et al; 2017 - Lee, S. K.et al; 2014)*

In this scenario, resective surgery has been recognized as an effective treatment for pharmacoresistant TLE/HS *(Wiebe, S. et al; 2001 – Wiebe, S. et al; 2012 – De Tisi, J. et al; 2011 – Engel, J et al; 2000)*, however, one third of the surgically treated patients presented unfavorable results. Therefore, TLE/HS is a heterogeneous condition and there are gaps in our understanding of its pathophysiological mechanisms, natural history and progression. *(Jardim, A. P et al; 2012)*

Considering the variety of treatment options that are available future efforts should focus on advocating for early referral of patients with drug-resistant TLE-HS for more comprehensive epilepsy management. *(Robin J. et al; 2018)*

Lateral temporal lobe epilepsy is often associated with detectable underlying structural pathology, the most common being a glioma, angioma, cavernoma, hamartoma, DNET, neuronal migration defect and post-traumatic change. Unlike mTLE, this condition is not associated with febrile convulsions. Unsurprisingly, there is considerable overlap between the clinical and electrophysiological features of mesial and lateral temporal lobe epilepsy, due presumably to the rapid spread of discharges between these two neighboring anatomical areas. However, subtle differences between lateral and mesial temporal lobe epilepsies are discernible. For example, during a lateral temporal lobe seizure auras include hallucinations that are often structured with visual, auditory, gustatory or olfactory forms, which can be crude or elaborate in nature or with illusions of size, shape, weight, distance or sound. Compared to mTLE, affective, visceral or psychic auras are far less frequent. Moreover, lateral temporal lobe seizures typically involve more motor activity; automatisms are unilateral and have more prominent motor manifestations. However, from a post-ictal perspective, mesial and lateral temporal lobe epilepsies are difficult to distinguish. The electrophysiological pattern between seizures (interictal pattern) shows spikes over the temporal region, maximal over the posterior or lateral temporal rather than inferomesial electrodes. In contrast to mTLE, hippocampal volumes and T2 measures (fluid attenuated inversion recovery; FLAIR) on MRI are usually normal *(Shorvon S. et al; 2010).*

CHAPTER 3: THE CONCEPT OF PHARMACORESISTANCE AND EPILEPSY

Despite the existence of numerous antiepileptic drugs, 30% of patients who develop epilepsy continue to experience seizures. *(Duncan JS et al; 2006)*

Although the new AEDs have contributed significantly to a better safety profile, they have not changed the percentage of people exhibiting drug refractoriness in any measurable way.

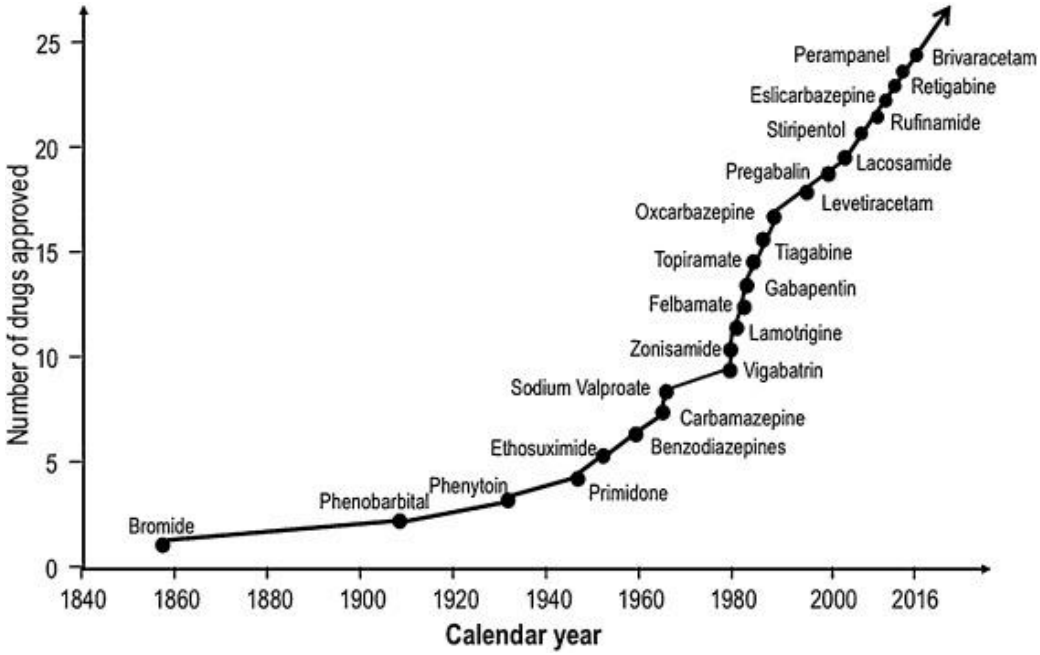

FIGURE 1: Chronological development of AEDs *Modified from: Kwan P. Drug development for refractory epilepsy: The past 25 years and beyond (2017)*

Kwan and Brodie (2000) demonstrated in a prospective study that 47% of patients with new-onset epilepsy became seizure-free on the first AED, 32% on the second AED, and 9% on the third AED. Forth and subsequent AEDs had at most a 5% chance of bringing seizure remission. *(Kwan P. et al; 2000, Golyala A. et al; 2017)*

Conventional antiepileptic drugs are antiictogenic (able to suppress seizures), but are not antiepileptogenic (able to prevent epilepsy, interfere with the process of epileptogenesis, or even cure epilepsy).

The capacity of current agents to be antiictogenic has been demonstrated in multiple animal models and in human brain tissue. This antiictogenic effect is achieved by reducing seizure occurrence via the following molecularly mediated mechanisms:

(1) voltage-gated sodium channel blockade;
(2) voltage-gated calcium channel blockade;
(3) c-aminobutyric acid (GABA)ergic system modification by direct effects on components of the GABA system (GABAA receptors, GAT-1 GABA transporter, and GABA transaminase);
(4) molecular targets including a2d, SV2A, and Kv7/ KCNQ/M potassium channels

A number of AEDs work by more than one of these biochemical effects.*(Rogawski MA. et al; 2008)* The International League Against Epilepsy (ILAE) recently defined pharmacoresistance in epilepsy as a "failure of an adequate trial of two tolerated, appropriately chosen and used AED schedules (whether as monotherapies or in combination) to achieve sustained seizure freedom". *(Kwan P. et al; 2010)*

3.1 Hypothesis of pharmacoresistance

Two major hypotheses have been proposed to explain the phenomenon of pharmacoresistance.

The first *"transporter hypothesis"* suggests that alterations in transport across the blood-brain barrier restrict uptake of antiepileptic drugs in the brain, resulting in inadequate concentrations at the putative site of action. The current hypothesis proposes that overexpression of efflux transporters in peripheral organs such as intestine, liver, and kidney decreases antiepileptic drugs plasma levels in refractory epileptic patients, thereby reducing the amount of drug available to cross the blood–brain barrier and reach the epileptic focus in the brain; this coincided with increased P-glycoprotein (P-gp) protein expression levels in endothelial cells, astrocytes, and neurons from the patient's resected brain tissue.

The second *"target hypothesis"* suggests that alterations occur in drug targets that reduce or eliminate the effectiveness of administered drugs.

These two hypotheses are not mutually exclusive and both mechanisms may contribute to the development of pharmacoresistance. *(Schmidt D. et al; 2005)*

In studies in humans and in animal models of epilepsy numerous changes in transporters, ion channels, neurotransmitter systems, synaptic receptors and neuronal network properties have been uncovered which are consistent with either of the two hypotheses.

In recent years there is also another hypothesis called "intrinsic severity hypothesis" which states that common neurobiological factors contribute to both epilepsy severity and

pharmacoresistance; in other words, pharmacoresistance is inherent to the disease severity, which could exist on a continuum ranging from mild to severe.

In particular in TLE the formation of new excitatory circuits as a result of progressive sprouting has been widely investigated, molecular evidence shows that the growth cone at the tip of an exon receives abnormally expressed guidance and signaling molecules in the epileptic brain.

Neurogenesis and astrogliosis in TLE could contribute to the development of abnormal neural networks and eventually pharmacoresistance.

However, the major weakness of this hypothesis is that alterations in the neural network do not lead to refractoriness in all epileptic patients, and therefore, further biological evidence on potential differences in the changes of brain plasticity between drug-responsive and drug-resistant epilepsy is needed to support this hypothesis .It is possible that more than one mechanism contributes, in different degrees, to the development of pharmacoresistance.

There are different proposed hypotheses for possible underlying mechanism(s) of antiseizure drug (ASD) resistance. *(Tang F. et al; 2017)*

(1) The Pharmacokinetic Hypothesis proposes that overexpression of drug efflux transporters in peripheral organs decreases ASD plasma levels, thereby reducing the amount of ASD available to enter the brain and reach the epileptic focus.

(2) The Neuronal Network Hypothesis states that seizure-induced degeneration and remodelling of the neural network suppresses the brain's seizure control system and restricts ASDs from accessing neuronal targets.

(3) The Intrinsic Severity Hypothesis proposes that common neurobiological factors contribute to both epilepsy severity and pharmacoresistance

(4) The Gene Variant Hypothesis states that variations in genes associated with ASD pharmacokinetics and pharmacodynamics cause inherent pharmacoresistance. These genes include metabolic enzymes, ion channels, and certain neurotransmitter receptors that are targets for ASDs.

(5) The Target Hypothesis postulates that alterations in the properties of ASD targets, such as changes in voltage-gated ion channels and neurotransmitter receptors (e.g., GABAA receptor), result in decreased drug sensitivity and thus lead to refractoriness.

(6) The Transporter Hypothesis states that overexpression of ASD efflux transporters at the blood–brain barrier in epilepsy leads to decreased ASD brain uptake and thus ASD resistance

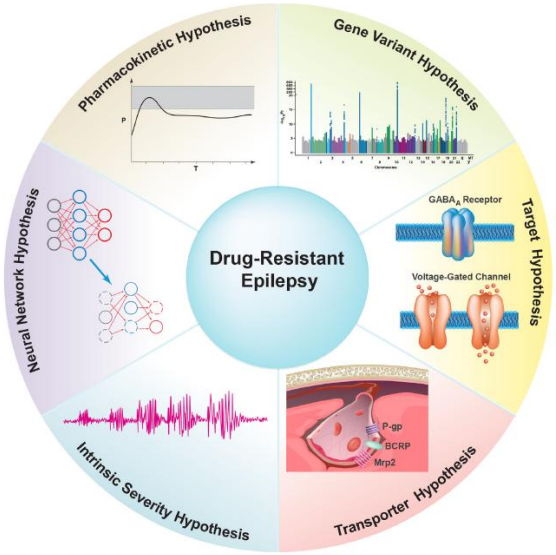

FIGURE 2 : Overview of proposed hypotheses for possible underlying mechanism(s) of antiseizure drug (ASD) resistance.

Modified from: Tang et al. Drug Resistance in Epilepsy (2017)

It is clear from current evidence that pharmacoresistance in epilepsy is a multifactorial phenomenon, but based on existing evidence more work is needed to reinforce and integrate the current theories with the ultimate goal of guiding the development of better epilepsy therapies.

Until more data become available, it is fair to say that transporter overexpression is most likely not the only factor that plays in pharmacoresistance and that the best evidence available only supports the plausibility for the clinical role of efflux transporters in refractory epilepsy.

Obviously, a better understanding of the relationship between epileptogenesis and the development of pharmacoresistance will help to treat epilepsy by preventing the development of pharmacoresistance against antiepileptic drugs.

CHAPTER 4: THE ROLE OF MICRORNAS IN EPILEPSY

MicroRNAs (miRNAs) belong to a class of endogenous 22-nucleotide noncoding RNAs that regulate posttranscriptional gene expression for the inhibition or activation of targeted protein translation, depending on the gene complementarity. In recent years more than 700 miRNAs in mice and approximately 2000 miRNAs in humans have been identified as important for many functions *(Berezikov, E. et al;, 2006)* .

miRNAs are transcribed through RNA polymerase II-dependent transcription to produce pre-miRNAs (hairpin structures) *(Lee, Y et al; 2004),* which are subsequently cleaved by the Drosha microprocessor to generate a 60 – 80-nucleotide stem-loop.

