Could Extracellular microRNAs act as a novel biomarkers of temporal lobe epilepsy?

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Could extracellular microRNAs act as novel biomarkers of temporal lobe epilepsy?

A dissertation submitted by

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To the School of Postgraduate Studies, Faculty of Medicine and Health Sciences, Royal College of Surgeons in Ireland in fulfilment of the requirements for the degree of Doctor of Philosophy (PhD)

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Supervisor: Prof. David Henshall.

Funded by the Ministry of Higher Education and Scientific Research/Iraq
This thesis is dedicated to my mother Rajaa
and to the memory of my father Mumtaz.
Candidate Thesis Declaration

I declare that this thesis, which I submitted to RCSI for examination in consideration of the award of a higher degree of Doctor of Philosophy, is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme, this is dually acknowledged in the text such that it is possible to ascertain how much of my work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took responsible care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed: Rana M Raoof

Student Number: 12194654

Date: December/2017
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Abstract

Epilepsy diagnosis is always a challenging process. A detailed clinical history is the main tool used for diagnosis and classification; however, it is often incomplete and misleading. Electroencephalography and neuroimaging have their role in assisting the diagnosis but, still, they cannot confirm or rule out epilepsy. This is reflected clinically as a high figure of epilepsy misdiagnosis, where about one third of patients are being misdiagnosed. The identification of a reliable molecular biomarker for epilepsy will not only allow for a definitive diagnosis of seizures and epilepsy but as well help in the disease classification, individualize the treatment options and predict prognosis and comorbidities.

MicroRNAs have recently emerged as a promising class of biomarkers. These are small, endogenous RNA molecules that regulate gene expression by interfering with the translation of their target mRNA. The identification of microRNAs in various body fluids, their stability as well as their tissue specificity opens up a new field for biomarker studies in epilepsy. The main aim of the present study was to investigate the expression and dysregulation of microRNAs in biofluid samples of temporal lobe epilepsy patients and evaluate their possible role as diagnostic biomarkers. To achieve this aim, the study began by investigating the stability and inter-personal variabilities of extracellular microRNAs in healthy controls. The first goal was to identify any difference in extracellular microRNA profiles between morning-afternoon and male-female samples and to confirm the stability of these molecules in plasma samples overtime. Standardization of sample collection and processing techniques, RNA extraction and profiling methods and defining a protocol for optimal data analysis of genome-wide microRNA profiles were also among the important goals. The obtained results showed that microRNA levels were very consistent between males and females and at different time points confirming their suitability for biomarker studies.
Next, a multiphase case-control study was performed to investigate the ability of plasma microRNAs to differentiate temporal lobe epilepsy patients from healthy controls as well as to track the occurrence of seizures. Plasma microRNA profile was explored using dual platform design (RNA-sequencing and QuantStudio™ 12K Flex OpenArray system) which increased the confidence in the identified signature. Furthermore, the inclusion of pre- and post-seizure samples in the study allowed the identification of microRNAs with biomarker potential for both temporal lobe epilepsy and seizure. The dysregulation of several microRNAs was identified in the discovery phase and confirmed in the validation phase of the study. The performance of the dysregulated microRNAs as biomarkers was also investigated with miR-654-3p was best in differentiating temporal lobe epilepsy from controls. Moreover, reduced plasma levels of miR-328-3p and miR-27a-3p in post-seizure samples indicated a high performance of both microRNAs in identifying patients presented after a recent seizure episode.

Finally, microRNA expression and dysregulation in cerebrospinal fluid of temporal lobe epilepsy and status epilepticus patients was explored. Cerebrospinal fluid offers a unique source of microRNA biomarkers with the advantage of being in close contact with the sites of pathology. Differential expression of 20 microRNAs was detected between patient groups and controls. A validation phase included an expanded cohort and samples from patients with other neurological diseases. The study identified lower levels of miR-19b-3p in temporal lobe epilepsy patients compared to controls, status epilepticus and other neurological diseases. Levels of miR-451a were higher in status epilepticus compared to other groups whereas miR-21-5p was different in status epilepticus compared to temporal lobe epilepsy but not to other neurological diseases.

In conclusion, the present study indicates the presence of a set of dysregulated microRNAs in plasma and cerebrospinal fluid of temporal lobe epilepsy patients that can be utilized clinically as diagnostic and prognostic epilepsy biomarkers.
Publications and Manuscripts

Published:


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2017
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<tr>
<td>AB</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ABCG2</td>
<td>Breast-cancer related protein</td>
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<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
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<td>dsRNA</td>
<td>double stranded RNA</td>
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<td>DUB</td>
<td>Dublin</td>
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<td>EAS</td>
<td>Epilepsy after-seizure sample</td>
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<td>EBS</td>
<td>Epilepsy baseline samples</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>ECoG</td>
<td>Electrocorticography</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>EEG</td>
<td>Electroencephalogram</td>
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<tr>
<td>EMU</td>
<td>Epilepsy monitoring unit</td>
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<td>FC</td>
<td>Fold changes</td>
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<tr>
<td>FDR</td>
<td>False discovery rate</td>
</tr>
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<td>FSE</td>
<td>Focal status epilepticus</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
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<td>Generalized epilepsy with febrile seizures plus</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GM</td>
<td>Geometric mean</td>
</tr>
<tr>
<td>GS</td>
<td>Glutamine synthetase enzyme</td>
</tr>
<tr>
<td>GTC</td>
<td>Generalized tonic–clonic</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HFO</td>
<td>High frequency oscillation</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High-mobility group box-1</td>
</tr>
<tr>
<td>hnRNPA2B1</td>
<td>Heterogeneous nuclear ribonucleoprotein A2B1</td>
</tr>
<tr>
<td>HS</td>
<td>Hippocampal sclerosis</td>
</tr>
<tr>
<td>HSP70</td>
<td>Heat shock protein 70</td>
</tr>
<tr>
<td>HSP-90</td>
<td>Heat shock protein 90</td>
</tr>
<tr>
<td>i.c.v</td>
<td>intra-cerebroventricular</td>
</tr>
<tr>
<td>IGE</td>
<td>Idiopathic generalized epilepsy</td>
</tr>
<tr>
<td>IL-1R1</td>
<td>Interleukin-1 receptor type-1</td>
</tr>
<tr>
<td>IL-1ß</td>
<td>Interlukin-1ß</td>
</tr>
<tr>
<td>ILAE</td>
<td>International league Against Epilepsy</td>
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<tr>
<td>Itgß1</td>
<td>ß1-integrin</td>
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<tr>
<td>K,A</td>
<td>Kainic-acid</td>
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<tr>
<td>kb</td>
<td>kilobases</td>
</tr>
<tr>
<td>Kir 4.1</td>
<td>K⁺ rectifying channels</td>
</tr>
<tr>
<td>LGI-1</td>
<td>Leucine rich glioma inactivated 1 gene</td>
</tr>
<tr>
<td>LNA</td>
<td>Locked nucleic acid</td>
</tr>
<tr>
<td>IncRNAs</td>
<td>Long non-coding RNAs</td>
</tr>
<tr>
<td>MAR</td>
<td>Marburg</td>
</tr>
<tr>
<td>MDR1</td>
<td>Multidrug resistance protein 1</td>
</tr>
<tr>
<td>MFS</td>
<td>Mossy fibre sprouting</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<tr>
<td>MRS</td>
<td>Magnetic resonance spectroscopy</td>
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<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>MTHFR</td>
<td>Methylene tetrahydrofolate reductase</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MW</td>
<td>Mann-Whitney</td>
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<tr>
<td>MYC</td>
<td>V-Myc Avian Myelocytomatosis Viral Oncogene Homolog</td>
</tr>
<tr>
<td>MYD88</td>
<td>Myeloid differentiation primary response protein 88</td>
</tr>
<tr>
<td>NB</td>
<td>Northern Blot</td>
</tr>
<tr>
<td>NCSE</td>
<td>Non-convulsive status epilepticus</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NGS</td>
<td>Next Generation Sequencing</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-d-aspartate receptor</td>
</tr>
<tr>
<td>NSE</td>
<td>Neuron-specific enolase</td>
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<tr>
<td>nt</td>
<td>Nucleotide</td>
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<tr>
<td>OA</td>
<td>OpenArray</td>
</tr>
<tr>
<td>PACT</td>
<td>Protein Activator of the interferon-induced protein kinase</td>
</tr>
<tr>
<td>PB</td>
<td>Phenobarbital</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
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<tr>
<td>PCA</td>
<td>Principle component analysis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PHC</td>
<td>Parahippocampal cortex</td>
</tr>
<tr>
<td>piRNAs</td>
<td>PIWI-interacting RNAs</td>
</tr>
<tr>
<td>PNEAs</td>
<td>Psychogenic non-epileptic attacks</td>
</tr>
<tr>
<td>PreAmp</td>
<td>Preamplification</td>
</tr>
<tr>
<td>pre-microRNA</td>
<td>Precursor microRNA</td>
</tr>
<tr>
<td>pri-microRNA</td>
<td>Primary microRNA</td>
</tr>
<tr>
<td>PTGS2</td>
<td>Prostaglandin-endoperoxide synthase 2</td>
</tr>
<tr>
<td>ran-GTP</td>
<td>Ran guanosine triphosphate</td>
</tr>
<tr>
<td>RBCs</td>
<td>Red blood cells</td>
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<tr>
<td>RISC</td>
<td>RNA induced silencing complex</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RNA-seq</td>
<td>RNA-sequencing</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver operating characteristic</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Quantitative reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>SCN1A</td>
<td>Neuronal sodium channel α 1 subunit gene</td>
</tr>
<tr>
<td>SCN</td>
<td>Suprachiasmatic nucleus</td>
</tr>
<tr>
<td>SE</td>
<td>Status epilepticus</td>
</tr>
<tr>
<td>SHUT</td>
<td>Stacking hybridized universal tag</td>
</tr>
<tr>
<td>sICAM5</td>
<td>Soluble intercellular adhesion molecule 5</td>
</tr>
<tr>
<td>snoRNAs</td>
<td>Small nucleolar RNAs</td>
</tr>
<tr>
<td>snRNAs</td>
<td>Small nuclear RNAs</td>
</tr>
<tr>
<td>SOD1</td>
<td>Superoxide dismutase 1</td>
</tr>
<tr>
<td>SOP</td>
<td>Standard operating procedures</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single stranded RNA-sequences</td>
</tr>
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</table>
SUDEP  Sudden unexpected death in epilepsy
TARC  Thymus and activation regulated chemokine
TBI  Traumatic brain injury
TE  Tris-EDTA buffer
TGF-βRs  Transforming growth factor β receptors
TLDA  TaqMan Low Density Arrays
TLE  Temporal lobe epilepsy
TLR  Toll-like receptor
Tm  melting temperature
TMM  Trimmed mean of M-values
TNF  Tumour necrosis factor
TP53  Cellular tumour antigen p53
TPM  Tags per Million
TRBP  Trans-activation response RNA binding protein
UTR  Untranslated region
VNS  Vagal nerve stimulation
VPA  Valproic acid
WG  Watson Grading system
1. General introduction
Epilepsy

Epilepsy is a chronic neurological disease that affects individuals of all ages. Currently, around 50 million people worldwide have epilepsy. The prevalence of epilepsy has been estimated to be between 0.5-1% (Bell and Sander, 2001) and the overall annual incidence to range from 50-70 cases per 100,000 in industrialized countries and up to 190 per 100,000 in developing countries (Wright et al., 2000, Forsgren et al., 2005, Banerjee et al., 2009). Epilepsy is associated with various somatic, psychological and neurodevelopmental conditions that affect the patient’s quality of life. Among them are depression, anxiety, dementia, migraine and heart diseases. These conditions are up to eight times more common in epilepsy patients than in the general population (Keezer et al., 2016). In addition, patients often report diminished socialization, negative self-image, diminished hope and ambition (Gillham et al., 2000). The mortality rate in people with epilepsy is two- to three-times higher than in the general population and life expectancy is reduced by 2-10 years (Tomson et al., 2005, Hitiris et al., 2007). Sudden unexpected death in epilepsy (SUDEP) is the most important direct epilepsy-related cause of death. Patients at higher risk for SUDEP are male, 20-40 years of age, with generalized seizures and pharmaco-resistant (Tomson et al., 2005, Johnston and Smith, 2007).

1.1 Historical overview

The earliest detailed clinical description of epilepsy was found in a Babylonian medical diagnostic series known as Sakikku “All Diseases” which may be dated to approximately the middle of the first millennium BCE. The Tablets which are part of the Babylonian Collection in the British museum (Figure 1.1), gave remarkably detailed clinical observations of most of the common seizure types and some of the rare epilepsy syndromes that are well recognized nowadays. Accurate details of the prodromal symptoms, auras, ictal and post ictal period were described (Wilson and Reynolds, 1990, Reynolds and Wilson, 2008, Reynolds and Wilson, 2014).
Figure 1.1: Tablet 26 on Epilepsy from the Babylonian collection in the British Museum, London. Obverse of BM47753. Adapted from Wilson and Reynolds (1990).

The Tablet is part of medical diagnostic series known as Sakikku. It gave an accurate description of most of the common seizure types and some of the rare epilepsy syndromes that are well recognized nowadays.
The Babylonian text described *miqtu* “the falling sickness or epilepsy” as a supernatural disorder that is due to invasion of the body by evil spirits (Wilson and Reynolds, 1990, Reynolds and Wilson, 2014). This view of epilepsy dominated the thinking until the 5th century BCE where the first steps toward understanding epilepsy began. In his book, *On the Sacred Disease*, Hippocrates was the first to challenge the supernatural view of epilepsy (Magiorkinis et al., 2010). Since then, the concept of epilepsy as a brain disorder began to evolve and by the 19th century it began to be widely accepted. This is the time when Bromide, the first effective antiepileptic drug (AED), was introduced and used widely in the USA and Europe (Goldensohn et al., 1997).

A remarkable progress was achieved in 1929 with the discovery of the human Electroencephalogram (EEG) by the German neurologist Hans Berger (1873-1941). This discovery confirmed the electrical basis of epilepsy that has been proposed before by physicians including Robert Bentley Todd (1809-1860) (Goldensohn et al., 1997, Magiorkinis and Diamantis, 2014). The second half of the 20th century brought great advances in the diagnostic and therapeutic aspects of the disease and was marked by expansion of interest in basic mechanisms underlying seizures and epilepsy. This interest was driven by developments in genetics, molecular biology, neurophysiology, functional imaging and many other techniques (Goldensohn et al., 1997).

Despite the above facts, large gaps still exist in our knowledge about the pathophysiology of epilepsy and the mechanisms of epileptogenesis. Researchers still need to investigate theories in order to fill these gaps. The ultimate goal of epilepsy research is to identify reliable diagnostic and prognostic biomarkers and to investigate novel therapeutic targets with the hope of modifying or curing the disease and not simply preventing seizures (Simonato et al., 2012, Baulac et al., 2015).
1.2 Definition of epileptic seizures and epilepsy

Epilepsy is not a single disease; in fact it includes broad categories of disorders all of which are associated with long lasting derangement of brain structure and/or function. This abnormality in the brain is manifested clinically as different types of seizures. Seizures, however, are the ultimate end result of a variably long process (epileptogenesis) where normal brain function and structure are altered leading to an enduring state that favours the generation of abnormal electrical activity and recurring seizures i.e. epilepsy (Goldberg and Coulter, 2013).

An epileptic seizure is defined as “the clinical manifestation of transient, abnormal, excessive, hypersynchronous discharge of a group of neurons” (Fisher et al., 2005). A detailed description of how seizure manifests clinically is difficult, because it depends on many factors such as site of the epileptic focus in the brain, patterns of seizure propagation and age of the patient (Fisher et al., 2005). Seizures can affect sensory, motor and autonomic functions and can range in severity from a brief sensory experience to a major convulsive status epilepticus (SE) (Chang and Lowenstein, 2003, Baca et al., 2011).

In 2014, the International League Against Epilepsy (ILAE) proposed a new practical and clinical definition of epilepsy to replace the earlier conceptual one created in 2005 (Fisher et al., 2014). The new definition described epilepsy as a disease of the brain that is characterized by any of the following conditions: 1) Two unprovoked (or reflex) seizures occurring more than 24 hours apart, 2) One unprovoked (or reflex) seizure with a high risk of recurrence (>60%) that is similar to the general recurrence risk, 3) Diagnosis of an epilepsy syndrome (Fisher et al., 2014).

The first noticeable change in the new definition is that it refers to epilepsy as a disease, rather than a disorder. This change was brought to emphasize the serious nature of epilepsy as the term disease conveys a more lasting derangement of normal function. The concept of two unprovoked seizures more than 24 hours apart remained unchanged in the latest definition. However, it included reflex seizures
that are provoked by an external factor, provided that there is an enduring abnormal predisposition to have seizures (Fisher et al., 2014). The task force retain the current thinking that seizures clustering within 24 hours should be considered to be a single event as it has the same risk for later seizures as does a single seizure (Neligan et al., 2012, Fisher et al., 2014).

The current definition also strongly emphasizes the risk of recurrence after the first unprovoked seizure. A patient with a brain lesion causing an enduring predisposition for unprovoked seizures, such as stroke, infection and traumatic brain injury (TBI), usually has a risk for recurrent seizure comparable to those who have had two unprovoked seizures. Diagnosis of epilepsy should be made earlier in such case in order for appropriate treatment options to be considered (Fisher et al., 2014). This earlier diagnosis is particularly useful to prevent risk of physical injury and to avoid any social consequences of seizure recurrence. The revised definition also included all patients diagnosed with epilepsy syndromes even if there is a very low risk of subsequent seizures (Fisher et al., 2014).

A cure for epilepsy, a situation where the risk of having seizures is the same in patients as in the general population, has not been achieved thus far. However, the new definition allows for resolution of epilepsy, meaning that the patient no longer has epilepsy even though there is no guarantee that this patient will never develop seizures in the future. However, the decision to stop anti-seizure medications should be individualised. Epilepsy is regarded to be resolved if the patient is seizure free for the last 10 years and off treatment for the last 5 years. Epilepsy is also considered to be resolved for patients who have an age-dependent epilepsy syndrome and are now passed the applicable age (Fisher et al., 2014).
1.3 Classifications of the epilepsies

Classifying epileptic seizures and epilepsies is the key tool used clinically to evaluate an individual who is presenting with seizures. Classification serves many purposes. It helps understanding the type of seizures that the patient has, the other seizure types that are more likely to occur in that individual, the potential triggers for their seizures, and often their prognosis. In addition, classification has its role in predicting the risks of comorbidities and mortality in epilepsy patients. It also guides the selection of antiepileptic therapies (Scheffer et al., 2017). The 2017 classification of epilepsy is a multilevel classification. It starts with determining seizure type, epilepsy type and finally epilepsy syndrome (Scheffer et al., 2017).

1.3.1 Classification of epileptic seizures

The former seizure classification systems classify seizures based on only two criteria: clinical features and EEG features (Epilepsy, 1981, Epilepsy, 1989). Seizures were either generalized or partial. Partial seizures were further subdivided into simple and complex depending on presence or absence of impairment of consciousness. With significant advances in understanding the pathogenesis of seizure and epilepsy, a continuous revision of the classification systems has occurred. A number of papers have been published proposing a revision of the previous, outdated seizure classification that was several decades old (Lüders et al., 1998, Blume et al., 2001, Engel, 2001, Engel, 2006).

In 2010, the ILAE Commission on Classification and Terminology released a new revised classification system in which the most important concept is the mode of seizure onset (Berg et al., 2010). Accordingly, seizures were classified as being focal, generalized or unknown (Berg et al., 2010). Further clarification of this system has been recently published (Fisher et al., 2017a, Fisher et al., 2017b) (Figure 1.2). This new system has the advantage of being easier and more practical which is reflected clinically in more confident diagnosis and the choice of appropriate AED based on the type of seizure (Berg et al., 2010, Fisher et al., 2017b).
Figure 1.2 Operational classification of seizure types by the International League Against Epilepsy. Adapted from Fisher et al., 2017b.
Focal onset seizures (Figure 1.2): are those originating in a specific area of the brain and are usually limited to one hemisphere. Depending on the level of impairment of awareness or consciousness, focal seizures were further divided into three categories: a focal aware seizures (previously referred to as simple partial seizures), a focal impaired awareness seizures (previously referred to as complex partial seizures) and a focal to bilateral tonic–clonic seizures (previously referred to as secondary generalized seizures) (Fisher et al., 2017b). Both focal aware seizures and focal impaired awareness seizures types can be further categorized depending on the earliest prominent feature into motor and non-motor seizures (Fisher et al., 2017b, Fisher et al., 2017a).

Generalized onset seizures (Figure 1.2): are those originating within bilaterally distributed networks and propagate rapidly to involve the subcortical or cortical structures of both cerebral hemispheres. These seizures are divided into either motor or non-motor (absence) seizures. The latest classification recognizes 8 subtypes of generalized motor seizures and 4 subtypes of generalized non-motor (absence) seizures (Fisher et al., 2017b, Fisher et al., 2017a).

Unknown (Figure 1.2): seizures with unknown mode of onset are those where it is still unclear whether the onset is focal or generalized. However they can still be classified as being either motor non-motor. An example of those seizure types is epileptic spasms and behaviour arrest (Berg et al., 2010, Fisher et al., 2017b, Fisher et al., 2017a).

1.3.2 Classification of epilepsy type

At the Epilepsy Type level, the disease can be classified into focal, generalized, combined generalized and focal or unknown. The diagnosis of epilepsy type is made on clinical grounds and supported by the finding of typical ictal and/or inter-ictal EEG findings (Scheffer et al., 2017).
1.3.3 Syndromic classification of epilepsy

Epilepsy Syndrome is a complex of signs and symptoms that define a unique epilepsy condition. Age-dependent features such as age at onset and remission, seizure triggers, diurnal variation in seizures are often identified to be specific for each syndrome. Moreover, distinctive comorbidities as well as specific aetiologic and prognostic features might be identifiable for each epilepsy syndrome (Scheffer et al., 2017). The latest classification system (Scheffer et al., 2017) has identified two major epilepsy syndromes which are: Idiopathic Generalized Epilepsies (IGEs) and Self-limited focal epilepsies. Under IGES, four well-established epilepsy syndromes have been identified which are childhood absence epilepsy, juvenile absence epilepsy, juvenile myoclonic epilepsy and generalized tonic–clonic seizures alone. Many self-limited epilepsies have also been characterized. For example, self-limited epilepsy with centrotemporal spikes, frontal, temporal and parietal lobe epilepsies (Scheffer et al., 2017).

1.3.4 Aetiology classification of epilepsy

The former classification of epilepsy into idiopathic, symptomatic and cryptogenic has been associated with considerable confusion and contradiction among epileptologists. Revised aetiological classifications have been suggested that focus on the mechanisms and causal factors of epilepsy (Shorvon, 2011). Seven classes of aetiologies were proposed by the 2017 ILAE report: genetic, structural, infectious, metabolic, immune and unknown (Scheffer et al., 2017). However, the cellular, molecular and genetic basis of epilepsy is quite variable. All these factors, either alone or in combination, can increase the risk of developing a seizure disorder and lead to establishment of an epileptogenic focus. The general agreement is that, whatever the pathophysiology of a seizure is, the main factor is the disturbances in the neuronal excitation–inhibition balance generating transient abnormal activity (McCormick and Contreras, 2001, Gatto and Broadie, 2010).
1.3.4.a Genetic aetiology

This term is applied where epilepsy is the direct result of a known or presumed genetic defect (Berg et al., 2010, Moshé et al., 2015). Pathological mechanisms converting a normal neuronal network into an epileptic one can be driven by altered expression and/or function of one or a set of crucial neuronal genes. Gene expression studies performed on resected brain tissue after epilepsy surgery as well as tissues obtained from experimental epilepsy models suggested the potential role of mutations in different gene families in the pathogenesis of epilepsy. In vivo, in vitro and in silico approaches are in use to identify dysregulated genes during epileptogenesis and epilepsy (Rossignol et al., 2014, Mirza et al., 2016).

Single gene mutations leading to dysfunction in voltage-gated (Na+, K+, Cl−, Ca2+) and/or ligand-gated (Nicotinic, Acetylcholine and GABAergic) ion channels predominated the thinking of epilepsy genetics for a long time (Gatto and Broadie, 2010). Most familial cases of epilepsy involved mutations in genes encoding ion channels and neurotransmitter receptors. This is particularly evident in the case of IGE where mutations in about 21 genes have been identified. The vast majority of these genes are related to ion channel function or involved in regulating excitatory and inhibitory neurotransmitters in the central nervous system (CNS) (Rees, 2010). Single gene mutations can also trigger focal epilepsies such as familial mesial temporal lobe epilepsy (TLE), familial lateral TLE and autosomal-dominant nocturnal frontal lobe epilepsy (Andermann et al., 2005).

The best known example of genetic epilepsy is Dravet syndrome in which >80% of patients have a pathogenic variant of the neuronal sodium channel α 1 subunit (SCN1A) gene (Scheffer et al., 2017). Mutations in the SCN1A gene were first described in two families having generalized epilepsy with febrile seizures plus (GEFS+) (Escayg et al., 2000). In addition to Dravet Syndrome, mutations in this gene have been identified in many epilepsy syndromes such as GEFS+ and intractable childhood epilepsy with generalized tonic clonic seizures. Mutations in SCN1A have been studied extensively and are well replicated in clinical tests and nowadays it has
become the basis of a diagnostic test (Berg et al., 2010). SCN1A encodes a voltage
gated sodium channel that is widely expressed in the brain particularly in the
neuronal cell body (Westenbroek et al., 1989). Approximately, 600 mutations have
been discovered in the SCN1A gene with 90% of them arising as de novo events. The
other 10% are familial with autosomal dominant inheritance (Mulley et al., 2005,

Other mutations in genes linked to different forms of epilepsy encode subunit
components of receptor channels which mediate neurotransmission. Examples of
these are SCN1B, SCN2A affecting Na⁺ channels, CSCNA1A, CACNB4 and CACNA1H
affecting Ca²⁺ channels and KCNQ2, KCNQ3 affecting K⁺ channels (Rees, 2010). The
link between these mutations and epilepsy was documented in animal models.
However, due to the fact that they are rare mutations, population-wide
perspectives are difficult and confirmation of their clinical relevance still requires
sophisticated testing (Rees, 2010).

Another example of a single gene mutations resulting in epilepsy is the mutations in
the leucine rich glioma inactivated 1 (LGI-1) gene which is associated with
autosomal dominant lateral-TLE with auditory features (ADLTEAF). It has been
estimated that 50% of ADLTEAF patients carry different forms of LGI-1mutations.
Non-familial sporadic cases with de novo LGI-1mutations have also been identified
(Nobile et al., 2009). Up to 25 mutations in the LGI-1gene have been identified in
ADLTEAF patients, 24 of these mutations segregated in families and demonstrate
autosomal dominant inheritance. Only one was found to be a de novo mutation
(Nobile et al., 2009). Mutations in LGI-1 are not regarded as a form of channelopathy
as this gene does not code for a channel subunit. However, the protein coded by
LGI-1 is tightly associated with K⁺ channel complex therefore mutations in this
gene can dramatically affect the function of K⁺ channel (Schulte et al., 2006).
Epileptic seizures can also be an intermediate manifestation of more complex genetic diseases. Disturbances in the excitation/inhibition balance are frequently encountered in these patients where epilepsy is co-morbid with other CNS pathologies. Some of these genetic defects can be found in patients with epilepsy syndromes and in epilepsies that are due to structural/metabolic causes (Pitkänen and Engel, 2014). A few examples include mutations in the MeCP2 gene associated with Rett syndrome, mutations in the UBE3A gene associated with Angelman syndrome and mutations in the FMR1 gene associated with Fragile X syndrome (Berg et al., 2010, Rees, 2010). The majority of these patients suffer from intractable epilepsy. The abnormal genetic constituent of those patients usually reflects the main CNS pathology.

Other nonspecific structural genomic abnormalities in the form of deletions, insertions or duplications of specific genomic sequence might also be associated with epilepsies and seizures. These abnormalities are called copy number variants (CNVs). They may occur in regions that contain no known genes or may contain multiple genes. Examples of these syndromes are 1p36 microdeletion syndrome (Deletion of 1p36), Wolf-Hirschhorn syndrome (Del 4p16.3), 6q terminal deletion syndrome (Deletion 6q) and others (Battaglia et al., 2009, Bahi-Buisson et al., 2008, Striano et al., 2006).

With recent advances in molecular techniques, mutations in at least fifteen other genes have been identified in association with different forms of epilepsies (Guerrini et al., 2014). Despite that, the reference to epilepsy as being genetic does not exclude the possibility of other environmental factors that may also contribute to the occurrence of the disease. Ideally, a certain gene defect should be identified in order to classify a disease as being genetic but this is not always possible in the case of epilepsy (Berg and Scheffer, 2011, Scheffer et al., 2017).
1.3.4.b Structural aetiology

Certain structural abnormalities of the brain are associated with a substantially increased risk of developing epilepsy. These structural abnormalities are usually visible on neuroimaging. An electro-clinical assessment of the patient should suggest the causal relationship between the abnormality and the patient’s seizures. Structural aetiologies may be acquired such as stroke, trauma, and infection, or genetic such as the many malformations of cortical development (Berg et al., 2010, Scheffer et al., 2017). An example of the well-recognized associations of epilepsies with a structural aetiology is the frequent finding of mesial temporal lobe seizures with hippocampal sclerosis (HS) (Scheffer et al., 2017). Recognition of this association is important to ensure that the patient’s imaging is carefully examined for a specific structural abnormality. This in turn highlights the need for consideration for epilepsy surgery should the patient fail medical therapy (Scheffer et al., 2017).

1.3.4.c Infectious aetiology

Epilepsy sometimes occurs as a direct result of a known infection. Common infectious diseases that might lead to epilepsy include neurocysticercosis, tuberculosis, HIV, cerebral malaria, subacute sclerosing panencephalitis, cerebral toxoplasmosis, and congenital infections such as Zika virus and cytomegalovirus. Identifying an infectious aetiology for epilepsy has important implications for the approach to treatment (Scheffer et al., 2017).

1.3.4.d Metabolic aetiology

A number of well-identified metabolic disorders in which certain biochemical changes occur throughout the body can lead to development of seizures and epilepsy. Frequently, a specific genetic defect can be identified in these patients. Correct identification and diagnosis of these metabolic syndromes is extremely important due to implications for specific therapies and potential prevention of intellectual impairment (Scheffer et al., 2017).
1.3.4.e Immune aetiology

A number of neurological autoantibodies have recently been described in association with autoimmune encephalopathy and/or epilepsy. The majority of these antibodies are directed against neuronal cell surface antigens, including synaptic neurotransmitter receptors, ion channels, or related proteins (Quek et al., 2012, Lancaster and Dalmau, 2012). The most clearly identified inflammatory conditions leading to epilepsy are anti-NMDA (N-methyl-d-aspartate) receptor encephalitis and anti-LGI1 encephalitis. Several other immune epilepsies have been identified recently all of which had characteristic presentations in both adults and children (Dubey et al., 2017, Scheffer et al., 2017).

1.3.4.f Unknown aetiology

The term unknown simply means that the nature of the underlying cause is not currently known. All types of epilepsies with normal imaging and no documented genetic, metabolic or immune aetiologies are included in this category (Berg and Scheffer, 2011, Moshé et al., 2015, Scheffer et al., 2017).

1.4 Temporal lobe epilepsy

TLE is the most common form of epilepsy that is attributed to an earlier brain insult (acquired epilepsy). TLE is regarded as the best characterized electro-clinical syndrome of all the epilepsies, where the temporal lobe seizures originate from the temporal neocortex. TLE is also very common compared to other epilepsy types, comprising about 40% of all epilepsies in adult patients (Tatum, 2012). It has been estimated that, 60-80% of patients referred to tertiary epilepsy centres due to refractory seizures have their focal seizures believed to originate from the mesial temporal lobe structures, hippocampus, amygdala and hippocampal formation (Scharfman et al., 2000).
Although temporal lobe epilepsies share a common site for seizure origin, they are highly heterogeneous in terms of aetiology, age of onset, prognosis and therapeutic response. An initial precipitating insult in the form of febrile seizure, hypoxia, infection and trauma especially before the age of 5 years, can be identified in the majority of patients (French et al., 1993). However, a definitive link between this insult and the development of TLE and HS is difficult to prove as it requires prospective studies with large number of patients and long follow up. Recently, a study has been conducted to understand the rate and pathogenesis of epilepsy among children aged 1 month to 6 years who presented with febrile SE. Neuroimaging evidence of acute hippocampal injury has been found in 9.6% of these children. This form of injury often evolves into HS after one year, thus suggesting a link between febrile seizures and the development of TLE (Lewis et al., 2014).

1.4.1 Mechanisms of TLE

The most consistently affected processes in acquired epilepsy are neuronal degeneration, neurogenesis, gliosis, inflammation, reorganization of neuronal microarchitecture and reorganization of the extracellular matrix. These mechanisms may act solely or in combination to produce the altered neuronal excitability. This concept suggests that manipulation of these pathways has the potential to modify the disease before the appearance of the first seizure or influence its progression by reducing seizures severity and/or frequency. However, it is more likely that these processes act simultaneously to produce seizures, as modification of one or more pathways only resulted in modest effects on the epileptogenesis process (Pitkänen and Sutula, 2002, Pitkänen and Lukasiuk, 2011b). These molecular and cellular changes may continue to progress after the establishment of epilepsy. A continuous remodelling of the neuronal circuits could occur due to the recurrent episodes of neuronal hyper-excitation leading to a progression of the disease (Pitkänen and Sutula, 2002, Pitkänen and Lukasiuk, 2011b).
Despite the above facts, we cannot definitely exclude the existence of a genetic predisposition for TLE. Cases of familial mesial TLE have been documented where patients presented with intractable seizures and HS with a clear family history of the condition. However, these cases do not show Mendelian inheritance. The complex inheritance pattern suggests a possible role of more than one gene defect in causing epilepsy (Cendes et al., 1998, Kobayashi et al., 2002, Thomas and Berkovic, 2014). Furthermore, certain genetic defects have been identified in mice that are associated with HS and TLE. Similar defects in humans can also lead to TLE (Kearney et al., 2001). Finally, a genetic predisposition to febrile seizures can lead to a seizure severe enough to produce hippocampal damage and TLE (Abou-Khalil et al., 2001, Engel Jr, 1996).

1.4.1.a Neuronal cell death in TLE

Neuronal loss is a common and consistent finding within the hippocampus of TLE patients. This type of damage was successfully reproduced in experimental animals through either the systemic or intracerebral administration of excitotoxic substances such as kainic acid (K.A) and pilocarpine (Leite et al., 1990, Ben-Ari et al., 1986) or through sustained electrical stimulation leading to SE (Sloviter and Damiano, 1981).

Based on histopathological and magnetic resonance imaging (MRI) studies, TLE can be distinguished into two types. The first, lesional TLE, is associated with HS and is the most common type (60-70% of patients). Second, non-lesional TLE, with no or only mild neuronal loss within the hippocampus (Mueller et al., 2011, Blumcke et al., 2002). The association of HS with TLE was identified early in the course of the disease and it is regarded as the most common histopathological abnormality in adult patients with drug resistant TLE (Blumcke et al., 2002).

According to ILAE classification of HS, type 1 is the most severe form and refers to severe neuronal cell loss and gliosis predominantly in CA1 (with >80% cell loss) and CA4 (with 40-90% cell loss) regions, whereas other segments (CA2 with 30-50% loss
and CA3 with 30-90% loss) show significant but less severe neuronal loss (Blumcke et al., 2013). In addition, the dentate gyrus (DG) is usually affected by 50–60% granule cell loss. The main types of neurons affected in these areas are the glutamatergic principal neurons and the gamma-aminobutyric acid (GABAergic) interneurons (Dingledine et al., 2014). Choi (1988) suggested that excessive glutamate release (the principal excitatory neurotransmitter in the brain) during seizure leading to intracellular sodium and calcium overload is the initiating mechanism leading to neuronal cell death. This cytotoxic excessive glutamate release is also associated with production of free radicals, organelle swelling, and disturbances of both intra- and extracellular homeostasis leading to cell lysis (Meldrum, 1993).

Once developed, neuronal loss can further increase the susceptibility to develop spontaneous seizures. First, by direct loss of inhibitory neurons or indirectly through formation of maladaptive new circuits in response to loss of synapses (Kobayashi and Buckmaster, 2003; Sloviter and Bumanglag, 2013). Second, by provoking reactive gliosis and inflammation (Vezzani et al., 2011). This characteristic pathological finding can be assessed non-invasively using MRI studies where atrophy of different areas of the mesial temporal lobe can be visualised and evaluated (Bernasconi et al., 2003).

The two main cell death processes activated by seizures and involved in neuronal cell death are apoptosis and necrosis (Henshall and Meldrum, 2010). However, the molecular distinctions between them are unclear, as many factors initially identified in apoptosis have also been found to contribute to necrosis. In contrast to necrosis, which is regarded as an uncontrolled, chaotic form of cell death that is initiated after an extreme form of cell injury, apoptosis is a well-controlled, programmed cell death process. Morphologically, necrosis is characterized by cellular and organelle swelling that leads eventually to rupture of the plasma membrane and cell lysis. Apoptosis, on the other hand, is associated with chromatin and cytoplasmic condensation with formation of apoptotic bodies (Dingledine et al., 2014).
Whether cell death after SE and brief seizures is derived by activation of the apoptotic or necrotic pathways is still a matter of debate. Several models of SE in adult brain showed that necrosis is the predominant form of neuronal death. Fujikawa (1999) and Fujikawa et al. (2000) examined multiple brain regions 24 and 72 hours after Lithium-pilocarpine or K.A induction of SE in rats. The presence of necrotic neurons, rather than apoptotic ones, was confirmed by light and electron microscopy, TUNEL staining and by agarose gel electrophoresis of DNA. No apoptotic morphology could be detected in the affected neurons. The same findings were reported in developing brain of post-natal day 14 rat pups, where electron microscopic examination of neurons, 24 hours after induction of SE, showed that 47 out of 50 injured CA1 neurons had a necrotic morphology. After 72 hours 100% of the injured neurons were necrotic (Niquet et al., 2007). However, alongside this observation, activation of caspase-3 in injured neurons 24 hours after SE was also reported, raising the possibility that neuronal death was mediated through a caspase-dependent programmed necrosis (Niquet et al., 2007).

Apoptosis, on the other hand, is mediated through two main molecular pathways (intrinsic and extrinsic pathways). The initial signal for the extrinsic pathway is the activation of cell-surface-expressed death receptors of the tumour necrosis factor (TNF) superfamily. Once activated, a series of intracellular events occur that eventually lead to the recruitment of one or more members of the caspase family of cell death proteases, typically caspase-8. Caspase-8 is an apoptosis initiator that will cleave and activate downstream effector caspases, in particular caspase-3 and -7, leading to enzymatic cleavage of key intracellular organelles and proteins, leading eventually to cell death (Henshall et al., 2001b, Taylor et al., 2008, Engel and Henshall, 2009).