The resulting pre-miRNA is transported via exportin-5 and Ran-GTP to the cytoplasm for processing through the RNAase III enzyme Dicer to form an immature duplex of 20–25 nt *(Obernosterer, G. et al; 2006).* Through helicase, one strand of this immature is typically degraded, whereas the other strand develops into mature miRNA, which subsequently binds to Argonaute (Ago) proteins to form the RNA-induced silencing complex. In addition to Ago proteins, GW182 proteins are required for inducing target gene silencing, and this process is highly conserved. miRNAs control protein translation through the formation of Watson-Crick base pairs with sequences in the 3 ′ untranslated region (UTR), which have also been identified in the 5 ′ UTR.

The pivotal binding site, called the ‘ seed ’ region, is a 5 – 8-nt sequence. Binding at the seed region results in either the inhibition of translation or the degradation of the mRNA target, depending on incomplete or complete complementarity to the target mRNA sequence *(Bartel, D.P. et al; 2004, Luciano, D.J. et al;2004, Vermeulen, A.et al; 2007, Kim, V.N. et al; 2009).*

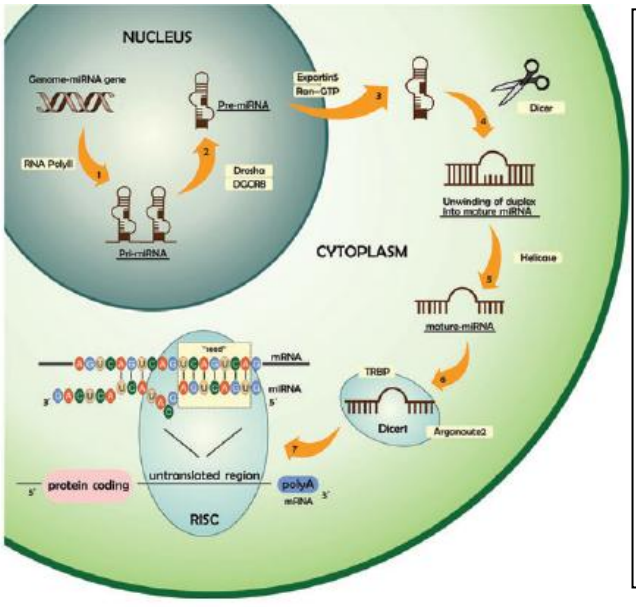

FIGURE 3

miRNA biogenesis pathway MiRNAs are transcribed by RNA PolyII to produce pri-miRNAs (hairpin structures), which are cleaved by the nuclease Drosha to generate pre-miRNA and is then transported via exportin-5 and Ran-GTP to the cytoplasm for further processing by Dicer to form mature miRNAs. After that, the single mature strand of miRNA is uploaded to the RISC complex, which contains Ago-2 and GW182 proteins. Binding of the miRNA to the mRNA usually occurs within a ‘ s eed’ region of 5– 8 nt sequence of 3 ′U TR. The result is either the inhibition of translation or the degradation of the mRNA target, depending on incomplete or complete complementarity to the target mRNA sequence.

Modified from *J. Gan et al.: microRNA, autophagy and epilepsy. Rev. Neurosci. 2015*

Through this mechanism, miRNAs have been implicated in the proliferation, differentiation, and death of cells, playing a significant role during various stages of growth and development. Recently, there has been increasing evidence implicating the underlying molecular mechanisms of miRNAs in neurological diseases, including epilepsy *(Bian, S. et al; 2011)*

4.1 Circulating miRNAs

Several studies have demonstrated that miRNAs are differentially expressed in presence of drug-resistant epilepsy, with a specific expression pattern in brain regions connected to the epileptogenic activity *(Hu, K. et al; 2011 - Li M et al; 2014).* Consequently epilepsy research has focused on the question that whether miRNA are altered by seizures during epileptogenesis or in chronic epilepsy.

Epileptogenesis is most commonly defined as the asymptomatic period between the precipitating insult such as traumatic brain injury, stroke or status epilepticus, and the first spontaneous seizure.

The boundaries of this time period are the cause of much debate as studies have shown that the first detectable convulsive seizure (and therefore end of epileptogenesis) is almost always preceded by numerous non-convulsive seizures. Additionally TLE can be progressive and therefore the epileptogenic period may not end at the time of first seizure but instead the phenotype (severity or frequency of seizures) continues to progress after establishment of the epileptic state.

Like the human condition, the latency to onset of the first spontaneous seizure differs between models although pathological features of epileptogenesis are often shared. These include chronic neuroinflammation, neuronal death/apoptosis, epigenomics, altered neurogenesis and others.

Understanding how the expressions of related genes are regulated could thus provide valuable insights into the molecular basis of epilepsy and illuminate the path toward novel therapeutic venues**.**

Expression levels of miRNAs in blood have been found to be reproducible and demonstrative of the disease state. Serum miRNAs derived from diversified tissues/organs are stable and resistant to nuclease digestion as well as other harsh conditions, including extended storage, freeze-thawcycles, boiling, low/high-pH *(Chen X. et al;2008).*

These miRNAs in biological fluids may come through the damaged blood–brain barrier after epilepsy onset or originate from controlled release in exosomes *(Choi J.W.et al 2017 – Gourlay J. et al 2017).*

Animal studies in epileptic rat models suggest that specific miRNA in blood plasma and sera reflected different types of brain injury, including dynamic change after seizure onset and a pattern unique to prolonged seizures *(Gorter J.A. et al., 2014 – Yan S. et al., 2016).*

Here a figure of recent findings on miRNAs in these pathogenic processes and their potential as targets for disease-modifying therapies.

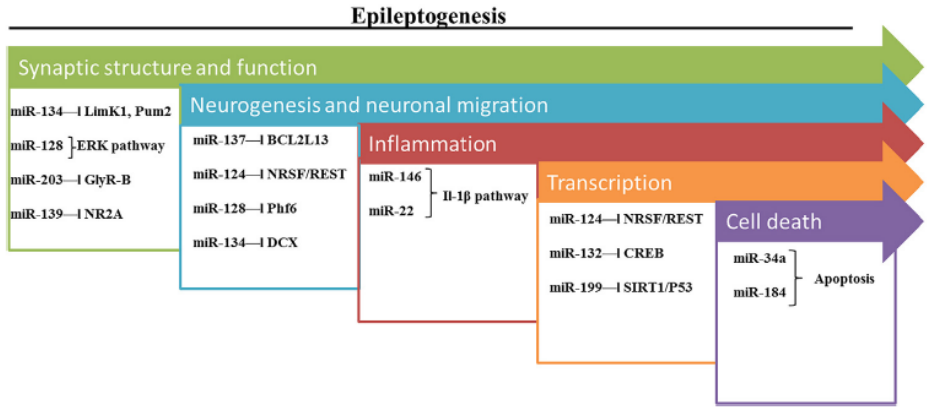

Figure 4

Examples of miRNAs and their mRNA targets and pathways linked to key processes in epileptogenesis

Modified from: G.P. Brennan, D.C. Henshall Neuroscience Letters (2017)

4.2 Exosomal miRNAs

Moreover miRNAs can be transmitted from one neuron to another across the synaptic cleft carried by exosomes. Because of the ability of exosomes to mediate drug efflux, it could be useful to study how they participate in the pathogenesis of drug-resistant epilepsy.

Then, taken into account all these evidences it could also be useful to study how exosomes participate in the pathogenesis of drug-resistance in epilepsy, a mechanism that is not completely clarified *(Gupta A. et al; 2014).*

Understanding the role of circulating miRNAs but also exosomal miRNAs could thus provide valuable insights into the molecular basis of epilepsy and illuminate the path toward novel therapeutic avenues.

By the way a number of studies have investigated the role of miRNAs in epilepsy; both acute neurological insults and prolonged seizures can regulate miRNA expression in brain *(Aronica et al., 2010; Hu et al., 2011).* Moreover several miRNAs have been found to be differentially

expressed in the hippocampus of temporal lobe epilepsy (TLE) or status epilepticus (SE) models.

Emerging evidence shows that epilepsy and epileptogenesis are controlled by epigenetic factors and gene products that regulate multiple genes and proteins on a system level. The Pichardo-Casas et al. (2012) study has demonstrated that the dynamic modulation in the local distribution of miRNAs from brain might contribute to controlling localized protein synthesis at the synapse. *(Pichardo-Casas I et al; 2012)*

4.3 Tissue "epilepsy-specific" miRNAs

Moreover recent profiling data show that a subset of miRNAs seems to be specifically altered in the epilepsy brain, either in brain tissue samples after SE in experimental epilepsy models *(Liu et al., 2010; Hu et al., 2011, 2012; Jimenez-Mateos et al., 2011; Pichardo-Casas et al., 2012; Bot A.M. et al., 2013; Gorter J.A. et al., 2014; Li M. et al., 2014; Kretschmann A. et al., 2015)* or in serum of patients with epilepsy.*(Wang et al., 2015).*

In particular studies on miRNAs in human epilepsy, in patients with TLE, *(Aronica et al., 2010; Jimenez-Mateos et al., 2012; Omran et al., 2012; Ashhab et al., 2013; Peng et al., 2013; Alsharafi and Xiao, 2015)*observed a subset of miRNAs are under investigation as potential regulators of a wide variety pathways involved in epilepsy such as neuroinflammation, blood brain barrier (BBB) dysfunctions, apoptosis, ion channels, tumors, axonal guidance, cell proliferations, neuronal function, and synaptic plasticity *(Kan A. A. et al., 2012; McKiernan R.C. et al., 2012; Kaalund S.S. et al., 2014; Zucchini S. et al., 2014; Wang et al., 2015)*

These "epilepsy-specific" miRNAs include different types of miRNAs such as: miR-128, that governs neuronal excitability, frequently downregulated – miR-134 up-regulated and alterated in epileptogenesis – miR-146 that regulates astrocytic inflammatory response, upregulated. *(Gary P. et al; 2017)*

The last study about "Whole miRNome profiling of human hippocampus" have identified and validated 20 miRNAs with altered expression in the human epileptic hippocampus; 19 miRNAs were up-regulated and one down-regulated in mTLE+HS patients. Because the expression of genes depends on the fine balance of miRNAs present in the cell, an abnormal up or down-regulation of specific miRNAs might influence genes and pathways, resulting in pathology. This study extends current knowledge of miRNA-mediated gene expression regulation in mTLE-HS by identifying miRNAs with altered expression in mTLE-HS, including nine novel abnormally expressed miRNAs and their putative targets. *(Bencurova et al,2017)*

Two direct strategies to develop miRNA-based therapeutics were identified: mimics or agomirs to restore a loss of function of miRNAs and increase their effective levels. The other called inhibitors or antagomirs which intended to block endogenous levels of miRNAs to increase expression of its mRNA targets.

Several functional studies using agomirs/antagomirs reported miRNAs as novel potential approaches to treat epilepsy. These observations further encourage the potential of microRNA-based biomarkers or therapies. *(Bencurova P. et al,2017)*

CHAPTER 5: THE ROLE OF HSPs IN EPILEPSY: Hsp27, Hsp70, Hsp60

Among the mediators of the stress response the heat-shock proteins (HSPs) are functionally implicated in the maintenance of protein homeostasis. HSPs are divided into subfamilies according to weight. They play a crucial role in functioning as chaperones to prevent protein misfolding and aggregation.

HSPs are expressed at low levels in most eukaryotic cells, but are induced by cellular stress such as increased temperature, radiation, exposure to chemicals, oxidative stress and various physiological and pathological stimuli *(Tsan MF. et al; 2004 - Multhoff G. et al; 2007)*

In addition to being chaperone proteins, the HSPs play a part in antigen presentation and cross-presentation *(Li Z. et al; 2002)* . They also function as cytokines to induce production of proinflammatory cytokines and promote dendritic cell maturation *(Wang Y. et al; 2002 - Asea A. et al; 2000)* .

A pathogenic role of HSPs has been suggested for some neurological disorders such as: **Myasthenia Gravis**, **Guillain-Barré Syndrome, Multiple Sclerosis, Neurodegenerative Disorders and stroke,** advances have been made in understanding this role.*(Fredrik R. et al; 2011)* In particular, in epilepsy **Hsp27** has been found to be highly expressed in epileptic neocortex obtained from patients during neurosurgery, being present in astrocytes and in cerebral blood vessel walls. Only low amounts of HSP-27 were detectable in control brains. This indicates that HSP-27 becomes induced in response to epileptic pathology, and it is a marker of cortical regions where seizures have caused a stress response. *(Bidmon HJ. et al; 2004)* Existing data also suggest the involvement of Hsps in neuronal damage caused by SE, although their role in neurodegeneration during epilepsy still remains uncertain.*(Rejdak K. et al; 2012)*

In animal models of epilepsy, increate **Hsp70** expression during acute and chronic phases has been documented. In TLE patients, complete remission of mesial TLE seizures postsurgery was associated with decreased Hsp70 expression in CA4 and subiculum and decreased Hsp90 expression in the granular layer. Higher Hsp70 serum levels in patients with TLE as compared to controls were observed, and were predictive of higher frequencies of seizures in the TLE group. Data from animal models showed that Hsp72 increased levels in specific hippocampal neuronal subpopulations correlate with limbic seizure intensity and duration and it is a highly sensitive and specific hippocampal marker for full development of pilocarpine-induced SE *(Rejdak K. et al; 2012).*

In contrast to other Hsps, **Hsp60** levels and expression have only been sporadically studied in animal models of CNS diseases or neurological patients. In fact, the mitochondrial chaperonin Hsp60 also named HSPD1 *(Kampinga H.H. et al., 2009)* was found in multiple subcellular sites and function in the folding and intracellular trafficking of many proteins *(Cappello F. et al., 2008; Deocaris C.C. et al., 2006)*. Hsp60 may be present in cell cytoplasm, cell membrane, free in the extracellular space or as cargo of extracellular vesicles like exosomes *(Cappello F. et al., 2008).*Hsp60 has been found elevated in a large number of human carcinomas, which opens novel perspectives for cancer diagnosis and therapy targeting Hsp60 *(Cappello F. et al., 2014; Tang H. et al., 2016).* The chaperonin can activate the immune system *(Marino Gammazza A. et al., 2013)* and can have both, pro-survival and pro-death functions, depending on tissue, cell type, and apoptosis inducers (*Cappello F. et al., 2008*).

Regarding the nervous system, Hsp60 was found increased in the brain stem after subarachnoid hemorrhage, forebrain or focal cerebral ischemia, and neonatal hypoxia-ischemia in rats *(Stetler, R. A.et al; 2010)*

Hsp60 was found in the protein aggregates typical of neurodegenerative diseases such as Parkinson's disease (PD) and Alzheimer's disease (AD), in which a fundamental role is played by oxidative stress and mitochondrial dysfunction *(Calabrese V. et al; 2007- Yang T. et al; 2009).*

Physiologically, neural expression of Hsp60 increases over the course of development, consistent with the changes of mitochondrial content in the brain *(D'Souza, S. M. et al; 1998).*

The association between Hsp60 in the central nervous system and TLE, the most common type of epilepsy in humans, is poorly understood.

Mitochondrial oxidative stress and dysfunction have been suggested to be contributing factors to the development of neurological disorders, TLE in particular, but their precise role (cause or consequence) in epileptic seizures has not yet been fully characterized *(Waldbaum S. et al; 2010)*. Mitochondrial dysfunction and oxidative stress are factors that not only occur acutely as a result of precipitating injuries such as SE, but may also contribute to neuronal cell death, epileptogenesis, and chronic epilepsy. Therefore, Hsp60, a constitutive mitochondrial protein with specific functions related to mitochondrial protein folding, especially in response to oxidative stress *(Sarangi U.et al; 2013),* might play an important role in the physiopathology of this type of epilepsy.

CHAPTER 6: AIMS OF THE THESIS

Aims of this research project is to discover an *"epilepsy-specific" miRNAs profiling* subset specifically altered in the epileptic brain involving neuroinfiammation, neurodegeneration, duration of the disease, seizures frequency and also in the mechanism of drug-resistance.

In particular the aim is to select patients with drug-resistant epilepsy, both with and without a brain structural lesion, eligible to epilepsy surgery, in order:

1) to evaluate the miRNAs serum expression in patients with focal non lesional drug-resistant epilepsy, comparing differences of miRNA expression in patients with focal lesional epilepsy, such as focal cortical dysplasia (FCDs) or temporal lobe epilepsy with hippocampal sclerosis (TLE-HS), using miRNAs serum expression as a biomarker of drug-resistance;

2) to evaluate miRNAs expression in exosomes isolated from patients with lesional and non lesional focal epilepsy, comparing the levels with those found free in the blood;

3) to investigate miRNA expression in specimens of hippocampus from patients with TLE-HS, comparing control hippocampal tissue obtained at autopsy from patients without history of seizures or other neurological; empathizing a role of miRNAs in epileptogenesis and drug-resistance.

4) to investigate Hsp60 involvement in epileptogenesis as a marker of oxidative stress and the correlation of its levels with the selected miRNAwith a direct effects on its expression.

CHAPTER 7: MATERIAL AND METHODS

7.1 Patient selection and enrolment

This research project is a multiphase case-control study designed to evaluate significant miRNAs expression profiling from patients with drug-resistant epilepsy and healthy controls.
The study was performed through a prospective and consecutive recruitment of patients belonging to the centre for the diagnosis and treatment of epilepsy of the department of clinical neurosciences at the University of Palermo and at the University "La Sapienza" of Rome from 2015 to 2018.
We selected 51 patients with a confirmed diagnosis of drug-resistant epilepsy according to the criteria of the International League against Epilepsy (ILAE 2010), 26 of theme (51%) had idiopathic focal epilepsy and 25 (49%) had lesional focal epilepsy (12 FCDs e 13 TLE-HS).
All these patients has been recruited, equally, from the Department of Experimental Biomedicine and Clinical Neurosciences, Epilepsy Center of the Neurology Unit, of the University of Palermo and from the Epilepsy Center of the University "La Sapienza" of Rome.
Then we also obtained 10 surgical specimens of hippocampus from patients with TLE-HS (Fondazione IRCCS IstitutoNeurologico Carlo Besta, Milan) and control hippocampal tissue was obtained at autopsy from 4 patients without history of seizures or other neurological diseases (Forensic Science, University of Palermo).

The clinical evaluation of patients was performed by neurologists with experience in the treatment of patients with epilepsy. All patients were interviewed using a structured questionnaire gathering information regarding age, onset of epilepsy, history of febrile seizure, family history of epilepsy and number of AEDs used. In addition, all patients underwent a neurological exam, serial interictal EEGs and high resolution MRI with a specific epilepsy protocol. Hippocampal atrophy and other MRI signs of hippocampal sclerosis (HS) were assessed by visual analyses and the images were classified as having normal findings or signs of HS. Patients with dual pathology or tumors were not included.

Demographic and clinical characteristics of selected patients are summarized in table 1 (Tab.1).
The control group, matched by age (+/- 5 years) and sex to patients group, consisted of volunteers from among college students, relatives and friends of the medical staff.
The local ethics committee approved the study protocol and the patients gave their consent for the execution of the study.

TABLE 1 : Demographic and clinical characteristics	
Patients	51
Age (years)	50,8
Males (%)	16 (31%)
Females (%)	35 (69%)
Duration of disease (years)	15 years
Focal idiopathic epilepsy (%)	26 (51%)
Focal lesional epilepsy (%)	25 (49%)
▪ **Focal cortical dyspalsia (FCDs) (%)**	12 (48%)
▪ **Hippocampal sclerosis (TLE-HS) (%)**	13 52%)

7.2 Extraction of miRNA from plasma

Blood samples has been withdrawn from patients was processed within 90 min after collection. Plasma was obtained by centrifugation at 1500g for 15min and stored at -80°C until needed. Total RNA including small RNA was isolated from aortic specimens using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) and from plasma using the miRNeasy Serum/Plasma kit (Qiagen) according to the manufacturer instructions. The quantity and quality of total RNA were determined by a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Milan, Italy).

7.3 Exosomes isolation from plasma and miRNA extraction.

Exosomes were isolated from plasma as previously described *(Campanella, C. et al.2015)*with some modifications. Blood samples of all patients were treated with EDTA and centrifuged at 4000 x g for 20 minutes to separate the plasma and then centrifuged at 11000 x g for 30 minutes to remove cell debris. The supernatant was diluted with PBS then filtered through a 0.2 µm filter (Millex GP, Millipore), followed by 2 step ultracentrifugation at 110,000 x g for 2 hours to pellet the exosomes. The exosomes were then washed in cold PBS and suspended in 50 µl of PBS. Total RNA content including small RNA was isolated from exosome samples using the miRNeasy Mini Kit (Qiagen) according to the manufacturer instructions.

7.4 Exosome characterization via transmission electron microscopy (TEM) and Dynamic Light Scattering (DLS)

Exosomes obtained from the plasma were examined by Transmission Electron Microscopy (TEM) and Dynamic Light Scattering (DLS) for their morphological evaluation. Pellets obtained by ultracentrifugation were resuspended in PBS with the addition of 100µl of freshly made fixative (2.5% glutaraldehyde in PBS) for 30 minutes. After fixation, the preparations were mounted on formvar nickel grids by layering grids over 10 ml drops of exosome preparations for 10 minutes at 24°C. Grid-mounted preparations were prepared for contrast staining by treating them with uranyl acetate (1%) for 5 minutes and with Reynolds' solution for 5 minutes and, finally, rinsing them eight times in distilled water for 2 minutes. After this procedure, the grids were ready to be observe at the electron microscopy (JEOL JEM 1220 TEM at 120kV). DLS measurements were performed using a Brookhaven Instrument BI200-SM goniometer. The temperature was controlled to within 24° C using a thermostated recirculating bath. The time autocorrelation functions (TCF) were measured by using a Brookhaven BI-9000 correlator and a 100 mW solid-state laser (Quantum-Ventus MPC 6000) tuned at λ = 532 nm. Measurements were taken at 90° scattering angle. All samples were filtered through 0.2 µm cellulose acetate (Millipore) syringe filters to remove gross contaminants.

7.5 Exosome characterization via western blotting analysis

Western Blotting analysis was carried out to characterize exosome isolated from plasma of TLE patients and controls, using recognized exosomal markers like Alix,Hsc70, and CD 81.Exosomal pelletswere washed twice in PBS and resuspended in 50 µl ice cold RIPA lysis buffer (0.3 M NaCl, 0.1% SDS, 25 mM HEPES pH 7.5, 1.5 mMMgCl2, 0.2 mM EDTA, 1% Triton X-100, 0.5 mM DTT, 0.5% sodium deoxycholate) containing proteases and phosphatase inhibitors (0.1 mg/ml phenylmethylsulfonylfluoride, 20 mg/ml aprotinin, 20 mg/ml leupeptin, 10 mg/ml NaF, 1 mM DTT, 1 mMsodiumorthovanadate, 20 mM ß-glycerol phosphate) to obtain lysates. A standard western blotting procedure was conducted to separate protein using a 12% polyacrylamide gel (SDS-PAGE), in which equal amounts of protein (50 µg) were added to 10X Laemmli buffer and heated for 5 min at 95 °C. Therefore samples were loaded in each well and transferred onto a nitrocellulose membrane (BioRad, Milan, Italy). The membranes were stained with Ponceau S to verify the quality of transfer and loading similarity. After blocking with 5% albumin bovine serum (Sigma Aldrich), membranes, with the spotted proteins, were analyzed to measure the protein levels, probing with specific primary antibodies (mouse anti-Alix, 1A12 clone, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA, diluted at 1:500; mouse anti-Hsc70, B-6 clone, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA, diluted at 1:500; mouse anti-CD81, B-11 clone, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA, diluted 1:1000.) for 12 hours followed by incubation with horseradish peroxidase-conjugated secondary antibody. The final detection was performed using the enhanced chemiluminescence (ECL) detection system, Western Blotting Detection Reagent (Amersham Biosciences, GE Healthcare Life Science, Milan, Italy), according to the manufacturer's instructions. Membranes were then exposed to X-ray film from few second to 5 min and the film was analyzed. Densitometric analysis of the bands was evaluated and quantified using the NIH Image J 1.40 analysis program (National Institutes of Health, Bethesda, MD). Each experiment was performed at least three times.

50µg of protein extracted from exosome were used, and the primary antibody used for Alix (mouse anti-Alix, 1A12 clone, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA, n° catalogue sc-53540) was diluted at 1:500; the one for Hsc70 (mouse anti-Hsc70, B-6 clone, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA, n° catalogue sc-7298) was diluted at 1:500; while the one for CD81 (mouse anti-CD81, B-11 clone, , Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA, n° catalogue: sc-166029) was diluted 1:1000.