The second apoptotic pathway, the intrinsic pathway, is activated after an intracellular stimulus such as DNA damage, endoplasmic reticulum stress or calcium overload. The upstream initiators of this pathway appear to be members of BCL-2 homology domain 3-only (BH3), a subgroup of the B-cell lymphoma protein (BCL-2)
family. These molecules either act as facilitators, in that they bind and neutralize the function of anti-apoptotic members of the BCL-2 family, or act as activators, in that they directly activate the assembly of the pro-apoptotic Bax/Bak complex within the outer mitochondrial membrane (Youle and Strasser, 2008). This in turn triggers the release of apoptogenic factors from the mitochondria, including cytochrome c and apoptosis inducing factor, into the cytosol which initiates the assembly of the apoptosome as well as the activation of caspase-9 and caspase-3 which will in turn propagate a proteolytic cascade inside the cell (Cain et al., 2002, Taylor et al., 2008, Engel and Henshall, 2009).

There is accumulating evidence for the activation of BCL-2-family related apoptotic pathways after SE and brief seizures. The first molecular evidence was reported by Zhang et al. (1998) who detected an increase in Bax mRNA in rat hippocampus following multiple kindling seizures. Other findings such as upregulation of Bax, levels of TNFα and increase in caspase activity were reported to further support the activation of apoptotic pathway after brief seizures induced by kindling (Akcali et al., 2005, Shandra et al., 2002, Pavlova et al., 2004).

Many of the molecular dysregulations associated with apoptosis were also detected in resected hippocampal tissue of TLE patients, including increased neuronal expression of the pro-apoptotic molecules; Bax (Nagy and Esiri, 1998, Uysal et al., 2003), caspase-2 (Narkilahti et al., 2007), caspase-3 (Pavlova et al., 2004b, Schindler et al., 2006), caspase-6, caspase-7 and caspase-9 (Yamamoto et al., 2006). The increase in the pro-apoptotic factors have been shown to be associated with increases in the anti-apoptotic factors BCL-xL,BCL-w which may be explained as a reactive neuroprotective mechanism in surviving neurons (Henshall et al., 2000, Murphy et al., 2007).

Other functional studies based on protein-protein interactions and studies on mice lacking specific genes provided more insight on how apoptosis-related genes can regulate seizure-induced neuronal death. Examples from those studies are mice lacking the pro-apoptotic Bim and Puma genes which were found to be associated
with less CA3 damage and milder epileptic phenotype after induced SE (Murphy et al., 2010, Engel et al., 2010). Knockout of other pro-apoptotic BCL-2 members such as Bid, Bad and Bmf was also tested with variable degrees of neuronal damage after induced SE. Loss of the anti-apoptotic Mcl-1 and BCL-w was associated with enhanced hippocampal damage and earlier onset of SE (Mori et al., 2004, Murphy et al., 2010, Henshall and Engel, 2013). Inhibition of specific members or all members of the caspase family was also reported to be protective after SE (Lopez-Meraz et al., 2010).

In summary, neuronal cell death is regarded as a hallmark for human TLE. Both apoptotic and necrotic pathways contribute significantly to the occurrence of neuronal loss after prolonged SE and after brief seizures. Furthermore, alterations to these signalling pathways have been proved to have a neuroprotective effect.

1.4.1.b Gliosis and altered glial functions

Neuroglial cells play a central role in supporting neuronal cytoarchitecture and providing homeostatic regulation. They also play an important role in modulating synaptic function and plasticity (Burda and Sofroniew, 2014). The term glial cells collectively refer to astrocytes, oligodendrocytes and microglia. Each has a specific function and responds to cellular injury caused by diverse aetiologies in a specific manner. Disturbances in the function of glial cells after brain injury are believed to cause seizure and initiate epileptogenesis (Wetherington et al., 2008).

Astrocytes are the most abundant cell type in the brain. In humans, a single, mature astrocyte extends about 40 large processes in all directions from the cell body allowing the modulation of function of roughly two million synapses (Oberheim et al., 2006). The main function of astrocytes is to maintain the water, ion, pH and transmitter homeostasis of the synaptic interstitial fluid. As well, astrocytes play an important role in the regulation of cerebral blood flow through their end-feet processes that wrap the entire cerebral vasculature (Sofroniew and Vinters, 2010, Robel, 2016). All astrocytic processes are rich in glutamate, purines (ATP and
adenosine) and GABA transporters and in potassium K^+ rectifying channels (Kir 4.1). These channels serve to clear different neurotransmitters and ions from the synaptic space, thus modulating synaptic function. After its uptake into astrocytes, glutamate will be converted by glutamine synthetase enzyme (GS) into glutamine which will be recycled back to synapses for reconversion into active transmitters. Glutamine itself is the substrate for the production of GABA for the inhibitory GABAergic neurons (Sofroniew and Vinters, 2010). Astrocytic processes are also rich in aquaporin 4 (AQP4) water channels. These channels are mainly clustered within the astrocytic end-feet processes and to a large extent, co-expressed with Kir 4.1 channels. A coordinated action of both channels is required to maintain water and K^+ homeostasis (Seifert et al., 2006).

Astrocytes react to any type of CNS insults with changes in their cellular, molecular and functional characteristics. Reactive astrogliosis involves progressive hypertrophy of the cell and in severe cases, proliferation and scar formation (Sofroniew and Vinters, 2010). In epilepsy, reactive astrocytes are found in putative epileptic foci and they play a central role in the epileptogenesis process (Robel and Buckingham, 2015). It is now widely acknowledged that astrogliosis alone, in the absence of other potentially cofounding pathological effects of brain injury such as inflammation and blood–brain barrier (BBB) disruption, is sufficient to promote neuronal hyperexcitability and cytotoxicity through several mechanisms (Ortinski et al., 2010). In TLE with HS, reactive astrocytes display a redistribution of AQP4 from the perivascular end-foot processes into the cytoplasm, thus affecting K^+ clearance leading to increased propensity for seizures (Eid et al., 2005). Another potential mechanism is impaired astroglial glutamate uptake from the synaptic cleft as a result of downregulation of glutamate transporters (EAAT1, EAAT2) (Sarac et al., 2009). This leads to both elevated glutamate levels in the synapse and a reduced rate of glutamate conversion into glutamine thus depriving inhibitory interneurons of an essential precursor of GABA synthesis (Eid et al., 2004, Wang et al., 2009b, Benedetti et al., 2011) leading to local network hyperexcitability. Further support to the role of astrogliosis in neuronal hyperexcitability came in 2010. Ortinski and
colleagues observed that adenovirally-induced astrogliosis via overexpression of green fluorescent protein (GFP) in the hippocampus of otherwise normal adult mice was sufficient to produce downregulation of the GS enzyme and impaired inhibitory transmission and hyperexcitation (Ortinski et al., 2010).

In another study, widespread, chronic reactive astrogliosis was genetically induced in mice by a conditional deletion of β1-integrin (Itgβ1) in glia. In these mice, astrogliosis was induced without the association of any gross brain abnormality or inflammation and it was sufficient for the development of spontaneous seizures by 6 weeks of age (Robel and Buckingham, 2015). In contrast to the previous study, GS level was comparable between the knockout and control brains. However, whole-cell patch clamp recordings revealed decreased glutamate uptake capabilities of reactive astrocytes in addition to functional impairments in the Kir4.1 potassium channel. These findings demonstrated that genetically induced chronic astrogliosis is sufficient to induce spontaneous, recurrent, GTC seizures in vivo (Robel and Buckingham, 2015).

1.4.1.c Inflammation

Activation of the brain’s innate and adaptive immune systems is acknowledged to contribute to the pathogenesis of epilepsy. The association between chronic brain inflammation and the ability of the brain to generate seizures was first observed in patients with Rasmussen encephalitis, a severe epileptic encephalopathy of childhood (Rasmussen et al., 1958) and evidence continues to emerge since that time. Neuropathological examination of resected hippocampal specimens from patients suffering from refractory TLE supported this view. These tissues displayed all of the hall-marks of a chronic inflammatory state such as infiltration of leukocytes, reactive gliosis and overexpression of cytokines and their target proteins. These findings, together with evidence of disease modification after administration of anti-inflammatory agents, suggest that neuroinflammation plays an important role in the development of TLE (Vezzani et al., 2016). The major contributors to the synthesis of these inflammatory mediators in TLE are activated
astrocytes, microglia and neurons. Endothelial cells of the BBB and leukocyte extravasation into brain parenchyma also contribute to brain inflammation (Maroso et al., 2011).

In 2002, Vezzani et al. reported a rapid (within 3 hours) and reversible induction of pro-inflammatory cytokines such as interleukin-1β (IL-1β) in the glial cells of the hippocampus after K.A induced SE. This upregulation was found mainly in areas of the hippocampus showing activated microglial cells. Astrocytes were also found to contribute to cytokine production but at a later time point (18 hours onward) (De Simoni et al., 2000, Vezzani et al., 2002). The pro-convulsant effect of IL-1β is mediated through several mechanisms. An increased expression of plasma membrane receptor for IL-1β (interleukin-1 receptor type-1 (IL-1R1)) was observed in neurons and astrocytes of epileptic tissue (Ravizza and Vezzani, 2006, Ravizza et al., 2008). In addition, IL-1β has been found to be critical for induction of reactive astrogliosis both in vivo and in vitro (Giulian et al., 1986, John et al., 2004).

Moreover, IL-1β markedly attenuates astrocytic glutamate uptake through a pathway that involves liberation of nitric oxide, leading to neuronal excitotoxicity (Ye and Sontheimer, 1996). IL-1β also potentiates NMDA receptor function in cultured hippocampal neurons by enhancing the NMDA-mediated neuronal Ca^{2+} influx (Viviani et al., 2003). A reduction in GABA-mediated inhibition was observed in cultured hippocampal neurons treated with IL-1β (Wang et al., 2000). Finally, IL-1β is believed to be involved in the synthesis and release of other cytokines such as IL-6 and TNF-α in astrocytes and microglia (Benveniste, 1992).

More recent studies support the activation of toll-like receptor (TLR) signalling pathways in epilepsy. TLR-4 and -2 are of particular importance as they are constitutively expressed in the CNS (Chakravarty and Herkenham, 2005, Maroso et al., 2010). TLRs are key receptors of innate immunity and they play an important role in pathogen recognition (Kawai and Akira, 2007). These receptors are activated by molecules released from injured cells (danger signals) such as the chromatin binding protein high-mobility group box-1 (HMGB1) that is passively released by
necrotic cells and actively secreted by cells in profound stress (Scaffidi et al., 2002). Increased HMGB1 was observed in activated microglia and astrocytes during seizure activity and a similar pattern was observed in human epileptic tissue from patients with temporal lobe epilepsy (Maroso et al., 2010). Activation of both IL-1R1 and TLRs, in neurons and activated astrocytes, alters neuronal excitability via activating the transcription factor nuclear factor-kappa B (NFkB), which in turn induces the transcription of genes encoding cytokines, chemokines and proteins of the complement system. Moreover, the transcriptional events induced by inflammatory mediators will contribute to a lasting decrease in seizure threshold by inducing the expression of genes involved in neurogenesis, cell death and molecular and synaptic plasticity (Vezzani et al., 2011). Given the role of IL-1R/TLR signalling in seizures, inhibition of this signalling may be useful in treating drug-resistant human epilepsy through reducing inflammatory processes in the epileptic brain, thereby counteracting neuronal hyperexcitability. Certain preclinical data support this hypothesis; a number of pharmacological agents targeting this pathway are now under clinical evaluation for treatment of sepsis and inflammatory diseases, and some are already in clinical use to treat chronic peripheral inflammatory conditions (Maroso et al., 2011). Experimental studies confirmed the upregulation of HMGB1 in the blood and brain, 4 and 24 hours respectively, after the induction of status epilepticus (Walker et al., 2014). Interestingly, a progressive increase in astrocytic HMGB1 was noted 1-3 hours after induction of seizure by intra-hippocampal injection of kainic acid. A similar but transient expression was also noted in microglia one hour after seizure induction. This increase was associated with substantial upregulation of TLR-4 in pyramidal neurons and astrocytes but not in microglial cells. The same results were reported in blood samples collected from drug refractory patients and in resected sclerotic hippocampal tissues of TLE patients (Maroso et al., 2010, Walker et al., 2014).

Finally, brain inflammation may also contribute to the disruption of the BBB. An intact BBB is dependent on the normal functioning of pericytes, perivascular microglia and astrocytes. During inflammation the secretion of cytokines by the BBB
endothelial cells, microglia and astrocytes contributes to increased permeability of the BBB and leads to accumulation of serum albumin and IgG in the brain parenchyma (Webb and Muir, 2000, Vezzani et al., 2013). Albumin itself has been shown to have a strong, long lasting hyperexcitability effect. Direct in vivo and in vitro exposure of brain to serum albumin has been shown to be associated with a receptor mediated albumin uptake into the astrocytes by the transforming growth factor β receptors (TGF-βRs). This uptake is followed by downregulation of Kir 4.1 channels in astrocytes predisposing to neuronal hyperexcitability (Ivens et al., 2006).

To summarize, inflammatory processes in brain tissue have been described in human epilepsy of various aetiologies and in experimental models of seizures. Certain anti-inflammatory therapies are now in clinical or experimental evaluation, further highlighting the importance of inflammation in the aetiology of epilepsy. The preclinical data available suggests that direct targeting of pro-inflammatory molecules such as IL-1β and TLR-4 by inhibitors might lead to protection against seizure development.

1.4.1.d Neuronal plasticity and altered neuronal architecture

An important ultrastructural finding consistently observed in human TLE and in the related animal models is the aberrant sprouting of the surviving neurons in the hippocampal subfields and in the dentate gyrus with associated dendritic and axonal reorganization (Sutula et al., 1989, Houser et al., 1990, Thom et al., 2009). The most extensively studied form of axonal sprouting is mossy fibre sprouting (MFS) which refers to the synaptic reorganization and growth of new axon collaterals from the dentate granule cells into the inner molecular layer of the dentate gyrus of the hippocampus (Represa et al., 1993, Okazaki et al., 1995).

The discovery of MFS in the sclerotic hippocampus led to its candidacy as a mechanism of hyperexcitability. Initially, the sprouting was suggested to affect the glutamatergic neurons with formation of new functional synapses and targeting
new regions leading to a major change in the circuitry. This hypothesis elegantly explains the formation of hyper-innervated and aberrant excitatory circuit within the epileptic brain that facilitate the seizure activity (Tauck and Nadler, 1985, Ribak et al., 2000, Wuarin and Dudek, 2001). However, Acsady et al. (1998) challenged this idea when they found that the majority of the sprouted mossy fibres project to the inhibitory GABAergic interneurons rather than to the excitatory principal cells. This raises the possibility that MFS may promote inhibition of dentate granule cells rather than excitation.

Recent data have identified the activation of the mammalian target of rapamycin (mTOR) signalling pathway as responsible for mossy fibre sprouting during epileptogenesis. Rapid and transient activation of mTOR was reported in the rat hippocampus shortly after the induction of status epilepticus. A second wave of activation was noted during epileptogenesis and persisted after epilepsy onset (Zeng et al., 2009). Treatment with an mTOR inhibitor, rapamycin, attenuated post status epilepticus mossy fibre sprouting (Zeng et al., 2009, Crino, 2011, Galanopoulou et al., 2012). Although previous reports showed that the complete inhibition of mossy fibre sprouting by rapamycin is neither anti-seizure nor anti-epileptogenic (Heng et al., 2013), a very recent study by Drion et al. (2016) indicated that mTOR inhibition by rapamycin has a strong seizure suppressive effect that continues to be effective as long as the blood level of the drug is sufficiently high. Once the treatment was discontinued, however, gradual recurrence of seizure in the following weeks was noticed. Thus, mTOR inhibition is not disease modifying, at least in this model (Drion et al., 2016).

Altogether these data suggest both a pro- and anti-seizure effect of the mossy fibre sprouting but it is not shown to be a requirement for epileptogenesis. Other changes that have been identified at the axonal and dendritic level are spine retraction or loss, reduction in spine area and reduction in spine density (Guo et al., 2012, Wong and Guo, 2013). The functional implication and clinical consequences of those pathologies are still unclear.
1.4.2 Clinical and Laboratory Diagnosis of TLE

Since the treatment of epilepsy depends on accurate diagnosis, determining if the patient’s attack was a seizure and diagnosing the type of seizure and type of epilepsy syndrome is regarded as the first critical step in the management of a patient.

The cornerstone for an accurate epilepsy diagnosis is careful history. A detailed recall of the signs and symptoms preceding, during and following the “event” is critical to differentiate between seizures and other episodes of altered consciousness. An eye witness or a video record of the event is often required. Sometimes the patient presents with more than one seizure semiology which requires careful attention (Engel, 2011a, Engel, 2011b, Galanopoulou and Moshe, 2011).

Obtaining a fully complete history from a patient is time consuming but it is very important in establishing the diagnosis of epilepsy. The clinician should have excellent communication skills to encourage the patient or the eye witness to recall the event accurately (Ferrie, 2006). The clinician should be able to discriminate between spontaneous unprovoked seizures and seizures that are provoked. Classification of epilepsy, the diagnosis of epilepsy syndrome and determining any aetiological factor should be clearly addressed while obtaining the patient’s medical history.

Neuropsychological and social assessment of patients is also important in making a definitive epilepsy diagnosis. Epilepsy is often accompanied by cognitive and developmental delays and is more likely to be accompanied with depression, anxiety and other forms of psychological illnesses. Epilepsy is associated with low self-esteem, discrimination and stigma (Kwong et al., 2016, Chong et al., 2016, Caplan, 2013). Psychological assessment is also important to differentiate epileptic seizures from other psychogenic non-epileptic attacks (PNEAs) which constitute the most common condition misdiagnosed as epilepsy (Benbadis, 2009).
A complete physical and neurological examination is essential for epilepsy diagnosis not only to determine any underlying causes of seizure episodes but also to exclude other medical conditions that might produce similar clinical features such as syncope, transient ischemic attacks and migraines (Ahmed and Spencer, 2004, Benbadis, 2009).

1.4.2.a Role and limitations of Electroencephalogram in epilepsy diagnosis

Since its discovery in 1929, EEG was widely used for diagnosis and classification of epilepsy syndromes. Routine and long-term EEGs are regarded nowadays as part of the standard care procedures for epilepsy patients and are recommended to be conducted as soon as possible after the first unprovoked seizure (Fisch et al., 2016).

The ability of the brain to generate seizures is regarded as the main functional disturbance in epilepsy. Functional brain data can be assessed by different forms of EEG; they have their role as diagnostic and prognostic epilepsy biomarkers as they can give an idea about the severity and frequency of the abnormal electrical discharge in the brain. In addition, EEG has an important role in diagnosing specific epileptic seizure types and epilepsy syndromes (Engel, 2011b). EEG recorded using either depth electrodes or surface electrocorticography (ECoG) gives high resolution data with maximum quality if compared with that obtained from scalp EEG recording. These recordings can provide precise data about the neuronal clusters causing the abnormal firing (Engel et al., 2013).

Potential functional biomarkers for epilepsy and epileptogenesis include several forms of abnormal EEG recordings such as inter-ictal spikes, high frequency oscillation (HFO) and abnormal changes in background such as slowing (Rakhade et al., 2007). One of the strongest parameters in this regard, is the pathological HFO. This abnormal EEG pattern was recorded during the inter-ictal stage from areas adjacent to the epileptic zone in TLE patients and it has been used to localize this zone prior to surgery (Pitkanen et al., 2016, Bragin et al., 1999). It was found that the removal of the area generating this HFO resulted in improvement in patient
condition (Haegelen et al., 2013). Interestingly, in an experimental model of
epilepsy, all rats in which HFO activity was recorded after SE developed recurrent
spontaneous seizures, whereas none of the rats without HFO developed seizures
(Bragin et al., 2004). A strong correlation was found between the time to develop
the HFO and the timing of the first spontaneous seizure (Bragin et al., 2004).
Together those findings might form the basis of utilizing EEG data as a predictive
biomarker for epilepsy development. Studies correlating the HFO with the correct
delineation of the epileptogenic zone in TLE patients were almost always performed
using invasive EEG techniques. Although the generated data was extremely useful,
the invasive nature of this technique made it difficult to apply and a non-invasive
recording for the incidence and the spectral properties of the pathological HFO still
needs to be established (Pitkanen et al., 2016).

Despite all the above facts, the usefulness of EEG in epilepsy diagnosis has been
questioned over several decades as EEG has a number of limitations. It is almost
never possible to diagnose or exclude epilepsy based on EEG alone (Fowle and
Binnie, 2000). The failure of EEG to definitely diagnose epilepsy has many reasons;
the electrical activity recorded by EEG is mainly generated in the more superficial
layers of the cortex. At the same time, propagation of electrical activity along
physiological pathways might give a misleading impression on the source of these
discharges (Smith, 2005). Routine outpatient inter-ictal EEG with recording time
approximately 30 minutes has a relatively low sensitivity in epilepsy diagnosis
(ranging between 25-56%). Specificity, on the other hand, is about 78-98%. It is well
known that a normal inter-ictal EEG does not rule out the diagnosis of epilepsy.
About 18% of epilepsy patients never exhibit epileptiform discharges despite
repeated EEGs over several months (Fowle and Binnie, 2000). As well, epileptiform-
like discharges can be noticed in EEG recordings of healthy adults at an incidence of
about 0.5%; a slightly higher incidence is found in healthy children (2-4%). A
substantial increase in this figure (10-30%) was noted in patients with cerebral
pathologies such as tumours, brain injury and after brain surgery. Careful attention
in interpreting EEG findings in these patients is required (Smith, 2005).
The sensitivity of routine inter-ictal EEG can be increased by repeating the EEG and with longer recording. Baldin et al. (2014) reported up to 53% cumulative yield of epileptiform activity after the first EEG which increased to 72% after the third EEG. EEG is more sensitive if it is obtained within the first 24 hours after a seizure where the ability to detect epileptiform discharge increases from 34% to 51% (King et al., 1998). The yield of routine EEG can be further increased by applying certain seizure provocative measures such as photo-stimulation, hyperventilation and sleep deprivation (Benbadis, 2015).

Long term EEG, on the other hand, has a much higher sensitivity and specificity in diagnosing epilepsy as it increases the probability of capturing seizures. Long term EEG include three modalities: (i) continuous prolonged video EEG lasting from several hours to several days, (ii) 24-hours ambulatory EEG, (iii) sleep and sleep deprived EEG lasting for at least 1-3 hours (Michel et al., 2015, Fisch et al., 2016). In-patient long term-video EEG monitoring performed in a specialized epilepsy monitoring unit (EMU) is the best and the gold standard for diagnosis of seizure disorder, classification of seizure types and localization of the epileptogenic region of the brain for the possibility of surgical resection (Lagerlund et al., 1996).

The main advantage of long term-video EEG is that it is conducted in a relatively controlled environment which provides the optimal conditions that allow for AED reduction to encourage seizures. Application of seizure provocative measures is easy and controlled as well as it allows for close medical and psychological support for the patient (Benbadis, 2015). However, this procedure still has limitations such as the limited availability with long waiting lists, the high cost (as it requires 24-hour nursing services) and the need for hospital admission as is it not always convenient and feasible for patient and family (Benbadis, 2015).
1.4.2.b Role and future directions of neuroimaging in epilepsy diagnosis

Neuroimaging is frequently used as a mean to support epilepsy diagnosis. The main focus of MRI and computerized tomography scan (CT scan) in epilepsy patients is to confirm or rule out any underlying tissue abnormality or structural lesion. Neuroimaging should not be interpreted alone, but in combination with the clinical history and EEG findings in order to establish epilepsy and a correct epilepsy syndrome diagnosis. The main advantage of MRI is being safe, non-invasive, quantifiable and repeatable which allows monitoring of disease onset and progression (Sitoh and Tien, 1998). It is advised that all patients with epilepsy should undergo an MRI. Certain epilepsy syndromes, however, do not have any neuroimaging correlates such as primary generalized epilepsy, absence epilepsy and myoclonic epilepsy. The role of neuroimaging in those patients is only to rule out other differential diagnostic conditions. Other types of epilepsy, chronic TLE with HS for example, show a characteristic pattern of damage in the brain which can be detected with different neuroimaging techniques (Gaillard et al., 2009, Crocker et al., 2016). A review by the ILAE subcommittee for paediatric neuroimaging showed that nearly 50% of children with new onset focal seizure showed abnormalities on MRI. In these children, 15-20% of imaging studies provided useful information on aetiology and/or seizure focus. In 2-4% of children, neuroimaging provided useful information that led to an immediate change in medical management (Berg et al., 2000, Gaillard et al., 2009).

In adults, a potentially epileptogenic lesion is detected in about 23% of patients presenting after their first seizure. The most common lesion type in these patients was gliosis (49%). Other abnormalities were also reported including tumours (15%), cavernomas (9%) or mesial temporal sclerosis (9%) (Hakami et al., 2013). A more recent study reported useful MRI findings in about 44% of adult patients presenting with acute seizure. However, 56% of patients did not have any abnormal imaging findings (Olszewska and Costello, 2014).
It is not always clear whether a certain lesion discovered by MRI should be considered epileptogenic or coincidental. Likewise, a negative MRI may reflect the lack of sensitivity of this test. Certain brain lesions such as neoplasms, vascular malformations, gliosis and sclerosis were recognized as definitively epileptogenic but others still raise a dilemma in the setting of epilepsy diagnosis for being non-epileptogenic such as microvascular lesions in the white matter which can be found in about half of elderly people (Hakami et al., 2013, Crocker et al., 2016).

The yield of MRI findings also depends on the level of expertise of the reading radiologist. The sensitivity of non-expert reports for focal epileptogenic lesions was found to be only 39% and HS was missed in 86% of cases. This sensitivity increased to 50% with expert reviewer and to 91% with an epilepsy dedicated MRI (Von Oertzen et al., 2002).

Early imaging changes during the epileptogenesis process are still not fully known. The epileptogenic lesions in epilepsy patients can be very subtle and below the current limits of MRI resolution. Efforts to improve the detection of lesions by MRI are ongoing. Examples of these include the addition of contrast to MRI to better probe tissue microstructure, increase MRI field strength, and multimodal imaging approaches (Crocker et al., 2016).

In an attempt to validate new modalities of MRI as a biomarker for epileptogenesis, Choy et al. (2014) used a high magnetic field MRI in combination with long term-video EEG in a rat model of febrile SE as a potential predictive marker for TLE development. They found a reduction in MRI T2 relaxation time in the amygdala that was detectable only hours after the febrile SE. This reduction successfully predicted rats that were going to develop TLE in the following months. Comparing these findings to data using low field MRI showed contrasting results. The most common MRI abnormality reported in children (and in experimental animals) after febrile SE is an increase in T2 relaxation in the hippocampus which is detectable 2-5 days after the event. However, this late detectable MRI abnormality did not
successfully predict TLE development (VanLandingham et al., 1998, Scott et al., 2002, Dubé et al., 2004, Jansen et al., 2008).

Non-invasive assessment of certain metabolic parameters through imaging methods is now possible. The response of hippocampal glial cells to induced SE in the form of activation and the associated neuronal death was assessed in rats using 1H magnetic resonance spectroscopy (MRS) which allows measurement of metabolite content in the hippocampus (Filibian et al., 2012). A progressive increase in glutathione and myo-inositol concentration (metabolites linked to astroglial activation) during the epileptogenesis phase was noted. This increase was maintained in the chronic epileptic rats. Glutathione level during epileptogenesis was negatively correlated with the frequency of spontaneous seizures in the chronic epileptic phase suggesting that these and other studied metabolites can be used as predictive biomarkers for epilepsy development (Filibian et al., 2012).

The association between glucose metabolism and epileptogenesis after TBI or SE was assessed in a longitudinal study using serial MRI and positron emission tomography (PET) imaging over 6 months post injury (Guo et al., 2009). Abnormalities in glucose metabolism were reported in different brain regions. The largest decrease was found in the limbic structures during the early post injury phase with the hippocampus and thalamus displaying persistent hypo-metabolism throughout the epileptogenesis phase. However, the sensitivity and specificity of those changes remain to be established.

Other modalities of structural and functional brain imaging are available. However, these have not yet been assessed for their value as epilepsy and epileptogenesis biomarkers. An example of these techniques is the diffusion tensor imaging (Gross, 2011). These techniques might revolutionize the ability to detect microstructural and large scale network changes related to cell death, reactive gliosis, axonal remodelling etc. before the occurrence of the first seizure (Pitkanen et al., 2016).
Despite improvements in imaging studies, MRI alone cannot give a definite diagnosis of epilepsy. The data generated should be analysed in light of other parameters; mainly a positive history that is suggestive of epilepsy and suggestive EEG data (Engel, 2011b). Other functional neuroimaging techniques for example PET, MRS and single photon emission computed tomography (SPECT) are gaining more and more attention particularly for their pivotal role in the assessment of patients with drug-resistant focal epilepsy who are being considered for surgical treatment (Cendes et al., 2016). Therapeutic options in epilepsy

The main goal for epilepsy treatment is to control seizures with minimum side effects and to improve the patient’s quality of life. Current treatment options involve pharmacological and non-pharmacological approaches.

1.4.2.3 Pharmacological therapy using antiepileptic drugs

After the introduction of the first effective anti-seizure drug (potassium bromide) in 1850 there has been a steady increase in the number of newly discovered compounds with anti-seizure effects. The first modern AED, phenobarbital (PB), was discovered in the early 20th century and it is widely used to this day (Yasiry and Shorvon, 2012). The association of PB with sedation and CNS depression led to the discovery and introduction of phenytoin in 1937 which has better tolerability and clinical efficacy compared to PB and since then it has become a major first line AED (Yasiry and Shorvon, 2012, Brodie, 2010). The next few years saw an increase in the number of licenced AEDs with the introduction of ethosuximide in 1958, carbamazepine, benzodiazepines and valproic acid (VPA) in the 1960s.

The modern era of AED discovery began with the introduction of the anticonvulsant drug development programme in 1975 in the USA. Large numbers of chemical compounds were screened for the possibility of anticonvulsant effects based on target-oriented design, structural modification of already established AEDs and most importantly, the intensive utilization of animal models of seizures and epilepsy (Brodie, 2010). All of the mentioned compounds share a common ability to reduce
neuronal excitation and/or enhance the neuronal inhibition through different pharmacological processes (Brodie, 2010, Kwan and Brodie, 2007). Second generation antiepileptic drugs include vigabatrin, zonisamide, lamotrigine, felbamate and many others. The most recently introduced AEDs (third generation) include lacosamide, retigabine, eslicarbazepine, perampanel, rufinamide and brivaracetam (Patsalos and Berry, 2012, Mula, 2016). These anticonvulsants are currently the mainstays of seizure treatment as they have fewer toxic effects, good efficacy and better tolerability. The choice of the first line AED depends mainly on the diagnosis of epilepsy syndrome, age, sex and possibility of drug interactions if the patient is under treatment for other medical conditions.

Despite the unprecedented expansion in the number of AEDs, it is estimated that seizure control is only achieved in 50% of newly diagnosed epilepsy cases after the introduction of the first AED. A further 10% of patients achieve seizure control after the use of second or third AED (Kwan and Brodie, 2000). In some patients, epilepsy is initially controlled but then gradually becomes refractory; this pattern is mainly seen in childhood epilepsy or in patients with HS (Berg et al., 2006).

At least 25-40% of patients will continue to have seizures despite optimum pharmacological treatment (Wilcox et al., 2013, Brodie et al., 2011). The main reasons for this high figure of drug resistance is either misdiagnosis of epilepsy, misdiagnosis of epilepsy type leading to inappropriate drug selection, wrong dose (too low) or poor compliance of the patient (Kwan et al., 2011a). Other factors that can contribute to the incidence of drug resistant epilepsy is the inadequate penetration of the drug across the BBB, acquired modification of the structure and/or function of the ion channel and neurotransmitter receptor targeted by the AED and an inherent resistance due to certain genetic variants of proteins involved in AED kinetics (Löscher and Sills, 2007, Schmidt and Löscher, 2005). However, de novo drug resistance is also very common where patients with newly diagnosed epilepsy for whom the first drug was ineffective had only an 11% probability of future success (Kwan and Brodie, 2000, Schmidt and Loscher, 2005). The main focus
for any new drug therapy should be directed toward preventing, delaying or modifying epilepsy rather than only preventing seizure occurrence. This will require more in-depth research and understanding of the pathophysiology of epilepsy (Bialer and White, 2010).

1.4.2.d Non-pharmacological treatment of epilepsy

Other treatment options with well-established safety and efficacy might help in controlling seizures in drug refractory epilepsy. Among those are surgical resection of the epileptic focus, vagal nerve stimulation (VNS) and ketogenic diet therapies.

The value of surgical treatment has been proven viable especially for patients with lesional epilepsy identified through imaging and/or electrophysiological studies. Nowadays, removal of the epileptic focus is regarded as the method of choice to treat medically intractable epilepsy, particularly refractory TLE (Wiebe et al., 2001, Bell et al., 2009). A prospective randomized-controlled study comparing surgical versus medical management clearly demonstrated a superior effect of surgery over medical treatment with more than 70% of patients were seizure free within the first 2-3 years after surgery (Thom et al., 2010). Surgery also significantly improved patient’s quality of life and socialization (Wiebe et al., 2001, Engel et al., 2012).

All published surgical series reported no incidence of mortality after temporal lobectomy (Georgiadis et al., 2013). However, other cognitive, neuropsychological and psychiatric complication has been reported in some cases (Gleissner et al., 2002, Shaw et al., 2004, Malmgren et al., 2002). Seizures recurrence was reported in approximately 30% of patients which could be due to inadequate resection of the epileptogenic focus or the presence of secondary lesions outside the resected area (Wieser et al., 2003, McIntosh et al., 2004). To date, there is a significant lack of robust predictive criteria for the surgical outcome. An early clinical or molecular biomarker that will predict surgical success rate and the possibility of seizure recurrence and other post-operative complication will help to determine
postoperative clinical management especially in terms of when to stop the AEDs (Jehi, 2013).

Another effective intervention for pharmaco-resistant patients is electrical brain stimulation through the vagus nerve. VNS is a safe and effective alternative to epilepsy surgery for adult and paediatric population over 12 years old (Connor et al., 2012). Applying intermittent electrical stimulation to the cervical vagus nerve is thought to cause desynchronization of the cerebral cortical activity leading to attenuation in seizure activity. Greater than 50% reduction in seizure frequency was reported in approximately 50% of patients. However, fewer than 10% of patients achieved seizure freedom with VNS therapy (Kuba et al., 2009, Ghaemi et al., 2010).

Another valuable option, especially in the treatment of childhood epilepsy, is the ketogenic diet. It has been estimated that 32% of patients had a greater than 90% reduction in seizure frequency after 6 months of starting the diet. Parents also reported an improvement in their child’s behaviour and attention (Freeman et al., 1998). The diet is thought to work by altering brain metabolism, although the exact mechanism remains incompletely understood (de Lima et al., 2014).

1.4.3 Epilepsy misdiagnosis and the need for epilepsy biomarkers

Misdiagnosis of epilepsy is an increasingly important issue; the incomplete or sometimes vague history from the patient and eyewitnesses, as well as the lack of sensitive and specific diagnostic tests for epilepsy (including EEG) can make it difficult to prove the accuracy of diagnosis. A population based study conducted in adults with primary diagnosis of epilepsy found (following a specialist review and investigations) a misdiagnosis rate of 23% (Scheepers et al., 1998). Among patients with refractory epilepsy, the misdiagnosis rate was reported to be 26.1% (Smith et al., 1999). In children, the rate of misdiagnosis is even higher due to the prevalence of other paroxysmal events. Hindley et al. (2006) found that epilepsy was the final diagnosis in only 23% of children referred to secondary care clinic.
The commonest condition misdiagnosed as epilepsy in both adults and children was syncope (42%). A motor phenomenon (brief muscle jerk or tonic phase) is extremely common and might be mistakenly diagnosed as convulsive activity (Lempert et al., 1994). Other relatively common conditions were PNEAS (8%), day dreaming (5%), night terrors (4%), migraine (3%), ritualistic movement (2%), and parental anxiety/fabricated illness (2%).

According to Smith et al. (1999), the main causes of epilepsy misdiagnosis were the failure to obtain a complete recall of the event from the patient and misinterpretation of the EEG. Others attribute the high misdiagnosis rate to the extensive list of differential diagnoses, especially in children. UK’s National Clinical Guideline (2012) lists up to 36 differential diagnoses for seizures in children. The clinician should pay particular attention to criteria that can distinguish between them. The lack of a sensitive and specific test to diagnose epilepsy, false perception about the risk of missing epilepsy and fear from the consequences of delaying treatment such as SUDEP further complicates the problem and may put pressure on clinicians for diagnosis (Smith et al., 1999).

A false positive epilepsy diagnosis may have severe psychological and socioeconomic consequences for the patient and economic implications on health and welfare services. The annual cost of such misdiagnosis in England and Wales alone was estimated to be about £29,000,000 (Juarez-Garcia et al., 2006). Most of the patients misdiagnosed as having epilepsy received AEDs. Patients with PNEAS are often exposed to dual or polytherapy for prolonged periods with drug side effects and symptoms of dose-related neurotoxicity (diplopia, ataxia, nausea and sedation). These patients lost their opportunity to receive appropriate treatment for their condition (Juarez-Garcia et al., 2006). Other negative social implications may greatly affect patient life such as loss of jobs and loss of driving licence (Smith et al., 1999).
All the above factors, in addition to the highly unpredictable course of the disease and timing of individual seizures reveals the urgent need to develop a biomarker for epilepsy diagnosis, prognosis and treatment response (Pitkanen et al., 2016).

A biomarker is a characteristic that can be objectively measured and evaluated as an indicator of a normal biological process, pathological process or pharmacological response to a therapeutic intervention. Clinically, biomarkers are used as predictive (risk indicators or treatment response), diagnostic (disease identification) and prognostic (disease outcome) tools (Biomarkers Definitions Working group, 2001, Strimbu and Tavel, 2010). In regards to epilepsy, both epileptogenesis biomarkers and epilepsy biomarkers are equally important for optimizing the care of epilepsy patients. A molecular biomarker that can be measured in biological fluids (blood and CSF) would be transformational in being more readily available and easily accessible when compared to other structural (MRI) or functional (EEG) biomarkers (Pitkanen et al., 2016). Consequently, identification of molecular biomarkers that can help to guide diagnosis and treatment of epilepsy has been at the centre of research efforts in the past decade. Past attempts to identify molecular biomarker candidates have focused on changes in blood concentration of neuroendocrine hormones, markers of CNS injury, inflammation etc., but no clinically accepted biomarker have been found (Hedge and Lowenstein, 2014).