7.6 MicroRNAs extraction from paraffin embedded tissue

Formalin-fixed paraffin-embedded hippocampus were sectioned at 7 μm, four sections were used for RNA isolation and were placed in 1.5 ml microcentrifuge tube DNase/RNase free, to avoid the nucleic acid degradation. Samples were dewaxed with xylene and rehydrated with alcohol scale at room temperature and in agitation. Then pellets were treated with Triazol to lysis tissues, following the manufacturing instruction (miRNeasy Mini Kit®, Qiagen, Hilden, Germany). Briefly, tissue samples were homogenized in QIAzol Lysis Reagent and after addition of chloroform, the homogenate was separated into aqueous and organic phases by centrifugation. RNA partitions to the upper, aqueous phase, while DNA partitions to the interphase and proteins to the lower, organic phase or the interphase. The upper, aqueous phase was extracted, and ethanol was added to provide appropriate binding conditions for all RNA molecules from 18 nucleotides (nt) upwards. The samples were then applied to the RNeasy Mini spin column, where the total RNA binds to the membrane and phenol and other contaminants are efficiently washed away. Then, miRNAs were enriched in a separate fraction through the RNeasy MinElute® Cleanup Kit (Qiagen, Hilden, Germany). Finally, RNA was dissolute in 50μl of RNase/DNase-free H2O and was determined using Thermo Scientific NanoDrop ND-2000 1-position Spectrophotometer (Thermo Scientific Massachusetts, USA).

7.7 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR).

Reverse transcription was performed using the miScript II RT kit and the miScript Reverse Transcriptase Mix (Qiagen) according to the manufacturer's instructions. Complementary deoxyribonucleic acid (cDNA) was preamplified using the miScriptPreAMP PCR Kit (Qiagen). qRT–PCR analysis was performed using the miScript SYBR Green PCR Kit (Qiagen) as previously described *(Barone R. et al; 2017)* and the primers indicated in Table 2. miRNA were selected from the literature based on their previously reported association with TLE (miR-8071, miR-663b, miR-146a, miR-124a) and with the regulation of Hsp60 (miR-1 and miR-206). miRNA levels were normalized to that of miR-16. Changes in the transcript level were calculated using the $2^{-\Delta\Delta CT}$method. The PCR was carried out using the Rotor-gene™ 6000 Real-Time PCR Machine (Qiagen).

Table 2. Primers used for qRT-PCR.

Name	Sequence
Hs_miR-8071_1	5'CGGUGGACUGGAGUGGGUGG
Hs_miR-663b_2	5'GGUGGCCCGGCCGUGCCUGAGG
Hs_miR-146a_1	5'UGAGAACUGAAUUCCAUGGGUU
Hs_miR-206_1	5'UGGAAUGUAAGGAAGUGUGUGG
Hs_miR-124a_1	5'UAAGGCACGCGGUGAAUGCC
Hs_miR-1_2	5'UGGAAUGUAAAGAAGUAUGUAU
Hs_miR-16_2	5'UAGCAGCACGUAAAUAUUGGCG

7.8 Immunohistochemistry

Sections (5 μm) of hippocampus from patients and controls were dewaxed in xylene for 10 minutes and rehydrated by sequential immersion in a descending scale of alcohols and transition in water for five minutes. Subsequently, the samples were immersed for 8' in Sodium Citrate Buffer (pH 6) at 95°C for antigen retrieval and, subsequently, immersed for 8’ in acetone at -20°C to prevent the detachment of the sections from the slide. After a wash with PBS for 5’, the sections were treated for 5’ with Peroxidase Quencing Solution (reagent A of Histostain®-Plus 3rd Gen IHC Detection Kit, Invitrogen) to inhibit any endogenous peroxidase activity. Another washing with PBS for 5’ was carried out and the sections were treated with a blocking protein (reagent B of Histostain®-Plus 3rd Gen IHC Detection Kit, Invitrogen) for 10’ to block non-specific antigenic sites. Subsequently, the sections were incubated overnight, with a primary antibody against human Hsp60 (mouse anti-Hsp60 monoclonal antibody, Sigma, St. Louis, MO, catalogue no. H4149, dilution 1:400) Appropriate positive and negative controls, were run concurrently. After a wash with PBS (Phosphate Buffered Saline pH7.4) for 5’, the sections were incubated with a universal biotinylated secondary antibody (Biotinylated Secondary Antibody reagent C Histostain®-Plus 3rd Gen IHC Detection Kit, Invitrogen) for 10’. After a subsequent washing with PBS for 5', the sections were incubated with streptavidin-peroxidase complex (Streptavidin-

Peroxidase Conjugate reagent D Histostain®-Plus 3rd Gen IHC Detection Kit, Invitrogen) for 10', and following a further washing in PBS for 5', the slides were incubated in the dark for 5' with the DAB chromogen (diaminobenzidine) (DAB chromogen reagents E1 and E2 Histostain®-Plus 3rd Gen IHC Detection Kit, Invitrogen). The slides were mounted with cover slips and images were taken with a Leica DM5000 upright microscope (Leica Microsystems, Heidelberg, Germany). ImageJ 1.41 software was used to calculate the density of Hsp60-immunoreactive neurons in the DG and hippocampus proper (CA3, CA1) of HC and TLE patients. The densitometry analysis was performed as described before *(Marino Gammazza et al., 2015)* on five fields per hippocampal sector, four sections per samples and in three samples for HC and three for TLE-HS patients. The objective lens used was 40X. The acquired images (RGB) were converted into grey scale images (32-bit) and inverted. Staining intensity of neurons was represented by a histogram and expressed as mean of pixel intensity (PI). The intensity of a pixel was expressed within a given range between a minimum (zero) and a maximum (255), where 0 corresponds to no positivity (black) and 255 to maximal positivity (in greyscale black and white, respectively). Sections stained only with hematoxylin were run concurrently; pixel intensity was measured and subtracted from the Hsp60 quantification. Finally, the slides were prepared for observation with coverslips with an aqueous mounting solution. The observation of the sections was performed with an optical microscope (Leica DM 5000 B) connected to a digital camera (Leica DC 300F).

7.9 ELISA

Elisa was performed using a specific commercial Hsp60 (human) enzyme immuno-assay (EIA) kit (StressMarq Biosciences, Victoria, Canada, USA). The standards were diluted in sample diluent to generate a standard curve with six points, ranging from 3.125 to 100 ng/ml. Sample diluent alone was used as 0 (zero) standard. First, 100 µl of prepared standards and undiluted plasma was added in duplicate to wells of the immunoassay plate precoated with mouse monoclonal antibody specific for Hsp60 and incubated at 23 °C for 1 h. The primary and the secondary antibodies were diluted according to the manufacturer's instructions. After washing, 100 µl of diluted horse radish peroxidase-conjugate secondary antibodies were added to the plates and incubated at 23 °C for 30 minutes followed by 100 µl of 3,3′,5,5′- tetramethylbenzidine substrate for 15 minutes in the dark. Finally, 100 µl of Stop Solution was added, and absorbance was measured at 450 nm in a microplate photometric reader (Microplate reader, Euroclone, Milan, Italy). Sample concentration was

calculated by interpolating the sample concentrations in the standard curve. The sensitivity of the human Hsp60, EIA kit was determined to be 3.125 ng/ml

Statistical analysis

The analysis was performed using the statistical software package GraphPadPrism4 (San Diego, CA). The data obtained were compared by unpaired *t*-test and One-way ANOVA analysis of variance. If a significant difference was detected by ANOVA analyses, this was further evaluated by Bonferroni post-hoc test. The data are expressed as means ± SD. The statistical significance threshold was fixed at $p < 0.05$.

CHAPTER 8: RESULTS

MiR-8071, miR-663 and miR-146a circulating levels significantly decreased in the plasma of epileptic patients (TLE-HS and IFE)while miR-124 levels did not changed.

As showed in *Figure 5*, plasma expression levels of miR-8071,miR-663 and miR-146a significantly decreased in FCD, TLE-HS and IFE patients compared to HC group ($p<0.001$) while miR-124circulating levels did not showed any significant changes. All the analysis were performed in quintuplicate.

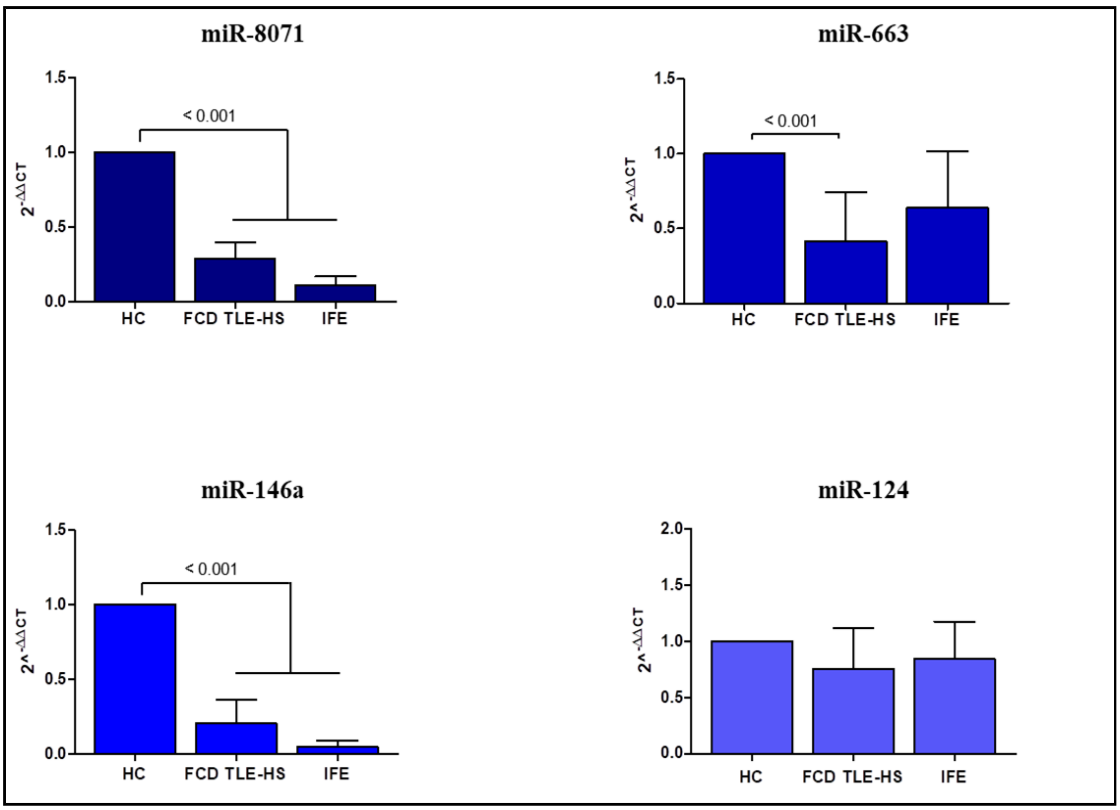

FIGURE 5. qRT-PCR evaluation of plasma circulating levels of miR-8071, miR-663, miR-146a and miR-124 in TLE patients and HC according to the Livak method $2^{-\Delta\Delta CT}$. Data are presented as the means ± SD. FDC TLE-HS: Temporal lobe epilepsy with hippocampal sclerosis and focal cortical dysplasia ; IFE: Idiopathic focal epilepsy; HC: Healthy control.

Exosomes isolated from plasma samples of TLE patients showed typical characteristics.

Exosome obtained from plasma of patients affected by TLE and from controls were analyzed by TEM (*Fig. 6A*). The observation at the electron microscope indicated that size and morphology of the obtained vesicles were in accordance with those described for exosomes. In addition, the results obtained by western blotting analysis for Alix and CD81 protein expression confirmed that the vesicles isolated were exosomes (*Fig.6 B*). Furthermore, the exosomes preparations were analyzed by DLS (*Fig.6C-E*). DLS measures the size and number of each particle by the direct observation of scattered light and the particle motion. As showed in *Figure 6C-D* and summarized in table 3, the vesicle populations isolated from plasma of TLE patients, both lesioned and idiopathic, have not significant differences then the controls either in size and in number. All the analysis were performed in quintuplicate.