1.4.3.a Epileptogenesis biomarker

Currently, there are no biomarkers that can be used to measure various aspects of epileptogenesis. Among various benefits, an epileptogenesis biomarker would help in identifying people at risk of developing epilepsy after an initial brain injury, as well reliably uncover the presence of an epileptogenic process before the first unprovoked seizure and validate the usefulness of any anti-epileptogenic intervention. Furthermore, it has the potential to provide insights into the underlying pathological mechanisms of epileptogenesis which ultimately help in identifying new anti-epileptogenic therapies (Pitkanen et al., 2016, Henshall et al., 2016).
A key issue for any molecular biomarker of epileptogenesis is that the molecule may change as a function of time during the process of epilepsy development. It is critical to determine when exactly this biomarker appears, and how long it persists. A biomarker that can successfully predict epilepsy a few hours after a brain insult might fail to do so if measured after a few days (Engel et al., 2013). However, if such biomarkers were discovered they could provide a means to successfully identifying the epileptogenesis process and permit its staging into different categories depending on the severity.

1.4.3.b Epilepsy biomarkers

The benefit of an ideal diagnostic epilepsy biomarker is mainly in providing a definitive diagnosis and differentiating epilepsy from other types of seizure due to other medical conditions so that appropriate treatment can be started immediately. Moreover, it can provide information about the extent and localization of the epileptogenic area (Engel, 2011a, Engel, 2011b). The relation of biomarker level to seizure is very important. It is critical to know how the biomarker behaves in the pre-ictal, ictal, post-ictal and inter-ictal stages. Equally important, is the relation of biomarker to physiological states such as sleep-wake cycles, age and sex and how the biomarker behaves in response to certain seizure provocative states such as sleep deprivation (Engel et al., 2013).

Prognostic biomarkers are as well of potentially high clinical importance. They have the potential to provide information about the progression or severity of epilepsy as well as may predict remission or provide an evidence of remission. A reliable biomarker might help in predicting the occurrence of cognitive, psychiatric and behavioural comorbidities or even predict the possibility of SUDEP (Pitkanen et al., 2016, Pitkänen and Engel, 2014, Henshall et al., 2016).

Epilepsy biomarkers will have a great potential in deciding the direction of clinical management of epilepsy patients. They may aid in the early identification of pharmaco-resistant cases, in individualizing and optimizing epilepsy treatment.
They can help in deciding the best treatment choice and after that, in assessing the
efficacy of treatment. So far, the approach in epilepsy treatment, despite the large
number of AEDs, remains trial-and-error with the only way to assess the efficacy of
the drug being to wait for another seizure to occur which can take weeks or months
(Engel, 2011b, Engel et al., 2013).

Furthermore, identifying a reliable epilepsy and epileptogenesis biomarker will
greatly enhance the power of antiepileptogenic and antiseizure clinical trials which
is extremely difficult now due to the heterogeneous nature of the epileptogenesis
process and of epilepsy itself (Pitkanen et al., 2016, Pitkänen and Engel, 2014).

1.4.4 New insights in epilepsy pathogenesis and their role in biomarker
discovery

There is growing interest in understanding the processes which orchestrate large
scale deregulation of gene expression in epilepsy. It is well known that certain genes
can be turned “on” or “off” in response to different environmental and/or
pathological stimuli. Gene expression can be regulated at different points and by
different mechanisms. Among them are biochemical modification of both
chromatin/DNA structure (epigenetic regulation) and RNA structure
(epitranscriptomic regulation) as well as post-transcriptional gene regulation and
translation repression (Kumar et al., 2006, Holoch and Moazed, 2015).

Epigenetics typically refers to the processes that influence gene transcription
through altering the chromatin state without changing the DNA nucleotide
sequence. This includes DNA methylation and histone modifications. RNA
interference (RNAi) through non-coding RNAs is also commonly included (Graff et
al., 2011, Holoch and Moazed, 2015).

With increasing research addressing the epigenetic mechanisms in epilepsy, the
awareness that dysregulation of these mechanisms play a pivotal role in the disease
is also increasing (Henshall and Kobow, 2015, Sweatt, 2013). Approaches that allow
DNA methylation refers to the covalent addition of a methyl moiety to the cytosine base of DNA generating 5-methylcytosine (5-mc). This methylation is associated with a gene silencing effect mediated either directly through inhibiting DNA binding to transcription factors (Iguchi-Ariga and Schaffner, 1989) or indirectly through recruiting methyl-binding proteins with transcriptional repression domains (Nan et al., 1998). Genome-wide changes in DNA methylation has been reported in animal models of epilepsy after induction of SE. More than 300 genes showed an alteration in DNA methylation, where the majority of altered genes underwent hypomethylation of its promoter allowing for increased gene transcription (Miller-Delaney et al., 2012). On the other hand, hypermethylation of genes was the predominant finding during epileptogenesis and in chronic epilepsy models and it was inversely correlated with gene expression (Kobow et al., 2013, Williams-Karnesky et al., 2013).

To look for convergence of DNA methylation among brain insults known to trigger epilepsy, the hippocampi of three different epilepsy models (focal SE, systemic SE and TBI) were analysed (Dębski et al., 2016). Remarkably, the authors were unable to identify a methylation event common to the three models. Only a few altered DNA methylation regions were found common to any two models. Nevertheless, alteration in DNA methylation clearly differentiated control from injured animals with a characteristic pattern of hypermethylation in gene areas and hypomethylation in non-gene areas (Dębski et al., 2016). These findings suggest that DNA methylation is broadly reflective of an epileptogenic injury but specific insults modulate specific DNA loci indicating model specific epileptogenic mechanisms.

DNA methylation is also altered in human TLE. A total of 146 protein-coding genes exhibited an altered DNA methylation state in human TLE hippocampal tissue when compared to controls with a predominant tendency toward promoter
hypermethylation (Miller-Delaney et al., 2015). Most of those genes were associated with neuronal, neurotransmitter/synaptic transmission, cell death and transcriptional regulation functions.

The second major epigenetic process investigated in epilepsy is the modification of histone proteins. Histones are a fundamental component of the nucleosome, the building block of chromatin. Modification of histones in the form of acetylation, methylation, phosphorylation and ubiquitination are commonly observed in epilepsy (Graff et al., 2011). Of all the histone modifications, methylation and acetylation are well-studied and have central epigenetic roles in the regulation of gene transcription (Chen et al., 2014). The effect on gene expression in the form of silencing or induction generally depends on the nature of modification; acetylation for example is typically associated with gene induction as it promotes a state of euchromatin. In contrast, deacetylation of histones by histone deacetylases contributes to transcriptional silencing and gene suppression (Chen et al., 2014). Methylation, however, has a dual role. It can either promote or repress transcription depending on the site and number of methylation events (Graff et al., 2011, Jakovcevski and Akbarian, 2012).

Rapid and reversible acetylation and phosphorylation of histones was reported early after induced status epilepticus in different epilepsy models (Huang et al., 2002, Tsankova et al., 2004, Crosio et al., 2003). Interestingly, these modifications were regionally and temporally specific in relation to seizure. Phosphorylation was restricted to the dentate granule cells whereas acetylation appeared later and spread throughout the hippocampus (Sng et al., 2006).

Attempts to explore the effect of histone deacetylase (HDAC) inhibitors in epilepsy gave further insight into the function of histone modifications as potential therapeutic targets in epilepsy. The administration of an HDAC inhibitor, trichostatin A, increased basal acetylation and gene expression (Sng et al., 2005) but it had only a minimal anticonvulsant effect (Hoffmann et al., 2008). The well-established antiepileptic drug, VPA, was found to have HDAC inhibitory activity
(Göttlicher et al., 2001). However, its antiseizure activity was deemed unlikely to arise through HDAC inhibition (Hoffmann et al., 2008). Despite having no anticonvulsant effects, HDAC inhibitors might retain use in modulating the progression of epilepsy through neuroprotection and attenuation of neuronal death (Rossetti et al., 2012) and reduction in aberrant neurogenesis (Jessberger et al., 2007).

Assessment of epigenetic markers (markers for DNA methylation and histone modification) in the peripheral blood of epilepsy patient provides an opportunity to develop a biomarker for treatment monitoring, prediction of response & prognosis. Only few reports were found investigating the peripheral blood methylation state in epilepsy patients. Ni et al. (2015) compared the level of DNA methylation between TLE patients and healthy controls and between epilepsy patients treated by different AEDs. Results demonstrated an alteration in the level of methylation of methylenetetrahydrofolate reductase (MTHFR) amplicon between AED-treated patients and controls. A positive correlation between serum folate levels and peripheral blood MTHFR amplicon methylation status was also observed.

In another study, prenatal exposure to lamotrigine was associated with differences in the methylation patterns of cord blood DNA (Smith et al., 2012). Moreover, Tremolizzo et al. (2012) investigated the effect of VPA administration at concentrations relevant for the treatment of epilepsy on the epigenome of peripheral blood mononuclear cells (PBMC). A significant increase in histone acetylation and a correlated decrease of global DNA methylation were shown at therapeutically relevant plasma concentrations of VPA. This effect was drug-specific, since it was not demonstrated in PBMC obtained from PB-treated patients. Moreover, a VPA dose–response curve was performed on PBMC obtained from healthy controls, demonstrating an increase of acetyl-histone H3 content, suggesting the beneficial role of these epigenetic properties to monitor treatment and predict response in epilepsy patients (Tremolizzo et al., 2012).
RNAi is another important paradigm for understanding gene regulation at both transcriptional and post-transcriptional levels. Within the nucleus, RNAi pathways can repress target genes by guiding other epigenetic chromatin modifications (Castel and Martienssen, 2013, Holoch and Moazed, 2015). In the cytoplasm, post-transcriptional regulation of genes is mediated through specific classes of non-coding RNA molecules particularly microRNAs (Table 1.1). These are short RNA molecules that inhibit mRNA translation or cause mRNA degradation (Castel and Martienssen, 2013). Their role in proliferation, differentiation and function of neuronal cells has been demonstrated in several experimental models. In recent years, large numbers of studies have established the pivotal role of microRNAs in the pathophysiology of epilepsy. MicroRNA research represents an exciting field for the discovery of both epilepsy biomarkers as well as epilepsy therapeutics.

1.5 MicroRNAs

MicroRNAs are a family of endogenous, naturally occurring non-coding RNAs, about 22 nucleotide (nt) in length. Their main function is post-transcriptional gene regulation in a sequence specific manner. About 60% of protein coding genes have been predicted to be under direct microRNA regulation (Friedman et al., 2009) and each individual microRNA can have dozens of targets. The combined action of large numbers of microRNAs in each cell will dramatically affect the rate of utilization and translation of mRNAs in that cell, thus determining different cellular phenotypes.

The most current database (miRBase 21, http://www.mirbase.org) lists 2588 unique mature microRNAs in humans. Around 50% of all identified microRNAs are expressed in the brain (Krichevsky et al., 2003). Analysis of cell type specific and brain region specific microRNA profiling allowed the identification of individual microRNAs that are deemed neuronal, astrocytic or microglial in origin as well as the identification of differential enrichment of microRNAs in different brain regions (Jovicic et al., 2013, Bak et al., 2008).
Table 1.1: The major, conserved RNA silencing pathways, which are involved in the cellular control of gene expression in eukaryotes

<table>
<thead>
<tr>
<th>Small RNA</th>
<th>Abbreviation</th>
<th>Size (nucleotide)</th>
<th>mechanism of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>MicroRNA</td>
<td>miRNA</td>
<td>18-22</td>
<td>post transcriptional gene regulation</td>
</tr>
<tr>
<td>Piwi-interacting RNA</td>
<td>piRNA</td>
<td>24-31</td>
<td>post transcriptional gene regulation</td>
</tr>
<tr>
<td>Small interfering RNA</td>
<td>siRNA</td>
<td>21-25</td>
<td>post transcriptional gene regulation</td>
</tr>
</tbody>
</table>

The three major regulatory small RNA silencing pathways identified and conserved in eukaryotes (reviewed in (Moazed, 2009))
1.5.1 MicroRNA discovery

The first report about animal microRNA dates back to 1993. Two experiments conducted at approximately the same time by Lee et al. (1993) and Wightman et al. (1993) led to the discovery of the first microRNA in Caenorhabditis elegans (C. elegans). The initial finding was that the lin-4 gene that was known to control C. elegans development does not code for a protein. Instead it codes for a small RNA molecule that has a very striking feature in having two different forms. A large (61 nt) RNA which has the ability to fold forming a stem loop (hairpin) structure, and a small (22 nt) RNA which is originally part of the larger transcript. The smaller transcript was then found to be complementary to multiple sites in the 3’ untranslated region (3’UTR) of the target mRNA lin-14 (Lee et al., 1993, Wightman et al., 1993). This sequence complementarity between lin-4 and lin-14 was later found to substantially reduce the amount of LIN-14 protein without a noticeable change in the level of the lin-14 mRNA (Wightman et al., 1993).

Several years later, in 2000, microRNA with similar characters, Let-7, was discovered in C. elegans from a study of developmental timing (Reinhart et al., 2000). The discovery of homologue Let-7 microRNA in frog, flies, mice and human was regarded as an important step forward. The sequences of all those small let-7 RNAs proved to be surprisingly similar indicating that microRNA mediated gene regulation might be more ancient and common than was previously thought (Lagos-Quintana et al., 2001, Lau et al., 2001, Lee and Ambros, 2001). Since that time, enormous effort has developed a field of study on microRNAs which changed the way we think about genes and how they are regulated inside the body.

1.5.2 Definition of microRNAs

A classic definition of microRNAs was implemented upon their discovery to differentiate them from other forms of small RNAs. This definition proposed that candidate microRNA should fulfil both expression and biogenesis characteristics to be annotated as a proper microRNA (Figure 1.3).
First: expression criteria, involve the detection of the mature 22 nt microRNA using different microRNA expression techniques such as RNA (northern) blot analysis or cloning from size fractionated small RNA libraries. Second: biogenesis criteria where the mature microRNA should originate from a precursor with a hairpin secondary structure where the mature microRNA occupies the stem part of the hairpin. In animals, this hairpin structure is usually about 60-80 nt in length. Third: Phylogenetic conservation of the mature microRNA-sequence and its hairpin primary structure is a strong and favourable point. Fourth: the mature microRNA should be processed by Dicer (Ambros et al., 2003) (Figure 1.3).

According to Ambros et al. (2003) who proposed the first system for microRNA classification and nomenclature, a mature microRNA-sequence would have a strong annotation when it’s 22 nt product is expressed in vivo, phylogenetically conserved and it shows a Dicer dependent biogenesis. However, if not all data about a microRNA are available, in vivo expression and phylogenetic conservation (of the microRNA and/or its precursor hairpin structure) is enough to consider this 22 nt sequence as a mature microRNA (Figure 1.3).

The confidence in this annotation system was challenged by many researchers who believed that the miRBase repository contains large numbers of false positive data generated by degradation and fragmentation of larger RNAs (Schopman et al., 2010). Next Generation Sequencing (NGS) was used to interrogate the accuracy of microRNA registry and less than one third of the human miRBase entries (only 523) were supported as bona fide microRNA genes, which highlights the need for a more strict criteria for annotating microRNAs (Hansen et al., 2011, Fromm et al.,
Figure 1.3: Basic structure of mature microRNA and its precursor molecules.

A short 22 nt length RNA molecule can be annotated as microRNA after fulfilling both expression and biogenesis criteria. The mature microRNA should originate from a Dicer dependent cleavage of a precursor molecule with a hairpin secondary structure where the mature microRNA occupies the stem part of the hairpin.
Due to the availability of data from an increased number of NGS studies, Fromm et al. (2015) and Hansen et al. (2011) proposed a new set of criteria for microRNA annotation. Annotation of any microRNA-sequence requires evidence of mature microRNA expression accompanied with the expression of two (20-26 nt long) reads from each of the two arms of the hairpin precursor with evidence of Dicer biogenesis (2 nt offset between the 5’ and 3’ arms).

Fromm et al. (2015) further highlighted the importance of phylogenetic conservation of both the mature and the precursor sequence. 340 human precursor microRNA-sequences were conserved between human and mice. All of these sequences show 5’ homogeneity and have the 2nt offset between the precursor arms. The median read length of hairpin precursor arms is 22 nt (varies between 20-60). At least 16 complimentary bases should be present between the two arms of the hairpin, with the median loop length being 15 nt (range of 8-38). These criteria of the precursor microRNA-sequence show an interesting conservation among other species such as chicken, zebrafish, fruit fly and nematode worms. The only variability was in loop length which could vary between species (Fromm et al., 2015). Using this definition, nearly two thirds of the miRBase entries was regarded as false positives.

However, by concentrating on the combined expression of both arms of the precursor microRNA arms as the main criteria for defining a microRNA, we might be unintentionally excluding a set of valid microRNAs that are processed by unusual (non-Dicer dependent) biogenesis pathways, for example miR-451 (Cifuentes et al., 2010). Low sequencing depth and rapid degradation of the other parts of the hairpin structure can also be causes of failure to sequence the whole precursor hairpin structure (Hansen et al., 2011).
1.5.3 MicroRNAs biogenesis and function

1.5.3.a MicroRNA genes

MicroRNA biogenesis starts in the nucleus with the microRNA “gene” (Figure 1.3 and 1.4). Those genes represent one of the most abundant and most conserved classes of genes with about 196 microRNA families being conserved between mammals (Chiang et al., 2010). The majority of microRNAs have dedicated genetic loci for their expression, about 50% of microRNA genes reside within the intergenic region, others (40%) are within introns of coding and non-coding genes or within exons of coding genes (10%) (Lin and Gregory, 2015, Kim et al., 2009, Lagos-Quintana et al., 2001). The intergenic localization of microRNA genes indicates an autonomous expression where microRNA genes have their own enhancer and promotor elements while the intronic and exonic microRNA genes are usually transcribed with their host gene (Baskerville and Bartel, 2005).

Several microRNA genes (approximately 50%) exist as clusters of 2-7 genes separated with short intervals of 0.1-50 kilobases (kb) from each other. Those genes display a similar expression pattern which suggested that their transcription is derived by a single promoter and it is under tight control by specific transcription factors and posttranslational chromatin modification (Lagos-Quintana et al., 2001, Baskerville and Bartel, 2005).

1.5.3.b Primary microRNA (pri-microRNA)

Most microRNA genes are transcribed by RNA Polymerase II enzyme (RNA Pol II) to generate a several kb long, 5’ capped and polyadenylated stem loop structure called the primary microRNA (Figure 1.3 and 1.4) (Cai et al., 2004, Lee et al., 2004, Lim et al., 2003). Each individual pri- microRNA can either produce a single microRNA or contain clusters of two or more microRNAs that are processed from a common primary transcript.
Pri-microRNA processing is a critical step in microRNA biogenesis as it defines the microRNA-sequence embedded within the long pri-microRNA by generating one end of the mature microRNA molecule (Han et al., 2006). The pri-microRNA will be recognized and cleaved at the stem of the hairpin structure by a multiprotein complex called the microprocessor. The core components of this complex are an RNAase III enzyme (Drosha) and the double-stranded RNA binding protein DiGeorge syndrome critical region 8 (DGCR8) (Gregory et al., 2004, Denli et al., 2004). Both components of the complex are necessary for the pri-microRNA processing. Drosha alone will have non-specific RNAase activity. DGCR8 acts to inhibit non-specific RNAase effects and promote Drosha’s pri-microRNA processing. In vivo and in vitro knock down of either of those molecules or both of them resulted in diminished mature microRNA expression and accumulation of the pri-microRNA (Gregory et al., 2004).

The recognition of pri-microRNA by the microprocessor complex is based on its specific structural and specific nt. sequence features. The typical structure of the pri-microRNA comprises an imperfectly paired, double stranded stem of about 33 base pairs, a terminal loop and long flanking single stranded RNA-sequences (ssRNA) on each side of the stem (Han et al., 2006, Kim et al., 2009). Both the terminal loop and the flanking sequence of pri-microRNA were proposed to be critical for cleavage site recognition. DGCR8 will interact with the ssRNA and with the stem sequences and assist Drosha to cleave the structure 11 base pairs (bp) away from the ssRNA-stem junction (Zeng et al., 2005, Lee et al., 2003).

Han et al. (2006) proposed another model based on evidence from computational and biochemical analysis. Based on this model, the pri-microRNA consists of an imperfect stem structure of about three helical turns. They found that Drosha cleaves the RNA molecule about 2 helical turns away from the terminal loop and about one helical turn away from the start of the flanking sequence. More specifically, the microprocessor will measure the distance (about 11 bp) from the junction between the flanking sequence and the stem, which represents the most
Figure 1.4: MicroRNA biogenesis and different forms of Extracellular microRNAs.

MicroRNA genes are transcribed by RNA Pol II to generate the pri-microRNA which will be recognized and cleaved by the microprocessor complex (Drosha/ DGCR8) to form the pre-microRNA. The pre-microRNA will be then rapidly transported into the cytoplasm where it will be subjected to a second processing step by Dicer followed by uploading of the mature microRNA sequence into either RISC complex or exosomes and microvesicles.
thermodynamically unstable position in the pri-microRNA-sequence (Han et al., 2006). However, recent evidence on viral pri-microRNAs showed that neither model alone is sufficient to determine the precise Drosha cleavage site and both the basal ssRNA-sequences (flanking sequences) and the terminal loop are able to influence cleavage site (Burke et al., 2014).

1.5.3.c Precursor microRNA (pre-microRNA)

The microprocessor complex cleaves the pri-microRNA, producing about 60-70 nt hairpin shaped intermediate known as pre-microRNA which bears the 2nt overhang in the 3’ end characteristic for RNAase III mediated cleavage (Denli et al., 2004, Lee et al., 2003). This protruding end will be recognized efficiently by Exportin-5 and later on by Dicer.

Exportin-5 then rapidly transports the pre-microRNA into the cytoplasm. The affinity of Exportin-5 for pre-microRNAs is greatly enhanced by Ran guanosine triphosphate (ran-GTP), and depletion of ran-GTP leads to a significant reduction in pre-microRNA export (Lund et al., 2004, Yi et al., 2003). In vitro analysis of the export mechanism showed that more than 90% of the pre-microRNA was efficiently transported to the cytoplasm within 30 minutes (Lund et al., 2004).

Once in the cytoplasm, the pre-microRNA is subjected to a second processing step by Dicer (cytoplasmic RNAase III enzyme), which cuts the pre-microRNA near the terminal loop to produce an imperfect microRNA: microRNA* duplex. Dicer is a large 220 kDa protein that is conserved in all eukaryotic cells. It contains multiple domains such as an RNA helicase domain, ATPase domain, PAZ domain, two RNAase III-like domains and a double stranded RNA binding domain (dsRBD). The binding of Dicer to dsRNAs and its role in cleaving them depends mainly on the dsRBD and the RNAase III-like domain (Zhang et al., 2002). The PAZ domain, on the other hand, recognizes and anchors the 2nt. 3’ overhang end of the pre-microRNA and this will facilitate positioning of dsRNA stem to the catalytic centre of Dicer (MacRae et al.,
Dicer will then measure a specific distance from the 3’ end of the dsRNA (about 22 nt.) and perform the cleavage (Zhang et al., 2002).

The rate of pre-microRNA cleavage by Dicer is enhanced by specific Dicer partners which are the human trans-activation response RNA binding protein (TRBP) and protein activator of the interferon-induced protein kinase (PACT). TRBP enhances pre-microRNA binding to Dicer and facilitates product release after cleavage (Chendrimada et al., 2005). In an in vitro TRBP knock out model, although Dicer stability was not affected, Dicer cleavage was compromised leading to generation of truncated iso-miRs (Lee and Doudna, 2012). MicroRNA abundance and Argonaute protein loading was also altered (Kim et al., 2014). TRBP was found to aid in the later formation of RNA induced silencing complex (RISC) by bridging Dicer with Argonaute proteins (Chendrimada et al., 2005). PACT is another ds-binding protein that is important for the function of RISC. The depletion of PACT by RNAi resulted in a reduction in mature microRNA production without accumulation of pre-microRNAs (Lee et al., 2006).

Interestingly, Dicer has the ability to distinguish different pre-microRNAs and process them at substantially variable rates. In an in vitro experiment, Lee and Doudna (2012) found more than a five-fold difference in the rate of Dicer processing of pre-miR-21 and pre-let-7a. The difference was more pronounced in the presence of TRBP (up to 11-fold change). This difference was mainly attributed to structural elements such as cleavage site structure, helical stem length and loop size (Lee and Doudna, 2012).

Whereas Drosha levels were normal in the hippocampus of TLE patients and in mice after status epilepticus (McKiernan et al., 2012a), a reduction in Dicer protein levels was reported in resected sclerotic hippocampal tissue from refractory TLE as well as in mice with chronic epilepsy. In human samples, this was accompanied by large scale reduction in mature microRNA expression (McKiernan et al., 2012a). In the same study, an analysis of Dicer levels in the adjacent neocortex lacking the characteristic pathological lesions and in hippocampal tissue from mice subjected to
non-harmful seizures, revealed normal Dicer levels and normal microRNA expression. This suggests that loss of Dicer function might be a characteristic pathophysiological feature of TLE with HS (McKiernan et al., 2012a).

1.5.3.d Assembly of RNA Induced Silencing Complex

Following Dicer cleavage, the dsRNA stem is loaded to the RISC and this guides the complex to a complementary sequence in the target mRNA. The microRNA strand with relatively lower thermodynamic stability of base pairing at its 5’ end will be incorporated into the RISC and function as a guide strand, whereas the microRNA* (passenger) strand is typically degraded (Schwarz et al., 2003). Once incorporated into RISC, the microRNA guides the complex to its target mRNA by base-pairing interaction (Du and Zamore, 2005).

The process of RISC assembly can be divided into two successive steps. The first being RISC loading, and the second being strand dissociation or unwinding. In humans, the process of RISC loading involves both Dicer and TRBP which together, along with Argonaute2 protein (Ago-2), comprises the core component of the RISC (Gregory et al., 2005). The role of the Ago-2 protein is to position the microRNA in a conformation that will facilitate target recognition (Pratt and MacRae, 2009). The 3’ end of the guide microRNA strand will fit into a pocket in the Ago PAZ domain, while the 5’ end will sit within a pocket of the Mid domain. This specific loading is essential for the overall functional efficacy of the RISC complex (Wang et al., 2008).

The 3D shape reconstitution of RISC loading complex showed that the Ago PAZ domain is placed in close proximity above TRBP and Dicer PAZ domain, thus making it possible, at least theoretically, for those domains to bind the opposite ends of the guide strand at the same time. With the help of TRBP, the microRNA duplex will be released from Dicer and loaded completely to Ago (Wang et al., 2009a). This transfer of microRNA to Ago protein was shown to be mediated by the multiprotein chaperon complex, heat shock protein 90 (HSP-90) which helps in keeping Ago proteins in an open state to accommodate the microRNA duplex (Johnston et al.,
In the next step of the process, the microRNA duplex is unwound; the passenger strand is removed with the support of Ago protein and active an RISC complex is now ready to bind with target RNAs (Chandradoss et al., 2015).

In human TLE resections, Ago-2 levels were reported to be higher both in the hippocampus and neocortex (McKiernan et al., 2012a). This might be an adaptive measure to maintain microRNA biogenesis after reduction in Dicer function. However, these findings were not observed in animal models of epilepsy where the Ago-2 level was reported to be normal even after prolonged seizures (Jimenez-Mateos et al., 2011, McKiernan et al., 2012a).

1.5.3.e MicroRNA effects on Post-Transcriptional Gene expression

In animals, base pairing between microRNA and their target mRNA usually leads to a change in protein levels through a variety of mechanisms that involve mRNA degradation or destabilization, translational repression or even sometimes translational activation (Vasudevan et al., 2007). Imperfect base pairing of mature microRNA within the RISC to the 3’ UTR of the target sequence can result in mRNA degradation through recruitment of a deadenylase complex that removes the poly(A) tail (Braun et al., 2011, Fabian et al., 2011). This mechanism of regulation is thought to account for most of the repression mediated by microRNAs in mammalian cells (Huntzinger and Izaurralde, 2011).

Other mechanisms involved in mRNA regulation include translational repression through either RISC mediated inhibition of translation initiation by interfering with Cap recognition step (Humphreys et al., 2005, Pillai et al., 2005) or through inhibiting a vital step in the translation process such as promoting ribosome drop-off or stimulating proteolysis of the newly formed peptide (Nottrott et al., 2006).

In contrast to the above general assumption, translational activation and microRNA-mediated upregulation of target transcripts have been reported. More interestingly the effect of a single microRNA might oscillate between repression and stimulation in response to specific cellular conditions (Vasudevan et al., 2007).
1.5.3.f MicroRNA target recognition

Accurate target recognition requires base pairing of nt. 2-8 of the 5’ end of the microRNA (seed region) with a specific complementary sequence, typically within the 3’ UTR of the target mRNA (Bartel, 2009). The importance of seed region for target recognition was established early through extensive comparative, genomic and experimental studies (Lee et al., 1993, Lewis et al., 2003, Doench et al., 2003). Although the 5’ location of microRNA seed region is the most common rule, other seed region sites were also identified such as centred sites and 3’ sites (Pasquinelli, 2012). This adds more complexity to the concept of how microRNAs recognise a specific sequence in their target mRNA. Other important factors in increasing microRNA target specificity is the number and position of the target site within the 3’ UTR as well as the A/U richness of those sites (Bartel, 2009). Effective target sequences preferentially reside near both ends of the 3’UTR but not within the first 15 nt downstream from the translational stop codon which is usually occupied by the ribosome and thus not recognized by microRNAs. Moreover, multiple closely spaced sites often have a synergetic effect on mRNA regulation. Sites highly enriched for A and U content were considered to be more functional relative to the non-functional sites (Grimson et al., 2007).

In Silico prediction of microRNA targets

Numerous bioinformatics tools for microRNA target prediction have been developed to support researchers such as Tarbase (http://diana.imis.athenainovation.gr/DianaTools), PicTAR (http://www.pictar.org/) and TargetScan (http://www.targetscan.org). Although most of those methods rely on sequence complementarity between microRNA and its target, this task is very complicated given the small size of the seed region. This means that each microRNA will have several (probably hundreds) of targets. Many mRNA targets can be found just by chance which leads to a high false positive prediction rate (Lewis et al., 2003).
To improve the specificity of target prediction, computational methods have used other criteria such as the presence of multiple binding sites and filtering by evolutionary conservation (Lewis et al., 2005, Krek et al., 2005, Enright et al., 2003). Other structural features such as the relative position of the complementary sequence within the 3’ UTR, local structure and AU content were also proposed to improve target prediction tools (Friedman et al., 2009, Grimson et al., 2007).

1.5.4 MicroRNA detection methods

The size of microRNAs presents a challenge for their detection and quantification. At the same time, in contrast to mRNAs they are less vulnerable to degradation. Consequently, numerous detection approaches have been developed, each with specific advantages and disadvantages.

The choice of microRNA profiling method depends most importantly on the research question to be answered, number of microRNAs under investigation and the numbers of samples included in the study. Researchers generally agree that each of the tools have their own strengths and weaknesses. For example, specificity may come at a cost of technical difficulty, experimental duration or capacity. So there is no perfect method of microRNAs investigation. The decision will depends mainly on cost, sensitivity, accuracy and other factors (Baker, 2010).

1.5.4.a Northern Blot (NB) analysis for microRNAs

This is a widely used technique to assess the level of microRNAs and was the first technique to be used in microRNA research after their discovery (Lau et al., 2001, Sempere et al., 2004). The basic concept of NB is size dependent RNA separation by electrophoresis using denaturing Urea-polyacrylamide gel followed by transfer to a membrane. The microRNA is then hybridized with radioisotope labelled probes, visualized and ultimately quantified (Varallyay et al., 2007).

The main advantages of this method is that it separates the RNA according to its size which enables the analysis of mature, pre-microRNA and pri-microRNA at the
same time (Hunt et al., 2015). However, in comparison to other widely available detection methods, NB suffers from low sensitivity (Kim et al., 2010), is low throughput and requires a high amount of input RNA (Hunt et al., 2015). NB is also very time-consuming so it is not suitable for microRNA detection in large clinical studies. The common need for radioisotope labelled probes in NB protocols and its low sensitivity encouraged the researchers to improve and modify the protocol by using digoxin labelled or locked nucleic acid (LNA) probes. LNA is a special nucleotide whose ribose backbone is chemically modified resulting in greater affinity between the probe and the target RNA. This allows a tenfold increase in sensitivity as compared to the traditional NB (Varallyay et al., 2007, Válóczi et al., 2004).

1.5.4.b Microarrays to detect microRNAs

Microarray is another technique that depends on sensitive and specific microRNA hybridization to complementary probes that are usually immobilized onto glass slides. It allows for a parallel analysis of a large number of microRNAs in each sample. After their isolation, microRNAs are labelled with a fluorescent dye and then hybridized to the microarray slide. The specific binding between the probe and the microRNA is then visualized and quantified through analysing the fluorescent signal (Li and Ruan, 2009).

One major drawback of this method is that each microRNA has a unique melting temperature ($T_m$), depending on its GC content and length. Based on this $T_m$, each microRNA will have a specific optimum hybridization temperature, yet all microRNAs in the microarray chip will undergo the same hybridization conditions. Thus, microarray hybridization efficiency will greatly differ between microRNAs leading to serious distortion in the fluorescent signal generated with a high rate of false positives and false negatives (Li and Ruan, 2009, Koshiol et al., 2010). Different strategies have been developed to account for this problem such as using LNA probes to increase and normalize $T_m$, which improved as well the detection sensitivity (Castoldi et al., 2006).
MicroRNA labelling techniques, both enzymatic and chemical, can introduce another level of detection bias. Among the different labelling methods that have been developed, the most widely used is direct microRNA conjugation with a fluorescent dye. However, each method has its own advantages and disadvantages with no ideal labelling approach (Li and Ruan, 2009). This difference was the main reason behind the lack of inter-platform reproducibility (Sato et al., 2009). To overcome this problem, certain microarray methods aimed to replace the traditional labelling methods with alternative approaches such as using biotin labelled-structure specific RNA binding protein to recognize the probe-miR binding or using a stacking hybridized universal tag (SHUT) have been developed. These methods have been reported to be highly sensitive and specific (Lee et al., 2010, Duan et al., 2011, Hunt et al., 2015).

In summary, the main strengths of microarray microRNA detection are its low cost and high-throughput screening capacity. Issues regarding imperfect specificity, reproducibility and inability to perform absolute quantification of microRNA abundance are among the limitations of this platform.

1.5.4.4 Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

This approach is based on reverse transcription (RT) of the microRNA into complementary DNA (cDNA), followed by quantitative polymerase chain reaction (qPCR) with real time monitoring of the reaction. Owing to its high sensitivity and specificity, low cost and low RNA requirement, RT-qPCR was considered for some time, as the gold standard in microRNA expression profiling (Hunt et al., 2015, Schmittgen et al., 2008, Pritchard et al., 2012a). It remains the most widely used technique to profile and to confirm results obtained by other genome wide microRNA profiling methods as it is highly convenient, reliable and a sufficiently precise method.

Another important advantage of RT-qPCR is the possibility of performing it in a high-throughput way to allow detection of large number of microRNAs simultaneously.
Commercially available predesigned qPCR plates and microfluidic cards can be used to profile hundreds of microRNAs. Customizable plates can be designed to examine smaller numbers of microRNAs (Mestdagh et al., 2008, Pritchard et al., 2012a).

Depending on the RT technique, four RT-qPCR methods have been developed for microRNA detection. Each method has its own advantages and disadvantages. These methods are: (i) stem-loop RT-qPCR, (ii) poly (A) tailing RT-qPCR, (iii) primer-extension RT-qPCR and (iv) miQPCR, with the stem-loop RT-qPCR (Mou et al., 2013).

The stem-loop RT-qPCR techniques uses specifically designed primers to perform the reverse transcription step. Stem-loop primers have a short single stranded portion that is complementary to the microRNA’s 3’ end, a double stranded portion (the stem) and finally, a loop that contain a universal primer binding sequence (de Planell-Saguer and Rodicio, 2013). Although stem-loop primers are more difficult to design, they provide the RT reaction with at least 100 times higher efficiency and with a better specificity (Chen et al., 2005, Benes and Castoldi, 2010). The specific complementary annealing of the primer to the microRNA allows for a better discrimination between similar microRNAs. The stem loop structure also prevents hybridization of the primer to microRNA precursors and to the genomic DNA (Chen et al., 2005).

Following the RT step, qPCR can be performed. qPCR is a method to amplify and quantify the abundance of a specific DNA/cDNA sequence. It is based on repeated cycles of denaturing of the template DNA, primer annealing to target specific sequence and extension of the complementary strand by thermostable DNA polymerase (Smith and Osborn, 2009). This eventually leads to exponential increase in the copy numbers of target sequence with a corresponding increase in the fluorescence signal associated with the target sequence. The most common fluorescent systems used in microRNA qPCR detection are the intercalating SYBR green assay and the TaqMan probe system (Benes and Castoldi, 2010, Van Rooij, 2011).
SYBR Green is an intercalating dye that binds to the minor groove of dsDNA. This binding leads to a more than 100-fold increase in the fluorescence intensity, allowing the detection of the accumulated PCR product (Benes and Castoldi, 2010, Smith and Osborn, 2009). However, accurate detection of the target sequence is usually hindered by the nonspecific binding of SYBR Green to other dsDNA molecules in the sample. This requires extensive optimization of primers to improve their specificity.

TaqMan probes, on the other hand, are characterized with higher sensitivity and specificity (Chen et al., 2005). In this method, qPCR is performed using microRNA specific forward and reverse primers and a specific fluorogenic-labelled probe (TaqMan probe) designed to hybridize to a specific region of the target amplicon sequence. TaqMan probes are dual labelled with a fluorescent reporter and a quencher in close proximity which prevents emission of fluorescent signal when the probe is intact. With each PCR extension cycle, the Taq polymerase cleaves the reporter dye from the probe leading to an increase in the fluorescence signal which allows for an accurate quantification of the target (Benes and Castoldi, 2010, Bernardo et al., 2012, Van Rooij, 2011). The combination of stem-loop RT and TaqMan PCR provides researchers with an ideal tool to investigate both high and low abundant microRNAs. The high sensitivity and specificity, in addition to a high dynamic range (up to seven orders of magnitude), made this method the most widely used in microRNA research (de Planell-Saguer and Rodicio, 2013).