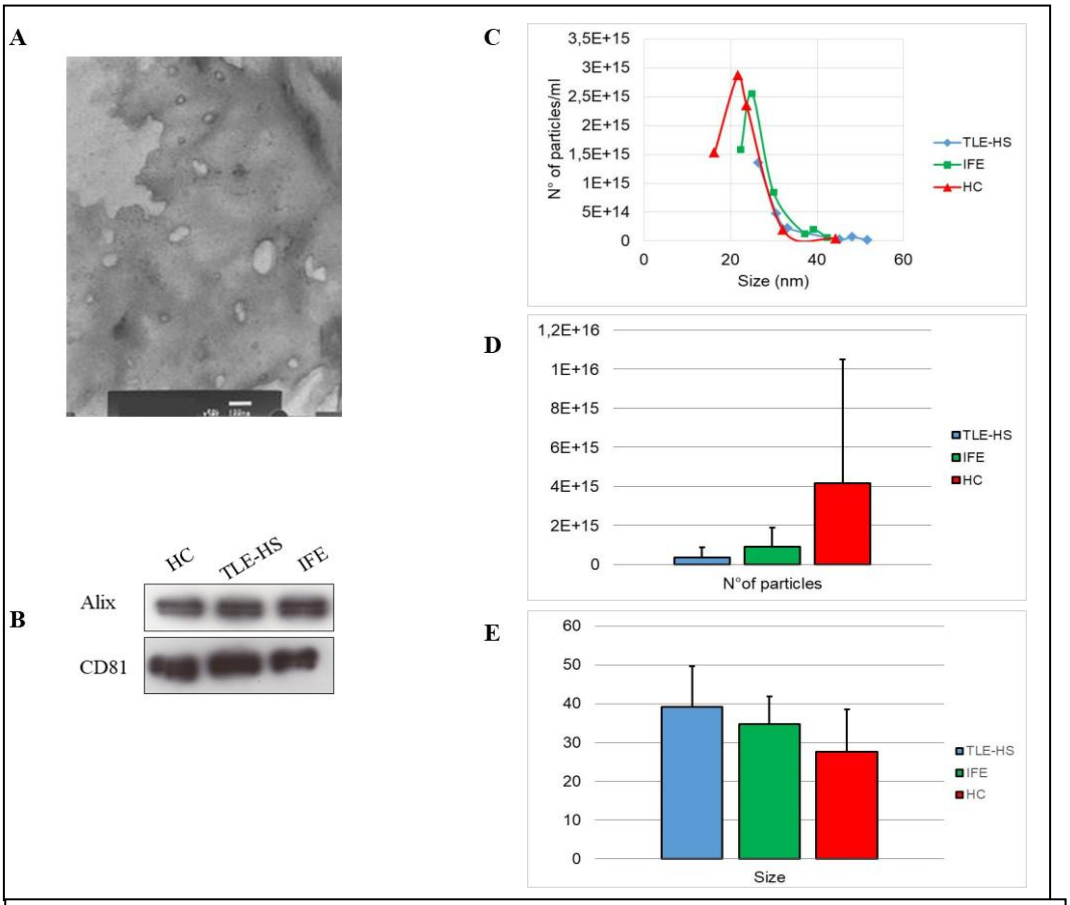

FIGURE 6.
A) An illustrative image (TEM) of exosomes purified from plasma of TLE patients, bar=200 nm.
B) Representative Western blotting bands for the exosomal markers Alix and CD81. C-E) Representative graphs of the DLS analysis of the isolated exosomes. Data are presented as the means ± SD. FDC TLE-HS: Temporal lobe epilepsy with hippocampal sclerosis and focal cortical dysplasia; IFE: Idiopathic focal epilepsy; HC: Healthy control.

Table 3

	Size (nm)	Number (N° of particles/ml)
TLE-HS	39.202 ± 10.431	3.62E+14± 5.179E+14
IFE	34.710 ± 7.127	8.95E+14±9.981E+14
Healthy control	27.635± 10.960	4.151E+15± 6.365E+15

MiR-8071 and miR-146a significantly increased in the exosome of TLE patients while miR-663 and miR-124 levels did not changed.

As showed in *Figure 7*, the exosomes cargo of miR-8071 significantly increased in epileptic patients (more in IFE than FCD and TLE-HS patients) compared to the control group ($p<0.05$). In the same manner, miR-146a levels significantly increased in the exosomes isolated from IFE patients compared to FCD, TLE-HS and controls ($p<0.001$). On the contrary, miR-663 and miR-124 levels did not showed any significant changes. All the analysis were performed in quintuplicate.

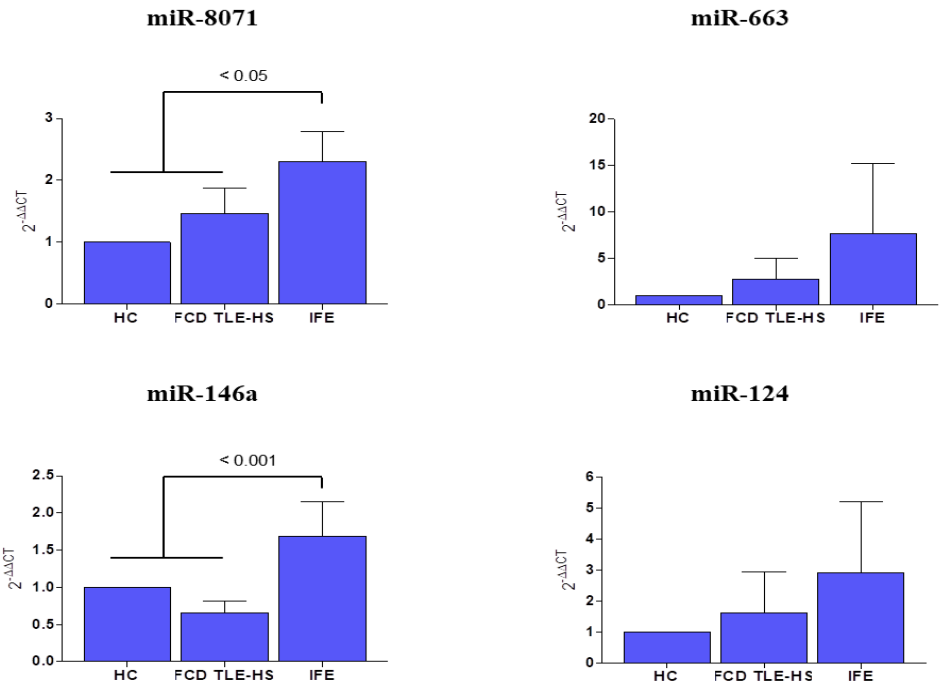

FIGURE 7. qRT-PCR evaluation of exosomal circulating levels of miR-8071, miR-663, miR-146a and miR-124 in TLE patients and HC according to the Livak method $2^{-\Delta\Delta CT}$. Data are presented as the means ± SD. FDC TLE-HS: Temporal lobe epilepsy with hippocampal sclerosis and focal cortical dysplasia; IFE: Idiopathic focal epilepsy; HC: Healthy control.

Hsp60 immunoreactivity significantly decrease in hippocampus of TLE-HS patients.

To determine the localization of Hsp60, immunostaining was performed.

In hippocampus of HC and TLE-HS patients Hsp60 was detected in all the hippocampal sectors and Hsp60 immunoreactivity was localized in the dentate gyrus granule cells, and in CA3 and CA1 pyramidal cells.

*Figure 8-A*shows representative microphotographs of CA3 e sector. There was also a diffuse neuropilar labeling of undetermined association with specific elements. As determined by the densitometry analysis (*Fig. 8-B*)in TLE-HS patients Hsp60 immunoreactivity decreased along the strata of the hippocampal sectors (*Fig. 8*, $p<0.05$). Interestingly, Hsp60 immunoreactivity in TLE-HS patients showed a preferential distribution pattern compared to HC, resembling mitochondria localization.

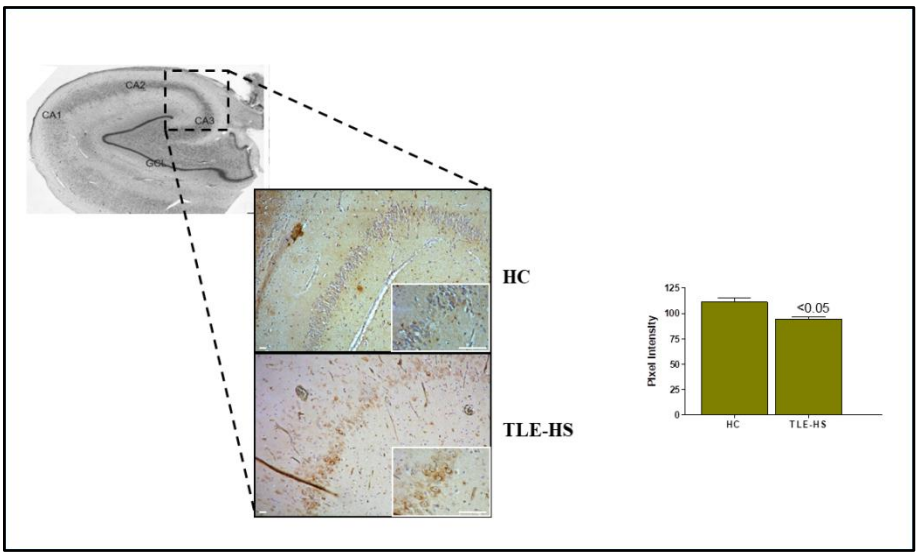

FIGURE 8.
A) Representative microphotographs of Hsp60 immunoreactivity in the hippocampus of control and TLE patients. Bar = 100 μm.
B) Representative histograms showing densitometric measurements of the Hsp60 staining intensity in controls and TLE subjects. Staining intensity is expressed as pixel intensity. TLE-HS: Temporal lobe epilepsy with hippocampal sclerosis HC: Healthy controls.

Hsp60 circulating levels increased in FCD TLE patients while decreased in IFE patients.

To determine the circulating levels of Hsp60 in plasma, Elisa test was performed. The difference between plasmatic levels of Hsp60 in patients affected by FCD TLE-HS(n=10) significantly increased compared to the circulating levels present in IFE (n=10) and HC (n=10) (*Fig. 9*; FCD TLE-HS *vs* HC $p<0.05$; FCD TLE-HS vs IFE $p<0.001$).

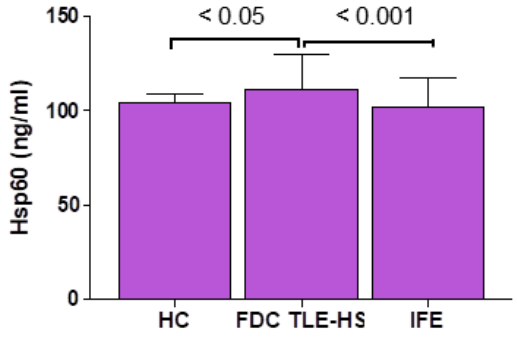

FIGURE 9. Histograms representing ELISA measurements of Hsp60 plasmatic levels in TLE patients and HC. Data are presented as the means ± SD. FDC TLE-HS: Temporal lobe epilepsy with hippocampal sclerosis and focal cortical dysplasia; IFE: Idiopathic focal epilepsy; HC: Healthy control.

MiR-8071 and miR-124 significantly increased in the hippocampus of TLE patients while miR-663 and miR-146adid not changed.

As showed in *Figure 10*, hippocampal expression levels of miR-8071 and miR-124 significantly increased in TLE-HS patients compared to HC group ($p<0.001$) while miR-663 levels did not showed any significant changes. After several attempts, it was not possible to determine the tissue levels of miR-146a. All the analysis were performed using n=5 samples per group.

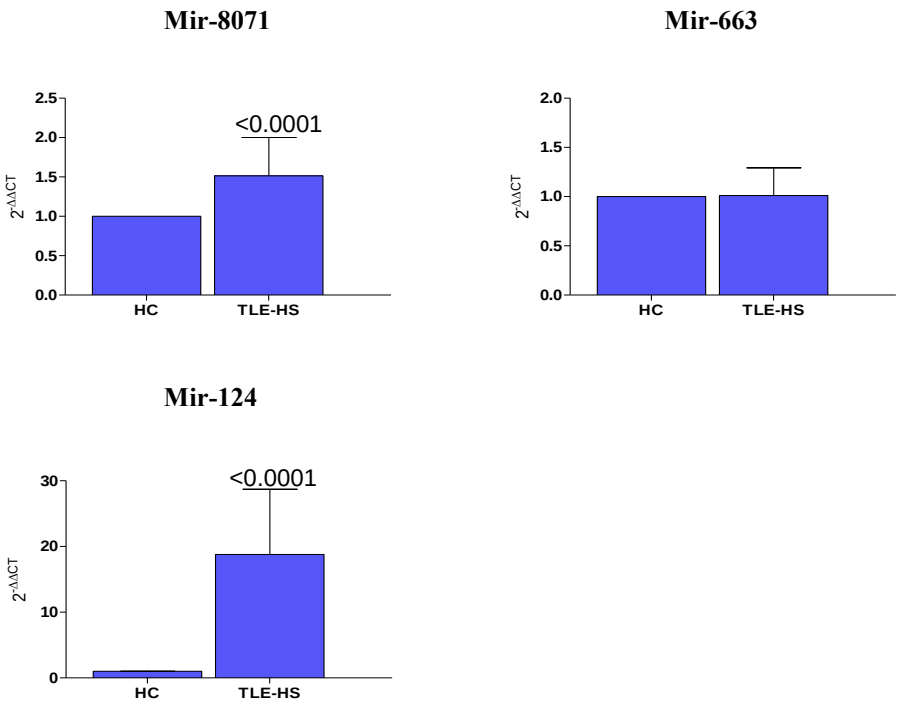

FIGURE 10. qRT-PCR evaluation of hippocampal levels of miR-8071, miR-663, and miR-124 in TLE patients and HC according to the Livak method $2^{-\Delta\Delta CT}$. Data are presented as the means ± SD. FDC TLE-HS: Temporal lobe epilepsy with hippocampal sclerosis and focal cortical dysplasia; IFE: Idiopathic focal epilepsy; HC: Healthy control.

MiR-1 and miR-206 significantly decreased in the hippocampus of TLE patients.

The tissue expression levels of two miRNAs correlated to Hsp60 expression were also investigated using Real Time PCR. (*Figure 11*)

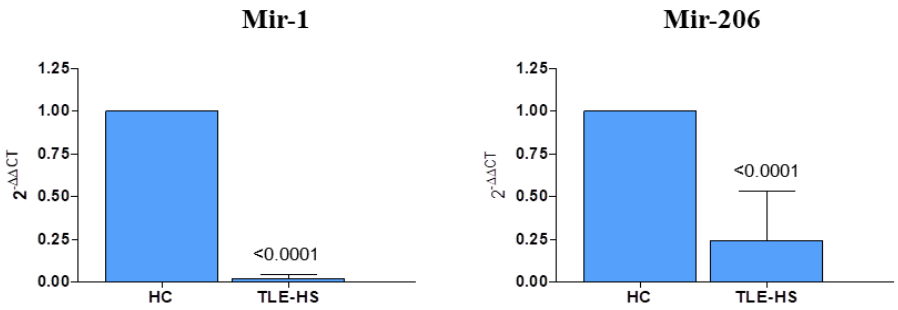

FIGURE 11. qRT-PCR evaluation of hippocampal levels of miR-1 and miR-206 in TLE patients and HC according to the Livak method $2^{-\Delta\Delta CT}$. Data are presented as the means ± SD. FDC TLE-HS: Temporal lobe epilepsy with hippocampal sclerosis and focal cortical dysplasia; IFE: Idiopathic focal epilepsy; HC: Healthy control.

CHAPTER 9: DISCUSSION

It has become increasingly clear that acute epileptogenesis, the process by which an insult leads to the development of spontaneous seizures, is complex and multifaceted. Roles have been described for inflammation *(Vezzani A. et al; 2011)* as well as to a variety of molecular and cellular changes *(Brooks-Kayal A R et al; 2009; Cacheaux LP et al; 2009).*

Therefore, we focused on circulating, exosomal and tissue miRNAs profile expression, such a mechanisms that might control several epilepsy-mediating pathways.

Firstly we investigated the role of circulating ***miR-146a, miR-8071, miR-663 and miR- 124*** levels in epilepsy.

We observed a down-regulation of ***miR-146a, miR-8071*** and ***miR-663,*** both in patients with idiopathic and lesional focal epilepsy, and compared them with healthy control.
It is well known that miR-146a is significantly up-regulated in the hippocampi obtained from patients with TLE, as well as in experimental TLE rats suggesting the possible role of miR-146a in epileptogenesis.*(Aronica E. et al., 2010;Song Y. et al., 2011;Hu G. et al; 2012;Omran A. et al; 2012;He X. et al; 2016).*
The fact that ***miR-146a*** plays an inhibitor role during the acute phase of an epileptic seizure *(Kretschmann A. et al; 2015)* can explain the reason why in our population of epileptic patients these levels are down-regulated, we suggest it should be an intrinsic marker of drug-resistance.

Similarly ***miR-8071*** has a good diagnostic value for mTLE-HS with 83.33% sensitivity and 96.67% specificity, and is well associated with seizure severity. *(Shaofeng Y. et al; 2017).*
The study indicates that these miRNAs, may be regulators for the seizure development in mTLE-HS, and can be used as potential biomarker for diagnosis in mTLE-HS.

So it is well known that in tissue, human or experimental material, these miRNAs are highly expressed, while their role in circulating plasma should be of less significance, as suggested by our results.

However considering the role of circulating ***miR-663*** no data are available in the field of epilepsy until now. Anyway its close correlation with mechanism of cellular differentiation and chronic inflammation *(Michaille J. et al; 2018)* should help in the identification of new targets inside the mechanism of drug-resistant epilepsy.

We did not observed any significant changes in ***miR- 124*** circulating levels between patients with idiopathic and lesional focal epilepsy compared with controls.

We already known that miR-124, a brain-specific microRNA, was originally considered as a key regulator in neuronal differentiation and the development of the nervous system. *(Wang W. et al 2016, Lagos-Quintana M. et al. 2002 - Deo M. et al. 2006 - Yu J.Y. et al. 2008).*

A recent study also showed that miR-124 could function at the growth cone or at synapses by modulating synaptic activity and neuronal connectivity *(Sun A.X et al 2013).* However, it remains unclear whether miR-124 is involved in regulation of neuronal excitability and seizures.

It was previously observed that miR-124 is downregulated in epilepsy, and it was also identified that miR124 regulated the CREB1 gene and CREB1 protein expression, which is a key regulator in epileptogenesis.*(Wang W. et al 2016)*

Our results are not statistically significant but they suggest a neuroprotective role of miR-124, more evident in idiopathic focal epilepsy, with a low intensity and severity of epileptic seizures, compared to lesional focal epilepsy.

These results should be considered as a previously unknown function of miR-124 in neuroprotection.

Secondly we investigated the role of ***exosomal miRNAs expression in epileptic patients***.

Exosomes, a specific subtype of secreted membrane vesicles that are approximately 30–100 nm in size, are involved in cell-to-cell communication and targeting cells by transferring exosomal molecules including proteins and microRNAs *(Montecalvo A. et al 2012; Hannafon B.N. et al; 2013).*

Exosomal miRNAs have been identified, both in physiological and disease conditions *(Stoorvogel W. et al; 2012).*

Furthermore, the quantity and composition of exosomal miRNAs are different between patients and healthy individuals *(Cullman SL et al 2016).*

There is growing evidence supporting miRNAs changes in the pathophysiology of epilepsy *(Wang J. et al; 2015).* However, to our knowledge, there is only one report that has already investigated exosomal miRNAs in the plasma of epileptic (mTLE-HS) patients. *(Shaofeng Y. et al; 2017)*

Shaofeng et al. (2017) reported that miR-8071 had the best diagnostic value for mTLE-HS with 83.33% sensitivity and 96.67% specificity, and was associated with seizure severity.

We found that not only ***miR-8071***, but also, ***miR-146a*** are significantly associated with epilepsy and in particular with focal, lesional and non lesional, drug-resistant epilepsy. We can speculate that there is an association with disease duration, seizure frequency and severity.

However we need to further study the function mechanism of exosomal miRNAs on epileptogenesis, these results indicates that exosomal miRNAs could be used as better biomarker of diagnosis of drug-resistance.

Finally the intent of our research project was also to evaluate the ***Hsp60 involvement in epileptogenesis,*** as a marker of oxidative stress, and indicate the correlation of its levels with the selected miRNAs expression.

We observed that plasmatic levels of Hsp60 in patients affected by FCD and TLE-HS (n=10) significantly increased compared to the circulating levels in IFE.

These data are in line with data reported in the paper *"Hsp60 response in experimental and human temporal lobe epilepsy" (Gammazza Marino A.et al; 2015)* in which Hsp60 in blood is a seemingly reliable diagnostic biomarker of disease.

Hence, we agree that there is a clinical practical utility to follow patient disease, including response to treatment, and it should be assessed routinely in the evaluation of epileptic patients.

Anyway our data about the tissue material are in contrast with these previous data in which there are high Hsp60 levels in affected tissues.

Our data reported that Hsp60 immunoreactivity decreased in TLE-HS patients along the strata of the hippocampal sectors. Similarly both miR-1 and miR-206, regulators of Hsp60 expression post-transcriptionally, significantly decreased in the hippocampus of TLE patients.

The difference may be explained because in the paper was used a rat model of status epilepticus, obtaining with acute electric kindling (the MDA model), and so with a clear relation to seizures, while our population is composed by chronic epileptic patients.

The different results may be expression of a different level of cellular stress, acute in SE rat model and chronic in our population of epileptic patients.

By the way data from animal models showed that Hsp72 increased levels in specific hippocampal neuronal subpopulations correlated with limbic seizure intensity and duration *(Gass P. et al; 1995)* and Hsp27 was found to be a highly sensitive and specific hippocampal marker for full development of pilocarpine-induced SE.*(Kirschstein T et al; 2012)*

As a result this study suggests a further investigations on the intrinsic molecular mechanisms of the chaperonin's involvement in epileptogenesis and disease progression.

In our opinion the most important data of this research project was the role of miRNAs tissue samples expression.

In line with previous studies of Shaofeng et al. (2017) we also demonstrated that hippocampal expression levels of ***miR-8071*** is significantly increased in TLE-HS patients compared to HC group ($p<0.001$).

In the same way our data suggest the role of exosomal ***miR-146a*** significantly associated with focal, lesional and non lesional, drug-resistant epilepsy.

These results indicates that exosomal miRNAs, more than circulating ones, could be used as biomarker of drug-resistance.

CHAPTER 10: CONCLUSION

More than being a potential therapeutic target, miRNAs also hold great promise as potential biomarkers in epilepsy.

Our data showed that exosomal miRNAs, more than circulating ones, could be used as non invasive biomarkers indicating disease states.

In particular our data showed the important role played by ***miR-8071*** which results overexpressed both in exosomal and in tissue samples of epileptic patients and therefore should be indicated as a biomarker of drug-resistance.

The aim of this study was to understand if there are differences in miRNA expression between surgical and serum sample. The role of ***miR-8071***would be of great importance for clinical practice using miRNAs as biomarkers in drug-resistant epilepsy associated to structural brain abnormalities.

Each drug-resistant patients, being considered for surgery epilepsy therapy, would be carefully evaluated just with a blood test, reducing the time that elapses between the diagnosis and the surgery treatment.

Moreover, by a therapeutical point of view, the recent observations indicating the ability of exosomes crossing the blood-brain barrier suggest they should be administered intravenously or in several others non-invasive ways.

Brain-specific miRNAs represent promising targets for therapeutic modulation.

However, further studies are required to determine whether targeting this interesting miRNA by its agomir/antagomircolud affect seizure variables.

Given the current limited evidence for miRNAbased anti-epileptic treatment, the future for miRNA-based therapies remains still uncertain and further studies on this domain are strongly suggested.

References

1. Aronica E., Fluiter K.,Iyer A., Zurolo E.,Vreijling J.,VanVliet E., Baayen J., Gorter J., *Expression pattern of miR-146a, an inflammation-associated microRNA,in experimental and human temporal lobe epilepsy*. European Journal of Neuroscience 2010. 31,1100–1107.
2. Asadi-Pooya A. A., Stewart G. R., Abrams D. J. & Sharan A. *Prevalence and Incidence of Drug-Resistant Mesial Temporal Lobe Epilepsy in the United States.* World Neurosurgery 2017. 99, 662–666.
3. Asea A, Kraeft SK, Kurt-Jones EA, Stevenson MA, Chen LB, Finberg RW, Koo GC, Calderwood SK. *HSP70 stimulates cytokine production through a CD14-dependent pathway, demonstrating its dual role as a chaperone and cytokine*. Nat Med 2000; 6: 435–442
4. Baker GA. *The psychosocial burden of epilepsy.*Epilepsia. 2002;43 Suppl 6:26-30
5. Barone R, Pitruzzella A, Marino Gammazza A, Rappa F, Salerno M, Barone F, Sangiorgi C, D'Amico D, Locorotondo N, Di Gaudio F, Cipolloni L, Di Felice V, Schiavone S, Rapisarda V, Sani G, Tambo A, Cappello F, Turillazzi E, Pomara C. *Nandrolone decanoate interferes with testosterone biosynthesis altering blood-testis barriercomponents.* J Cell Mol Med.2017 Aug;21(8):1636-1647.
6. Bartel D.P. *MicroRNAs: genomics, biogenesis, mechanism, and function.* Cell 2004. 116 , 281 – 297.
7. Bartel DP. *MicroRNAs: target recognition and regulatory functions***.**Cell. 2009 Jan 23;136(2):215-33.
8. Bencurova P., Baloun J., Musilova K., Radova L., Tichy B., Pail M., Zeman M., Brichtova E., Hermanova M., Pospisilova S., Mraz M., Brazdil M. *MicroRNA and mesial temporal lobe epilepsy with hippocampal sclerosis: Whole miRNome profiling of human hippocampus*. Epilepsia. 2017.58(10):1782–1793.
9. Berezikov E., van Tetering G., Verheul M., van de Belt J., van Laake L., Vos J., Verloop R., van de Wetering M., Guryev V., and Takada S. *Many novel mammalian microRNA candidates identified by extensive cloning and RAKE analysis.* Genome Res. 2006. 16 , 1289 – 1298.
10. Bian S. and Sun T. *Functions of noncoding RNAs in neural development and neurological diseases*. Mol. Neurobiol. 2011. 44 , 359 – 373.
11. Bidmon HJ, Görg B, Palomero-Gallagher N, Behne F, Lahl R, Pannek HW, Speckmann EJ, Zilles K. *Heat-shock protein-27 is upregulated in the temporal cortex of patients with epilepsy*. Epilepsia 2004; 45: 1549–1559
12. Blümcke I, Thom M, Wiestler OD. *Ammon's horn sclerosis: a maldevelopmental disorder associated with temporal lobe epilepsy.*Brain Pathol. 2002 Apr;12(2):199-211. Review.