1.5.4.d MicroRNA detection by RNA-sequencing (RNA-seq)

RNA-seq is nowadays regarded as an invaluable tool in microRNA research. In addition to the simultaneous sequencing of millions of RNA molecules, it has the superiority in combining discovery of novel microRNAs with quantitative expression analysis of the already known microRNAs. It has the added advantage of not depending on prior sequence information, therefore has the ability to obtain information on all RNAs found in the sample not simply those of known sequence (Bernardo et al., 2012, Creighton et al., 2009). However, a key challenge in RNA-seq
experiments is the requirement of at least 10µg of high quality RNA, which can be
difficult in certain types of samples (Creighton et al., 2009). The relatively high cost,
the complexity involved in data analysis, as well as sequence specific bias related to
small RNA cDNA library preparation that favour capture of some microRNAs over
the others are regarded as additional limitations to RNA-seq experiments (Hunt et
al., 2015, Jin et al., 2013).

The first step in RNA-seq is the preparation of small RNA cDNA library from the RNA
sample. Different protocols were described for different RNA-seq platforms. The
most critical step in library preparation is the adaptor ligation to the 3’ and 5’ ends
of the microRNA. These adaptors provide the template for primer-based cDNA
synthesis, amplification and eventually sequencing. The efficiency of adaptor
ligation can vary significantly between the available kits, leading to significant bias
in results of RNA-seq experiments (Tian et al., 2010, Baran-Gale et al., 2015). Each
library preparation approach preferentially captures a distinct set of microRNAs.
This was reflected as a large difference (up to 30 fold) in microRNA read frequency
that prevented the accurate estimation of microRNA expression (Linsen et al., 2009,
Baran-Gale et al., 2015).

Library preparation is then followed by parallel sequencing of millions of the
generated cDNA molecules. Finally, bioinformatics analysis of microRNA, which
involves transcript quantification, normalization and differential expression analysis,
is performed. Several quality control measures are also applied at different stages
during analysis to ensure reproducibility and reliability of the results (Pritchard et
al., 2012a, Conesa et al., 2016).

During analysis, the ligated adaptor sequences are first trimmed, and the read
length distribution is computed which is usually around 22-23 nt. Reads must then
be aligned to a reference genome (Human genome) using certain bioinformatics
tools. Small RNA reads are then simply counted to obtain the expression value
(Conesa et al., 2016).
1.5.5 Extracellular microRNAs

Extracellular release of microRNAs was first discovered in cell culture medium (Valadi et al., 2007). Interestingly, microRNAs (and other mRNA species) were released from the donor cells into the extracellular medium and transferred to other recipient cells via exosomes. One year later, several studies confirmed the existence of extracellular microRNAs in blood (Chen et al., 2008, Chim et al., 2008, Lawrie et al., 2008, Mitchell et al., 2008) and then in various body fluids including urine, saliva, cerebrospinal fluid (CSF), semen and breast milk (Weber et al., 2010). An equally important discovery was that extracellular microRNA profiles differ considerably between healthy and diseased individuals, suggesting a wide range of clinical applications especially in the field of biomarker discovery (Etheridge et al., 2011).

1.5.5.a Stability of extracellular microRNAs in biofluids

Biomarker stability is an important prerequisite for use in a clinical setting. Previous biomarker candidates such as proteins and mRNAs have been shown to be quite unstable; therefore translation into a clinical setting would be unfeasible. Many investigators have confirmed that extracellular microRNAs are relatively resistant to degradation by endogenous RNAase activity and they are also resistant to freeze-thaw cycles, extreme variation in pH and harsh storage conditions.

Mitchell et al. (2008) introduced three synthetic microRNAs into human plasma either before or after the addition of RNAase inhibitor. Subsequent microRNA analysis of the synthetic microRNAs and three endogenous microRNAs showed that the synthetic microRNAs were rapidly degraded when added directly to the plasma when compared to their level after adding the RNAase inhibitor. However, the level of the endogenous microRNAs remained stable in both conditions indicating that those endogenous microRNAs are found in the plasma in a form that is resistant to RNAase activity.
The stability of circulating microRNAs at room temperature and the effect of freeze-thaw cycles on their level have been also investigated. Gilad et al. (2008) found that the level of different microRNAs in unfrozen serum did not change over 4-hour incubation at room temperature. This time has been extended up to 24 hours by Mitchell et al. (2008) and also displayed no significant change in microRNAs level. In the above two experiments, serum and plasma were also subjected to a number of freeze-thaw cycles (2 and 8 respectively) and they found that those cycles had a minimal effect on the level of microRNAs.

In 2011, Li et al confirmed the earlier view about microRNA stability in plasma when they studied a number of liver cancer-related microRNAs. They found that microRNA levels do not vary significantly after 3 hr. incubation under different temperatures (from -80 up to 37 °C), or up to 24 hr. incubation at room temperature, after RNAase digestion, 10 freeze-thaw cycles and 5 different pH values also failed to significantly alter microRNA levels (Li et al., 2011c).

This surprising stability of microRNAs in body fluids has inspired researchers to find an explanation. The first hypothesis in this regard was that both free and Ago-bound microRNAs were released from cells in membrane-bound vesicles which protected them from RNAase activity. Two types of vesicles have been proposed to be involved in microRNAs circulation in body fluid, exosomes and microvesicles. Exosomes are 50-90 nm membrane vesicles of endocytic origin that are released into the extracellular environment through fusion of multivesicular bodies (MVB) with the plasma membrane (van Niel et al., 2006). The first report about the presence of RNA inside exosomes (including small RNAs) was by Valadi et al (2007). Their results showed that exosomes carry approximately 121 microRNAs and some of these microRNAs were expressed at higher levels in exosomes than in their donor cells. This meant that microRNA uploading into the exosome is a selective process (Valadi et al., 2007, Pigati et al., 2010). This was confirmed by Gallo et al. (2012) who found that the majority of biofluid microRNAs are enclosed in exosomes
this was the case for fresh and frozen samples, serum and saliva and under health and disease conditions.

Microvesicles are larger membrane bound vesicles (up to 1 µm in diameter) that shed from the plasma membrane into the extracellular environment to facilitate communication between cells (Heijnen et al., 1999). Hunter et al (2008) was the first to identify and define microRNA expression in circulatory plasma microvesicles of healthy donors. They found 104 microRNAs were significantly expressed in microvesicles, with miR-223 and miR-484 being the most highly expressed ones. Those microRNAs were proposed to regulate the cellular differentiation of blood cells and to modulate immune responses.

Despite previous reports, other researchers have found that the majority of microRNAs are indeed not found inside vesicles, but rather are bound to protein or lipid complexes. In 2011, Arroyo et al. reported that vesicle-encapsulated microRNAs represent only a minority of circulating microRNAs. This study found that 90% of microRNAs in the circulation are found in a non-membrane bound form. They are protected from RNAase digestion by binding to a protein complex in which Ago-2 is the main component and that only a minority of microRNAs were enriched in vesicles (Arroyo et al., 2011). In agreement with the above study, Turchinovich et al. (2011) demonstrated that most of the circulating microRNAs in the plasma were bound to Ago-2 protein. Further, they found that microRNAs might be associated with other Ago proteins such as Ago-1, Ago-3 and Ago-4. Researchers argue that the previous reporting of microRNA excretion in a vesicle bound form depends on ultracentrifugation of the sample to precipitate the vesicles and large protein complexes such as RISC can co-purify with the precipitated vesicles (Turchinovich et al., 2016).

In another study, Vickers et al. (2011) suggested that microRNAs circulate in the blood in association with high density lipoprotein (HDL). Their findings showed that HDL carried a set of microRNAs that differ from those associated with exosomes. The most abundant HDL-microRNAs in normal subjects were miR-135a, miR-188-5p
and miR-877. On the other hand, HDL from patients with hypercholesterolemia carries a microRNA signature that differs from healthy subjects where the most abundant microRNAs were miR-223, miR-105 and miR-106a.

1.5.5.b Origin of extracellular microRNAs

The origin and the biological role of the extracellular microRNAs remains a matter of debate. There has been a substantial effort to explain why, when and how microRNAs are released from the cell and what is the significance of this release. Generally, there are two main explanations for the origin of both vesicle-encapsulated and protein-associated extracellular microRNAs. First is the “cell-cell communication hypothesis” and second is the “cellular by-product hypothesis”. These are not mutually exclusive, however, with accumulating evidencing supporting both theories.

The cell-cell communication hypothesis proposed that microRNAs are selectively packed into exosomes and microvesicles, subsequently released into the extracellular environment, transferred to a recipient cell and ultimately alter the gene expression in that cell to provoke functional effects. Increasing evidence suggests that this form of communication is vital for regulation of different physiological processes such as immune response (Mittelbrunn et al., 2011), cellular migration (Zhang et al., 2010). It may also participate in development and progression of pathological conditions such as tumour metastasis (Skog et al., 2008).

A key support for the cell-cell communication hypothesis was the selective enrichment of microRNAs inside exosomes. MicroRNA content of exosomes was found to be significantly different from that of parental cells and this was confirmed both in vitro and in vivo (Valadi et al., 2007, Skog et al., 2008, Mittelbrunn et al., 2011). Subsequent reports demonstrated that exosomes and their microRNAs are released from the cell through ceramide dependent secretory machinery (Kosaka et al., 2010b). These exosomes are transferred through biofluids to recipient cells
where they will be released into their cytosol following fusion of the exosome to the cell membrane. More importantly, these microRNAs were able to repress target mRNAs within the recipient cells (Mittelbrunn et al., 2011, Montecalvo et al., 2012, Kosaka et al., 2010b). This, by far, is the strongest evidence for the cell-cell communication theory.

However, the exact mechanism of the selective sorting of microRNAs into exosomes is still poorly understood (Kosaka et al., 2010a). In 2013, Villarroya-Beltri et al. (2013) identified two short sequence motifs (GGAG and CCCU) that are overrepresented in exosomal microRNA and are thought to be involved in the control of microRNA loading into exosomes. The GGAG sequence was found in the 3’ half of the microRNA in 75% of cases. On the other hand, three different sequence motifs were enriched in cellular microRNAs. Furthermore, a proteomic analysis of exosomal proteins detected direct and specific binding of heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) to the exosomal microRNAs, allowing control of their loading into exosomes (Villarroya-Beltri et al., 2013). More recently, it has been found that microRNAs packaged into exosomes have specific editing features (3’ end uridylation) when compared to intracellular microRNAs (3’ end adenylation) suggesting that these posttranscriptional modifications may contribute to direct microRNA loading into exosomes (Lau et al., 2001a).

In the cellular by-product hypothesis, microRNA release is viewed as a consequence of cellular physiological and/or pathological activity; either apoptosis or necrosis. MicroRNAs in such cases are passively (non-specifically) released from dead or dying cells into the extracellular environment and then to the blood. These microRNAs usually do not carry any significant biological function (Turchinovich et al., 2016). In support of this hypothesis, is the notable increase in global and tissue specific circulating microRNAs after different forms of tissue injury and toxicity (Laterza et al., 2009, Baker et al., 2015). The high stability of the microRNAs in the circulation (as mentioned previously) renders them detectable for prolonged periods after the death of parental cells (Turchinovich et al., 2011). Furthermore,
there are no current indications of active and selective release of Ago-bound microRNAs from cells or supporting their uptake by recipient cells.

Although during the process of cell death, microRNAs are released into the circulation in Ago-bound form, the association of microRNAs with exosomes and microvesicles can be explained by the well-known capacity of RNA binding proteins to bind to membranes or by random packaging of cellular components into vesicles during cell death process (Turchinovich et al., 2016). Another significant challenge to the idea of specific enrichment of microRNAs within exosomes came from a quantitative and stoichiometric analysis of microRNA content within exosomes using a novel nanoparticle tracking analysis (Chevillet et al., 2014). Their results showed that even the most abundant microRNAs are present at far less than one copy per exosome indicating that most exosomes are actually devoid of microRNAs. This indicates that exosomes may not have the potential to serve as vectors for extracellular cell-cell communication.

1.5.6 Extracellular microRNAs as disease biomarkers

Extracellular microRNAs have the potential to serve as diagnostic and prognostic biomarkers for different disease states. In general, an ideal biomarker should be tissue and disease specific, functional, easily detectable and quantifiable and easily accessible through minimally invasive procedures. MicroRNAs fulfil most of these criteria. Their expression differs substantially between normal and abnormal tissue and biological fluid samples (Lawrie et al., 2008, Skog et al., 2008), they are relatively stable in various body fluids, their expression is tissue specific or biological stage specific and their expression level can be easily assessed by different methods including the highly sensitive and specific RT-qPCR (Etheridge et al., 2011).

Despite that, microRNAs have unique features that make them challenging molecules to analyse. This includes their short length with highly divergent sequences and large variation in GC content, the presence of several microRNAs that differ only in a single nucleotide and also their low abundance in plasma or
serum samples (Zampetaki and Mayr, 2012). Plasma is also regarded as a challenging biofluid to study microRNAs because of the low RNA yield and the relative high protein and lipid content which may interfere with RNA isolation. The presence of microRNAs in the circulation in different forms (either bound to protein/lipoprotein or contained within vesicles) may have a specific effect on microRNAs yield and microRNAs expression results (Zampetaki and Mayr, 2012, Blondal et al., 2013).

1.6 MicroRNAs in epilepsy

In recent years, large numbers of studies have established the pivotal role of microRNAs in regulating every cellular process, such as differentiation, proliferation, growth, development and function (Houbaviy et al., 2003, Rogelj and Giese, 2004, Sempere et al., 2004). Furthermore, almost all disease states are associated with an aberrant microRNA expression profile. Clinically, the aberrant microRNA expression levels in each condition has a great potential in developing novel biomarkers for disease diagnosis, prognosis and therapeutic response (Etheridge et al., 2011). This abnormal expression can be viewed as either a disease causing factor or a consequence of the disease state. Manipulation of tissue microRNAs level using microRNAs inhibitors in the form of antisense oligonucleotides (antagomirs) or microRNA replacement (mimics) can represent a potential therapeutic approach to alter the course of the disease (Henshall et al., 2016).

Experimental and human epilepsy were found to be associated with genome-wide dysregulation of protein coding genes through prominent transcriptional suppression (Gorter et al., 2006), an effect that can be mediated through microRNAs. Aberrant microRNA expression or function has been linked to epilepsy development and research supports a role of microRNAs as critical gene expression regulators during epileptogenesis and epilepsy (Jimenez-Mateos and Henshall, 2013).
The first study to analyse the brain and blood microRNA profiles after seizure was published in 2010. Liu et al. (2010) compared the blood and hippocampal microRNA expression in rats following different brain insults. Several microRNAs were found to be up or down regulated after K.A induced seizures. The signature was unique and differed from other brain insults (TBI and ischemic stroke). 21 and 15 microRNAs were upregulated (>1.5 fold) in the hippocampus and blood respectively. miR-298 was the only microRNA upregulated both in the brain and blood samples. On the other hand, 39 and 43 microRNAs were downregulated in the hippocampus and blood with 7 microRNAs in common between the brain and the blood.

Shortly after, Aronica and colleagues performed the first study of microRNA dysregulation in human epilepsy and reported an increase in hippocampal levels of miR-146a, a microRNA targeting the inflammatory response, in tissue samples collected from TLE patients compared to autopsy controls (Aronica et al., 2010). Subsequently, with an increasing interest in the role of microRNAs in epilepsy, microRNA expression profiles were extensively studied in different animal models of epilepsy such as systemic K.A (Pichardo-Casas et al., 2012) (McKiernan et al., 2012b), intra-amygdala K.A (Jimenez-Mateos et al., 2011) and systemic pilocarpine (Hu et al., 2011, Hu et al., 2012). Altogether, these studies confirmed the highly selective changes in microRNA expression in epilepsy indicating a tight relationship between microRNA level and epilepsy progression (Jimenez-Mateos and Henshall, 2013).

1.6.1 Genome-wide microRNA profiling in experimental models of TLE

Several experimental studies were performed to investigate the global changes in microRNA expression both in hippocampal tissue and biofluids during epileptogenesis and epilepsy. The first study used a microRNA microarray technology and found 19 upregulated and 7 downregulated microRNAs in rat hippocampus, 24 hours following lithium-pilocarpine induced SE (Hu et al., 2011). The expression of four dysregulated microRNAs (miR-34a, miR-22, miR-125a and
miR-21) were then confirmed using RT-qPCR. Interestingly, a similar trend in microRNA expression alteration was noted in post status blood samples collected from those animals (Hu et al., 2011).

In the same year, the hippocampal CA3 region was analyzed in injury (mice after K.A induced SE), tolerance (mice with seizure preconditioning before the onset of K.A induced SE) and control groups. CA3 microRNAs were profiled using TaqMan® low-density arrays (Jimenez-Mateos et al., 2011). The injury group showed upregulation of 54% of microRNAs and downregulation of 21% of microRNAs. The scale of microRNA up-regulation was dramatic for several microRNAs, including >20-fold for miR-132, miR-219 and miR-323 and between 45-fold and 144 fold for miR-21, miR-507, and miR-518d. The expression level of nearly half of those microRNAs was different in the tolerance group when compared to injury group, being either unchanged or even showed the opposite regulation pattern (Jimenez-Mateos et al., 2011).

The first study to look at the time point dependent and brain region dependent (spatio-temporal) changes in microRNA profile in experimental animal models of epilepsy was performed by Gorter et al. (2014). MicroRNA expression levels at different time points after induction of SE, (1 day, 1 week and 3-4 months), and in distinct brain areas (CA1, DG and parahippocampal cortex-PHC) were analysed using Exiqon microRNA arrays. Results showed that the number of upregulated microRNAs is more than the number of downregulated microRNAs at each time point and in each brain region (except in the PHC). Analysis of the upregulated microRNAs in the acute phase after SE showed that 31 microRNAs had an increased expression in the PHC compared to 18 in CA1 and 20 in DG. There were no downregulated microRNAs in the CA1 and DG. However, the PHC had 68 microRNAs with decreased expression in the acute phase. The plasma levels of three inflammation-associated microRNAs were assessed and a significant increase in the plasma level of miR-21-5p one week after SE was found. This upregulation was similarly observed in the examined brain tissues. The two other microRNAs under
investigation, miR-142-5p and miR-146a-5p, showed elevation in plasma shortly after status epilepticus and in the chronic epileptic rat respectively (Gorter et al., 2014).

A number of other studies have followed, with analysis of global microRNA expression levels in different models of acute and chronic seizures (Kretschmann et al., 2014, Li et al., 2014, Song et al., 2011). microRNA dysregulation was analysed in different brain regions and in specific hippocampal subfields with specific pathological changes (Risbud and Porter, 2013, Bot et al., 2013, Schouten et al., 2015, Roncon et al., 2015). The relationship to treatment response (Moon et al., 2014) and effect of seizure preconditioning (McKiernan et al., 2012b) was also analysed.

1.6.2 Genome-wide microRNA profiling in human TLE

In 2012, two large scale gene-profiling studies on microRNAs in human epilepsy emerged. In the first study, a hippocampal microRNA profile of autopsy controls was compared to microRNA profiles in hippocampal tissues (with and without HS) obtained from pharmaco-resistant epilepsy patients. Subsets of microRNAs were differentially expressed in hippocampal tissues of TLE patients. Interestingly, a unique microRNA signature differentiated the sclerotic from non-sclerotic hippocampi and from controls (Kan et al., 2012). In the second study, McKiernan et al. (2012a) reported an expression of about 200 microRNAs in healthy human hippocampus. However, when working with tissue obtained from patients with TLE+HS, a large-scale reduction of microRNA expression was found (51% of microRNAs tested expressed at lower levels than in controls and about 24% not detectable in epileptic tissue). Lower levels of Dicer were also observed in resected sclerotic hippocampus from epilepsy patients in this study (McKiernan et al., 2012a). For this reason, the authors argued that this large scale reduction in microRNA levels might be due to a general failure in the production of mature microRNA transcripts in this brain region.
Researchers have also identified altered microRNA profiles within specific cells in a subpopulation of epileptic brains (hippocampal granule cells). In an effort to overcome the problem of cellular heterogeneity in the brain and to investigate the impact of different pathological features on hippocampal microRNA profile, the coexistence of HS with type 2 granule cell pathology was compared to sclerotic tissue samples that lack this characteristic feature. Laser microdissection of the granule cell layer of the hippocampus allowed the identification of 12 microRNAs that were differentially expressed between the two groups (Zucchini et al., 2014). Moreover, the alteration in hippocampal microRNA profile was found to be correlated with the severity of HS that was determined according to Watson Grading (WG) system (Watson et al., 1996). Miller-Delaney et al. (2015) reported a total of 18 microRNAs to be differentially expressed in TLE versus autopsy controls. Among these 18, miR-363, miR-876-3p and miR-483-3p were uniquely dysregulated in association with mild HS (WG1). Whereas, miR-138, miR-873 and miR-876-3p were uniquely dysregulated in association with severe HS (WG4) (Miller-Delaney et al., 2015).

Progress has also been made on understanding the roles of specific microRNAs in models of epilepsy and in human material. Particular focus has been on microRNAs with a presumed role in inflammation, neuronal death and neurogenesis. miR-146a is one of the most extensively studied microRNAs in relation to epilepsy. Significant up-regulation of miR-146a was detected in hippocampal tissues obtained from TLE patients (Aronica et al., 2010, Omran et al., 2012). Attention had focused on miR-146a due to its role in modulating innate immunity through regulation of TLR signaling and cytokine responses. miR-146a has been implicated in regulation of astrocyte-mediated inflammatory response (Iyer et al., 2012). In addition, in vitro experiments showed a significant up-regulation of miR-146a in astrocytes when exposed to IL-1ß stimulation, which is known to be up-regulated in the acute phase of some animal models of TLE (Aronica and Crino, 2011). Recent work showed that intra-cerebroventricular (i.c.v) delivery of miR-146a mimics reduced neuronal excitability and arrested epilepsy progression in mice (Iori et al., 2017). Another
microRNA that has been associated with inflammatory pathways in TLE is miR-155 (Ashhab et al., 2013). An increase in the expression of miR-155 in hippocampal tissue from children with TLE, as well as in an immature rat epilepsy model has been demonstrated. The observed increase in miR-155 expression correlated with an increase in TNF-α in the tissue (Ashhab et al., 2013).

The expression profiles of individual microRNAs have been also studied in the hippocampus of children with TLE and their levels were compared with hippocampal tissues obtained from normal controls. Results showed upregulation of miR-9 and miR-181a and downregulation of miR-138, miR-221 and miR-222. Similar expression patterns of those 5 microRNAs were found in the hippocampus of an immature rat model of TLE (Peng et al., 2013).

In summary, there is growing evidence supporting dysregulation of microRNA expression in the pathogenesis of epilepsy. Time point specific, brain region specific and pathological feature specific signatures of microRNAs were confirmed in experimental and human TLE and during both epileptogenesis and chronic epilepsy. Those studies suggest the potential use of extracellular microRNAs as a biomarker for epilepsy diagnosis and prognosis. However, until now, there is a considerable paucity in research focusing on extracellular microRNA expression profiles in patients with epilepsy. More studies using human samples are still needed which will provide the bases to translate preclinical studies to clinical ones.

1.6.3 In vivo functional manipulation of microRNAs and their role as therapeutic targets in epilepsy

The first study to functionally interrogate a specific microRNA in a seizure model in vivo was reported by Jimenez-Mateos et al. (2011). The strong up-regulation of miR-132 after SE (> 22 fold) suggested a possible pathological role of this microRNA in epilepsy. To further investigate its role in epilepsy, anti-miR-132 oligonucleotides (antagomirs, chemically engineered molecules for microRNA silencing) were injected intra-cerebroventricularly before induction of SE. Results were compared
to mice injected with a scrambled oligonucleotide sequence. The study found that mice injected with the antagomirs targeting miR-132 developed less hippocampal damage after SE. However, the antagomir injection did not alter the severity or duration of SE (Jimenez-Mateos et al., 2011). Despite the previous report, work by Huang et al. (2014) has suggested that targeting miR-132 can reduce the neuronal damage after SE as well as suppress the spontaneous seizures (Huang et al., 2014).

The first report showing a direct effect of a microRNA manipulation on seizure severity and duration was by Jimenez-Mateos et al. (2012). miR-134 expression was found to be upregulated mainly in the hippocampal pyramidal neurons and hilar interneurons after induction of SE (Jimenez-Mateos et al., 2012). The severity and duration of SE was strongly reduced in mice that were treated 24 h earlier with an antagomir targeting miR-134. Hippocampal neuronal damage was also dramatically reduced (Jimenez-Mateos et al., 2012). The reduced severity of SE in mice treated with miR-134 antagomirs was later reproduced in the pilocarpine model (Jimenez-Mateos et al., 2014).

The role of microRNA manipulation (in the form of silencing or activation) in reducing the severity of both the induced SE and the subsequent recurrent spontaneous seizures as well as in protecting the neurons from seizure induced damage is gaining more and more attention. To date at least a dozen microRNAs have been shown to affect seizure thresholds or neuropathology when targeted in animal models of epilepsy. Among those tested microRNAs were antagomirs targeting miR-34a (Hu et al., 2012), miR-181a (Ren et al., 2016), miR-199a (Wang et al., 2016a), miR-210 (Chen et al., 2016) and miR-203 (Lee et al., 2016).

In a complementary approach, microRNAs mimics have been used to upregulate microRNAs before or after SE. Alleviation of seizure phenotype have been observed for mimics that increase the level of miR-128 (Tan et al., 2013), miR-219 (Zheng et al., 2014), miR-23b-3p (Zhan et al., 2016), miR-124 (Wang et al., 2016b) and miR-22 (Jimenez-Mateos et al., 2015). This form of microRNA functional restoration has its role in neuronal protection and minimizing neuronal hyperexcitability.
MicroRNA dysregulation in epilepsy is thought to regulate a wide variety of cellular and molecular mechanism that are involved in epilepsy pathophysiology. miR-134, for example, have been linked to altered volume and function of dendritic spines on excitatory neurons through targeting LIM domain kinase (Jimenez-Mateos et al., 2012, Jimenez-Mateos et al., 2014). miR-146a, miR-221 and miR-222 are involved in controlling the immune response through targeting IL-1ß and cellular adhesion molecules (Aronica et al., 2010, Kan et al., 2012, Iyer et al., 2012).

However, the exact transcriptional control which leads to a microRNA deregulation in epilepsy is still largely unknown. Recently, miR-22 dysregulation after SE was suggested to be under control of transcription factor specificity protein 1 (SP1) (Jimenez-Mateos et al., 2015). However, for most microRNAs we do not know the exact mechanism behind their regulation. Direct deregulation through epigenetic mechanisms leading to more or less active transcription of microRNA genes has been linked to microRNA dysregulation (Miller-Delaney et al., 2015). Other indirect mechanisms such as change in the number and function of viable neurons or glial cells after brain insult and in epilepsy can be accompanied by changes to the levels of microRNAs in these cells (Henshall et al., 2016).
1.7 Remit of this thesis

This thesis concerns the promising role of extracellular microRNAs in epilepsy diagnosis.

Study hypotheses to be tested:

1. Extracellular microRNAs are stable in healthy controls and display little inter-individual variability over time.
2. Extracellular microRNA signatures differ in patients with epilepsy when compared to healthy controls.
3. Extracellular microRNA levels change in epilepsy patients after seizure.

Study objectives:

- **Study I: stability of microRNAs in plasma of healthy individuals**
  - Pre-profiling standardization of methods.
  - Morning-afternoon variation in microRNA profile.
  - Day-day variation in microRNA profile.
  - Sex difference in microRNA profile.

- **Study II: plasma microRNAs as biomarkers of temporal lobe epilepsy**
  - Determine plasma microRNA signature in patients with temporal lobe epilepsy compared to controls
  - Determine whether plasma microRNA profile changes after a seizure

- **Study III: role of cerebrospinal fluid microRNAs as potential biomarkers of temporal lobe epilepsy and status epilepticus**
  - Determine CSF microRNA signature in patients with temporal lobe epilepsy compared to controls
  - Determine the ability of CSF microRNAs to distinguish TLE patients from patients with other neurological diseases.
2. Patients, materials and methods
2.1 Ethical approval:

This study represents an extensive collaboration between several clinical and research centres across Europe: RCSI-Dublin, Department of Neurology, Beaumont Hospital-Dublin, the University of Magdeburg, The Philipp University of Marburg and The Friedrich-Alexander-University Erlangen-Nurnberg.

Ethical approval was obtained from the local medical ethics committees at each centre. Dublin’s part of the study was approved by Research Ethics Committee of RCSI (REC859b) and Beaumont hospital (13-79). Following a detailed discussion of the study, patients were given a written information leaflet further describing the study design and rationale. Consent was obtained according to the Declaration of Helsinki from all participants. This included informed written consent given by the participating healthy volunteers and patients or their legal representatives if the patients were obtund.

2.2 Patients and healthy controls:

2.2.1 Study I: stability of microRNAs in plasma of healthy individuals

Twenty healthy non-fasting male and female volunteers were recruited for the study divided into three groups. The first group, the morning-afternoon (AM-PM) group, was used to assess the effect of blood collection timing on microRNA profile in healthy volunteers. Blood was collected from 10 healthy volunteers at 2 time points on the same day, between 9:30 am and 10:30 am and between 4:00 pm and 5:00 pm. The second group, the day-to-day group, was used to check for microRNA stability in healthy volunteers over a period of time. Blood was collected from 10 healthy volunteers on 4 days over one-month period (day 1, day 2, day 7 and day 28) between 9:30 am and 10:30 am. The third group, the male-female group, was used to check for a difference in microRNA expression between males and females. The subjects in this group were included in the previous two groups, but only the
morning samples for the AM-PM group and the day 1 samples from the day-to-day group were used (Table 2.1A).

2.2.2 Study II: plasma microRNAs as biomarkers of TLE

To uncover potential circulating microRNA biomarker for TLE, 32 patients attending the EMU/Beaumont hospital/Dublin (DUB group) and the epilepsy centre Hessen/department of Neurology/ Marburg (MAR group) were recruited. The majority of patients were diagnosed with refractory TLE prior to admission and were on poly-drug therapy for TLE. The reasons for admission were mainly to confirm the diagnosis, assess pharmaco-resistance and to assess the suitability of the patient for surgical resection of temporal lobe. Continuous EEG/CCTV monitoring was performed for each patient using a standard international system electrode placement. Continuous computerized seizure detection was performed throughout the recording period. The entire recording from the monitoring session was manually reviewed by a neurologist. From each patient, a baseline blood sample (epilepsy baseline samples-EBS) was collected 24 hours after admission provided that the patient had no seizures during this period. An epilepsy after-seizure sample (EAS) was then collected exactly 24 hours after experiencing an electro-clinical seizure. In addition, 32 non-fasting male and female healthy volunteers were recruited in both centres. From these subjects, blood was collected and processed by the same personnel who collected and processed the patient’s blood. Major exclusion criteria for the study were age (below 18 years old), severe cognitive impairment, history of autoimmune diseases, allergic response, immune deficiency disorder, diabetes, heart disease, stroke, atherosclerosis, psychiatric illness, malignancy, or a systemic or CNS infection 2 weeks before sample collection. Summary of the demographics for both the patient and healthy control groups can be viewed in table 2.1B.
2.2.3 Study III: role of cerebrospinal fluid microRNAs as potential biomarkers of temporal lobe epilepsy and status epilepticus

For this study, patients were recruited from three different centers during clinical workup: The University of Magdeburg, The Philipp University of Marburg and The Friedrich-Alexander-University Erlangen-Nurnberg. In total, 83 adults were recruited for the study. 45 of them were included in the discovery phase where genome-wide microRNA profiling of their CSF samples was performed. These included 15 patients who presented with non-specific headache (CH) from whom CSF was collected to exclude infectious causes, which was confirmed negative, 15 patients suffering from refractory TLE with or without HS and 15 patients who experienced SE. The underlying cause of SE was unclear in 4 patients. However, a wide range of causes were responsible for the SE episodes in the others, including for example, alcohol withdrawal, post-stroke and encephalitis. SE was focal in 10 patients, GTC in 3 and non-convulsive (NCSE) in 5. In the second phase of the study, validation phase, microRNA expression was validated in CSF samples from 25, 18 and 15 controls, TLE and SE patients respectively. A fourth group of 25 patients diagnosed with other neurological diseases (CND) was included in the validation phase as a second control group. This group included 9 patients with Alzheimer’s disease (AD), 10 with multiple sclerosis (MS) and 6 patients with other neurological disorders including primary or secondary brain tumours. A summary of demographics and clinical details for patients recruited for study III are provided in table 2.1C and table 2.2.
Table 2.1 Summary of demographics for healthy volunteers and patient groups included in each study.

### Study I

<table>
<thead>
<tr>
<th>Gender</th>
<th>AM-PM</th>
<th>M</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM-Pm</td>
<td>5 (36.6 ± 2 yrs)</td>
<td>5 (35.2 ± 5 yrs)</td>
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</tr>
<tr>
<td>Day-to-day</td>
<td>5 (37.2 ± 4 yrs)</td>
<td>5 (29.0 ± 1 yrs)</td>
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<tr>
<td>M-F</td>
<td>10 (36.9 ± 2 yrs)</td>
<td>10 (32.1 ± 3 yrs)</td>
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### Study II

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<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>8 (41.2 ± 4 yrs)</td>
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<tr>
<td>TLE</td>
<td>8 (40.2 ± 5 yrs)</td>
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</tbody>
</table>

### Study III

<table>
<thead>
<tr>
<th>Group</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td>CH</td>
<td>10 (29.8 ± 2.2 yrs)</td>
<td>18 (39.9 ± 2 yrs)</td>
</tr>
<tr>
<td>TLE</td>
<td>10 (37.70 ± 5 yrs)</td>
<td>6 (46.3 ± 2.9 yrs)</td>
</tr>
<tr>
<td>FSE</td>
<td>4 (72.2 ± 7.3 yrs)</td>
<td>6 (72.8 ± 4.7 yrs)</td>
</tr>
<tr>
<td>GTC</td>
<td>3 (76 ± 4.7 yrs)</td>
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<tr>
<td>NCSE</td>
<td>3 (68 ± 10.4 yrs)</td>
<td>2 (63.5 ± 10.5 yrs)</td>
</tr>
<tr>
<td>AD</td>
<td>6 (73.3 ± 2.7 yrs)</td>
<td>3 (72 ± 3.6 yrs)</td>
</tr>
<tr>
<td>MS</td>
<td>2 (62 ± 19 yrs)</td>
<td>8 (32.3 ± 2 yrs)</td>
</tr>
<tr>
<td>Others</td>
<td>3 (74.6 ± 2.1 yrs)</td>
<td>3 (60.6 ± 3.8 yrs)</td>
</tr>
</tbody>
</table>

Data are represented as Number (mean age ±SD)

Key: Am-Pm, Morning-afternoon group; AD, Alzheimer’s disease; CH, control (headache); DUB, Dublin group; F, female; FSE, focal status epilepticus; GTC, generalized tonic clonic seizures; M, male; MAR, Marburg group; MS, multiple sclerosis; NCSE, nonconvulsive status epilepticus; SE, status epilepticus; TLE, temporal lobe epilepsy.
Table 2.2 Detailed demographics and clinical data for control and patients groups recruited for study III.

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<tbody>
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<td>------------</td>
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<td>OA, V, A/E</td>
</tr>
<tr>
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<td>OA, A/E</td>
</tr>
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<td>TLE</td>
<td>OA, V, A/E</td>
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<tr>
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<td>67</td>
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</tr>
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<td>83</td>
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<td>53</td>
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</tr>
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<td>M</td>
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<td>NCSE</td>
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</tr>
<tr>
<td>M</td>
<td>88</td>
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### Control-Headache (CH)

<table>
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<tbody>
<tr>
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<td>V</td>
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<td>35</td>
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<td>V</td>
</tr>
<tr>
<td>F</td>
<td>36</td>
<td>chronic headache</td>
<td>V</td>
</tr>
<tr>
<td>F</td>
<td>37</td>
<td>chronic headache</td>
<td>V</td>
</tr>
<tr>
<td>F</td>
<td>40</td>
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<td>OA, V</td>
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<tr>
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<td>V, A/E</td>
</tr>
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<td>F</td>
<td>43</td>
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<td>OA, A/E</td>
</tr>
<tr>
<td>F</td>
<td>44</td>
<td>chronic headache</td>
<td>OA, V</td>
</tr>
<tr>
<td>F</td>
<td>44</td>
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<td>V, A/E</td>
</tr>
<tr>
<td>F</td>
<td>46</td>
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<td>OA, V, A/E</td>
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<td>37</td>
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<tr>
<td>M</td>
<td>41</td>
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<td>OA, A/E</td>
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### Control-Neurological diseases (CND)

<table>
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<tbody>
<tr>
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</tr>
<tr>
<td>F</td>
<td>70</td>
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<td>V, A/E</td>
</tr>
<tr>
<td>F</td>
<td>79</td>
<td>Alzheimer's disease</td>
<td>V, A/E</td>
</tr>
<tr>
<td>M</td>
<td>65</td>
<td>Alzheimer's disease</td>
<td>V, A/E</td>
</tr>
<tr>
<td>M</td>
<td>68</td>
<td>Alzheimer's disease</td>
<td>V, A/E</td>
</tr>
<tr>
<td>M</td>
<td>69</td>
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<td>V, A/E</td>
</tr>
<tr>
<td>M</td>
<td>78</td>
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<td>V, A/E</td>
</tr>
<tr>
<td>M</td>
<td>79</td>
<td>Alzheimer's disease</td>
<td>V, A/E</td>
</tr>
<tr>
<td>M</td>
<td>81</td>
<td>Alzheimer's disease</td>
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</tr>
<tr>
<td>F</td>
<td>26</td>
<td>multiple sclerosis</td>
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<tr>
<td>F</td>
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<td>V, A/E</td>
</tr>
<tr>
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<td>29</td>
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<tr>
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<tr>
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<td>64</td>
<td>undefined gait disorder</td>
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<td>65</td>
<td>sinus vein thrombosis</td>
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</tr>
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<td>choroidal melanoma with possible brain metastasis</td>
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<td>73</td>
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</tr>
<tr>
<td>M</td>
<td>79</td>
<td>right hypoglossal paresis</td>
<td>V</td>
</tr>
</tbody>
</table>

CH= control headache; CND= control/neurological diseases; M = Male; F = Female; FSE = Focal SE; GTC = generalized tonic clonic; NCSE = non convulsive SE; OA, samples used for genome-wide microRNA profiling; V, samples used for RT-qPCR validation; A/E, samples used for Ago/Exo analysis.
2.3 Biofluid sample collection and processing:

2.3.1 Mouse terminal blood collection:

After terminal anaesthesia of mouse using isoflurane, the animal was placed in dorsal recumbency and a wide gauge needle was inserted and advanced slowly into the heart chamber (just below and slightly to the left of the xiphoid cartilage at the base of the sternum, at a 15° to 20° angle). Once inside the heart 2.5 ml of blood was aspirated. Cervical dislocation was then performed to ensure death of the animal.

2.3.2 Human plasma and serum collection and processing:

Sample collection: from each donor, at each time point, 10 ml of peripheral blood was collected from the antecubital vein using a syringe with a wide-gauge needle, into K2 ethylenediaminetetraacetic acid (K2EDTA) tubes (BD Bioscience) for plasma generation or into serum separator tube (BD Bioscience) for serum generation. Blood for serum generation was incubated at room temperature for at least 30 minutes to allow for the coagulation process.

Plasma/serum processing and quality control: Within one hour of blood collection, plasma/serum was prepared by centrifuging the tubes at 1300 xg, for 10 min, at 4°C. The supernatant was collected into an RNAase free tube and was kept in -80 °C. A post-freezing centrifugation step was then performed at 1940 xg for 10 min at 4°C to further reduce cellular and platelet contamination (Cheng et al. 2013). The level of haemolysis in samples was assessed by spectrophotometric analysis using Nanodrop 2000 spectrophotometer. Absorbance at 414 nm was checked and any sample with an absorbance >0.25 was excluded from the study due to possibility of haemolysis (Kirschner et al. 2013a).
2.3.3 CSF collection and processing:

CSF samples were collected by the standard lumber puncture procedure (Teunissen et al., 2009) centrifuged within one hour of collection at 300 xg for 10 min at 4°C to remove contamination or cellular debris. Finally, the supernatant was collected and stored at −80°C until further use.

2.4 MicroRNA expression in biofluid samples:

2.4.1 Total RNA extraction from biofluid samples:

Upon commencing this study, microRNA isolation from biofluid samples presented a significant challenge. No standardised protocol existed, and the methods described in the early publications in this emerging field of microRNA research were vague. Here, in order to standardize RNA extraction method, various techniques were compared including column based, Trizol based and a combination of both techniques. RNA yield from serum and plasma samples was also compared in order to identify which circulating medium was optimal for blood based microRNA studies. Total RNA in the samples was isolated using different kits and according to the manufacturer protocol for each case.

2.4.1.a Protocol I: miRCURY RNA Isolation Kit–Biofluids (Exiqon):

miRCURY RNA Isolation Kit–Biofluids (Exiqon) represents a column based RNA isolation method that is optimized for the purification of all RNAs smaller than 1000 nt from various biofluid samples. Using this kit, membranized particles within the sample are first lysed using lysis solution. Proteins are then precipitated and removed from the sample without the need for phenol or chloroform using precipitation solution. The resultant supernatant was then mixed with isopropanol and loaded into a spin column which binds only the RNA. This was followed by two respective wash steps followed by a centrifugation for 2 minutes at 11,000xg to dry the column membrane completely. The final purified RNA was eluted in 25 µl RNAase free water.
2.4.1.b Protocol II: miRNeasy Serum/Plasma Kit (Qiagen):

miRNeasy Serum/Plasma Kit (Qiagen) involves a combined column and phenol based extraction. First, 1 ml of QIAzol lysis reagent was added to 200 µl of plasma. Phase separation was performed by adding 200 µl of chloroform to the sample followed by centrifugation at 12000xg for 15 minutes. The upper aqueous phase was then mixed with ethanol and loaded into spin columns which bind the RNA molecules. This was followed by two washing steps and the final RNA was eluted using 14 µl RNAase free water.

2.4.1.c Protocol III: TRIZol® LS (ThermoFisher Scientific) extraction

In this method, a chloroform-mediated phase separation was performed using 200 µl of chloroform. In brief, 200 µL plasma was mixed with 700 µL TRIZol lysis reagent and 200 µL of chloroform, and then centrifuged at 12000xg for 15 minutes at 4°C. The upper aqueous phase was collected and transformed into a new tube and followed by isopropanol mediated precipitation of RNA molecules which was then washed using 75% ethanol and reconstituted using 25 µl RNAase free water.

2.4.2 Genome-wide extracellular microRNA expression analysis:

2.4.2.a micropRNA profiling using the QuantStudio™ 12K Flex OpenArray (OA) system (ThermoFisher Scientific):

OA technology is a high-throughput, real-time PCR–based method for microRNA detection that enables simultaneous running of hundreds of TaqMan microRNA assays in a plate format. Each OA panel enables quantitation of microRNA expression in 3 samples. Up to 4 panels can be cycled simultaneously (which allow analysis of 12 samples within a single run). The OA system allows the amplification of 755 human microRNAs in each sample. Negative control (ath-miR159a) as well as endogenous control genes (RNU44, RNU48, U6) were measured for each sample. The OA workflow involves three steps; RT, preamplification (preAmp) and PCR amplification. To prepare samples for OA, mature microRNAs in the RNA samples
were reverse transcribed first using Megaplex™ RT primers in a set of two pre-defined pools (Human Pool A v2.1, Human Pool B v3.0). 3 μL of total RNA extracted from each biofluid sample (plasma and CSF) were used as a template for the RT reaction (for each pool) in a 7.5μl final volume. In addition to RNA and RT primers, RT reaction mix contains 0.15μl dNTPs with dTTPs (100 mM), 1.5 μl multiscribe reverse transcriptase enzyme (50U/μl), 0.75 μl of 10X RT Buffer, 0.9 μl MgCl2 (25 mM) and 0.09 μl RNAase inhibitor (20U/ μl). Reverse Transcription reaction was performed in Applied Biosystem thermal cycler, cycling conditions for the RT reaction is provided in table 2.3A.

Second, to increase the quantity of cDNA prior to PCR and to significantly increase the ability to detect low abundance transcripts a preamplification of RT product was performed. The entire 7.5 μl of RT product was used in a final volume of 40 μl. In addition, the preAmp reaction mix contain Megaplex™ preAmp Primers (Human Pool A v2.1, and Human Pool B v3.0) and 20µl of 2X TaqMan preAmp master mix. The preAmp reaction was performed in Applied Biosystem thermal cycler, cycling conditions for the preAmp reaction is provided in table 2.3B.

Last, genome-wide microRNA profiling was performed. The preAmp product was first diluted with 0.1X Tris-EDTA (TE) buffer to a ratio of 1:40 and then diluted 1:2 with 2X TaqMan OA Real-Time PCR Master Mix. 5 μl of the diluted reaction mixture for each sample were aliquoted into each of eight wells on an OA 384-well sample plate where each well corresponds to one subarray on OA panel. The OA panels were automatically loaded by OpenArray AccuFill™ System and finally, samples were run on the QuantStudio 12K Flex Real-Time PCR System with OA Block (ThermoFisher Scientific).

MicroRNA expression levels were calculated in cycle threshold values (ct), which estimates the amplification cycle at which the fluorescence levels for each of the analysed microRNAs exceeded the background fluorescence threshold.
Table 2.3 Openarray reverse transcription and preamplification cycling conditions

<table>
<thead>
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<th>OA reverse transcription reaction conditions</th>
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</thead>
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<tr>
<td></td>
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<tr>
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<td>50°C</td>
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<tr>
<td>Hold</td>
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<table>
<thead>
<tr>
<th>B</th>
<th>OA preamplification reaction conditions</th>
</tr>
</thead>
<tbody>
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<td>Temp</td>
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<td>Hold</td>
<td>72 °C</td>
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<tr>
<td>Cycle (16 cycle)</td>
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<td></td>
<td>60 °C</td>
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<tr>
<td>Hold</td>
<td>99.9 °C</td>
</tr>
<tr>
<td>Hold</td>
<td>4 °C</td>
</tr>
</tbody>
</table>

All reactions were performed in Applied Biosystem thermal cycler (Thermofisher Scientific).
2.4.2.b microRNA profiling using RNA-sequencing

The experiment was conducted at Exiqon Services, Denmark. After total RNA extraction using miRCURY RNA Isolation Kit–Biofluids (Exiqon), 6μl of total RNA was converted into microRNA NGS libraries using NEBNEXT library generation kit (New England Biolabs Inc.) according to the manufacturer’s instructions. Each individual RNA sample had adaptors ligated to its 3’ and 5’ ends and was converted into cDNA. Then the cDNA was pre-amplified with specific primers containing sample specific indexes. After 18 preamp cycles the libraries were purified on QiaQuick columns and the insert efficiency evaluated by Bioanalyzer 2100 instrument on high sensitivity DNA chip (Agilent Inc.). The microRNA cDNA libraries were size fractionated on a LabChip XT (Caliper Inc.) and a band representing adaptors and 15-40 bp insert excised using the manufacturer’s instructions. Samples were then quantified using qPCR and concentration standards. Based on quality of the inserts and the concentration measurements the libraries were pooled in equimolar concentrations (all concentrations of libraries to be pooled are of the same concentration). The library pool(s) were finally quantified again with qPCR. Optimal concentration of the library pool was used to generate the clusters on the surface of a flowcell before sequencing according to the manufacturer instructions (Illumina Inc.). Samples were sequenced on the Illumina HiSeq system (for Mar Samples) and on Illumina NextSeq (for DUB samples). Intensity correction, base calling and assigning of Q-scores were then performed as part of quality control measures to assess the quality of the data obtained.

After sequencing adapters were trimmed off as part of the base calling. Trimming of adapters from the dataset revealed distinct peaks representing microRNA (~18-22nt), and longer sequences of other origin (i.e. rRNA, tRNA and mRNA fragments, ~30-50nt). microRNA expression level was measured as Tags Per Million (TPM). TPM is a unit used to measure expression in NGS experiments. The number of reads for a particular microRNA is divided by the total number of mapped reads and multiplied by 1 million. Mapping of the sequencing reads followed which is
regarded as the first part of the data analysis. It also represents a useful quality control step in the NGS data analysis pipeline as it can help to evaluate the quality of the samples. Next, the differential expression analysis was done using the EdgeR statistical software package (Bioconductor, http://www.bioconductor.org/).

Differential expression analysis investigates the relative change in expression (i.e. counts) between different samples. For normalization the trimmed mean of M-values method (TMM normalization) was used. This method is based on log-fold and absolute gene wise changes in expression levels between samples. This normalization is primarily concerned with compensating for sample specific effects (generally caused by the variation in sequencing depth between samples). Additionally, the normalization step offsets under-sampling effects (due to highly expressed microRNAs dominating the read set) by identifying scaling factors that minimize log fold changes between samples across the majority of microRNAs.

2.4.3 Individual microRNA expression analysis using Real-Time qPCR:

RT-qPCR for individual microRNAs was adapted from (Mitchell et al., 2008). All microRNA RT reactions were carried out using the high capacity RT kit (ThermoFisher Scientific) in addition to microRNA-specific RT primers (ThermoFisher Scientific). During the RT step, a master mix containing, 0.5 µl 10X RT buffer, 0.063 µl RNAase inhibitor, 0.33 µl multiscribe reverse transcriptase enzyme, 0.05 µl dNTPs and 0.6 µl microRNA specific RT primer was prepared and added to 1.7 µl of total RNA. A final volume of 5 µl was reverse-transcribed with the following conditions: 30 min at 16°C to anneal primers, 30 min at 42°C for the extension phase, 5 min at 85°C to stop the reaction.

For real-time PCR amplification, the generated cDNA was diluted in a ratio of 1:6.5, mixed with 2.5 µl of 2X TaqMan Fast Universal PCR Master Mix (AB) and 0.25 µl of 2X microRNA-specific PCR primers. The amplification was done in triplicate and a negative control was included for each primer. The PCR reaction was performed in the QuantStudio™ 12K Flex PCR system using 96 well plates in the following cycle
conditions: 95°C for 20 sec. followed by 40 cycles of 95°C for 1 sec. and 60°C for 20 sec.

2.4.4 Analysis of CSF microRNAs within exosomes and complexed to Ago-2:

To indicate whether the differentially expressed microRNAs in study III (CSF microRNA biomarkers) are protein bound (Ago-2) or enclosed within exosomes, CSF samples were pooled into 5 control samples, 5 TLE samples, 5 SE samples, 3 MS samples and 3 AD samples. Each pool was then divided into three parts: 300 µl was allocated for exosome precipitation; 300 µl for Ago-2 immunoprecipitation (IP) and 200 µl for total RNA extraction.

Exosome precipitation from CSF samples was performed using the ExoQuick™ Exosome Precipitation Solution (System Biosciences-SBI). Briefly, 75.6 µl of precipitation solution was added to 300 µl of CSF. The mix was incubated for 24 hours at 4°C, followed by centrifugation at 1500 x g for 30 minutes. After centrifugation the exosomes appear as a white pellet at the bottom of the tube. To remove any excess precipitation solution a second quick centrifugation step at 1500 x g for 5 minutes was performed. Exosomal pellet was then reconstituted in 200 µl of RNAase free water and total RNA was extracted using miRCURY RNA isolation kit-biofluid as described previously.

Ago-2 IP from pooled CSF samples was adapted from (Jimenez-Mateos et al., 2012). Briefly, 300 µl of CSF was incubated overnight at 4°C with 10 µg of antibodies raised against Ago-2 (C34C6, Cell Signaling Technology). Protein A-agarose beads (Santa Cruz Biotechnology) were added and utilized to pulldown Ago-2 containing nucleoprotein complexes, following an incubation step (4 hours at 4°C) and centrifugation step (16000 x g, 15 min., 4°C). The Ago IP pellet was washed three times with IP buffer containing 300 mM NaCL, 5 mM MgCl2, 0.1% NP-40, 50 mM Tris HCl. Total RNA was extracted from Ago-2 IP pellet using 200 µl of Trizol reagent and 50 µl of chloroform. The final RNA pellet was dissolved in 12 µl RNAase free water.
To investigate microRNA expression after exosomal precipitation and Ago-2 IP, a customized RT and preAmp primer pools were prepared by pooling all microRNA-specific stem-loop primers of interest. In brief, for the RT step, microRNA-specific primers were pooled and diluted in 1X TE buffer to obtain a final concentration of 0.05X each. An RT mix was prepared containing 3 µl Multiscribe reverse transcriptase enzyme (50 U/µl), 1.5 µl 10X RT buffer, 0.19 µl RNAase inhibitor (20 U/µl), 0.3 µl dNTPs, 6 µl RT primer pool and 1.01 µl dH2O. For a 15 µl RT reaction, 3 µl RNA was mixed with 12 µl RT mix. RT cycling condition follows the same protocol as for individual microRNA analysis.

To increase the quantity of desired cDNA before performing PCR, a preAmp step was performed for each sample and for each microRNA. A preAmp primer pool mix was prepared by combining the TaqMan microRNA assays of the microRNAs studied and diluting it with 1X TE buffer to a final concentration of 0.2X. A preAmp reaction mix is then prepared from 3.75 µl of preAmp primer pool, 2.5 µl RT product, 12.5 µl of 2X TaqMan preAmp master mix and finally dH2O was added to a final volume of 25 µl. PreAmp cycling condition follows the same protocol as for OA microRNA profiling. RT-qPCR was then performed in 96 well plates following the same protocol described earlier in the individual microRNA expression analysis.

2.4.5 Absolute quantification of microRNA copy number using The QuantStudio™ 3D Digital PCR System:

Digital PCR (dPCR) was performed to obtain copy number estimates for CSF microRNAs. The QuantStudio™ 3D Digital PCR System is based on a silicon chip that allow detection and absolute quantification of nucleic acid. Each chip is divided into 20000 individual reaction wells. An automatic chip loading system allows for a random and uniform distribution of sample into the reaction wells in such a way that each well contain either one (positive) or zero (negative) target molecules. Within each well an individual endpoint PCR is performed. The fraction of positive and negative reaction wells is used to calculate an estimation number of target molecules present.
For Study III, a set of three representative CSF samples from each group (CH, TLE, SE and CND) were selected for the experiment. dPCR was performed according to manufacturer instruction. Briefly, total RNA was reverse transcribed using microRNA-specific RT primers following the protocol previously described for individual RT-qPCR. The optimal digital range of dPCR reaction was determined first for each microRNA under investigation. For this, the generated cDNA was serially diluted over the range 1:2, 1:4, 1:8, 1:16 and 1:32. Different dilutions were used as a template for dPCR using the QuantStudio 3D Digital PCR System (ThermoFisher Scientific). A dilution of 1:2 was determined as optimum for the three microRNAs (using this dilution, most of dPCR reaction wells contain either zero or one target molecule). For the dPCR reaction, 16 µl reaction mix was prepared containing 7.25µl dPCR master mix, 0.725 µl microRNA-specific TaqMan assays, 1.525 µl nuclease-free water and 6.5 µl diluted cDNA. The mix was then loaded automatically onto the individual QuantStudio v2 dPCR chips using the QuantStudio 3D Digital PCR Chip Loader. The PCR was performed in a Proflex 2X flat block thermal cycler (ThermoFisher Scientific) using standard conditions: 96 °C for 10 min; 40 cycles of 60 °C for 2 min and 98 °C for 30 sec and 60 °C for 2 min. Chips were then primarily processed using the QuantStudio 3D instrument to obtain the number of wells with an amplified target (FAM positive) and number of empty wells (FAM negative). Copy numbers were determined using the QuantStudio 3D AnalysisSuite Cloud software.

2.4.6 *In situ* hybridization (ISH) detection of microRNA

In order to localise microRNA expression *in vivo*, ISH analysis for CSF microRNAs was performed on formalin-fixed, paraffin-embedded sections using LNA probes (Exiqon). Hippocampal tissue sections were obtained after surgical resection from TLE patients or as postmortem tissue sections from patients died from severe intractable SE. The ISH protocol was adapted from Jimenez-Mateos et al. (2012). Briefly, miR-19b-3p, miR-21-5p and miR-451a signals were localized after an overnight incubation with digoxigenin (DIG) labelled, LNA detection probes (Exiqon). Secondary detection of the probe-microRNA interaction was performed using anti-
Dig antibody (Anti-DIG-POD Fab fragment) (Roche). Tyramide signal amplification plus fluorescence (Cy3) kit (Perkin Elmer) was used to amplify the in situ labelling of the target microRNA. Fluorescence labelling of astrocytes was performed using antibodies against the astrocyte marker, glial fibrillary acidic protein (GFAP, Sigma-Aldrich Ireland) and AlexaFluor488 secondary antibodies (ThermoFisher Scientific). Fluorescent signals were imaged using LSM 202 710 confocal laser scanning microscope (Zeiss).

2.5 Filtering and statistical analysis of the OA microRNA profiling data

All analyses were performed in R Bioconductor (http://www.R-project.org/) (Huber et al., 2015) with the help of Dr. Catherine Mooney (PhD). In general, the same OA data analysis steps were followed for the whole study. However, parameters for each step were changed according to the type of data obtained. These steps are: data filtering, normalization and differential expression. To ensure good quality detection and to avoid false-positives the OA data were filtered according to three criteria, cycle threshold (ct), amplification score (AmpScore) and quantification cycle confidence (cqconf) provided by the ExpressionSuite software (ThermoFisher Scientific).

2.5.1 Data analysis: Study I

The criteria for data filtering was set as ct < 35, Ampscore < 1.24 and cqconf < 0.8. The ct value of any microRNA that fails these criteria was set to 40. Next, to be included in further analysis, a microRNA should be expressed in >= 80% of samples in each study comparison. Using these criteria, 647 miRNAs were filtered out leaving 108 miRNAs in the final data analysis. Missing data points (ct = 40) were then imputed (Bioconductor package “Non-detects” (McCall et al., 2014)). Data were normalised using the deltaCt method (Livak and Schmittgen, 2001) as implemented in Bioconductor package “HTqPCR” (Dvinge and Bertone, 2009). The average expression of six endogenous control (EC) microRNAs, selected using a
Consensus between the top 10 most stable miRNA ranked by NormFinder (Andersen et al., 2004) and geNorm (Vandesompele J et al., 2002), was used for delta ct normalization (Table 2.4).

The following formula was used to obtain the normalized ct value (∆ct) for each microRNA (note that ct values are inversely related to expression level i.e. a lower ct value corresponds to higher microRNA expression): $\Delta ct = ct_{\text{miRNA}} - ct_{\text{EC}}$

Differential expression analysis was performed by applying a Student’s t-test to the normalized ct values between the two conditions and the p-values were adjusted for multiple testing by controlling the false discovery rate (FDR) according to the method of Benjamini and Hochberg (Benjamini and Hochberg, 1995). An estimation of fold change of a specific microRNA in specific biological state relative to a calibrator group was calculated using the comparative delta ct ($\Delta\Delta ct$) method:

$$\text{Fold change (FC)} = 2^{-\Delta\Delta ct}$$

where $\Delta\Delta ct = ((Ct_{\text{miRNA}}) - (Ct_{\text{EC}}))_{\text{time X}} - ((Ct_{\text{miRNA}}) - (Ct_{\text{EC}}))_{\text{time 0}}$

Time (X) refers to the PM sample (in the AM-PM comparison) or to day 2, 7 or 28 in Day-to-Day comparison. Time (0) refers to the calibrator sample for each comparison (AM or day 1 sample in AM-PM and Day-to-day study respectively). A microRNA was considered to be significantly differentially expressed if its log₂ fold change was > 1 and with adjusted p-value ≤ 0.05. The limma package (Ritchie et al., 2015) was used for analysis of the day-to-day samples.

2.5.2 Data analysis: Study II

To investigate for a common microRNA signature of TLE in MAR and DUB samples, data generated from both experiments were first combined and analysed. Initially, the same filtering and analysis criteria (as in Study I) were applied. However, an obvious biasing effect due to difference in sample origin was identified. This necessitated the application of more stringent data filtering and analysis criteria.
Table 2.4 microRNAs used for delta ct normalization method (Study I)

<table>
<thead>
<tr>
<th>NormFinder</th>
<th>geNorm</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-150b-5p</td>
<td>miR-150b-5p</td>
</tr>
<tr>
<td>miR-17-5p</td>
<td>miR-17-5p</td>
</tr>
<tr>
<td>miR-24-3p</td>
<td>miR-24-3p</td>
</tr>
<tr>
<td>miR-30c-5p</td>
<td>miR-26b-5p</td>
</tr>
<tr>
<td>miR-126-5p</td>
<td>miR-27a-3p</td>
</tr>
<tr>
<td>miR-1274b</td>
<td>miR-30c-5p</td>
</tr>
<tr>
<td>miR-223-3p</td>
<td>miR-106a-5p</td>
</tr>
<tr>
<td>miR-331-3p</td>
<td>miR-126-5p</td>
</tr>
<tr>
<td>miR-532-3p</td>
<td>miR-331-3p</td>
</tr>
</tbody>
</table>

Top 10 most stable microRNAs identified by NormFinder and geNorm. Six microRNAs were common to both methods. An average of these microRNAs was used for delta ct normalization.
Only microRNAs with a ct value <25 were kept for further analysis. This, in addition to batch correction using Combat (within the Bioconductor package –R) (Müller et al., 2016) and normalization, reduced the site-specific effect on microRNA expression. Three microRNAs (miR-19b, miR-26a and miR-26b) were identified by NormFinder and geNorm methods as being the most stable microRNAs in the study samples. An average of these microRNAs was used for delta ct normalization. Differential expression analysis followed in the same way as previously described for Study I.

2.5.3 Data analysis: Study III

The first step in Study III data analysis was data filtering by applying a ct cutoff of 35, in addition to AmpScore and cqconf filtering. This gave a general idea about microRNA expression in CSF. More in depth data analysis was then performed by applying a more stringent “present”/“absent” filtering step. This allowed the identification of microRNAs that are exclusively expressed (present) or exclusively unexpressed (absent) in CSF samples in one or more study groups. Accordingly, a microRNA was considered “present” in a set of samples if it was expressed in 60% of the samples with a ct cutoff of 28. Similarly, a microRNA was considered “absent” if it was undetermined in 80% of the same samples with the same ct cutoff. MicroRNAs were then removed from subsequent analysis unless they were either present in both control/TLE or control/SE samples, or if they were absent from one set of samples and present in the other. Missing data points were imputed using the Bioconductor package “non-detects” (McCall et al., 2014). Due to the low rate of microRNA detection in CSF samples, no microRNA was identified as stable across the study groups. Therefore, data was normalised to the geometric mean (GM) as implemented in Bioconductor package “HTqPCR” (Dvinge and Bertone, 2009) and by applying the following formula:

$$\Delta Ct = ct_{microRNA} – GM$$
Differential expression analysis of microRNA was then performed using the limma package. \( P \)-values were adjusted for multiple testing by controlling the FDR according to the method of Benjamini and Hochberg. A microRNA was considered to be differentially expressed if the adjusted \( p \)-value was < 0.05.

### 2.5.4 Validation of genome-wide microRNA results:

A number of differentially expressed microRNAs were selected for validation using individual RT-qPCR assays. For Study II, microRNA levels were normalised to average \( \text{ct} \) of miR-19b, miR-26a and miR-26b using the \( \Delta \text{Ct} \) method described above. For Study III, miR-24 was used for normalization (as in (Baraniskin et al., 2012)). All validations were done in triplicate. ANOVA followed by Tukey’s post-hoc test was carried out to test for any significant differences between the means. A microRNA was considered to be differentially expressed if the adjusted \( p \)-value was \( \leq 0.05 \).

In Study III, relative expression of Ago-2 bound and exosomal microRNA relative to total microRNA expression in samples was calculated as \( 2^{(\text{miR-Total} - \text{miR-AGO})} \) and \( 2^{(\text{miR-Total} - \text{miR-EXO})} \) respectively. Correlations were determined by computing Spearman’s rank correlation coefficient (\( \rho \)).

Receiver operating characteristic (ROC) curve analyses were performed using the R pROC package (Robin et al., 2011). Logistic regression analysis of the combined microRNA was carried out with the R.glm package using the normalised expression of microRNAs from qRT-PCR experiments as independent variable, and TLE or SE status as the dependent variable.

### 2.6 Pathway analyses and bioinformatics

For Studies II and III, experimentally validated targets for the differentially expressed microRNAs were retrieved from miRTarBase (Chou et al., 2016). Only human targets with strong evidence were retained (reporter assay, western blot, qRT-PCR or qPCR). Validated targets were cross-checked against two databases of epilepsy gene mutations: CarpeDB (http://carpedb.ua.edu) and epiGAD
microRNA-target interaction network was generated using Cytoscape (Shannon et al., 2003) with the help of Dr. Niamh M. Connolly (PhD). Genes identified as validated targets were uploaded to Enrichr (gene set enrichment analysis web server) (Kuleshov et al., 2016) to explore the gene ontology biological processes, molecular function and pathway involvement of the targets. The significantly associated gene ontology terms (adjusted p-value < 0.05) were imported to REVIGO where they were clustered based on their relatedness and any redundancy was removed (Supek et al., 2011). Significantly enriched PANTHER pathways (Mi et al., 2016), with adjusted p-value < 0.001 were exported from Enrichr and plotted in Microsoft Excel.

For Study III, validated targets of miR-19b-3p, miR-21-5p and miR-451a were further compared with validated human targets of 15 additional microRNAs previously implicated in epilepsy (miR-22-3p, miR-23b-5p, miR-34a-5p, miR-124-3p, miR-128-3p, miR-132-3p, miR-134-5p, miR-146a-5p, miR-155-5p, miR-184, miR-199a-5p, miR-203a-3p, miR-210-3p, miR-219a-5p, miR-324-5p), which each have in vivo functional data showing effects on seizures and/or pathology in rat or mouse epilepsy models (reviewed in (Henshall et al., 2016)). Both microRNA:microRNA and microRNA:target interactions network was generated using Cytoscape (Shannon et al., 2003) with the help of Dr. Niamh M. Connolly (PhD).
3.1 Introduction

Although circulating microRNAs have been proposed as biomarkers for several diseases (Wang et al., 2015c), we still lack the sufficient basic knowledge required to translate promising microRNAs into a clinically valid tests. The effect of different normal biological states and different experimental settings on microRNA expression needs to be studied extensively to ascertain that the change in microRNA level, undoubtedly, reflects the underlying pathological state. The observed variation in the results of different microRNA biomarker studies and the difficulty to reproduce them is still considered as a major obstacle. The reason behind these variations is mainly the large number of analytical and biological variables that affect microRNA detection and expression. Together, these variables have led to both false positive and false negative microRNA association (McDonald et al., 2011, Becker and Lockwood, 2013, Nair et al., 2014).

3.1.1 Experimental variables affecting microRNA expression patterns

A large number of preanalytical and analytical variables can affect the level of microRNAs in biofluid samples, particularly the type of sample, sample collection and processing variables, detection method, and data analysis methods (Kroh et al., 2010, McDonald et al., 2011, Zampetaki and Mayr, 2012, Moldovan et al., 2014). There is general agreement that all those variables need to be standardized in order to avoid any source of bias in the study.

The first critical step in the analysis of circulating microRNAs is sample collection and handling. The way blood is collected and processed for cell-free, circulating microRNA analysis can markedly affect study results (Nair et al., 2014). Important points to consider here are collection site, type and calibre of the needle/cannula used, type and size of the collection tube, time between collection and further processing, conditions of sample processing (centrifugation speed and time) and also storage conditions of the sample (Kirschner et al., 2013b). All those variables need to be standardized to avoid any effect on microRNA expression level.
Sample quality is of optimum importance. A number of factors (for example haemolysis and cellular contamination) might have a big impact on extracellular microRNA profiles. Red blood cells (RBCs) are the most abundant cellular fraction in blood (constitute about 55% of blood volume) and the release of their intracellular microRNAs due to haemolysis has a dramatic effect on the circulating microRNA profile that was initially underappreciated. Krischner et al. (2011) and McDonald et al. (2011) examined the effect of haemolysis on the expression levels of a limited number of microRNAs in the plasma or serum. Both concluded that rupturing of RBCs that usually occurs during blood collection or sample processing can have a profound effect on the level of many microRNAs. miR-16, miR-451, miR-15b, miR-92a were among the microRNAs that are highly susceptible to haemolysis. However, the level of liver-specific microRNA (miR-122) was not affected by haemolysis. In another study, Kirschner et al. (2013a) profiled the microRNA content of plasma samples with varying degrees of haemolysis and compared it to non-haemolysed samples. They found that the level of 26 microRNAs were increased at least two fold and their increase correlated with the degree of haemolysis. These findings emphasize it can be difficult to distinguish whether any increase in microRNA level is due to a controlled release from its tissues of origin or merely an effect of haemolysis.

Concerns remain that the majority of plasma microRNAs are blood cell-derived. In an experiment performed to evaluate the contribution of blood cell-derived microRNAs to the overall circulating microRNAs profile, Pritchard et al. (2012b) showed that about 58% of microRNAs previously reported to be biomarkers for different disease processes were highly expressed in one or more blood cell types. Indicating that microRNA expression represented cellular microRNA profiles rather than cell-free, disease specific profiles. Moreover, 91% of those microRNAs were also found in a significant amount in healthy plasma. Furthermore, they observed a significant positive correlation between blood cell-specific microRNAs and the blood cell count. They suggested that the changes in the level of those microRNAs reflect a secondary effect on blood cells rather than a disease specific effect.
In another study, Cheng et al. (2013) assumed that the differences in sample processing and low centrifugation speed will lead to inadequate removal of blood cells from the sample and this in turn, will significantly hamper microRNA downstream analysis. To check this, they performed a stepwise processing of plasma and serum from healthy donors to generate platelet concentrate, platelet rich sample, standard sample and platelet poor sample. The relative abundance of 365 microRNAs was then measured using RT-qPCR array. The results showed that the processing of standard samples into platelet poor samples removed 80-90% of residual platelets. However this had no effect on the exosomes and microparticle content. The levels of 72% of the detected microRNAs was affected by this extra processing step (specifically miR-142-3p, Let-7a, miR-223 and miR-16 were among the highly affected microRNAs). These results were in agreement with other work (Moret et al., 2013). Both studies indicated that an additional quality control step such as filtration or second centrifugation can minimize and control any bias resulting from platelet contamination.

The RNA isolation step may represent the least reproducible step and introduce most of the intra-assay imprecision (McDonald et al., 2011). The three commonly used methods for RNA extraction are a column-based purification using kits such as miRCURY biofluids kits (Exiqon) or a phenol-based technique that rely on the use of organic solvents, phase separation, and recovery of RNA by precipitation or a combined phenol and column-based technique that utilizes phenol and chloroform to separate RNA from other constituents, and a column for RNA adsorption, for example mirVana™ PARIS™ Kit and miRNeasy Serum/Plasma Kit (Qiagene). Recently, McAlexander et al. (2013) compared 5 commercially available kits/methods for RNA extraction from plasma; they also tested the effect of including glycogen as a carrier during the extraction. Their results indicated that the Exiqon miRCURY biofluids kit outperforms other isolation methods especially for isolating small RNAs from plasma. In another study Kim et al. (2012) reported an inefficient and a specific loss of microRNAs with low GC content during Trizol RNA extraction from low cell count samples (which is the case in biofluid samples).
According to this, Kirschner et al. (2013b) suggested that all studies investigating cell-free microRNAs should provide details about sample volume from which RNA was isolated, reagent or kit used, the use of a carrier and its concentration, the use of any spike-ins and their concentration and finally the volume used for re-suspension of the final RNA sample. For microRNA detection, a key problem is the low abundance of RNA in biofluid samples. A spectrophotometric analysis is the easiest and most common method used to quantify and assess RNA quality in a sample. However, biofluids RNA concentration is often under the detection limit of spectrophotometric devices (Kroh et al., 2010, Kirschner et al., 2013b) indicating that it will be difficult to use a fixed concentration of RNA as a template for the subsequent reverse transcription reaction. To overcome this problem, researchers have used an equal input volume of total RNA into the RT reaction (Mitchell et al., 2008).

The choice of microRNA profiling method will influence the experiment which can be performed and the results that will be obtained making comparisons between studies difficult (Mooney et al., 2015). A recent study compared the reproducibility, specificity, sensitivity, and accuracy of different profiling methods, across 12 platforms from 9 different vendors (Mestdagh et al., 2014). A better sensitivity and a higher overall detection rates was observed using RT-qPCR based methods (this included our current platform) compared to hybridization platforms. The RT and qPCR steps were regarded as the most reproducible steps and they contributed minimally to overall assay imprecision (McDonald et al., 2011). The minimum details that should be provided in this regard were amount of RNA used as an input for RT reaction, RT procedure, reagents used, and reaction conditions, details of the instrument used, any dilution of cDNA and amount of cDNA used for qPCR, all reagents and concentrations used for qPCR as well as reaction conditions and details on instrument used for qPCR (Kirschner et al., 2013b).

Data normalisation and analysis is regarded as a very critical step in any microRNA study regardless of the profiling method used. Its purpose is to minimize any
technical variability between different sample preparations (Nair et al., 2014). Using a fixed volume rather than a fixed amount of RNA as a template to study extracellular microRNA expression have a considerable effect on microRNA quantification as we are comparing microRNA expression in samples that differ in their RNA concentration (Kirschner et al., 2013b). To solve this problem several normalization strategies have been proposed by researchers. However, there is no general consensus for the best method.

The most common normalization method used is the normalization to either an endogenous reference gene or to known quantities of exogenous spiked-in microRNAs. A frequently used endogenous reference gene is miR-16 which was used for normalization by Kroh et al. (2010), Fayyad-Kazan et al. (2013), Bhatnagar et al. (2014) and many others. Using miR-16 for normalization has been criticized by the fact that its level is affected by presence of haemolysis in the blood sample (Krischner et al., 2011), and its association with at least 10 non-neoplastic diseases (Haider et al., 2014). Other studies used variable combination of microRNAs for normalization of circulating microRNA levels. However, the stable expression of these reference genes in health and disease conditions has not been established yet and studies are needed to find a suitable reference gene to normalize the circulating microRNA expression data.

On the other hand, it is now a common practice to spike-in samples with exogenous, non-human microRNAs (cel-miR-39 for example) to address technical variations particularly during the RNA extraction step. Normalization to known quantities of exogenous spiked-in microRNAs will allow normalization as well as estimating the efficiency of microRNA extraction and the RT step (Moldovan et al., 2014). This method of normalization was used by many researchers such as Mitchell et al. (2008) Brase et al. (2011) and Heegaard et al. (2012).

Large scale microRNA profiling studies require the use of more sophisticated normalization methods due to the large numbers of genes being studied. Some of these methods depend on choosing a three or more of the most stable microRNAs
throughout the study samples to be regarded as endogenous reference genes. Others found that the global mean (or the geometric mean) of microRNAs within each sample is a more stable normalizer when compared to one or a set of stable microRNAs (Wylie et al., 2011, Qureshi and Sacan, 2013). Other methods involve more complicated data handling such as quantile normalization method which assume that the overall distribution of signal does not change between samples and thus equalizing the distribution of expression intensities across many arrays will compensate for any technical source of variability (Meyer et al., 2010).

3.1.2 Biological variables affecting microRNA expression patterns

Stability of microRNAs in healthy controls is a prerequisite for their confident utilization as disease biomarkers. Moreover, any observed increase or decrease in microRNA expression should only reflect the underlying pathological state. However, the correct interpretation of these changes in microRNA expression as being clinically relevant is still very complex due to the large number of biological variabilities affecting microRNA levels which make it very difficult to replicate findings and discover true association. Numerous studies have described efforts to characterize variation in microRNA expression associated with different physiological states. Concerns have been expressed as to the effect of diurnal and day-day variation on circulating microRNA profiles (Hunter et al., 2008, Shende et al., 2011), the effect of aging (Hooten et al., 2013, Sawada et al., 2014), ethnic differences (Bovell et al., 2013), sex and in females difference throughout the menstrual cycle (Sawada et al., 2014, Morgan and Bale, 2012, Rekker et al., 2013), smoking (MacLellan et al., 2014), exercise (Baggish et al., 2011) and fasting (Kroh et al., 2010, MacLellan et al., 2014).

The well-documented oscillation of certain plasma proteins, electrolytes and hormones in response to the 24-hour light-dark cycle is mainly a reflection of rhythmic changes in gene expression that varies in circadian fashion. These changes are mainly derived by epigenetic and posttranscriptional mechanisms and microRNA regulation was suggested as an important effector in this regard (Azzi et
al., 2014, Kojima et al., 2011). Changes in microRNA expression over time are also essential to be identified especially if the microRNA biomarker was intended to monitor disease progression or treatment response. Only few studies have looked at the possible change in extracellular microRNA profile over time in healthy volunteers. These studies showed a minimal change in circulating microRNA levels when measured over several weeks to months (MacLellan et al., 2014, Rekker et al., 2013). Awareness of such difference in circulating microRNA level is of an obvious importance for biomarker studies. However, very little is known about the circulating microRNA circadian rhythmicity and day-day variation.

Age is another factor that might affect circulating microRNA profiles. At present, only few studies have identified significant age-related difference in microRNA levels both in tissues and in blood. Data obtained from experimental aging models have shown that specific microRNAs exhibited tissue-specific, age-dependent expression patterns (Ibáñez-Ventoso et al., 2006, Hooten et al., 2010, Li et al., 2011a). To identify age related changes in circulating microRNAs, Hooten et al. (2013) compared microRNA profile in serum from young and old individuals using RNA-seq technology. Their results showed that the serum level of several microRNAs significantly decreased with age. In separate studies, miR-21 in human plasma and miR-34a in mice plasma have been validated as an age related microRNAs where their level significantly increase with age (Li et al., 2011b, Olivieri et al., 2012). Knowing exactly which microRNA increase or decrease with age is very important particularly when searching for biomarkers for age related diseases such as cancer, cardiovascular and neurological disease.