13. Bot AM, Debski KJ, Lukasiuk K. *Alterations in miRNA levels in the dentate gyrus in epileptic rats.* PLoS One. 2013. 8:e76051.
14. Brennan G.P., Henshall D.C. *microRNAs in the pathophysiology of epilepsy*. Neuroscience Letters. 2017.
15. Brooks-Kayal AR, Raol YH, Russek SJ. *Alteration of epileptogenesis genes*. Neurotherapeutics. 2009; 6:312–318.
16. Bukau B, Horwich AL. *The Hsp70 and Hsp60 chaperone machines*. Cell 1998; 92: 351–366
17. Cacheaux LP, Ivens S, David Y, Lakhter AJ, Bar-Klein G, Shapira M, Heinemann U, Friedman A, Kaufer D. *Transcriptome profiling reveals TGF-beta signaling involvement in epileptogenesis*. J. Neurosci. 2009; 29:8927–8935.
18. Calabrese, V. *In vivo induction of heat shock proteins in the substantia nigra following L-DOPA administration is associated with increased activity of mitochondrial complex I and nitrosative stress in rats: regulation by glutathione redox state*. J. Neurochem. 2007.101, 709–717.
19. Campanella C, Rappa F, Sciumè C, Marino Gammazza A, Barone R, Bucchieri F, David S, Curcurù G, Caruso Bavisotto C, Pitruzzella A, Geraci G, Modica G, Farina F, Zummo G, Fais S, Conway de Macario E, Macario AJ, Cappello *Heat shock protein 60 levels in tissue and circulating exosomes in human large bowel cancerbe fore and after ablative surgery.* Cancer.2015 Sep 15;121(18):3230-9.
20. Cappello F., Conway de Macario E., Marasa L., Zummo G., Macario A.J.L., *Hsp60 expression, new locations, functions and perspectives for cancer diagnosis and therapy,* Cancer Biol. Ther. 2008. 7. 801e809.
21. Cappello F., Marino Gammazza A., . Palumbo Piccionello A, Campanella C., Pace A., Conway de Macario E. *Hsp60 chaperonopathies and chaperonotherapy: targets and agents*. Expert Opin. Ther. Targets 2014. 18. 185e208.
22. Chang B.S. and Lowenstein D.H. *Epilepsy*. N. Engl. J. Med. 2003. 349 , 1257 – 1266.
23. Chen X., Ba Y., Ma L., Cai X., Yin Y., Wang K., Guo J., Zhang Y., Chen J., Guo X. *Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases*. Cell Research. 2008. 18, 997–1006.
24. Chen Y., Liu W., Chao T., Zhang Y., Yan X., Gong Y., Qiang B., Yuan J., Sun M., Peng X. *MicroRNA-21 down-regulates the expression of tumorsuppressorPDCD4 in human glioblastoma cell T98G.* Cancer Letters. 2008. 272,197–205.
25. Choi J.W., Kim S. C., Hong S. H., and Lee H. J. *Secretable small RNAs via outer membrane vesicles in periodontal pathogens.* J. Dent. Res. 2017. 96, 458–466.

26. Cullman SL, Isola AL, Chen S. *Exosomes: The Messengers of Health and Disease*. Curr Neuropharmacol. 2016.
27. D'Souza S. M. & Brown I. R. *Constitutive expression of heat shock proteins Hsp90, Hsc70, Hsp70 and Hsp60 in neural and non-neural tissues of the rat during postnatal development*. Cell Stress Chaperones. 1998. 3, 188–199.
28. De Oliveira G. N. M. et al. *Psychiatric disorders in temporal lobe epilepsy: An overview from a tertiary service in Brazil*. Seizure 2010.19, 479–484.
29. De Tisi J. et al. *The long-term outcome of adult epilepsy surgery, patterns of seizure remission, and relapse: A cohort study*. Lancet 2011. 378, 1388–1395.
30. Deo M. et al. *Detection of mammalian microRNA expression by in situ hybridization with RNA oligonucleotides.* Developmental Dynamics 2006. 235, 2538-2548 21.
31. Deocaris C.C., Kaul S.C., Wadhwa R., *On the brotherhood of the mitochondrial chaperones mortalin and heat shock protein 60,* Cell Stress Chaperones .2006. 11. 116e128
32. Duncan JS, Sander JW, Sisodiya SM, Walker MC. *Adult epilepsy.*Lancet. 2006 Apr 1;367(9516):1087-1100. Review.
33. el Bahh B, Lurton D, Coussemacq M, Sundstrom L, Rougier A. *Seizure-linked hippocampal plasticity and protection against excitotoxicity: possible role of neuropeptide-y* Neurochirurgie. 1998 Mar;44(1):31-7 .
34. Elger CE, Schmidt D. *Modern management of epilepsy: a practical approach.*Epilepsy Behav. 2008 May;12(4):501-39.
35. Engel, J. et al. *Practice parameter: Temporal lobe and localized neocortical resections for epilepsy.* Epilepsia 2003. 44, 741–751.
36. Fisher RS, Acevedo C, Arzimanoglou A, Bogacz A, Cross JH, Elger CE, Engel J Jr, Forsgren L, French JA, Glynn M, Hesdorffer DC, Lee BI, Mathern GW, Moshé SL, Perucca E, Scheffer IE, Tomson T, Watanabe M, Wiebe S. *ILAE official report: a practical clinical definition of epilepsy.* Epilepsia.2014 Apr;55(4):475-82.
37. Fisher RS, van Emde Boas W, Blume W, Elger C, Genton P, Lee P, Engel J Jr. *Epileptic seizures and epilepsy: definitions proposed by the International League Against Epilepsy(ILAE) and the International Bureau for Epilepsy (IBE).*Epilepsia. 2005 Apr;46(4):470-2.
38. Fredrik R., Geir H., Nils E., *Heat-Shock Proteins in Clinical Neurology*. Eur Neurol 2011;66:65–69
39. Gass, P., Prior, P. & Kiessling, M. *Correlation between seizure intensity and stress protein expression after limbic epilepsy in the rat brain*. Neuroscience. 1995. 65, 27–36.

40. Golyala A, Kwan P. *Drug development for refractory epilepsy: The past 25 years and beyond.* Seizure. 2017. Volume 44 January, Pages 147-156
41. Gorter J. A., Iyer A., White I., Colzi A., van Vliet E. A., Sisodiya S. *Hippocampal subregion-specific microRNA expression during epileptogenesis in experimental temporal lobe epilepsy*. Neurobiol. Dis. 2014. 62,
42. Gourlay J., Morokoff A. P., Luwor R. B., Zhu H. J., Kaye A. H., and Stylli S. S.*The emergent role of exosomes in glioma.* J. Clin. Neurosci. 2017. 35, 13–23.
43. Gupta A, Pulliam L. *Exosomes as mediators of neuroinflammation.* J Neuroinflammation. 2014 Apr 3;11:68.
44. H.H. Kampinga, J. Hageman, M.J. Vos, H. Kubota, R.M. Tanguay, E.A. Bruford. *Guidelines for the nomenclature of the human heat shock proteins.* Cell Stress Chaperones. 2009. 14 (1) 105e111.
45. Hannafon BN, Ding WQ. *Intercellular Communication by Exosome-Derived microRNAs in Cancer.* Int J Mol Sci. 2013; 14:14240–14269.
46. Hannafon BN, Ding WQ. *Intercellular Communication by Exosome-Derived microRNAs in Cancer.* Int J Mol Sci. 2013; 14:14240–14269.
47. He X, Tang R, Sun Y, Wang YG, Zhen KY, Zhang DM, Pan WQ. *MicroR-**146** blocks the activation of M1 macrophage by targeting signal transducer and activator of transcription 1 in hepatic schistosomiasis.*EBioMedicine. 2016. Nov;13:339-347.
48. Hu K., Zhang C., Long L., Long X., Feng L., Li Y., Xiao B. *Expression profile of microRNAs in rat hippocampus following lithium–pilocarpine-induced status epilepticus.* Neuroscience Letters 2011. 488,252–257.
49. Hu,G.,Yao,H.,Chaudhuri,A.,Duan,M.,Yelamanchili,S.V.,Wen,H. *Exosome mediated shuttling of microRNA-29regulates HIVTat andmorphine-mediated.* Neuronaldysfunction. 2012. CellDeathDis. Aug 30;3:e381
50. Isola AL, Chen S. *Exosomes: The Messengers of Health and Disease***.** Curr Neuropharmacol.2017;15(1):157-165.
51. Jacoby A. *Stigma, epilepsy, and quality of life.*Epilepsy Behav. 2002 Dec;3(6S2):10-20.
52. Jardim, A. P. et al. *Temporal lobe epilepsy with mesial temporal sclerosis: hippocampal neuronal loss as a predictor of surgical outcome*. Arq. Neuropsiquiatr. 2012. 70, 319–24.
53. Jiang J, Quan Y, Ganesh T, Pouliot WA, Dudek FE, Dingledine R. *Inhibition of the prostaglandin receptor EP2 following status epilepticus reduces delayed mortality and brain inflammation.* Proc. Natl. Acad. Sci. USA. 2013; 110:3591–3596.

54. Kaalund SS, Venø MT, Bak M, Møller RS, Laursen H, Madsen F, Broholm H, Quistorff B, Uldall P, Tommerup N, Kauppinen S, Sabers A, Fluiter K, Møller LB, Nossent AY, Silahtaroglu A, Kjems J, Aronica E, Tümer Z. *Aberrant expression of miR-218 and miR-204 in human mesial temporal lobe epilepsy and hippocampal sclerosis-convergence on axonal guidance.* Epilepsia.2014 Dec;55(12):2017-27.
55. Kan AA, van Erp S, Derijck AA, de Wit M, Hessel EV, O'Duibhir E, de Jager W, Van Rijen PC, Gosselaar PH, de Graan PN, Pasterkamp RJ. *Genome-wide microRNA profiling of human temporal lobe epilepsy identifies modulators of the immune response***.** Cell Mol Life Sci.2012 Sep;69(18):3127-45.
56. Kim, V.N., Han, J., and Siomi, M.C. *Biogenesis of small RNAs in animals.* Nat. Rev. Mol. Cell Biol. 2009. 10 , 126 – 139.
57. Kirschstein, T. et al. *The 27-kDa heat shock protein (HSP27) is a reliable hippocampal marker of full development of pilocarpine-induced status epilepticus*. Epilepsy Res. 2012. 98, 35–43.
58. Kretschmann A., Danis B., Andonovic L., Abnaof K., vanRikxoort M., Siegel F., *Different microRNA profiles in chronic epilepsy versus acute seizure mouse models.* J.Mol.Neurosci. 2015. 55,466–479.
59. KretschmannA.,Danis,B.,Andonovic,L.,Abnaof,K.,vanRikxoort,M.,Siegel,F.,et al. *Different microRNA profiles in chronic epilepsy versus acute seizure mouse models*. J.Mol.Neurosci. 2015. 55,466–479.
60. Kwan P, Arzimanoglou A, Berg AT, Brodie MJ, Allen Hauser W, Mathern G, Moshé SL, Perucca E, Wiebe S, French J. *Definition of drug resistant epilepsy: consensus proposal by the ad hoc Task Force of the ILAECommission on Therapeutic Strategies.* Epilepsia. 2010 Jun;51(6):1069-77.
61. Lagos-Quintana M. et al. *Identification of tissue-specific microRNAs from mouse*. Current Biology. 2002. 12, 735-739 20.
62. Lee S. K. *Treatment strategy for the patient with hippocampal sclerosis who failed to the first antiepileptic drug.* J Epilepsy Res 2014. 4, 1–6
63. Lee Y., Kim M., Han J., Yeom K.H., Lee S., Baek S.H., and Kim V.N. *MicroRNA genes are transcribed by RNA polymerase II.* EMBO J. 2004. 23 , 4051 – 4060.
64. Lewis DV, Shinnar S, Hesdorffer DC, Bagiella E, Bello JA, Chan S, Xu Y, MacFall J, Gomes WA, Moshe SL, Mathern GW, Pellock JM, Nordli DR Jr, Frank LM, Provenzale J, Shinnar RC, Epstein LG, Masur D, Litherland C, Sun S, Team FS. *Hippocampal sclerosis after febrile status epilepticus: The FEBSTAT study.* Ann Neurol 2014;75:178–185.