Sex specific signatures in circulating microRNA profiles of male and female individuals have also been reported. Sex difference in microRNA expression in somatic tissues have been well characterised and the brain is the most extensively studied organ in this regard. Ziats and Rennert (2014) identified 40 microRNAs with significant sex-biased expression in different brain regions of normal human donors. A different study showed that 62% of microRNAs were differentially expressed
between male and female mouse neonatal brain (Morgan and Bale, 2012). Liver
expression of miR-26a was also reported to be higher in females than in males (Ji et
al., 2009). Other studies have found a difference in circulating microRNA profiles
between males and females. Duttagupta et al. (2011) profiled circulating microRNAs
in samples collected from 8 males and 10 females finding 4 microRNAs to be at
higher levels in females. Furthermore, male specific (miR-100, miR184 and miR-923)
and female specific (miR-222 ) microRNAs were identified in serum samples (Chen
et al., 2008).

Life style factors have also been investigated for their effect on extracellular
microRNA profiles. Smoking is one of the most studied factors, with variable results.
While MacLellan et al. (2014) demonstrated a minimal or even no effect of smoking
on circulating microRNAs in heathy individuals, others have found that cigarette
smoking significantly alters plasma microRNA profiles. Takahashi et al. (2013) found
an increased level of 43 microRNAs in smoker’s plasma when compared to non-
smokers while one microRNA was downregulated. Interestingly, within one month
of quitting smoking plasma levels of the upregulated microRNAs returned to the
non-smoking levels suggesting that plasma microRNAs were unambiguously
affected by smoking. Another study analysed the microRNA content of plasma
microvesicles and found that smoking led to changes in their microRNA content
(Badrnya et al., 2014). Altered microRNA signatures have also been reported after
exposure to many other factors including air pollution, nanoparticles and chemical
reagents (Reviewed in (Vrijens et al. (2015)).

Taken together, research has indicated the importance of microRNAs as disease
biomarkers. However, their expression is prone to a significant bias originating from
large number of analytical and preanalytical variables. Identification of these
variables and optimization of microRNA profiling methods are important factors for
any microRNA study.
3.1.3 Chapter summary

Hypothesis: Plasma microRNAs are stable in healthy controls and display little intra-individual variability over time.

Aims: to characterise microRNA stability in plasma of healthy controls focusing on morning-afternoon and day-to-day variation. Sex difference in microRNA profile will also be analysed. Genome wide microRNA profiling will be carried out using a recently developed high throughput screening platform, the QuantStudio™ 12K Flex PCR system (ThermoFisher Scientific) that utilizes TaqMan OA human microRNA panels. The platform developed from the earlier TaqMan Low Density Arrays (TLDA) cards (AB) which our group successfully used to profile microRNAs in brain tissues of experimental model of epilepsy.

In this first chapter of results, I sought to gain experience in processing and analysing different sample types, different RNA extraction methods, in the genome wide microRNA profiling using the OA platform and the best data analysis conditions. Here, commonly used RNA extraction methods have been compared for their efficiency in microRNA isolation from different blood sample types and from varying amounts of input material. The assessment of microRNA recovery has been evaluated by RT-qPCR using miR-specific primers. In particular, the performance of a phenol- (Trizol® LS), a column- (miRCURY™ biofluids kits) and a combined phenol-and column-based (miRNeasy Serum/Plasma Kit) RNA isolation techniques was evaluated. In addition, the impact of the amount of starting material on the efficiency of microRNA extraction was investigated. The effect of other experimental variables such as sample quality and effect of haemolysis on circulating microRNA profiles will also be discussed at the same time as learning about the stability of plasma microRNA profiles in healthy controls.
3.2 Results

3.2.1 Pre-profiling standardization of methods

The first goal was to characterise the effect of different experimental settings and conditions on microRNA expression profile. Starting with type of sample used and sample quality control and continuing through microRNA profiling and data analysis.

3.2.1.a Effect of sample type

The first decision to make when profiling circulating microRNA is the type of sample to use. Both plasma and serum are widely used clinical samples due to their ease of collection. Here we compared plasma and serum expression of microRNAs that are known to be more abundant/less abundant in blood (miR-92a and miR-103 respectively). First, 2500µl of mouse blood was collected and divided into 5 tubes, each contain different concentration of the anticoagulant EDTA (ranging from 0.5 to 2 mg/ml). The level miR-92a and miR-103 was checked using miR- specific primers. Results showed that the more EDTA in the sample (plasma), the lower the ct value for both microRNAs and hence reflecting more RNA concentration (Figure 3.1A). To determine whether this difference is real and was not due to selective loss of RISC complex in response to different EDTA concentration of each sample, protein was extracted and the concentration of Ago-2 protein in samples was analysed with no observed difference (Figure 3.1B).

Second, EDTA has been reported as a PCR inhibitor due to its ability to chelate Mg$$^{++}$$ ions thus reducing the activity and fidelity of Taq polymerase in PCR reaction. Increasing Mg$$^{++}$$ concentration in the sample can compensate for this effect (Khosravinia and Ramesha, 2007). To test this, blood was collected into 2 EDTA tubes containing 2mg/ml EDTA. 2mM MgCl$_2$ was then added to one tube after plasma collection and RNA was extracted from both tubes side by side (Figure 3.1C). Successful and matching amplification of miR-185, miR-22 and miR-134 was observed in both EDTA and EDTA+Mg samples suggesting a minimal inhibition and a valid PCR reaction protocol.
Figure 3.1: Effect of sample type on extracellular microRNA expression.

(A) Expression of miR-92a and miR-103 in blood samples collected in increasing concentrations of EDTA (0.5-2.0 mg/ml) (n=1 for each concentration). Increased EDTA is associated with more microRNAs in the sample. (B) level of Ago-2 protein in the same samples used for (A), no difference in Ago-2 protein levels was noticed indicating that the observed difference in RNA concentration is not due to altered of RISC complex in the sample. (C) To assess the efficiency of PCR reaction in EDTA sample, MgCl2 was added to one sample and expression levels of miR-185, miR-22, miR-140 and miR-134 was compared to another sample without MgCl2. No significant difference was observed. (D) Raw ct values of miR-16 and miR-146a in plasma and serum samples collected from one healthy volunteer. RNA extraction and microRNA expression was performed simultaneously by two researchers (A and B) to assess reproducibility. Raw ct values were consistently lower in plasma compared to serum, reflecting more RNA in plasma samples.

Ago-2: Argonaute 2 protein; ct: cycle threshold; P: plasma, P+: plasma with MgCl2, S: serum.
Third, trying to replicate these outcomes in human samples showed similar results. Blood was collected from one healthy subject (female, 35 years old); 4 ml of blood was directly collected either into EDTA tubes (containing 1.8 mg/ml K2EDTA) to generate plasma or into a serum separator tubes for serum. Samples were analysed for miR-16 and miR-146a content by 2 researchers side by side to assess reproducibility. miR-16 was chosen due to its abundance in both plasma and serum samples. miR-146a was chosen due to previous reports of its role in epilepsy and other inflammatory conditions (Omran et al., 2012). When using plasma samples, microRNAs were expressed at slightly lower ct values and with better reproducibility, suggesting that plasma samples might contain more RNA concentration and at the same time minimal effect of EDTA on PCR reaction (Figure 3.1D).

3.2.1.b Effect of haemolysis

The effect of haemolysis (defined as 414 Absorbance > 0.20 nm) on microRNA in plasma samples of 20 healthy volunteers was investigated. The level of 3 haemolysis sensitive microRNAs (miR-16, miR-92 and miR-451) and 3 haemolysis non-sensitive microRNAs (miR-127, miR-324-5p and miR-130b) based on work of Kirschner et al. (2013a), was measured in 12 haemolysis free (H-) and 8 samples with variable degrees of haemolysis (H+). As expected, higher variation in fold change of haemolysis sensitive microRNAs was found in H+ samples compared to H- (Figure 3.2A and B). Accordingly, only the samples that were haemolysis free (414 absorbance by Nanodrop <0.20) were included for further analysis.
Figure 3.2: Effect of haemolysis on microRNA expression.

Expression of 3 haemolysis sensitive (miR-16, miR-92, miR-451a) (A) and 3 haemolysis non-sensitive (miR-127, miR-324-5p, miR-130b) (B) microRNAs were measured in 12 haemolysis free (H-) and 8 samples with variable degrees of haemolysis (H+). Higher variability in microRNA expression was detected in miR-16, miR-92a and miR-451a compared to miR-127, miR-324-5p and miR-130.

H-: haemolysis free samples, H+: haemolysed samples.
3.2.1.c RNA extraction method

Previous work by Kim et al. (2012) have argued whether different methods of total RNA preparation would efficiently yield all types of RNA molecules regardless the extraction method used. Their work demonstrated a specific loss of certain microRNAs (with low-G-C content) during TRIzolLS extraction from low density cell culture media. Plasma is regarded as a cell-free biological sample and thus it might be vulnerable to the same bias when extracting RNA and to our knowledge no one has tested this before. To examine this, 4 ml of plasma were collected from one healthy subject (female, 35 years old) and the yield of TRIzolLS RNA extraction was compared to that of a column based RNA extraction method, miRCURY™ RNA Isolation Kit-biofluid (Exiqon). The average expression of low (miR-141) and moderately (miR-140 and miR-22) abundant G-C microRNAs was examined. While the RNA extraction method has only a minimal effect on miR-140 and miR-22 levels, a gain in miR-141 was noted when using the miRCURY™ RNA Isolation Kit-biofluid from Exiqon (Figure 3.3A). Thus the miRCURY™ RNA Isolation Kit-biofluid is not vulnerable to G-C bias.

Next, the RNA yield of a column based RNA extraction method (miRCURY™ RNA Isolation Kit-biofluid from Exiqon) was compared to that of a combined column and phenol based extraction method (miRNeasy Serum/Plasma Kit from Qiagene). The level of two abundant microRNAs (miR-16 and miR-92a) were analysed in plasma samples collected from 3 healthy volunteers (Figure 3.3 B). Both microRNAs were consistently expressed at a slightly lower ct values when using the miRCURY™ RNA Isolation Kit-biofluid. However, it must be considered that these results were obtained from 25 µl elution volume for miRCURY™ RNA Isolation Kit-biofluid versus only 14 µl for miRNeasy Serum/Plasma Kit, which means that either the total RNA was not appropriately recovered from the columns due to low elution volume or a lower extraction efficiency of the Qiagen kit.
Figure 3.3: Effect of different RNA extraction methods and different starting plasma volumes on the total RNA yield from plasma samples.

(A) Using plasma sample collected from one healthy volunteer, TRIzol extraction was compared to miRCURY RNA Isolation Kit-biofluid. The low G-C microRNA (miR-141) was expressed in a higher ct using Trizol suggesting loss of this microRNA by this method. In (B) miR-16 and miR-92a levels were compared in three plasma samples collected from healthy volunteers. From each sample, RNA was extracted using either miRCURY RNA Isolation Kit-biofluid or miRNeasy Serum/Plasma Kit. A slightly lower ct values were obtained with miRCURY RNA Isolation Kit-biofluid. In (C) the effect of different starting plasma volumes was assessed. RNA was extracted from 200, 100, and 50 µl of 4 plasma samples using miRCURY RNA Isolation Kit-biofluid. miR-16 and miR-134 levels in the samples increased as the starting volume increase. Ct: cycle threshold; E: miRCURY RNA Isolation Kit-biofluid/Exiqon, Q: miRNeasy Serum/Plasma Kit/Qiagene.
An additional experiment was then performed to assess the impact of different starting plasma volume on microRNA yield of the miRCURY™ RNA Isolation Kit-biofluid. Plasma was collected and processed from 4 individuals, total RNA was extracted from lowest, medium and highest plasma volumes recommended by the manufacturer (50, 100 and 200µl respectively). A very clear trend was noticed for the tested microRNAs (miR-16 and miR-134). The more volume we used the lower the ct values (Figure 3.3 C). We did not exceed 200 µl as this volume is the highest volume to be used due to the limited capacity of the column. This is particularly important since the use of a large amount of starting material was proposed to reduce the RNA in the sample through column saturation.

All together, these data demonstrated that the miRCURY™ RNA Isolation Kit-biofluid allowed a better detection of microRNAs from plasma when compared to other different RNA extraction methods. In addition, the miRCURY™ Kit protocol is faster, less technically demanding and subjected to less technical variation due to the lack of phase separation step so we decided to use it for all our subsequent experiments.

3.2.2 Genome-wide microRNA profiling using the QuantStudio™ 12K Flex OpenArray system

3.2.2.a Similarity to plasma profiles on other platforms

Twenty plasma samples (10 males and 10 females) were collected from healthy volunteers and profiled using the QuantStudio™ 12K Flex OA system. The obtained results have identified 108 individual microRNAs in at least 80% of all samples. The list of microRNA detected using the OA was then compared to results obtained from 6 other microRNA profiling studies that used healthy volunteer’s plasma or serum (Table 3.1 and 3.2). A large degree of variation between the lists of microRNA being detected by different platforms was found indicating a big impact of the profiling method on results of microRNA studies.
Table 3.1: Number of microRNAs commonly expressed in the present study and in 6 other microRNA profiling studies in healthy volunteers blood samples.

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(A) the present study; (B) Blondal et al. (2013); (C) Chen et al. (2008); (D) Mitchell et al. (2008); (E) Wang et al. (2012a); (F) Wang et al. (2012a); (G) Weber et al. (2010).
Table 3.2: list of 50 microRNAs that were detected in our profiling and in most of the other 6 profiling studies.

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(A) the present study; (B) Blondal et al. (2013); (C) Chen et al. (2008); (D) Mitchell et al. (2008); (E) Wang et al. (2012a); (F) Wang et al. (2012a); (G) Weber et al. (2010).
Interestingly, two experiments used Exiqon panels for microRNA detection. While Wang et al. (2012a) displayed the least similar results to our list with only 46 microRNAs in common between the two, Blondal et al. (2013) displayed the highest similarity with 68 microRNAs in common (Table 3.1). These results would support the importance of independent validation of any potential biomarkers by using a different platform. Furthermore, we have detected is a set of about 50 microRNA (Table 3.2) that are common to most of the studies most of the time. The top 10 most abundant plasma microRNAs in our study were miR-16-5p, miR-19b-3p, miR-24-3p, miR-92a-3p, miR-146a-5p, miR-20a-5p, miR-451a, miR-106-5p, miR-126-3p and miR-223-3p.

3.2.2.b Predicted cellular origin of microRNA in plasma samples

The possible cellular origin of each of the 108 microRNA in our data set was examined by cross checking them against the 100 most highly expressed microRNA in each of 18 unique cell types generated by Haider et al. (2014) and reflecting a broad range of most major cell types: epithelial, endothelial, mesenchymal, hematopoietic, and muscle cells (Table 3.3). From our 108 microRNAs, 25 were ubiquitously expressed and found in most cell types. 78 microRNAs were found in at least one and 38 were found in all of the seven hematopoietic cell types (centroblast, memory B cell, monocyte, naive B cell, Natural-killer cell, plasma B cell and RBCs). 13 microRNAs were not found in any of the haematopoietic cell types but had possible cellular origin in at least one of the other 11 cell types (Table 3.4).
### Table 3.3: Possible cellular origin of miRNA identified in plasma samples.

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**Key:**
a—acinar cell; b—adipocyte; c—ductal cell; d—endothelial; e—epithelial cell; f—fibroblast; g—hepatocyte; h—lymphatic EC; i—myocyte; j—neutrophil; k—smooth muscle cell; l—centroblast; m—memory B cell; n—monocyte; o—naive B cell; p—NK cell; q—plasma B cell; and r—red blood cell.

An asterisk is placed in the column if the miRNA is found in the top 100 of miRNA expressed in that cell type. Expression profiles for all cells taken from Haider et al. (2014). miRNA are included if they are identified in at least one cell type.
Table 3.4: Predicted cellular origin of microRNA in plasma samples.

<table>
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<th>All blood cell types</th>
<th>Not blood cell</th>
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From 108 detectable microRNAs using our platform, 25 were ubiquitously expressed and found in most cell types and 38 were found in all of the seven haematopoietic cell types. 13 microRNAs were not found in any of the haematopoietic cell types but had possible cellular origin in at least one of the other 11 cell types.
3.2.2.c Effect of storage on microRNA expression

To assess the effect of freezing and long-time storage on microRNA expression in RNA samples, single plasma sample was collected from a 28-year-old male volunteer. RNA was extracted and the sample split into two parts (R1 and R2). Both parts were processed and profiled using the OA system, the only difference being that the RNA from second part (R2) was frozen (at −80°C) for six months before processing and profiling. The data was filtered by CqConf and AmpScore, was not normalised and the missing values were not imputed. 122 microRNAs were detected in sample R1 and 128 microRNAs were detected in sample R2, 91 were common to both. A scatterplot of the Ct values in sample R1 against sample R2 showed a high degree of correlation between the Ct values in the two samples (Pearson’s correlation coefficient, r = 0.89) (Figure 3.4A), which increased when only high confidence Ct values, Ct ≤ 25, were included (r = 0.95) (Figure 3.4B).

3.2.3 Inter and intra individual variability of microRNAs in plasma of healthy individuals

3.2.3.a Morning-afternoon variation in microRNA profiles

Timing of blood collection (day or night) has been suggested to have an effect on microRNA expression. This is important in the context of biomarker discovery in epilepsy because we may need to process patient’s plasma samples at different time points in relation to seizures. Furthermore, a day/night pattern of seizure frequency is evident in patients with TLE and is associated with an additional interaction with sleep/wake state (Pavlova et al., 2004a). To assess the impact of morning-afternoon variation on microRNA profile, blood was collected from 10 healthy volunteers at two time points. A morning sample was collected between 9:30–10:30 am and an afternoon sample was collected between 4:00–5:00 pm. A principal component analysis showed no clustering of the samples based on timing of blood collection (Figure 3.5). More in depth analysis showed no significantly differentially expressed microRNAs between the morning and afternoon samples. This suggests that timing of blood collection should not interfere with biomarker studies.
Figure 3.4: Effect of storage on microRNA expression
Pairwise scatterplot of un-normalized data obtained from 2 OA runs (R1 and R2). Data were filtered by CqConf and AmpScore, without imputing the missing values. 122 microRNAs were detected in sample R1 and 128 microRNAs were detected in sample R2. 91 microRNAs were common to both. A high degree of correlation was observed between the two runs. (A) Ct > 35 are not included in the calculation of the correlation (B) Ct > 25 are not included in the calculation of the correlation.

3.2.3.b Day-to-day variation in microRNA profiles
To evaluate microRNA profile stability in healthy individuals over a period of time, plasma was collected between 9:30 and 10:30 am, on 4 days over a one month period (day 1, day 2, day 7 and day 28). This group included 5 males and 5 females.
No significant difference in microRNA expression over this one month was noticed (see principal component analysis Figure 3.6A). Although there was a slightly higher mean variance across the microRNA in the female samples compared to the male sample (2.15 in females, 1.8 in males), this was not found to be statistically significant. Figure 3.6B shows the variation in Ct values over the four sample times for the most abundant microRNA in these samples.

3.2.3.c Male-female difference in microRNA profiles

To check for a possibility of differential microRNA expression between males and females, microRNA expression was compared in 10 males to 10 females. Data analysis showed that there was no significant difference between the male and female samples both after performing a principle component analysis (PCA) (Figure 3.7) and differential expression analysis. There was no significant difference in the number of microRNA detected in the male or female samples. We did not find any microRNA unique to either all male or female samples. The top 12 most abundant microRNA were the same for the male and female samples which were: miR-223-3p, miR-16-5p, miR-19b-3p, miR-106a-5p, miR-17-5p, miR-20a-5p, miR-191-5p, miR-24-3p, miR-126-3p, miR-451a, miR-92a-3p and miR-146a-5p.
Figure 3.5: Morning-afternoon variation in microRNA profile.

Principle component analysis (PCA) showing the morning (AM) and afternoon (PM) samples collected from 5 females and 5 males. No obvious clustering was noticed (Plot created with R package ggbiplot).
Figure 3.6: Day-to-day variation in microRNA profile.

(A) Principle component analysis showing the samples from 5 females and 5 males collected over one month period. No obvious clustering was noticed (Plot created with R package ggbiplot). (B) Variation in Ct values over the four time points for the most abundant microRNA in these samples also show minimum differences. Statistical analysis of the normalized ct values of these microRNAs failed to identify any significant difference in their expression. This was performed using ANOVA and the p-values were adjusted for multiple testing by controlling the false discovery rate (FDR) according to the method of Benjamini and Hochberg.
Figure 3.7: Sex differences in microRNA profile.

Principle component analysis showing the samples collected from 10 females and 10 males. No obvious clustering was noticed (Plot created with R package ggbioplot).
3.3 Discussion

Extracellular microRNAs have emerged as a promising class of biomarker due to their tissue specificity, availability in biofluids and most importantly due to their stability in those fluids (Gilad et al., 2008; Mitchel et al., 2008). However, utilizing extracellular microRNAs as biomarkers for diseases faces significant challenges arising from their low abundance as well as the existence of many preanalytical and analytical variables that mask or bias microRNA expression profiles in health and disease (McDonald et al., 2011). The key questions of this chapter tried to address these challenges. First, the low copy number of microRNAs in biofluids necessitates the use of a highly sensitive method for their accurate detection that generates a minimum false positive data. For this purpose, the performance of the QuantStudio™ 12K Flex OA system (ThermoFisher Scientific) was investigated and compared to results of other studies using other platforms. Next, the effect of different normal biological states (mainly sex and blood collection time) and experimental conditions on plasma microRNA profile was investigated. The answers to both questions is vital for the subsequent work in relation to role of extracellular microRNAs as biomarkers for TLE.

The QuantStudio™ 12K Flex TaqMan OA system used here to profile plasma microRNAs utilizes a nanoliter fluidic panel which allows measurement of 755 microRNAs in the sample in addition to negative controls and endogenous controls (RNU48, RNU44 and U6 rRNA). TLDA cards (AB) and Exiqon panels systems allow measurement of 383 and 668 microRNAs respectively, including the negative and endogenous controls (Wang et al., 2012a). Thus, the present platform offers one of the most comprehensive analysis techniques for studying microRNAs. It covers the majority of microRNAs reported to be expressed in healthy control plasma in addition to a wide range of tissue specific and tissue enriched microRNAs including brain microRNAs. Technically, the OA has the advantage of being exceptionally fast with a number of quality control measures to monitor the performance and precision of the experiment. The automated system available for panel loading
further simplify the associated work and most importantly reduce the bias originating from this step.

In the present study, the average number of detectable microRNAs in the samples was 268 (range 198-363). 108 microRNAs were common in more than 80% of samples (after filtering depending on the AmpScore and CqConf values). This Figure is in line with other microRNA genome wide profiling studies. Wang et al. (2012a) compared plasma and serum microRNA expression profile using two methods, TaqMan cards and Exiqon panels, in 3 males and 3 females healthy volunteers. Using TaqMan cards the number of detectable microRNAs in the plasma ranged from 137-234, only 106 microRNAs could be detected in all samples. With Exiqon panels the average number of detectable microRNAs was 181 (range 123-296) but only 90 microRNAs were common in all the plasma samples. The top 5 most abundant plasma microRNA using TaqMan cards and Exiqon panels were (miR-19b, miR-223, miR-92a, miR-146a and miR-126) and (miR-451, miR-223, miR-16, miR-19b and miR-92a). Notably, these microRNAs were among the top 10 most abundant microRNAs in the present study. Thus our study shares a good concordance with other microRNA profiling work using a similar platform and compares well to Exiqon platform as well. In the present study, the average ct of all microRNAs in the samples profiled was 23.5 (range 22.0-25.6). miR-223 was the most abundant microRNA in all samples with an average ct value of 10.5. Of note, miR-146a was one of the top 10 most abundant microRNAs expressed in the plasma of healthy controls with an average Ct of 16.6. It was ranked the top fourth microRNA by Wang et al. (2012a). Brain levels of miR-146a have been found to be upregulated after status epilepticus in animal models of epilepsy (Aronica et al., 2010, Omran et al., 2012, Gorter et al., 2014). The increased circulating level of this microRNA have been proposed as biomarkers in TLE (Aronica et al., 2010), sepsis (Wang et al., 2010), peripartum cardiomyopathy (Halkein et al., 2013) and for colorectal tumour localization (Omrane et al., 2014). However the high level of this and other highly abundant microRNAs in the plasma of healthy volunteers could hamper their use as disease biomarkers. A careful approach needs to be done when utilizing those highly abundant microRNAs as biomarkers.
A comprehensive assessment of different microRNA profiling platforms using quantifiable performance metrics was reported in 2014 involving all the major vendors of microRNA profiling technologies including 7 qPCR, 5 hybridization and 7 Sequencing technologies (Mestdagh et al., 2014). All the technologies were assessed for reproducibility, accuracy, sensitivity, specificity and detection rate. 196 microRNAs were identified to be expressed on all the platforms. However, a great deal of difference in the list of microRNAs detected and in sensitivity and reproducibility between the platforms has been observed. This in part could be explained by the difference in the amount of input RNA specific for each method, however, difference in performance metrics between platforms are as well responsible for this variability.

In a study published recently, (Farr et al., 2015) performed a thorough assessment of the performance of the OA system for studying plasma microRNAs and compared it to three other qPCR based platforms including the gold standard 96 well plate. All the technologies were assessed for reproducibility, accuracy, sensitivity, and specificity. In addition, the ability to detect a disease signature was then compared to results from RNA-seq. Their analysis showed that the OA system was the most reproducible with the least inter- and intra-run variability which correlates well with our results. However, one should be careful when analysing the low abundance microRNAs transcripts (ct > 30) as the variability in replicates was very evident in all the high throughput platforms. This was also detected in our analysis; a higher degree of correlation was detected when including only the microRNAs with high confidence ct values (< 25) in the analysis (Pearson’s correlation coefficient, $r = 0.95$).

Another key objective for the present study was to assess an appropriate method for data normalization to be implemented for subsequent analyses. The main goal of data normalization is to minimize the effect of systemic experimental bias and technical variation on the study results, thus only the results that represents a true biological difference related to the condition being studied will be evident (Meyer et al., 2010, Pradervand et al., 2009). It has been suggested that rigorous normalization of microRNA data may be more critical than that of other RNA classes.
since relatively small changes in microRNA expression may be biologically and clinically significant (Peltier and Latham, 2008, Chang et al., 2010).

Many normalization techniques have been tried and validated by researchers in the field of microRNA studies. However, no agreement is reached about the best method (Meyer et al., 2010, Rai et al., 2012, Mohammadian et al., 2013). Wylie et al. (2011) compared seven methods for the normalisation of microRNA expression from biofluid and found that methods that focus on a restricted set of microRNAs tended to perform better than methods which focus on all microRNAs. The choice of this set of microRNA is challenging as so called “housekeeping genes” which may be stable in a given cell type or experimental condition can vary considerably under disease conditions and between different tissues or biofluids, and no universally invariant microRNA or any other small RNA molecule has been found to date (Weber et al., 2010, Moldovan et al., 2014, Kroh et al., 2010, Mestdagh et al., 2009).

In the present study, NormFinder and GeNorm algorithms were used to identify the top 10 most stable microRNAs in the plasma samples. 6 microRNAs were found to be common to both algorithms (miR-15b, miR-24, miR-30c, miR-126-5p, miR-331 and miR-17). These microRNAs were used for delta ct normalization method.

The next key objective of this project was to establish with certainty how stable microRNAs are in healthy people focusing on the morning-Afternoon variation and day-day variation of microRNAs expression in healthy controls. The reasoning here was to obtain comprehensive data on how plasma microRNAs behave normally. Indeed, it has been suggested by researchers that blood collection timing (day or night) might have an effect on microRNA expression (Moldovan et al., 2014). This is important in the context of epilepsy where seizures can occur at any time and any biasing factor must be known. Thus, awareness of a morning-afternoon change in circulating microRNA levels is very important. Earlier studies provided evidences for the presence of rhythmically expressed microRNAs in various tissues. Cheng et al. (2007) examined the role of two brain specific microRNAs (miR-219 and miR-132) in modulating the circadian clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus of mouse brain. Their results showed a significant higher level of miR-219 in the SCN during the day than during the night. Likewise, miR-132
exhibited a modest but also significant rhythm with the peak level occurring during the day.

In another study, Na et al. (2009) performed a microarray expression profile study of both microRNA and mRNA in mouse liver, 85 hepatic microRNAs were found to be expressed in circadian manner. Verified targets of those microRNAs were oncogenes and cell cycle regulatory proteins. Inversely correlated circadian expression pattern between miR-181d and miR-191 and their targets (Clock and Bmal1) were observed. More recently, Kinoshita et al. (2014) investigated the diurnal variation in Glutathione level in mouse central nervous system and the role of microRNAs in this diurnal variation. They first observed rhythmic diurnal expression of glutathione in the mesencephalon. The highest and lowest levels were observed at midnight and midday respectively. To look for a possible role of microRNAs in this rhythmic expression, they examined microRNA expression in the mesencephalon at 6 hours intervals. Their search identified 20 microRNAs with diurnal oscillation (more than 1.5 fold changes). Three microRNAs (miR-96-5p, miR-199a-5p and miR-200a-3p) were then chosen as candidates for further evaluation (they target the 3’-UTR of EAAC1, a key protein in Glutathione synthesis pathway). Their results showed that levels of those microRNAs oscillate in a diurnal manner. miR-96-5p reached a maximum one hour before lights were on, and a minimum at one hour before lights were off. miR-199a-5p and miR-200a-3p had larger amplitudes, with more than 3 fold changes and peaks 5 hours after lights on and 2 hours after lights off, respectively.

The first evidence supporting circadian fluctuation in circulation microRNA levels came in 2011. Levels of certain microRNAs predicted to target the clock gene, Bmal1, were found to fluctuate in mouse serum in a circadian rhythm dependent fashion (Shende et al., 2011). Of those microRNAs, levels of miR-152 and miR-494 were marked by diurnal oscillation with bimodal peaks in expression occurring near the middle of the day and 8-12 hour later during the night. However, human data are still very little in this regard. In the present study, to investigate for such an effect, a morning and an afternoon plasma sample was collected from 10 healthy volunteers. The results demonstrated a minimal difference between these two time
points, implying that this type of variability in plasma microRNAs should not interfere with an epilepsy biomarker study. The levels of the previously reported microRNAs were specifically checked and no significant changes were found. However, a very recent study performed by Heegaard et al. (2016) analysed the level of expression of 96 microRNAs in plasma samples of 24 healthy male volunteers collected every 3 hours over 24 hours. MicroRNAs were chosen based on a previously observed fluctuation of their levels in brain tissue samples in animal studies or a predicted targeting for the clock genes by literatures and bioinformatics screening. Other microRNAs known to be abundant in plasma and/or CNS were included in the study as well. Bimodal oscillation (only 2 peaks over 24 hours) was observed in the levels of 26 microRNAs. 12 and 14 of them displayed a nocturnal (between 23:00-8:00) and a diurnal (between 8:00-23:00) expression peaks respectively. A consistent 12 hours interval between the maximum and the minimum expression level has been observed which may explain why we couldn’t identify this fluctuation, effect of other environmental factors (such as timing of food intake, exercise, etc.) is another possible explanation.

Collectively, those studies provide evidence for the presence of a number of rhythmically expressed microRNAs in various animal tissues and as well in human plasma. However, none of the observed changes were detected in our study. This of course can be attributed to the difference in the study design. In order to observe the circadian rhythmicity in the level of any molecule, the subjects included in the study should be kept within a controlled environment for certain amount of time before sampling to escape any profound effect of any environmental factor (which is the case in experimental animals). This, however, was difficult to be achieved in our study.

An important aspect to consider in every biomarker study is the possibility of changes in biomarker levels over time in the healthy population. In order to evaluate plasma microRNA variability in healthy individuals, we measured the levels of microRNAs in plasma samples taken from healthy controls over a period of one month. No significant difference in the level of the screened microRNAs was observed over this one month period. This is in agreement with a study by
MacLellan et al. (2014) using serum from healthy volunteers across 2 time points (range between 7-15 months). They found a strong correlation between samples collected from the same person and no microRNAs were found to be significantly deregulated between time matched samples. Moreover, no significant differences were found in plasma microRNA levels in women throughout different phases of menstrual cycle (Rekker et al., 2013). This further confirms the suitability of microRNAs to be implemented as disease biomarkers.

In regard to sex variation in microRNA expression, similar to Hunter et al. (2008) we did not observe any significant differences between male and female microRNA profiles. However, other groups have detected differences. Four microRNAs were found to be significantly upregulated in females (miR-548-3p, miR-1323, miR-940 and miR-1292) in another study, Chen et al. (2008) identified miR-222 as female specific microRNA and miR-100, miR-184, and miR-923 as male specific microRNAs. In our data, miR-548-3p, miR-1323, miR-940, miR-1292, miR-184 and miR-923 were not identified as being dysregulated due to sex difference. In addition, we identified miR-222 in 9 out of 10 of both the female and male samples and miR-100 was identified in 5 female samples and 2 male samples.

In addition to the effect of biological factors on circulating microRNA levels, the effect of different experimental variables has been also investigated. In the clinical setting, both plasma and serum were used to profile circulating microRNAs with no preference of one of them over the other. The essential difference between plasma and serum is the presence and absence, respectively, of fibrinogen and clotting factors. More importantly, platelets contain a wide spectrum of microRNAs that will be released into the serum during coagulation (Wang et al., 2012a).

Comparing serum and plasma microRNA profiles side-by-side was performed early after the discovery of circulating microRNAs. However, data are not consistent. Some studies reported a more RNA yield and more number of detected microRNAs from serum samples compared to plasma (118 and 106 respectively) when doing a genome-wide microRNA profile (Wang et al., 2012a). The higher RNA concentration in serum samples was interpreted on the bases of the coagulation process which is
considered as a stressful environment leading to stimulated release of platelet associated microRNAs into the circulation. Others reported higher microRNA concentration in plasma which is in agreement with our findings (McDonald et al., 2011). In contrast to above findings, a strong correlation of microRNA’s raw ct value between plasma and serum samples was reported by Mitchell et al. (2008). In our study, we analysed the expression of a set of microRNAs in plasma and serum samples. Results showed a consistent lower ct values for the studied microRNAs when using plasma samples. However, the observed differences were minimal and not significant. The choice of sample type to profile circulating microRNAs usually depends on the researcher preference. In our experience, we found that plasma preparation is technically less demanding, faster and more standard. Serum, on the other hand, requires the sample to be incubated for at least 30-60 minutes before processing in order for the coagulation process to be completed, a step that may introduce some bias in microRNA expression. In agreement with our view, Wang et al. (2012a) suggested the use of plasma for circulating microRNAs studies since the coagulation process increase sample-sample variation and induce changes in repertoire of circulating microRNAs. Furthermore, some circulating microRNAs in both serum and plasma displayed a differential expression upon platelet activation (Willeit et al., 2013).

The impact of blood cell lysis on microRNA profile is well documented and it is regarded as the most important source of bias in circulating microRNA studies. It has been reported that each blood cell (RBCs, WBCs, platelets) has a unique intracellular microRNA profile (Ramkissoon et al., 2006, Bruchova et al., 2007). The first reports on the effect of RBCs lysis demonstrated a significant impact on the level of expression of certain circulating microRNAs (Kirschner et al., 2011, McDonald et al., 2011, Pritchard et al., 2012b). Three studies identified miR-16 and miR-451 to be the most abundant microRNAs in RBCs and their level is highly affected by haemolysis, a result that is successfully replicated in the present study. More thorough investigation on the effect of haemolysis was performed by Kirschner et al. (2013a). They found that up to 65% of circulating microRNAs were significantly elevated in haemolysed samples. Together, these observations suggest
that an assessment of haemolysis should be included in any study of a potential biomarker candidate as the enrichment of these miRNAs in RBCs complicates the interpretation of biomarker studies making it difficult to determine the exact origin of these molecules. In the present study, haemolysis in plasma samples was assessed by a spectrophotometric analysis of the sample optical density at 414 nm (the absorbance peak of free haemoglobin). This method is simple and cost effective and it was first described by Kirschner et al. (2013a). However, a second haemolysis assessment method may be used which depends on expression levels of miR-451 (a highly haemolysis sensitive microRNA) and miR 23a (a microRNA known to be unaffected by haemolysis). A deltaCt (miR-23a-miR451) >7 indicates high risk of haemolysis (Blondal et al., 2013). This method is particularly useful if the original sample is no longer available and only purified RNA is available.

Different methods and protocols are now available for extracting total RNA from cell-free biofluid and several efforts have been made toward the comparison and optimization of RNA extraction methods (McDonald et al., 2011, Tiberio et al., 2015, Sourvinou et al., 2013, Burgos et al., 2013). In the present study, different extraction methods were compared. First, TRizolLS extraction was compared to a biofluid specific, column based extraction method (miRCURY™ RNA Isolation Kit-biofluid). Our results showed a lower expression of miR-141 (a low G-C microRNA) after TRizol extraction which raised a concern of less RNA recovery using this method or at least a bias toward certain microRNA types. This effect was initially reported by Kim et al. (2012) while extracting from low density cell culture media. Biofluids are regarded as cell free samples and they might be subjected to the same issue. Apart from this effect, studies have consistently reported a lower overall microRNA expression when comparing TRizol with other column based techniques for extraction (McAlexander et al., 2013, El-Khoury et al., 2016, Sourvinou et al., 2013).

The present study also compared the yield of different column based RNA extraction kits (with and without phase separation by Trizol). Here the recovery of RNA after miRCURY™ RNA Isolation Kit-biofluid extraction was superior compared to that after miRNeasy Serum/Plasma Kit from Qiagen. Our results complement other researches that found a better performance for the miRCURY™ Kit-biofluid
over miRNeasy Serum/Plasma Kit (McAlexander et al., 2013), although others found that both kits performed equally (Tan et al., 2015, El-Khoury et al., 2016). On a different note, Cheng et al. (2014) demonstrated that miRNeasy Serum/Plasma Kit was slightly more efficient than miRCURY™ RNA Isolation Kit-biofluid when extracting microRNAs from urinary exosomes. The presence of high level of contaminants in the phenol based preparations has been suggested, at least partly, to account for such inconsistency in RNA recovery from different kits (Channavajjhala et al., 2014).

Another issue addressed here was in relation to optimizing starting volume of plasma. Interestingly, (McAlexander et al., 2013) observed a less efficient recovery of microRNAs by using miRCURY™ RNA Isolation Kit-biofluid when using 200 µl of plasma compared to 50 and 100 µl. This was attributed to more protein-mediated clogging of the columns and/or increased quantities of different PCR inhibitors on the samples. In our experience, this difference was not observed. Experiments here showed that the recovery of miR-16 and miR-134 was correlated with the amount of starting material, a finding in keeping with the work by El-Khoury et al. (2016).

Overall, the results obtained in this study may identify factors that should be taken in consideration when studying and selecting a microRNA biomarker for epilepsy. In order for plasma microRNA to be implemented in a clinical setting, a number of preanalytical and analytical variables that may affect its level should be standardized and its stability in healthy volunteers overtime should be thoroughly investigated. Results here showed that, microRNA levels were very consistent between individuals, males and females, and at different time points. MicroRNAs which were found to be highly expressed were consistent with previous studies of plasma from healthy controls using other platforms. These results confirmed the superiority of plasma over serum samples, the miRCURY™ RNA Isolation Kit-biofluid as RNA extraction method over other methods as well as the suitability of the QuantStudio 12K Flex OA Real-Time PCR System for microRNA profiling and biomarker discovery.
4. Chapter 4: Expression and potential biomarker role of plasma microRNAs in temporal lobe epilepsy
4.1 Introduction

Currently, the main clinically utilized tools for TLE diagnosis and prognosis are EEG and neuroimaging. These methods, despite their wide use, suffer from low sensitivity. For this reason, the process of TLE diagnosis is always challenging and time consuming. Despite all efforts made by physicians to correctly diagnose epilepsy, the rate of misdiagnosis remains high (Scheepers et al., 1998). Moreover, the involvement of different and variable molecular mechanisms in epilepsy and epileptogenesis complicates the chances of identifying a sensitive and specific circulating biomarker for epilepsy (Pitkanen et al., 2016).

In searching for a TLE biomarker, it should be considered that many aspects of the disease can be targeted for clinical biomarker discovery. The main clinical symptom in epilepsy is the recurrent unprovoked seizures. The first task to be addressed during the process of epilepsy diagnosis is to differentiate this seizure from other causes of disturbed consciousness and abnormal movement. Basically, an epilepsy biomarker can reflect different aspects related to seizure, for example, seizure semiology, severity and reduced seizure threshold. However, these concepts are very dynamic. TLE patients, sometimes, present with different seizure semiologies despite having a single epileptogenic focus. At the same time, seizures arising from different epileptogenic zones may give rise to the same seizure semiology in different patients (Tufenkjian and Lüders, 2012). Furthermore, TLE patients usually have a reduced seizure threshold. Certain external and life style factors such as alcohol consumption and lack of sleep as well as many internal factors, for example the diurnal cycle and in females the menstrual cycle may further reduce seizure threshold leading to seizure (Engel et al., 2013). This reduction in seizure threshold is usually transient; therefore, a complete knowledge about any potential seizure biomarker in terms of its window of assessment (i.e., the amount of time that the marker remains detected in the blood before or after seizure) is required.

A more consistent component of epilepsy that can be targeted for an epileptogenesis and/or epilepsy biomarker discovery is the specific epileptogenic abnormality that is found in TLE patients. The characteristic pathological lesion in
TLE (in form of HS) may produce a specific signal that can be detected with minimally invasive techniques (Engel et al., 2013, Pitkänen and Engel Jr, 2014). Nevertheless, this feature may vary over time from after the initial precipitating brain insult, through the epileptogenesis process and after the establishment of epilepsy. An ideal biomarker that reflects the underlying pathology should provide information regarding the progression and severity of the disease as well as the extent and localization of the epileptogenic lesion (Pitkanen et al., 2016).

An epilepsy biomarker may as well reflect the presence of other comorbid conditions in these patients. These comorbidities may share many pathologic mechanisms with epilepsy. The common association of TLE with other psychological and behavioural problems such as anxiety, depression and obsessive-compulsive disorder and other structural brain lesions further complicates the discovery of a specific biomarker for epilepsy but at the same time the identification of a specific biomarker for these comorbidities within the context of epilepsy will help in their early detection, prevention and follow-up (Kanner et al., 2014, Ravizza et al., 2016).

4.1.1 Proposed circulating biomarkers in Epilepsy

Blood level of certain metabolites can be measured and utilized as epilepsy biomarkers (Henshall et al., 2016). High throughput screening methods for genes, RNAs, proteins and other metabolites are widely used nowadays as they provide the opportunity to study thousands of molecules within a specific biological sample. In epilepsy, changes in such molecules might reflect the altered physiological mechanisms responsible for generating seizures, for example inflammation, neuronal death and differential gene expression (Hedge and Lowenstein, 2014, Pitkanen et al., 2016). These molecules can be measured in biofluids in a minimally invasive, accurate, rapid and affordable way. Another important advantage of biofluid biomarkers is the possibility of re-measuring its level to follow the changes over time and therefore relate these changes to the disease state and development and frequency of seizures (Hedge and Lowenstein, 2014, Pitkanen et al., 2016).

The discovery of certain genetic mutations as a possible cause of TLE and the ease of obtaining DNA samples from epilepsy patients have suggested a profitable role of
genetic markers as TLE diagnostic tests. Mutations in genes involved in neurotransmission, neuronal excitability, inflammation, cell-cell interaction and protection against oxidative stress have been identified in DNA samples of TLE patients (Pitkanen et al., 2016). However, most of the association studies between these variants and TLE were small and none have been replicated and up to now, no genetic biomarker achieved clinical translation for TLE diagnosis and/or prognosis.

Different epileptogenic insults were found to be associated with a rapid onset inflammatory response within vulnerable brain regions. Biochemical measurement of inflammatory mediators (such as inflammatory cytokines, chemokines and danger signals) in blood is characterized by being rapid, accurate and affordable which make them reasonable targets for biomarker discovery. Pelto la et al. (1998) demonstrated a reversible increase in IL-6 and IL-1RA after seizure in patients with chronic epilepsy. Serum levels of IL-6 have been found to peak 6-12 hours after both temporal lobe and tonic clonic seizures. In addition, a level of IL-6 was found to be positively correlated with the duration and severity of the seizure (Peltola et al., 1998, Peltola et al., 2000, Lehtimaki et al., 2004, Liimatainen et al., 2009). Other inflammatory mediators were also investigated. Levels of TNF-α was found to increase in serum of patients with drug resistant epilepsy (Alapirtti et al., 2009). Despite these findings, it was argued that many factors could hamper the value of these molecules as biomarkers. The main challenge was the difficulty in correlating the circulating level of these inflammatory markers with the extent of brain inflammation. This is mainly due to interference of peripheral (non-CNS) sources such as liver and lymphoid organs and peripheral leucocytes to their measured circulating level (Vezzani and Friedman, 2011, Pitkanen et al., 2016). Moreover, these molecules usually have a very short half-life in the circulation which impedes their accurate detection in peripheral biofluids (Aronica and Crino, 2011, Vezzani and Friedman, 2011). To overcome the problem of simultaneous release of inflammatory mediators from other sources, molecules that are CNS-specific and can be measured easily in the blood was suggested as biomarkers. A promising candidate is the soluble intercellular adhesion molecule 5 (sICAM5). This is an anti-inflammatory protein of strictly CNS tissue origin. It is expressed specifically by
glutamatergic neurons. In 2012, Pollard et al. (2012) reported a reduction in the level of sICAM5 with a concomitant elevation of other inflammatory mediators (IL-1β, IL-2 and IL-8) in blood samples of drug resistant epilepsy patients. None of the tested interleukins were able to discriminate between TLE patients and controls, however, ROC analysis showed that plasma ratio between sICAM5 and TARC (Thymus and activation regulated chemokine) was predictive of seizure activity.

The possible identification of auto-antibodies directed against ion channels and other specific neuronal antigens in serum samples of epilepsy patients has been studied in the past decade (McKnight et al., 2005, Brenner et al., 2013). These studies demonstrated the potential of autoantibodies as biomarkers for underlying limbic encephalitis which is thought to be a precipitating event in adult onset TLE with HS (Bien et al., 2007, Niehusmann et al., 2009). However, confirmation of these results in larger cohorts of well-phenotyped TLE patients and in patients with other epilepsy syndromes are required to validate the ability of inflammatory markers and/or autoantibodies to accurately identify patients with epilepsy (Pitkanen et al., 2016).

Elevated markers of apoptosis signalling pathways in hippocampus and temporal lobe cortex from TLE patients raised the question of whether these molecules can be utilized as biomarkers of epilepsy associated neuronal death (Galanopoulou and Moshe, 2011). In support of this, the serum level of the anti-apoptotic BCL-2 protein was found to be elevated in adult TLE patients and in children with non-lesional TLE compared to control. BCL-2 level was found to be positively correlated with frequency of seizures and severity of the disease and negatively correlated with IQ, suggesting that this molecule may serve as a surrogate marker for apoptosis caused by or leading to epileptic seizure (El-Hodhod et al., 2006, Kilany et al., 2012).

The role of several other molecules as biomarkers of TLE has been investigated. Neuron-specific enolase (NSE) and protein S100β are specific markers of brain damage and neuronal death. NSE is a dimeric isoenzyme of the glycolytic enzyme enolase, is found in the cytoplasm of neurons and cells with neuroendocrine differentiation (Barone et al., 1993). S100β is part of a large and diverse family of
Ca\textsuperscript{2+}-binding proteins predominantly found in astrocytes and Schwann cells (Schafer and Heizmann, 1996). Increased serum levels of NSE have been reported after GTC and complex partial seizures during inpatient video–EEG monitoring (Rabinowicz et al., 1996, Palmio et al., 2008) suggesting its role as a biomarker in TLE. The neurotrophic cytokine S100\textbeta is an astrogliosis marker that has been identified in hippocampal tissues of TLE patients as well as of experimental animal models of epilepsy (Griffin et al., 1995). An elevated level of S100\textbeta was reported in plasma of TLE patients compared to controls (Lu et al., 2010, Chang et al., 2012).

Heat shock protein 70 (HSP70) is another molecule investigated for its role in differentiating TLE patients from controls. Previous reports suggested an overexpression of HSP70 in hippocampal tissue of SUDEP cases indicating an acute neuronal injury occurring in the ante-mortem stage (Thom et al., 2003). Higher HSP70 levels have been also reported in TLE patients when compared with controls. This increase in HSP70 was associated with higher seizure frequency, memory dysfunction and poor cognitive performance in these patients (Chang et al., 2012). Despite their suggested role as TLE biomarkers, the value of all previously mentioned molecules in epilepsy diagnosis requires more follow-up studies with larger sample size. Inclusion of other epilepsy syndromes and/or other neurological disease will greatly improve the power of the detected biomarker (Engel, 2011b).

4.1.2 Circulating microRNAs and their role as TLE and/or seizure biomarkers

In this chapter, the potential role of microRNAs as diagnostic biomarkers in TLE will be investigated. This role was suggested based upon the known dysregulation in the level of many microRNAs following an acute brain insult (in the form of SE) and in chronic epilepsy animal models. Human data also confirmed this dysregulation in the hippocampal tissues of refractory epilepsy patients undergoing temporal resection (both animal and human data were reviewed in chapter one).

Further supporting this hypothesis is the presence of a set of microRNAs that were found to be enriched in different brain areas and different brain cells (neurons and glial cells) (Sempere et al., 2004, Smirnova et al., 2005, He et al., 2012, Jovicic et al., 2013). Levels of these microRNAs might increase or decrease in the circulation
reflecting the underlying pathophysiological mechanisms of epilepsy and/or the response to seizure.

Despite the promising role of microRNAs for biomarker studies, it should be kept in mind that a number of factors were confirmed to affect the level of detection of these molecules in the circulation. These factors may explain the inconsistencies in circulating microRNA profiling results noticed in other disease conditions. For example, results obtained after a genome-wide microRNA profiling (biomarker discovery phase) was suggested to be specific to the population studied and the platform used. A microRNA (or a set of microRNAs) that was validated to have a role as diagnostic biomarker within a certain cohort of population may fail to do so when its level is measured in another population. These results were not only attributed to the diversity of ethnic background between studied population which was confirmed by several researches (Rawlings-Goss et al., 2014, Wang et al., 2014) but even different life style parameters among population of the same ethnicity might affect microRNA levels in the blood. Addressing this problem is very difficult but very valuable for microRNA biomarker studies. Collaboration of many research groups around the world is required to overcome this issue. Extensive discussions in order to first implement standard operating procedures (SOP) for blood collection and sample preparation and second and most importantly, to reach an agreement regarding the disease diagnostic criteria which may differ in each centre is essential. This was achieved for TLE under the EU-funded EpimiRNA project that included more the 16 research centres around the world (www.epimiRNA.eu). The main objectives of this consortium are to explore the role of microRNAs in the development, treatment and diagnosis of epilepsy.

To achieve the goals of the present study, blood samples were collected from patients admitted to two epilepsy centres (Department of Neurology, Beaumont hospital, Dublin/ Ireland and Epilepsy Center Hessen, Department of Neurology, Baldingerstr, Marburg/ Germany). To minimise any bias from blood collection step, samples were collected and plasma was processed according a standard SOP for both centres. Samples were kept in -80 until shipment to RCSI/Dublin. Samples from two centres will be included in the discovery phase and confirmation phases.
Searching for a common epilepsy microRNA signature in plasma samples collected from both centres will help in identifying biomarkers that are robust in diagnosing epilepsy in different cohorts of populations.

Another important factor that affect microRNA biomarker discovery is the platform used in the initial discovery phase. Currently, the two main genome-wide microRNA profiling techniques that are used for the discovery of microRNA biomarker candidates are either a qPCR or RNA-seq based technologies. A recent study assessed the performance of 12 different technologies for quantitative microRNA profiling (hybridization, RT-qPCR and RNA-seq) and addressed several problems in genome-wide microRNA studies in relation to platform sensitivity, specificity, detection of differential expression signature and others (Mestdagh et al., 2014). What is important in the concept of biomarker studies is the ability of the platform to detect the difference in microRNA level between samples. This detection is affected by the parameters mentioned. Surprisingly, only 3% of dysregulated microRNAs were detected by all the investigated platforms (Mestdagh et al., 2014). Furthermore, the concordance rate between any 2 platforms was of about 79%. However, when concentrating on the differentially expressed microRNAs only, the concordance rate drops to only 54.6%. This means that each platform has the ability to detect an additional specific list of dysregulated microRNAs (in addition to commonly detected ones) (Mestdagh et al., 2014). For this reason and to improve the detection of differentially expressed microRNAs the authors have suggested to validate the results obtained by one platform by using another one (Mestdagh et al., 2014). In the present chapter, in order to obtain a full knowledge about the spectrum of microRNA differential expression in TLE patient’s plasma, all samples that will be included in the discovery phase will be profiled using the OA platform, the results will be compared and contrasted to results generated from profiling pools of the same samples by RNA-seq method.
Plasma samples were collected from 16 healthy controls and 16 patients admitted to two EMUs (Dublin or Marburg) for having refractory TLE. Global microRNA profiling was performed either on pooled samples using RNA-seq technology or on individual samples using the QuantStudio™ 12K Flex OA system (ThermoFisher Scientific). microRNAs that show a common dysregulation pattern between the two platforms were chosen for validation.

TLE: temporal lobe epilepsy; OA: openarray; RNA-seq: RNA sequencing
4.1.3 Chapter summary

**Hypothesis:** a unique circulatory microRNA signature can differentiate TLE from controls. This signature may differ in response to seizure.

**Aims:** to determine plasma microRNA signature in patients with temporal lobe epilepsy compared to controls and to investigate whether plasma microRNA profile changes after a seizure.

To achieve this aim, the study will be conducted in two phases. Phase one, *(discovery phase)* where the global microRNA expression will be profiled in TLE patients (both in baseline sample and in post-seizure sample) and compared to controls. After identification of differentially expressed microRNAs, phase two *(confirmation phase)* will follow. MicroRNA dysregulation will be confirmed in the same cohort of patients and controls that were used for phase one using individual microRNA assays.
4.2 Results

4.2.1 Patient characteristics

The majority of patients (11) recruited at MAR/EMU were diagnosed with TLE and showed the typical electrographic (ictal and inter-ictal EEG abnormalities) and neuroimaging features (HS) of the disease. The other 5 patients had unspecified seizure focus. Two TLE patients had a previous history of hippocampal resection and one with suspected cortical dysplasia. Patients were either on mono or polytherapy for refractory epilepsy. MAR/ group included 8 males (average age 40.2 ± 5 yrs) and 8 females (average age 36.7 ± 3 yrs). Healthy controls recruited at MAR were 8 males (average age 41.2 ± 4 yrs) and 8 females (average age 34.7 ± 3 yrs). No significant differences of age were identified in MAR set of samples.

Patients recruited in DUB/EMU included 10 males (average age 44.2 ± 6 yrs) and 6 females (average age 37.0 ± 7 yrs). All patients were on a poly AED therapy on admission. Two patients had a history of previous temporal resection and in two patients the seizure focus was in the temporal cortex with features of dysplasia on MRI. Seizure semiology in these patients ranged from simple focal seizures into major GTC seizures. DUB/ healthy controls included 10 males (average age 36.4 ± 3 yrs) and 6 females (average age 35.0 ± 3 yrs). No significant differences of age were identified in DUB set of samples. Majority of patients recruited for the study showed typical electrographic and neuroimaging features of TLE.

4.2.2 Phase I, discovery phase

To investigate the potential of microRNAs as diagnostic biomarkers for TLE, two plasma samples were collected from each patient, an epilepsy-baseline sample (EBS) collected 24 hours after admission provided that the patient has no seizure during this period and an epilepsy after-seizure sample (EAS) collected at exactly 24 hours after an electroclinical seizure (complex partial seizure without secondary generalization). Circulating microRNA profiles of these patients were compared to those of healthy controls. Samples were either profiled individually using the OA
platform or pools from each group and for each centre were generated (using 150 µl from each sample) and profiled using RNA-seq (Figure 4.1).

**4.2.2.a OpenArray genome-wide microRNA profiling in plasma of TLE patients and healthy controls**

In total, 96 samples from both DUB and MAR centres (including 32 EBS, 32 EAS and 32 C plasma samples) were profiled individually by the OA platform. In order to have a general idea about circulating microRNA profile in all of the 96 samples, raw data was first filtered using the AmpScore and Cqconf values with a maximum ct cut-off t of 35. Out of 755 microRNAs that can be profiled by OA, over half (419 microRNAs) were not expressed in any sample while 336 were expressed at least in one sample (Table 4.1 A). On average, 220 microRNAs (29.1%) were detected at ct value <35 per plasma sample for both DUB and MAR sites (range 110-336) (Table 4.1 A).

In general, the number of microRNAs detected in DUB samples was higher than those from MAR. In addition, the number of microRNAs detected in DUB/EAS samples was lower than DUB/C and DUB/EBS with p-value of 0.039 and 0.048 respectively (Table 4.1 B). Comparing the average ct values of 5 of the highly expressed microRNAs in each group for MAR and DUB (Figure 4.2 A and B), showed a more consistent expression in MAR samples. PCA of the raw ct values of all samples collected from both centres revealed a clustering based on collection site (Figure 4.2 C). Among the top 20 most abundant microRNAs, 14 were common to both DUB and MAR, although with slight difference in ranking (Table 4.2), indicating that, in general, samples from both sites carry a common microRNA signature.

Next, our analysis focused only on microRNAs with high confidence ct values (<25 ct). When this filter was applied, sample clustering based on collection site was less evident. With a ct 25 cutoff, an average of 128 microRNAs was detected per sample (58.18% of all microRNAs detected with <35 ct value) (Table 4.1 A and B). Still, DUB samples were associated with higher microRNA detection rates when compared to MAR.
Table 4.1: Average number of microRNAs detected in plasma samples profiled with OA

<table>
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<tr>
<th>Ct value</th>
<th>Number of microRNAs</th>
<th>% of total screened</th>
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<tr>
<td>&lt;25</td>
<td>128</td>
<td>16.9%</td>
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<td>25-35</td>
<td>92</td>
<td>12.2%</td>
</tr>
<tr>
<td>&gt;35</td>
<td>116</td>
<td>15.3%</td>
</tr>
<tr>
<td>Not detected in any sample</td>
<td>419</td>
<td>55.4%</td>
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<table>
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<tr>
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<tr>
<td>Ct&lt;= 35</td>
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<tr>
<td>C</td>
<td>207±10</td>
<td>258±12</td>
<td>0.004</td>
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<tr>
<td>EBS</td>
<td>192±11</td>
<td>255±11</td>
<td>0.001</td>
</tr>
<tr>
<td>EAS</td>
<td>191±12</td>
<td>216±15</td>
<td>0.193</td>
</tr>
<tr>
<td>Ct&lt;= 25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>119±11</td>
<td>152±12</td>
<td>0.05</td>
</tr>
<tr>
<td>EBS</td>
<td>108±9</td>
<td>161±9</td>
<td>0.0003</td>
</tr>
<tr>
<td>EAS</td>
<td>109±9</td>
<td>120±13</td>
<td>0.49</td>
</tr>
</tbody>
</table>

(A) Distribution of ct values for the detected microRNAs in both DUB and MAR plasma samples. The percentage shown is the number of detected microRNAs in each category divided by the total number of microRNAs that can be detected by OA. (B) Average number of microRNAs detected in each group and for each centre after filtering using a ct threshold of either 35 or 25. Statistical difference between the number of microRNAs detected in DUB versus MAR samples was determined using student t-test.

C: Control; Ct: cycle threshold; DUB: Dublin centre; EAS: epilepsy post-seizure sample, EBS: epilepsy baseline sample; MAR: Marburg centre.
To adjust for this difference in microRNA detection rates between sites and to minimize any technical variability between the samples, only microRNAs that were detected in >= 80% of samples (77 samples out of 96) with a ct <=25, were kept for further analysis. In total, 63 microRNAs fulfilled these criteria. This analysis was followed by delta ct normalization, imputing the missing values and batch correction for sample site. The effect of normalization in minimizing the technical variabilities between samples can be observed in figure 4.3 A. Before normalization, a large variability in ct values of all samples was observed. This difference was minimized after delta ct normalization (Figure 4.3A). By applying these steps, the site-specific clustering of samples was also reduced (Figure 4.3B). After these quality control steps, we looked for differences in microRNA expression between both sites combined either before or after seizure and compared to controls (i.e. including 32 C, 32 EBS and 32 EAS samples). Despite the identification of several microRNAs with an increased or decreased levels between the three groups, none of them reached statistical significance.

To further investigate the presence of specific microRNA signatures in epilepsy patients, a separate analysis (using the same filtering and quality control steps) was conducted for samples collected from each site. Analysing DUB-samples only revealed that 92 microRNAs were expressed in >= 80% of samples using a ct threshold of 25. However, no microRNA was found to be significantly dysregulated between the three studied groups. Analysing MAR-samples revealed that 55 microRNAs were commonly expressed in >= 80% of samples. Among these microRNAs, 6 were found to be significantly differentially expressed in one or both TLE samples versus controls using t-test and/or Mann-Whitney-test (MW-test) (Table 4.3). Hypothesis test outcome using limma confirmed the dysregulation of 4 of these microRNAs in EAS samples compared to controls. These microRNAs are miR-335-5p (FC= 1.94, adj. p-value= 0.011), miR-328-3p (FC= 1.69, adj. p-value= 0.019), miR-27a-3p (FC= 1.68, adj. p-value= 0.03), and miR-126-5p (FC= 1.86, adj. p-value= 0.035). miR-335-5p was also upregulated in EBS samples versus controls (FC= 1.91, adj. p-value <0.001). This dysregulation will be confirmed in the next phase of the study.
Mean ct value of 5 of the most abundant microRNAs in C, EBS and EAS plasma samples collected in MAR (n=16) (A) and DUB (n=16) (B) centres. More consistent expression across the three studied groups was observed in MAR samples. No significant difference in the ct values of these microRNAs was identified (within each centre) after ANOVA and Bonferroni post hoc test. (C) PCA of the raw ct values (before filtering) revealed a site specific clustering of samples. No clear separation of samples was observed based on disease state. The figure was generated with the help of Dr. Catherine Mooney, PhD.

C: Control; Ct: cycle threshold; DUB: Dublin centre; EAS: epilepsy post-seizure sample, EBS: epilepsy baseline sample; MAR: Marburg centre.
Table 4.2: Top 20 most abundant microRNAs in control plasma samples collected from both MAR and DUB centres and profiled individually by the OA. MicroRNAs that are in MAR only column were also abundantly expressed in DUB cohort but outside the top 20 and vice versa.

<table>
<thead>
<tr>
<th>MAR+DUB</th>
<th>MAR only</th>
<th>DUB only</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-223</td>
<td>hsa-miR-222</td>
<td>hsa-miR-21</td>
</tr>
<tr>
<td>hsa-miR-24</td>
<td>hsa-miR-30a-5p</td>
<td>hsa-miR-221</td>
</tr>
<tr>
<td>hsa-miR-19b</td>
<td>hsa-miR-126-5p</td>
<td>mmu-miR-451</td>
</tr>
<tr>
<td>hsa-miR-146a</td>
<td>hsa-miR-186</td>
<td>hsa-miR-320</td>
</tr>
<tr>
<td>hsa-miR-191</td>
<td>hsa-miR-331</td>
<td>hsa-miR-328</td>
</tr>
<tr>
<td>hsa-miR-126-3p</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa-miR-484</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa-miR-92a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa-miR-20a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa-miR-106a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa-miR-17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa-miR-150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa-miR-30b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa-miR-16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa-miR-30c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa-miR-222</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa-miR-221</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa-miR-21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa-miR-320</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa-miR-30a-5p</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Key:* DUB: Dublin centre; MAR: Marburg centre.
Figure 4.3: Pre-differential analysis filtering and quality control steps for the individual OA microRNA profiling data (combined MAR and DUB samples).

(A) Effect of normalization method in minimizing the technical variabilities between samples. The average ct value of microRNAs was plotted before and after delta ct normalization for each sample. The observed variability in ct values was much reduced after normalization. (B,C) PCA of the OA data after filtering, normalization and batch correction. Site-specific clustering of samples was less evident (B) and again no clustering based on disease state (C). The figure was generated with the help of Dr. Catherine Mooney, PhD.

C: Control; Ct: cycle threshold; DUB: Dublin centre; EAS: epilepsy post-seizure sample, EBS: epilepsy baseline sample; MAR: Marburg centre.
### Table 4.3: Differentially expressed microRNAs after individual OA profiling of MAR/C, EBS and EAS samples.

<table>
<thead>
<tr>
<th></th>
<th>microRNA</th>
<th>FC</th>
<th>adj.p.value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EBS vs C (MW-test)</strong></td>
<td>hsa-miR-335-5p</td>
<td>1.92</td>
<td>0.041</td>
</tr>
<tr>
<td><strong>EBS vs C (t-test)</strong></td>
<td>hsa-miR-335-5p</td>
<td>1.92</td>
<td>0.017</td>
</tr>
<tr>
<td></td>
<td>hsa-miR-199a-3p</td>
<td>1.4</td>
<td>0.017</td>
</tr>
<tr>
<td><strong>EAS vs C (MW-test)</strong></td>
<td>hsa-miR-335-5p</td>
<td>1.94</td>
<td>0.010</td>
</tr>
<tr>
<td></td>
<td>hsa-miR-199a-3p</td>
<td>1.46</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td>hsa-miR-27a-3p</td>
<td>1.68</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>hsa-miR-126-5p</td>
<td>1.86</td>
<td>0.038</td>
</tr>
<tr>
<td></td>
<td>hsa-miR-21-5p</td>
<td>1.33</td>
<td>0.049</td>
</tr>
<tr>
<td><strong>EAS vs C (t-test)</strong></td>
<td>hsa-miR-335-5p</td>
<td>1.94</td>
<td>0.011</td>
</tr>
<tr>
<td></td>
<td>hsa-miR-328-3p</td>
<td>1.69</td>
<td>0.035</td>
</tr>
<tr>
<td></td>
<td>hsa-miR-126-5p</td>
<td>1.86</td>
<td>0.035</td>
</tr>
<tr>
<td></td>
<td>hsa-miR-27a-3p</td>
<td>1.68</td>
<td>0.045</td>
</tr>
<tr>
<td><strong>EAS vs EBS (MW-test)</strong></td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td><strong>EAS vs EBS (t-test)</strong></td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Six microRNAs were found to be significantly dysregulated (adj. p-value <0.05) in TLE samples compared to controls using MW-test and/or t-test.

**Key:** C: controls; EBS: epilepsy baseline sample; EAS: epilepsy post-seizure sample; FC: fold change; MW: Mann-Whitney. The table was generated with the help of Dr. Catherine Mooney, PhD.
4.2.2.b Small RNA-sequencing profiling of TLE patients and healthy controls

plasma

To complement the OA profiling, an additional genome-wide microRNA profiling method (RNA-seq) was performed. Due to high cost of sequencing, samples used for this experiment were generated through pooling the plasma samples previously profiled using the OA. This pooling generated one control (C), one pre-seizure (EBS) and one post-seizure (EAS) sample for each centre. Two RNA-seq experiments (for MAR and DUB cohort) were conducted at Exiqon Services, Denmark. Several pre-sequencing quality control steps were initially performed to confirm suitability of plasma samples for the experiment as well as to monitor the efficiency of RNA extraction and library preparation steps. Expression level of four exogenous spiked-in controls (Cel-miR-39-3p, UniSp100, UniSp101 and UniSp6) (Figure 4.4 A and B) and five endogenous microRNAs (miR-103a-3p, miR-191-5p, miR-23a-3p, and miR-30c-5p) that are known to be expressed in plasma samples at a steady level (Figure 4.4 C and D), were checked in plasma samples. A stable expression of these microRNAs and spike-in controls was observed within samples of each cohort, indicating a similar extraction efficiency and successful RT and qPCR for all the samples with a minimum effect of qPCR inhibitors (Figure 4.4).

Next, small RNA libraries were prepared from these samples followed by RNA-seq using the Ilumina platform either by HighSeq instrument (for MAR) or NextSeq (for DUB) samples. Following sequencing, the quality of the reads obtained and the accuracy of base calling were assessed by measuring the quality scores (Q-scores) for each sample sequenced. All samples showed a good data quality with the vast majority of the data obtained present a Q-score > 30 which means a probability of incorrect base calling less than 0.001 (Figure 4.5 A and 4.6 A). The two most common read lengths obtained after removing the sequencing adaptors ranged between 18-24 (representing microRNAs) and 30-34 nt (representing other RNA species i.e. rRNA, tRNA and mRNA fragments)(Figure 4.5 B and 4.6 B). The total number of reads obtained from each sample is shown in Figure 4.5 C and 4.6 C. An average of 36.6 and 8.6 million reads were obtained for MAR and DUB samples respectively.
Figure 4.4: Pre-sequencing quality control steps for MAR and DUB pooled samples.

A stable expression of four exogenous controls was observed in plasma samples collected at MAR (A) and DUB (B) centres indicating similar extraction efficiency between samples of each centre (n=1 for each group and for each centre). A stable expression of five endogenous microRNAs was observed in MAR (C) and DUB (D) samples indicating successful RT and PCR for all the samples with the minimum effect of PCR inhibitors.

Key: C: Control; Ct: cycle threshold; DUB: Dublin centre; EAS: epilepsy post-seizure sample, EBS: epilepsy baseline sample; MAR: Marburg centre.
Bioinformatics tools were then used to map “align” the RNA-seq reads on the reference genome (human). Mapping of the reads showed that, on average, for MAR and DUB samples respectively, 32.8 million (90%) and 8.0 million (82%) reads were perfectly mapped per sample. This difference in the overall reads was due to the use of a different sequencer for the MAR study compared to the DUB study. From these mapped reads 24% and 12% correspond to microRNAs (as registered in miRBase 20). An average of 255 and 172 microRNAs were identified in each set of samples (MAR versus DUB) with more than 10 Tags per Million (TPM) (Figure 4.7A). Despite the observed difference in the total number of reads and in microRNA counts between DUB and MAR samples, the ability to detect microRNA signature was not affected. Among the top 20 most abundant microRNAs in MAR control samples, 17 were also found to be among the top 20 most abundant in DUB controls. The other three microRNAs were as well abundantly expressed and were ranked 21st, 25th and 39th (Table 4.4).

Differential expression analysis was conducted separately on each set of samples. For this, data were normalized first using the trimmed mean of M-values method (TMM normalization). Expression levels are measured as Tags per Million (TPM) where the number of reads for a particular microRNA is divided by the total number of mapped reads and multiplied by 1 million. A differential expression analysis was then performed using the EdgeR statistical software package (Bioconductor, http://www.bioconductor.org/). This analysis investigates the relative change in expression of mapped reads (i.e. counts) between different samples.

Unsupervised hierarchical clustering of microRNAs and samples, including the top 50 features with the highest coefficient of variation (%CV) revealed a clear separation of the samples based on their biological difference (Figure 4.7B). Comparing results from the two sequencing experiments, 11 microRNAs were found to have a common expression pattern with fold change > 1.5 or <-1.5 (Figure 4.7 C). The differential expression of these microRNAs will be confirmed in the second phase of the study.
Figure 4.5: MAR-sequencing data quality control.

(A) The average reads Q-scores for Mar/C, EBS and EAS samples. The vast majority of the data has Q score greater than 30 which equals an accuracy of 99.9% for the base-calling. (B) Read length distribution after filtering of the adaptors, the samples have the expected peak around 18-23 nt. representing microRNAs. (C and D) Number (C) and fraction (D) of the mapped reads. About 90% of reads were successfully mapped to human genome. Quarter of them mapped to known (miRBase 21) microRNA-sequence. C: Control; EAS: epilepsy post-seizure sample, EBS: epilepsy baseline sample; MAR: Marburg centre.
Figure 4.6: DUB-sequencing data quality control.

(A) The average reads Q-scores for Mar/C, EBS and EAS samples. The vast majority of the data has Q-scores greater than 30 which equals an accuracy of 99.9% for the base-calling. (B) Read length distribution after filtering of the adaptors, the samples have the expected peak around 18-23 nt. representing microRNAs. (C and D) Number (C) and fraction (D) of the mapped reads. About 82% of reads were successfully mapped to human genome. 12% of them mapped to known (miRBase 21) microRNA-sequence. C: Control; DUB: Dublin centre; EAS: epilepsy post-seizure sample, EBS: epilepsy baseline sample.
Table 4.4: Top 20 most abundant microRNAs in control plasma samples collected from both MAR and DUB centres and profiled by RNA-seq.

17 microRNAs (indicated by red dots in MAR list) were common to the top 20 most abundant microRNA list for both centres.

<table>
<thead>
<tr>
<th>MAR</th>
<th>DUB</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-30d-5p</td>
<td>hsa-miR-151a-3p</td>
</tr>
<tr>
<td>hsa-miR-451a</td>
<td>hsa-miR-423-5p</td>
</tr>
<tr>
<td>hsa-miR-26a-5p</td>
<td>hsa-miR-30d-5p</td>
</tr>
<tr>
<td>hsa-miR-21-5p</td>
<td>hsa-miR-148a-3p</td>
</tr>
<tr>
<td>hsa-miR-486-5p</td>
<td>hsa-miR-486-5p</td>
</tr>
<tr>
<td>hsa-miR-146a-5p</td>
<td>hsa-let-7i-5p</td>
</tr>
<tr>
<td>hsa-miR-423-5p</td>
<td>hsa-let-7b-5p</td>
</tr>
<tr>
<td>hsa-miR-151a-3p</td>
<td>hsa-miR-451a</td>
</tr>
<tr>
<td>hsa-miR-191-5p</td>
<td>hsa-miR-126-3p</td>
</tr>
<tr>
<td>hsa-miR-126-3p</td>
<td>hsa-miR-122-5p</td>
</tr>
<tr>
<td>hsa-let-7i-5p</td>
<td>hsa-miR-26a-5p</td>
</tr>
<tr>
<td>hsa-miR-320a</td>
<td>hsa-miR-21-5p</td>
</tr>
<tr>
<td>hsa-miR-122-5p</td>
<td>hsa-miR-320a</td>
</tr>
<tr>
<td>hsa-miR-92a-3p</td>
<td>hsa-miR-92a-3p</td>
</tr>
<tr>
<td>hsa-miR-22-3p</td>
<td>hsa-miR-128-3p</td>
</tr>
<tr>
<td>hsa-let-7b-5p</td>
<td>hsa-miR-25-3p</td>
</tr>
<tr>
<td>hsa-miR-423-3p</td>
<td>hsa-miR-423-3p</td>
</tr>
<tr>
<td>hsa-miR-148a-3p</td>
<td>hsa-miR-146a-5p</td>
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<tr>
<td>hsa-miR-24-3p</td>
<td>hsa-miR-146b-5p</td>
</tr>
<tr>
<td>hsa-let-7g-5p</td>
<td>hsa-miR-191-5p</td>
</tr>
</tbody>
</table>

*Key: DUB: Dublin centre; MAR: Marburg centre*
Figure 4.7: Normalization and differential expression of RNA-seq data.

(A) Number of known microRNAs identified in > 1TPM and in >10 TPM in each sample and for each centre (microRNAs are identified according to entries in miRBase release 20). (B) Heat Map and unsupervised hierarchical clustering by sample and microRNA shows a clear separation between samples. The clustering was performed on the top 50 microRNAs with highest %CV based on normalized microRNA counts. (C) microRNAs with a common expression pattern in both RNA-seq experiments. C: Control; DUB: Dublin centre; EAS: epilepsy post-seizure sample, EBS: epilepsy baseline sample; MAR: Marburg centre; TPM: Tag per million.
4.2.2.c Similarity of plasma microRNA signature obtained using OA and RNA-seq

Comparing OA and RNA-seq data revealed a moderate concordance between the two platforms. Comparing the top 20 most abundant microRNAs in control samples for both sequencing and OA datasets, revealed that seven were common to both. These microRNAs were miR-146a, miR-191-5p, miR-92a-3p, miR-126-3p, miR-451a, miR-320a and miR-26a-5p. The most abundant microRNA detected using the OA platform (miR-223-3p) was ranked 55th and 116th in MAR and DUB sequencing experiments respectively (using the control samples for comparison).

Despite this lack of concordance, more than half of the microRNAs that were found to be differentially expressed using the OA platform (4 out of 6) were found to have a similar expression pattern in RNA-seq experiment. miR-328-3p which was upregulated in EAS samples versus controls in MAR-OA dataset was found to be upregulated in DUB-seq (1.5 folds) and MAR-seq (1.9 folds). Two other microRNAs (miR-335-5p and miR-199a-3p) were found to be upregulated (>1.5 folds) in EBS samples versus C in both DUB-seq and MAR-OA datasets. In addition, miR-21-5p which was upregulated in EAS/C comparison in MAR-OA dataset was found to be upregulated in EBS/C in DUB-seq.