65. Lhatoo SD, Sander JW. *The epidemiology of epilepsy and learning disability.* Epilepsia. 2001;42 Suppl 1:6-9; discussion 19-20
66. Li MM., Li XM., Zheng XP., Yu JT., Tan L. *MicroRNAs dysregulation in epilepsy.* Brain Res. 2014 Oct 10;1584:94-104.
67. Li Z, Menoret A, Srivastava P. *Roles of heat shock proteins in antigen presentation and cross-presentation.* Curr Opin Immunol 2002; 14: 45–51
68. Luciano D.J., Mirsky H., Vendetti N.J., and Maas S. *RNA editing of a miRNA precursor.* RNA 2004. 10 , 1174 – 1177.
69. Marino Gammazza A, Campanella C, Barone R, Caruso Bavisotto C, Gorska M, Wozniak M, Carini F, Cappello F, D'Anneo A, Lauricella M, Zummo G, Conway de Macario E, Macario AJ, Di Felice V. *Doxorubicin anti-tumor mechanisms include Hsp60 post-translational modifications leading tothe Hsp60/p53 complex dissociation and instauration of replicative senescence.* Cancer Lett.2017 Jan 28;385:75-86..
70. Marino Gammazza A, Colangeli R, Orban G, Pierucci M, Di Gennaro G, Lo Bello M, D'Aniello A, Bucchieri F, Pomara C, Valentino M, Muscat R, Benigno A, Zummo G, de Macario EC, Cappello F, Di Giovanni G, Macario AJ. *Hsp60 response in experimental and human temporal lobe epilepsy.*Sci Rep.2015 Mar 24;5:9434.
71. Marino Gammazza A., Rizzo M., Citarrella R., Rappa F., Campanella C., Bucchieri F. *Elevated blood Hsp60, its structural similarities and crossreactivitywith thyroid molecules, and its presence on the plasma membrane of oncocytes point to the chaperonin as an immunopathogenic factor in Hashimoto's thyroiditis.* Cell Stress Chaperones. 2013. 19 343e353
72. Maroso M, Balosso S, Ravizza T, Liu J, Bianchi ME, Vezzani A. *Interleukin-1 type 1 receptor/Toll-like receptor signalling in epilepsy: the importance of IL-1beta and high-mobility group box 1.* J. Int. Med. 2011; 270:319–326.
73. McClelland S, Flynn C, Dube C, Richichi C, Zha Q, Ghestem A, Esclapez M, Bernard C, Baram TZ. *Neuron-restrictive silencer factor-mediated hyperpolarization-activated cyclic nucleotide gated channelopathy in experimental temporal lobe epilepsy*. Ann Neurol. 2011; 70:454–464.
74. McKiernan R.C. *Expression profiling the microRNA response toepileptic preconditioning identifies miR-184 as a modulator ofseizure-induced neuronal death*. Exp. Neurol. 2012. 237 (2) 346–354.
75. McNamara J.O., Huang Y.Z., and Leonard A.S. *Molecular signaling mechanisms underlying epileptogenesis.* Sci. Signal. 2006

76. Michaille JJ, Piurowski V, Rigot B, Kelani H, Fortman EC, Tili E. *M*iR-663, a MicroRNA Linked with Inflammation and Cancer That Is under the Influence of Resveratrol. Medicines. 2018 Jul 9;5(3). Review.
77. Montecalvo A, Larregina AT, Shufesky WJ, Beer Stolz D, Sullivan MLG, Karlsson JM, Baty CJ, Gibson GA, Erdos G, Wang Z, Milosevic J, Tkacheva OA, Divito SJ, et al. *Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes.* Blood. 2012; 119:756–766.
78. Multhoff G. *Heat-shock protein 70 (Hsp70): membrane location, export and immunological relevance.* Methods 2007; 43: 229–237
79. Obernosterer G., Leuschner P.J., Alenius M., and Martinez J. *Post-transcriptional regulation of microRNA expression*. RNA 2006. 12 , 1161 – 1167.
80. Omran A., Peng J., Zhang C., Xiang Q.L., Xue J., Gan N.*Interleukin-1ßandmicroRNA-146a in an immature rat model and children with mesial temporal lobe epilepsy*. Epilepsia. 2012. 53,1215–1224.
81. Pati S, Alexopoulos AV. *Pharmacoresistant epilepsy: from pathogenesis to current and emerging therapies.* Cleve Clin J Med. 2010 Jul;77(7):457-67.
82. Pichardo-Casas I. ,Goff L.A., Swerdel M.R., Athie A.,Davila J., Ramos-Brossier M., Lapid Volosin M., Friedman W.J., Hart R. P., Vaca L. *Expression profiling of synaptic microRNAs from the adult rat brain identifies regional differences and seizure-induced dynamic modulation.* Brain Research. 2012. 1436, 20–3.
83. Rejdak K. *Neurofilament heavy chain and heat shock protein 70 as markers of seizure-related brain injury*. Epilepsia. 2012. 53(5):922–927.
84. Rikako S., Akishi O., Chieko K., Rieko M., Satoshi W. Yuki M., Shoichi I., Shinji U., Toshiyuki K., Ryosuke M., Yoan C., Yoshihiro U., Dai W., Mineo K., Toshihide Y.& Takahisa F. *miR-124a is required for hippocampal axogenesis and retinal cone survival through Lhx2 suppression.* Nature Neuroscience. 2011. Vol.14.
85. Robin J, Rai Y, Valli M, Olsen RK. *Category specificity in the medial temporal lobe: A systematic review.*Hippocampus. 2018 Aug 28.
86. Rogawski MA, Johnson MR. *Intrinsic severity as a determinant of antiepileptic drug refractoriness.* Epilepsy Curr. 2008 Sep-Oct;8(5):127-30.
87. Sanuki R, Onishi A, Koike C, Muramatsu R,Watanabe S, Muranishi Y, et al. *miR-124a is required for hippocampal axogenesis and retinal cone survival through Lhx2 suppression*. Nat Neurosci. 2011;14(9):1125-34.

88. Sarangi, U. *Hsp60 chaperonin acts as barrier to pharmacologically induced oxidative stress mediated apoptosis in tumor cells with differential stress response*. Drug Target Insights 2013.
89. Schmidt D, Löscher W. *Drug resistance in epilepsy: putative neurobiologic and clinical mechanism.* Epilepsia. 2005 Jun;46(6):858-77. Review.
90. Shaofeng Y., Hua Z., Wenyan X., Fangang M., Kai Z, Yin J, Xin Z. and Jianguo Z. Altered *microRNA profiles in plasma exosomes from mesial temporal lobe epilepsy with hippocampal sclerosis*. Oncotarget. 2017 Jan 17; 8(3): 4136–4146.
91. Shorvon S, Guerrini R. *Acute symptomatic seizures--should we retain the term?* Epilepsia. 2010 Apr;51(4):722-3.
92. Song,Y.J.,Tian,X.B.,Zhang,S.,Zhang,Y.X.,Li,X.,Li,D.,et AL. *Temporal lobe epilepsy induces differential expression of hippocampal miRNAs including let-7e and miR-23a/b*. BrainRes. 2011. 1387,134–140.
93. Stetler, R. A. *Heat shock proteins: cellular and molecular mechanisms in the central nervous system.* Prog. Neurobiol. 2010. 92, 184–211.
94. Stoorvogel W. *Functional transfer of microRNA by exosomes*. Blood. 2012; 119:646–648.
95. Sun A.X., Crabtree G.R. and Yoo A.S. *MicroRNAs: regulators of neuronal fate.* Current Opinion in Cell Biology. 2013. 25(2), 215-221.
96. Sun Z, Yu JT, Jiang T, Li MM, Tan L, Zhang Q, Tan L. *Genome-wide microRNA profiling of rat hippocampus after status epilepticus induced by amygdala stimulation identifies modulators of neuronal apoptosis.* PLoS One. 2013 Oct 25;8(10):e78375.
97. Tang F, Hartz A.M. S, and Bauer B. *Drug-Resistant Epilepsy: Multiple Hypotheses, Few Answers.* Front Neurol. 2017; 8: 301.
98. Tang H., Chen Y., Liu X., Wang S., Wu Y. *Downregulation of HSP60 disrupts mitochondrial proteostasis to promote tumorigenesis and progression in clear cell renal cell carcinoma*. Oncotarget 2016. 25.
99. Tsan MF, Gao B. *Heat-shock protein and innate immunity.* Cell Mol Immunol 2004; 1: 274–279
100. Vermeulen A., Robertson B., Dalby A.B., Marshall W.S., Karpilow J., Leake D., Khvorova A., and Baskerville S. *Double stranded regions are essential design components of potent inhibitors of RISC function.* RNA. 2007. 13 , 723 – 730.
101. Vezzani A, French J, Bartfai T, Baram TZ. *The role of inflammation in epilepsy*. Nat. Rev. Neurol. 2011; 7:31–40.
102. Waldbaum S. & Patel M. *Mitochondrial dysfunction and oxidative stress: a contributing link to acquired epilepsy.* J. Bioenerg. Biomembr. 2010. 42, 449–455.

103. Waldbaum, S. & Patel, M. *Mitochondrial dysfunction and oxidative stress: a contributing link to acquired epilepsy.* J. Bioenerg. Biomembr. 2010. 42, 449–455.

104. Wang J, Tan L, Tian Y, Ma J, Tan CC, Wang HF, Liu Y, Tan MS, Jiang T, Yu JT. *Circulating microRNAs are promising novel biomarkers for drug-resistant epilepsy.* Sci Rep. 2015; 5.

105. Wang W. *The microRNA miR-124 suppresses seizure activity and regulates CREB1 activity.* Expert Reviews in Molecular Medicine. 2016. Vol. 18.

106. Wang Y, Kelly CG, Singh M, McGowan EG, Carrara AS, Bergmeier LA, Lehner T. *Stimulation of Th1-polarizing cytokines, C-C chemokines, maturation of dendritic cells, and adjuvant function by the peptide binding fragment of heat-shock protein 70.* J Immunol 2002; 169: 2422–2429

107. Wiebe S. & Jette N. *Pharmacoresistance and the role of surgery in difficult to treat epilepsy.* Nat. Rev. Neurol. 2012. 8, 669–677.

108. Wiebe S., Blume W. T., Girvin J. P., Eliasziw M. *T. L. E. S. A randomized, controlled trial of surgery for temporal-lobe epilepsy.* N. Engl. J. Med. 2001. 345, 311–318.

109. Yan S., Zhang H., Xie W., Meng F., Zhang K., Jiang Y. *Altered microRNA profiles in plasma exosomes from mesial temporal lobe epilepsy with hippocampal sclerosis*. Oncotarget. 2016. 8, 4136–4146.

110. Yang T. T., Hsu C. T. & Kuo Y. *M. Cell-derived soluble oligomers of human amyloid-beta peptides disturb cellular homeostasis and induce apoptosis in primary hippocampal neurons*.

111. Yu J.Y. et al. *MicroRNA miR-124 regulates neurite outgrowth during neuronal differentiation.* Experimental Cell Research 2008. 314, 2618-2633

112. Zucchini,S.,Marucci,G.,Paradiso,B.,Lanza,G.,Roncon,P., Cifelli,P. *Identification of miRNAs differentially expressed in human epilepsy with or without granule cell pathology*. PLoSONE 2014. 9:e105521.