4.2.3 Phase II, confirmation phase

To confirm the profiling results, validation of 6 and 8 microRNAs were performed after their initial identification by OA (Figure 4.8) and RNA-seq (Figure 4.9) respectively (miR-122-5p was excluded for being liver specific). A total of 96 samples were included in this phase; these are the same samples that were profiled initially using the OA platform. An external control gene (cel-miR-39) was spiked-in each sample to monitor the efficiency of RNA extraction step. To assess the quality of the ct values obtained, each qPCR reaction was performed in triplicate using microRNA specific TaqMan assays. An average of miR-19b, miR-26a, miR-26b and cel-miR-39 ct values was used for normalization. The differential expression of these microRNAs was assessed relative to controls using the \(2^{-\Delta\Delta ct}\) method and the generated \(p\)-values were adjusted for multiple comparisons by controlling the FDR according to the method of Benjamini and Hochberg.
4.2.3.a Circulating microRNAs with possible role as TLE biomarkers

For a microRNA to act as a TLE biomarker, its level must be either increased or decreased in both EBS and EAS plasma samples compared to C. In general, among the 15 microRNAs that were investigated in study phase II, the level of many microRNAs was confirmed to be higher in TLE samples compared to controls. This increase was (in the majority of microRNAs) significant only when comparing EBS samples versus controls. Interestingly, results of the confirmation phase identified a microRNA with a possible role as TLE biomarker. Level of miR-654-3p was found to be upregulated in both EBS and EAS samples compared to controls (Figure 4.9G). This upregulation was significant in EBS versus C (FC=3.0, p-value= <0.001) and near significant in EAS versus C (FC=1.8, p-value=0.06). Another microRNA with a similar trend but with lower fold change was miR-543 (Figure 4.9F). The increase in expression of this microRNA was only significant in EBS samples versus controls (FC=1.43, p-value=0.04).

4.2.3.b Circulating microRNAs with possible role as seizure biomarkers

Seizure biomarkers are clinically as important as TLE biomarkers. An accurate detection of seizure allows for a better monitoring of the disease and a better differentiation of the attack from other conditions that resemble seizures. For a microRNA to act as an accurate seizure biomarker, its level should be dysregulated in EAS samples compared to both C and EBS. In the confirmation phase, the level of miR-27a-3p (Figure 4.8C) and miR-328-3p (Figure 4.8A) was found to be comparable in C and EBS samples. However, in samples collected 24 hours after an electro-clinical seizure, their level found to significantly decrease in plasma indicating a potential value in diagnosing seizure episodes.
Figure 4.8: Confirmation of OA profiling data.

Boxplots showing the distribution of normalized ct following validation using individual TaqMan microRNA assays. Level of five microRNAs was confirmed to be significantly dysregulated after t-test in one or both TLE samples compared to healthy controls. (* = adj p-value < 0.05; ** = adj. p-value < 0.01; *** = adj. p-value < 0.001). Note: lower ct indicates higher expression. C: Control; Ct: cycle threshold; EAS: epilepsy post-seizure sample, EBS: epilepsy baseline sample; OA: openarray.
Figure 4.9: Confirmation of RNA-seq profiling data.

Level of seven microRNAs was confirmed to be significantly dysregulated after t-test in one or both TLE samples compared to healthy controls. (* = adj p-value < 0.05; ** = adj. p-value < 0.01; *** = adj. p-value < 0.001). Note: lower ct indicates higher expression. C: Control; Ct: cycle threshold; EAS: epilepsy post-seizure sample, EBS: epilepsy baseline sample; RNA-seq: RNA sequencing.
In addition to miR-27a-3p and miR-328-3p, the level of 5 other microRNAs were found to significantly decrease in TLE patient’s plasma after seizure. These microRNAs are miR-199a-3p (FC=0.7, p-value=0.03) (Figure 4.8E), miR-126-5p (FC=0.7, p-value= 0.04) (Figure 4.8D), miR-339-5p (FC=0.66, p-value=0.04) (Figure 4.9D), miR-654-3p (FC=0.61, p-value=0.04) (Figure 4.9G) and miR-4433b-3p (FC=0.53, p-value=0.04) (Figure 4.9H). These microRNAs displayed an increased expression level in EBS samples compared to both controls and EAS. The detected post-seizure reduction in microRNA level is particularly important to investigate the seizure occurrence within the TLE patients, and most importantly to monitor the treatment response in these patients.

4.2.4 Performance of dysregulated microRNAs as biomarkers for TLE and seizure

To evaluate the ability of the dysregulated microRNAs to accurately differentiate TLE patients from healthy controls, a ROC curve analysis was performed. From the 9 microRNAs that were significantly increased in EBS samples versus controls, the best performing microRNAs in differentiating TLE from healthy controls were miR-654-3p with an area under the curve (AUC) of 0.86 and miR-126-5p with AUC of 0.83 (Figure 4.10E and G). The AUC for the other 7 microRNAs varied between 0.59 up to 0.76 (Figure 4.10). On the other hand, microRNAs with a seizure biomarker potential displayed a higher discriminatory value in differentiating EAS from C and/or EBS samples. ROC curve analysis indicated that miR-328-3p was most valuable in differentiating EAS from C only, EBS only and EBS+C (Figure 4.11 A,B,C) with an AUC of 0.74, 0.93 and 0.83 respectively. Similarly, miR-27a-3p has its best performance in differentiating EAS from EBS samples with AUC of 0.89 (Figure 4.11E), its performance dropped slightly to 0.84 and 0.87 when differentiating EAS from C samples or EAS from EBS+C samples (Figure 4.11D and F). The discriminatory value of two other microRNAs (with a significant dysregulation between EAS and EBS samples) was tested as well. Both miR-339-3p (AUC= 0.72) and 4433b-3p (AUC=0.74) displayed a good ability to differentiate EAS from EBS samples (Figure 4.11 G and H).
Figure 4.10: Performance of dysregulated microRNAs as biomarkers for TLE.

ROC curve analysis was performed on 9 microRNAs with a significant dysregulation in EBS samples versus controls. The best performing microRNAs were miR-654-3p (E) and miR-126-5p (G). AUC: area under the curve; ROC: receiver operating characteristic.
Figure 4.11: Performance of dysregulated microRNAs as seizure biomarkers.

ROC curve analysis was performed on microRNAs with a significant dysregulation in EAS versus controls and/or EBS samples. The best performing microRNAs were miR-328-3p (B) and miR-27a-3p (E) in differentiation EAS from EBS samples. AUC: area under the curve; EAS: epilepsy post-seizure sample, EBS: epilepsy baseline sample; ROC: receiver operating characteristic.
4.2.5 Target recognition of the dysregulated microRNAs

In order to obtain an additional mechanistic evidences for the potential role of dysregulated microRNAs as TLE biomarkers, their human targets that were experimentally validated using qPCR, western blot or luciferase reporter assays (strong evidence validation) were retrieved from miRTarBase (Chou et al., 2016). Three of the identified microRNAs (miR-342-5p, miR-323a-3p and miR-4433b-3p) did not have any strongly validated targets, however, a total of 133 mRNA targets were identified for the other 9 microRNAs (Table 4.6 and figure 4.12).

Cross referencing the identified genes with the data available online on epilepsy genetics (CarpeDB: http://carpedb.ua.edu and epiGAD: http://www.epigad.org) identified three targets that were directly associated with epilepsy pathophysiology (Figure 4.12). The first gene is a multidrug transporter gene (breast-cancer related protein (ABCG2)) which belongs to the ATP-binding cassette (ABC) transporter superfamily at the BBB. This gene was suggested to play an important role in drug resistance among TLE patients (Kwan et al., 2011b). In the present data, ABCG2 was predicted to be targeted by miR-328-3p. In addition, apolipoprotein E (APOE) and prostaglandin-endoperoxide synthase 2 (PTGS2) genes were identified as targets for miR-199a-3p. Neuronal PTGS2 was identified to contribute to post-SE mortality and cognitive deficit in mice through mediating neuro-inflammation and neuro-degeneration (Levin et al., 2012). APOE gene was also identified as one of the susceptibility genes for TLE that is associated with an earlier onset of the disease (Kauffman et al., 2010).
Table 4.5: List of experimentally validated targets (with strong evidence) for
each of the differentially expressed microRNAs, extracted using miRTarBase.
miR199a3p

miR126-5p

miR144-3p

miR335-5p

miR328-3p

miR339-5p

ADAM9

CFTR

AKT1

ABCA1

MMP13

ARPC5L

ABCG2

BACE1

PTK2

CDKN1A

CXCL12

EZH2

APOE

MMP7

FGA

CAV2

APC

MSTN

BCL2L2

BACE1

BCL6

TWIST1

EXOG

BNIP3

MYT1

BIRC5

CD44

PTP4A1

MYC

FGB

CD44

CAB39L

NFE2L2

BRCA1

H2AFX

PTPN7
SLC45A3

FGG

DNAJA4

CCND1

PAX3

DAAM1

MMP16

MET

FUT4

CCNYL1

PDS5B

DAAM2

PTPRJ

MTOR

HGF

CNN3

PHB

EPN2

SFRP1

NFE2L2

IGF1

CPPED1

PIK3CG

FMN2

NOTCH1

KRT7

CRISP2

PPARG

FMNL3

PLAG1

MAPK1

DPYD

PSAP

FOXA2

PTEN

MAPK14

EGFR

RMND5A

ID4

TGFB1

MAPK8

ENDOU

SEMA6A

IGF1R

TTN

MAPK9

FBXW7

SEMA7A

LRG1

ZFX

MET

FOXO1

SLC7A11

MAPK1

MTOR

GATA2

SP1

MERTK

PTGS2

GRB2

SP3

MYC

SMRCA2

HIPK2

SP4

POU5F1

STK11

HOXA10

SPRY2

PTPRN2

ZHX1

IFNG

THRB

RASA1

IFNR

TP53

RB1

IGF1

WDR77

ROCK1

KRAS

WEE1

RUNX2

LDLR

WNK1

SOX17

LPIN1

YWHAZ

SOX4

MAP2K
4

ZBTB10

SP1

miR-27a-3p

MET

TFF2
TNC
UBE2F
ZEB2

200

miR543

miR654-3p


Target transcripts for the differentially expressed microRNAs with strong experimental evidence were retrieved from miRTarBase. Three of the identified microRNAs were not included as they do not have strongly validated targets. Edge thickness corresponds to the number of publications that validated the microRNA-mRNA interaction. Pink coloured targets were identified as being epilepsy related by CarpeDB and epiGAD databases. The network was generated using Cytoscape software with the help of Dr. Niamh M. Connolly, PhD.
4.3 Discussion

In this chapter, a multiphase case-control study was performed to investigate the ability of plasma microRNAs to both differentiate TLE patients from healthy controls and to track the occurrence of seizure. This is the first multi-centre study to investigate the differential expression of microRNAs in plasma of TLE patients using dual platform approach. The current study has the advantage of including patients admitted to two EMUs in Europe. In addition, to increase the confidence in the identified microRNA signature, two different genome-wide profiling methods was utilized to identify a common plasma microRNA signature in TLE. Moreover, by comparing microRNA profiles in pre and a post-seizure samples, the current study was able to identify microRNAs with a possible role as both TLE and/or seizure biomarkers.

Despite the many difficulties encountered during the experiment in regard to low sample number as well as the inherent heterogeneity in many experimental aspects, a n important finding in the present study was the identification of a number of microRNAs that are dysregulated in TLE when compared to healthy controls. The level of miR-654-3p was increased in both pre- and post-seizure samples, indicating a strong candidate TLE biomarker. The increased circulating level of this microRNA could reflect a contribution to one or more pathological mechanisms of TLE. However, to date, there has been no study to link this microRNA to epilepsy (Mooney et al., 2016). More investigation is required to establish this role and to confirm that this up-regulation is not related to AED administration by these patients. miRTarBase search for validated human targets with strong evidence for miR-654-3p identified two genes; Cyclin Dependent Kinase Inhibitor 1A (CDKN1A) and Exo/Endonuclease G (EXOG). No direct pathophysiological role was reported for alteration of these genes in TLE. CDKN1A gene was believed to regulate of cell cycle progression at G1. Downregulation of CDKN1A in the dentate gyrus and sub-ventricular zone was found to induce premature differentiation of neuronal stem cells into mature astrocytes at the expense of neurons (Porlan et al., 2013), thus, this target has a potentially
important role in astrogliosis. This could, in theory, be a mechanistic link to the observed biomarker finding. Notably, astrocytes respond to neuronal hyperexcitability by increase in their size and number (Shapiro et al., 2008). Moreover, it has been documented that reactive astrocytes have the ability to modify the brain microvasculature leading to capillary dilatation through Ca\(^{2+}\)-dependent signalling (Mishra et al., 2016). Hence, it could be speculated that both astrocyte activation and the associated capillary dilatation may enhance the delivery of microRNA-loaded astrocytic-exosomes into the circulation rendering them amenable to be used as biomarkers. However, more investigations are required to identify the cellular origin of miR-654-3p (neuronal versus astrocytic) and to confirm its loading into released exosomes upon excitation or activation of these cell types.

Other microRNAs were identified for having a seizure biomarker potential. Circulating levels of miR-328-3p and miR-27a-3p was found to be reduced only in post-seizure samples when compared to healthy controls and baseline epilepsy samples. This was rather unexpected since the hypothesis originally was that seizure activity may trigger a release of material from the brain that can be detected in the circulation. These microRNAs may have a significant clinical value in both detecting and confirming seizures in patients already diagnosed with TLE and in patients presenting after their first seizure attack. Moreover, TLE patients sometimes experience both epileptic and “psychogenic” non-epileptic attacks. It is usually very difficult to differentiate between them depending on the clinical history only. An admission of the patient to an EMU with continuous EEG monitoring is essential in these patients in order to characterize the nature of the attack and to accurately determine the frequency of “true” seizures, thus modify treatment regimes. A seizure biomarker would clearly be beneficial in diagnosing these patients. ROC curve analysis of the normalized ct value of miR-328-3p and miR-27a-3p indicated a high performance of these microRNAs in identifying patients presented after a recent seizure episode. Furthermore, there are encouraging mechanistic links for these microRNAs to epilepsy pathogenesis. Dysregulation of both microRNAs in relation to TLE have been identified previously. A reduced
hippocampal expression of miR-328-3p was previously identified in rats 48 hours following pilocarpine induced SE (Risbud and Porter, 2013). It was also reported to be reduced in blood and hippocampal tissues of chronic epileptic rats (Liu et al., 2015). This reduction was observed to be closely associated with impaired memory function and reduced learning abilities in these rats (Liu et al., 2015). On the other hand, upregulation rather than down regulation of miR-27a-3p was observed in resected hippocampal tissue of TLE patients (Kan et al., 2012, Alsharafi and Xiao, 2015) and in hippocampi of chronic animal models of epilepsy (Alsharafi and Xiao, 2015). However, in the present study, a similar expression level of miR-27a-3p was observed comparing TLE and controls. Reduction in miR-27a-3p expression was only identified after seizure episodes, a condition that cannot be observed or replicated in resected tissue.

The present study also identified another important set of dysregulated microRNAs. These microRNAs exhibited a unique plasma signature in the three studied groups. A significant higher level was observed in baseline TLE samples when compared to both controls and post-seizure samples. The observed tendency toward upregulation in baseline samples followed by downregulation after seizure might have an important diagnostic value. The interpretation and confirmation of the cause of this change requires extensive studies to specifically identify seizure-microRNA relationship. The initial upregulation can be viewed either as having a seizure-protective (which might be related mainly to the administration of AEDs) or seizure-inducing effect. Time window between baseline sample collection and seizure recording might give a clue about these two possibilities. The identification of this association will be very important for individualizing therapeutic options and monitoring treatment response in TLE patients. Nevertheless, these microRNAs cannot be utilized as TLE biomarkers as their level fluctuate up and down in TLE plasma depending on seizure occurrence. It is also important to determine whether this reduction is actually a response to seizure and not a secondary response to reduced levels of AEDs in these patients.
Previous reports about the biomarker role of microRNAs in TLE have identified microRNAs with dysregulated blood levels in both human and experimental animal samples. Recently, two genome-wide microRNA profiling studies have been conducted and the potential role of several microRNAs as markers of epilepsy have been investigated (Wang et al., 2015a, Wang et al., 2015b). In both studies, Wang and colleagues used RNA-seq technology to profile microRNA expression in serum of either TLE patients (Wang et al., 2015b) or drug-resistant TLE (Wang et al., 2015a). In the former study, the downregulation of miR-15a-5p and miR-194-5p and the upregulation of Let-7d-5p, miR-106b-5p, -130a-3p, and -146a-5p were validated in human epilepsy relative to normal controls. Moreover, miR-106b-5p was proposed to be the best diagnostic biomarker for epilepsy due to its high sensitivity and specificity (AUC=0.88). However, this study ignored the effect of seizure on circulating microRNA profile as it profiled microRNAs in blood samples irrespective of seizure timing, an effect that was clearly identified in the present study. In the latter study, (Wang et al., 2015a), levels of miR-194-5p, miR-301a-3p, miR-30b-5p, miR-342-5p, and miR-4446-3p were confirmed to be reduced in drug resistant group when compared to drug-responsive patients and control group. No microRNA was confirmed to be upregulated. Of these 5 microRNAs, miR-301a-3p was reported as the best diagnostic biomarker for drug-resistant epilepsy with AUC=0.89. Comparing results of these two studies and the present study revealed a poor concordance. Differences in the study design and in patient ethnicity could very well account for the variable results between studies (Rawlings-Goss et al., 2014). Notably, in the present study, the level of miR-342-5p was found to be increased in TLE baseline samples when compared to controls. Provided that all patients included in the present study were drug refractory, this dysregulation was inconsistent with that observed by Wang et al. (2015a) study where miR-342-5p was reduced in drug resistant TLE patients compared to both controls and drug responsive patients.
In a third study that was recently published, pre and post-ictal microRNA expression was compared in serum samples collected at different time points after focal seizure propagation into secondary GTC seizures (time points were within 30 min, 3-6 hours, 20-28 hours and within 3-6 days). In contrast to the present data, no significant change in microRNA profile was detected in most of time points studied. MicroRNA dysregulation was only evident in serum samples collected within 30 min after GTC seizures. Validation of 20 dysregulated microRNAs identified four (miR-143-3p, miR-145-3p, miR-365a-3p and miR-532-5p) with an increased postictal level (Surges et al., 2016). However, the very early and transient upregulation of microRNAs identified by Surges et al. (2016) may reduce their benefit as biomarkers. It may also be speculated that the analysis approach applied in the present study is more sensitive and thus was able to detect subtle post-seizure changes at later time points. Moreover, from a practical point of view, it is very difficult to obtain postictal blood samples within this tight window (30 min), unless the patient is already hospitalized. Therefore, microRNAs with a dysregulated level that can be detected several hours after seizures are more feasible clinically. Furthermore, the identified microRNAs were only studies within the context of GTC seizures. This seizure semiology is characterized by the tandem occurrence of muscle spasm (in the tonic phase) followed by violent muscle contractions (in the clonic phase). Hence, the circulating microRNA profile in these patients might reflect either the abnormal electrical brain discharge or the rapid release of muscle related microRNAs into the circulation after this muscle activity. This possibility should be investigated. Inclusion of a separate group of patients presenting with other seizure semiologies and even patients with psychogenic GTC episodes might be helpful in this regard.

Further investigation about the specific origin of the identified dysregulated circulating microRNAs in TLE is required. The observed dysregulation may represent an altered intracellular production (either increased or decreased) of that specific microRNA in response to either the pathological condition and/or in response to drug administration. In such case, a similar dysregulation pattern of microRNAs should be observed in the tissue of origin (neurones and/or glial cells) and in the
circulation. However, it might be speculated that (due to the role of microRNAs in remote cell-to-cell communication) the increased level of microRNA production might be associated with a very efficient uploading of this microRNA into exosomes and microvesicles and with rapid delivery into the circulation. In such case, an increased level of this microRNA might be observed in the circulation; however, its level in tissue samples might be lower. On the other hand, microRNA dysregulation might be unrelated to the disease itself, reflecting instead either a secondary phenomenon of the disease process (for example muscle contractions in GTC seizures), comorbid pathology or even a side effect of drugs administered on other body organs.

After identifying their origin, more studies will be required to determine the cellular function of the dysregulated microRNAs in neurons and glia as well as any changes in their level over the course of the disease. This will allow the proper utilization of these microRNAs not only as diagnostic markers for TLE and/or seizure but as well as prognostic and theranostic markers. Using the EpimiRBase database (http://www.epimirbase.eu/) (Mooney et al., 2016), all of the dysregulated microRNAs in the present study (except miR-654-3p, miR-543 and miR-4433b-3p) were identified in at least one study as being dysregulated in blood samples and/or hippocampal tissues of both TLE patients and experimental epilepsy models. However, these studies were performed on hippocampal tissue homogenates which completely erased cell type distinction. Thus, identification of cell-of-origin remains critical.

In summary, several microRNAs were found to be differentially expressed in TLE plasma samples when compared to controls supporting the potential role of these molecules as diagnostic and prognostic biomarkers in TLE. However, data clearly showed that the population studied and the platform used for genome-wide microRNA profiling both have a profound effect on the obtained results. Only three microRNAs (miR-335-5p, miR-328-3p and miR-199a-3p) were found to have a common deregulation signature using the RNA-seq and OA platforms. Study phase II confirmed that dysregulation and showed that these microRNAs followed exactly the RNA–seq identified pattern of deregulation rather than the OA one. Further
validation of the obtained results in a larger cohort of TLE patients is still required. Including plasma samples collected from other epilepsy syndromes, PNEAs and other neurological diseases will enhance the confidence of these biomarkers. Moreover, correlation of the obtained results with patient’s clinical data will help in identifying biomarkers for treatment response, seizure frequency, seizure severity and others.
5. Cerebrospinal fluid microRNAs are potential biomarkers of temporal lobe epilepsy and status epilepticus
5.1 Introduction

TLE patients usually present to clinicians with a wide range of symptoms that require attention in order to correctly identify seizure semiology. Seizure episodes vary greatly between patients in regard to their severity and duration. Seizures can be very mild, with brief motor or sensory experience and without any disturbances in concentration (aura/simple partial seizure). A major tonic-clonic convulsion represents the most severe form of seizures (Chang and Lowenstein, 2003). In regard to their duration, seizures usually last from few seconds to few minutes. Any form of prolonged seizure regardless its semiology and severity is referred to as SE (Trinka et al., 2015). The close association between TLE and SE raised the possibility of a common molecular mechanism for seizure generation. Failure of seizure termination in TLE and/or the initiation of other mechanisms responsible for abnormally prolonged seizures may continue to progress leading to SE (Trinka et al., 2015).

Identifying the effect of both extremes of seizures (in relation to severity and duration) on biofluid microRNAs signature can provide an important insight for their potential role as a diagnostic, prognostic and theranostic biomarkers in TLE and SE. Furthermore, it provides more insight into the common molecular pathways and targets that are shared between the two conditions which in turn allows for a better understanding and possible identification of novel therapeutic targets (Henshall, 2013, Baran-Gale et al., 2015).

5.1.1 Status epilepticus

SE is one of the most common neurologic emergencies; it is associated with high mortality rate which was reported to be up to 20% (DeLorenzo et al., 1996, Betjemann and Lowenstein, 2015). Patient’s age has a dramatic effect on the incidence of SE, an incidence up to 86/100000/year was reported in older age population (60 years and older) compared to 5.2-28/100000/year in younger adults (DeLorenzo et al., 1996, Chin et al., 2004).
5.1.1.a Definition and classification of SE

The first definition and classification of SE was dated back to 1970 (Gastaut, 1970). SE was defined as ‘‘A seizure that persists for a sufficient length of time or is repeated frequently enough that recovery between attacks does not occur’’. An important pitfall of the former definition and classification systems is that they did not define the duration after which the seizure is prolonged, nor there was a clear description of the type of SE (semiology). This problem was addressed by Lowenstein et al. (1999) who proposed a more practical definition for SE by specifying time points after which a seizure is regarded as prolonged (seizure activity that continues for more than five minutes or two or more individual seizures with incomplete recovery between them).

Based on the current knowledge of pathophysiology of SE, the ILAE task force on classification of SE proposed a new definition in 2015 that took into consideration all types of clinical SE as well as specifying when the seizure is regarded as prolonged (Trinka et al., 2015). Thus, SE is currently defined as ‘‘a condition resulting either from the failure of the mechanisms responsible for seizure termination or from the initiation of mechanisms, which lead to abnormally prolonged seizures (after time point t1). It is a condition, which can have long-term consequences (after time point t2), including neuronal death, neuronal injury, and alteration of neuronal networks, depending on the type and duration of seizure’’ (Trinka et al., 2015). This definition is more practical as it specifies two important time points on which the decision of when to start treatment and how aggressive the treatment should be depends. Both time points were determined based on animal studies and clinical research. Time point 1 (t1), is the time when a seizure should be regarded as abnormally prolonged. It was set at 5 min for generalized convulsive seizures, and at 10 min for focal seizures with impairment of consciousness and at 10-15 min for absence seizures. Time point 2 (t2) is the time during the ongoing status after which there is risk of neurological consequences (30 min for generalised convulsive SE, >60 min for focal SE, and still unknown for absence SE). Treatment at (t2) time point should be more aggressive to stop the seizure activity (Trinka et al., 2015, Trinka et al., 2016).
The ILAE task force also proposed a new classification for SE that takes into consideration SE semiology, aetiology, EEG correlates and age. Classification of SE depending on seizure semiology can be assessed immediately upon presentation where SE is divided into two major categories (i) with prominent motor symptoms: this include convulsive SE, myoclonic SE, focal motor SE, tonic status and hyperkinetic SE (ii) without prominent motor symptoms or referred to as nonconvulsive SE. This include NCSE with coma and NCSE without coma. It should be noted that each one of the above categories includes many other subtypes that were discussed in detail in the ILAE report (Trinka et al., 2015).

The diagnosis and prediction of outcome in SE is complex and mainly based on clinical features and aetiology of SE (Rossetti et al., 2006, Hocker et al., 2013). To date, there is no reliable biomarker to diagnose and predict SE outcomes. In convulsive SE, EEG is of little diagnostic value. The clinical presentations in these patients are clear and the artefact from muscle contraction usually obscures the specific EEG findings. However, in NCSE, the correct diagnosis is impossible without EEG findings especially if the patient is in a state of coma (Bauer and Trinka, 2010, Trinka et al., 2016). Other circulatory markers of brain injury, such as NSE and S100ß protein have been measured in patients with SE with controversial findings (Rabinowicz et al., 1995, Büttner et al., 1999, Palmio et al., 2001). A sensitive and specific biomarker will be very valuable in this context as it will allow for an accurate and rapid diagnosis leading to initiation of effective and individualized treatment options thus reducing the mortality and morbidity. It may also help in predicting treatment response and outcome for these patients (Trinka et al., 2016).

5.1.1.b Aetiology of SE

About 40% of patients presenting with SE have a clear history of epilepsy. The most common cause of SE in those patients is a dramatic drop in serum level of AEDs due to noncompliance (Barry and Hauser, 1993, Trinka et al., 2012). These patients are usually younger and SE outcome has a good prognosis with low mortality rate (4-8.6%) (Towne et al., 1994). Approximately 12% of epilepsy patients presented with SE as their first clinical manifestation (Hesdorffer et al., 1998, Hauser, 1990).
However, in 50-70% of patients, the sustained excitatory synaptic activity leading to SE is usually a consequence of an acute CNS pathology such as cerebrovascular disorders, trauma and infection. These patients are usually older and carry a higher (up to 38%) mortality rate when compared to SE patients secondary to TLE (DeLorenzo et al., 1996). Other uncommon causes of SE include immunologically mediated encephalitis and encephalopathies, mitochondrial diseases, uncommon infective disorders, genetic disorders and drugs or toxins (Trinka et al., 2012).

5.1.1.c Pathophysiology of SE

As with epilepsy, the initiation phase of SE (or might be described as impending SE) is presumed to be mainly due to an imbalance between excitatory and inhibitory neurotransmission leading to multiple and discrete seizures. This phase is clinically manifested as a seizure activity lasting more than 5 minutes. After the initiation phase the abnormal neuronal discharge accompanied by ineffective recruitment of inhibitory neurons and/or excessive neuronal excitation leads to a further propagation of seizures and transition from initiation into maintenance phase leading to established SE (Lowenstein and Alldredge, 1998, Chen et al., 2007).

It is likely that numerous mechanisms are involved in SE initiation and maintenance such as a reduction in inhibitory neurotransmitters (Kapur et al., 1989), activation and inactivation of ion channels, receptor trafficking (mainly GABA, NMDA and glutamate receptors) (Goodkin et al., 2008, Naylor et al., 2005, Naylor et al., 2013) as well as increased expression of pro-convulsive neuropeptides and decreased availability of inhibitory ones (N’Gouemo and Rogawski, 2006).

Long term changes in gene expression have been reported within hours to days after the onset of SE (Chen et al., 2007). At a molecular level, several cell death, neuroprotective and plasticity-associated signalling pathways are triggered after SE. SE is associated with a rapid increase in the generation of reactive oxygen species leading to oxidative stress and damage to different cellular components (Burgess, 2006, Ribak et al., 2006).
5.1.1.d Nonconvulsive Status epilepticus

NCSE refers to a heterogeneous group of diseases with different aetiology, prognosis and treatment. Recently, Rohracher et al. (2016) reported that NCSE was the mode of presentation in about 25-50% of SE patients older than 60 years. This seizure semiology is very subtle and represents a diagnostic challenge to physicians. An added level of difficulty is the effect of associated comorbidity and the often poor baseline cognitive function in these patients which further delays diagnosis and complicates assessment leading to a delay in treatment initiation for days or even weeks (Witt et al., 2014, Rohracher et al., 2016). A diagnosis of NCSE should be considered in all patients with otherwise unexplained changes in consciousness or behaviour. This diagnosis demands rapid further diagnostic work up including detailed history, clinical examination, neuroimaging studies, CSF analysis and an EEG (Rosenow et al., 2012).

5.1.2 Cerebrospinal fluid and its role in biomarker discovery for TLE and SE

5.1.2.a CSF characteristics

CSF is a clear colourless fluid that surrounds the brain and spinal cord. Its main functions are to provide mechanical support for the brain and maintain the neuronal metabolic homeostasis through regulating extracellular fluid physiology. In addition, CSF permeates the brain parenchyma and flushes waste products from the intercellular space into the systemic circulation (Puntis et al., 2016).

Normal CSF volume in an adult human is estimated to be around 150 ml. The majority of CSF is secreted by the choroid plexuses in the lateral, third and fourth ventricles (Oreskovic and Klarica, 2010, Sakka et al., 2011), around 30% of CSF is secreted by ependymal cells of the ventricles. After circulating within the ventricles and cranial/spinal subarachnoid spaces, CSF returns to the peripheral circulation via reabsorption by arachnoid granulations in venous sinuses of the brain (Lehtinen et al., 2013)
5.1.2.b CSF as a source of biomarkers in TLE and SE

CSF has been regarded as an excellent source of molecular biomarkers of numerous neurological diseases. The direct and intimate relationship between CSF and brain tissues may precisely reflect changes in brain structure and function induced by different pathological stimuli. Thus, it can provide critically important diagnostic information related to a number of neurological conditions (Gallego et al., 2012, Schwarz et al., 2012).

Analysis of the molecular and cellular content of CSF is widely used as a diagnostic test in inflammatory and infectious conditions affecting the CNS. The main focus of these diagnostic tests is leucocyte count, cytological findings in addition to analysis of several proteins and antibodies (Huhmer et al., 2006). In addition, CSF contains small membrane particles (extracellular vesicles and exosomes) that are released into it from CNS-constituting cells (Ozsolak and Milos, 2011, Chugh and Dittmer, 2012) providing an extra source of information about neurological pathologies and representing a novel reservoir for biomarker discovery.

Several studies reported up or down regulation of proteins such as gelsolin, fibronectin, superoxide dismutase 1 (SOD1) and α-synuclein in CSF of patients with intractable epilepsy (Peng et al., 2011, Chen et al., 2012, Rong et al., 2015, Xi et al., 2015). Other studies reported an increase in the release of certain CSF membrane particles (CD133-enriched membrane particles) in patients with chronic epilepsy compared to healthy controls (Huttner et al., 2012). In addition, different inflammatory mediators were reported to be upregulated in CSF such as IL-1β and IL-6, TNFα and IL-1R1 (reviewed in (de Vries et al., 2016)). However, none of these studies were replicated in a larger cohort of patients Thus they were not translated into clinically useful biomarkers for epilepsy diagnosis and prognosis.
5.1.2.c CSF microRNAs as potential novel biomarker candidates

As in peripheral blood samples, CSF microRNAs are easily detected and characterized with a remarkable stability when exposed to repeated freeze-thaw cycles and long-term storage (Baraniskin et al., 2012). The first report about the role of CSF microRNAs as a disease biomarker was by Cogswell et al. (2008) who identified the presence of a set of differentially expressed microRNAs in CSF of AD. Several studies followed reporting alteration in a specific set of CSF microRNAs in a number of neurodegenerative diseases such as AD (Kiko et al., 2014), MS (Ahlbrecht et al., 2015), parkinson's diseases (PD) (Gui et al., 2015), and in patients with different brain malignancies (Teplyuk et al., 2012). For example, an increased level of the pro-inflammatory microRNAs (miR-146a and miR-155) was found in CSF of AD patients compared to age matched controls (Alexandrov et al., 2012). In addition, the diagnostic value of CSF microRNAs in glioma patients was assessed in a small pilot study. Out of the six microRNAs studied, miR-21 and miR-15b were found to have a promising diagnostic potential. Both microRNAs were specifically expressed in glioma patients; combination of both microRNAs increased the diagnostic accuracy to 100%. However, confirming these results is essential to endorse microRNA value as biomarkers (Baraniskin et al., 2012).

Genome-wide microRNA analysis studies have also been conducted on CSF for some neurological diseases. These studies are valuable in that they provide a general idea about CSF microRNAs in term of their detection numbers, abundance, expression stability and effect of several disease pathologies. However, most of these studies were performed in the context of biomarker discovery for AD and dementia (Burgos et al., 2013, Denk et al., 2015, Sørensen et al., 2016). This makes a comparison with other studies difficult due to the difference in the age spectrum of the studied individuals. Large differences in microRNA profiles were also noticed within studies analysing the same pathology. For example, while Denk et al. (2015) reported the highest number of detected microRNAs in CSF of AD (441), Haghkia et al. (2012) reported dramatically lower level of detection with only 52 microRNAs detected. This highlights the need for more studies to establish a baseline CSF microRNA profile in healthy individuals and its relation to other factors such as sex and age.
Recently, Gui et al. (2015) analysed exosomal microRNA content in CSF samples collected from AD and PD patients using TaqMan microRNA arrays and compared it to that of age matched controls. Results indicated a detectable level (ct <35) of 132 microRNAs in CSF exosomes. A number of these microRNAs were found to be altered in AD and/or PD when compared to controls suggesting a potential role of CSF exosomal microRNAs as biomarkers of both conditions (Gui et al., 2015).

The possible role of CSF microRNAs as biomarkers in TLE and SE has not been explored. The repetitive or prolonged seizures in TLE and SE might alter microRNA expression in CSF reflecting the underlying neuronal injury, activation of glia and interruption of the integrity of the blood- and/or CSF-brain barrier. Identification of a unique microRNA signature in CSF of TLE and SE patients may help in developing a sensitive and specific biomarker test for both conditions.
5.1.3 Chapter summary

Hypothesis: CSF microRNAs differ in TLE and SE patients when compared to controls.

Aims: to investigate microRNA expression profiles in CSF of TLE and SE patients and compare these to controls and to other neurological diseases. The study was conducted in two phases. First, a profiling phase for the global detection of microRNAs in CSF. Second, a validation phase to confirm the profiling results where the dysregulation of microRNAs was confirmed by both absolute and relative microRNA quantification techniques. Here we focused on a set of three microRNAs with a significant dysregulation when comparing TLE and SE CSF with that of controls and other neurological diseases. Different combinations of these microRNAs were analysed to obtain the best performing test in regard to both sensitivity and specificity. To further investigate their role in TLE and SE, predicted targets for these microRNAs were identified and their presumed impact on different cellular processes and pathways in relation to epilepsy were investigated.
5.2 Results

5.2.1 Genome-wide microRNA profiles in CSF of TLE and SE patients

High throughput screening of microRNA expression in CSF samples collected from 15 controls, 15 TLE, 15 SE patients using the QuantStudio™ 12K Flex OA system indicated that, on average, 51 microRNAs were detected per CSF sample (range: 6 - 100) (Figure 5.1A). In eight samples (5 controls, 2 TLE and one SE), fewer than 25 microRNAs have been detected, these samples were removed from subsequent analysis due to very low microRNA expression, leaving 37 samples (10 controls; 13 TLE and 14 SE).

In general, a significantly higher number of microRNAs were detected in the SE samples (average 74 per sample, p < 0.001). 42 of these microRNAs were identified in at least 50% of all SE samples (Ct < 35). The number of detected microRNAs in control and TLE CSF samples was comparable (43 and 56 on average per sample, respectively) for both conditions (Figure 5.1B).

The most abundant microRNAs identified in CSF were: miR-24-3p, miR-30b-5p, miR-30c-5p, miR-150-5p, miR-204-5p, miR-223-3p, miR-320a and miR-483-5p. Overall, the most abundant and most consistently detected microRNA in each of the three sets of samples were very similar. A PCA (Figure 5.1C) and a heatmap showing hierarchical clustering of the normalised Ct values in the samples in two dimensions (Figure 5.1D) failed to show any clear separation of the three groups, further supporting that the CSF microRNA profiles of TLE and SE patients are broadly similar to controls.

Although the PCA and heatmap clustering did not indicate a large difference in microRNA profiles, a differential expression analysis revealed a number of significantly regulated microRNAs between the three groups. This included five differentially expressed in TLE compared to controls (one down and four upregulated) and 15 microRNAs in SE samples compared to controls (seven down and eight upregulated) (Table 5.1).
Figure 5.1: Genome-wide microRNA profiles in CSF of TLE and SE patients.

(A and B) Bar plots showing the number of microRNA detected in each sample before (A) and after (B) filtering to remove any microRNA not present in at least 50% of samples. (C) PCA plot showing clustering of samples from the three groups. The PCA was performed on samples after imputing missing values, normalisation of Ct values and batch correction for sample origin. (D) Heat map and unsupervised two-way hierarchical clustering by sample and microRNA. Dendrograms were constructed using complete linkage method with Euclidean distance measure.

(The figure was generated with the help of Catherine Mooney, PhD.). CH: Control headache; TLE: temporal lobe epilepsy; SE: Status epilepticus.
Table 5.1: $\log_2(\text{FC})$ and adjusted $p$-value for significantly differential expressed microRNA after screening by QuantStudio™ 12K Flex OA PCR system.

### CH and SE samples

<table>
<thead>
<tr>
<th>microRNA</th>
<th>Log$_2$(FC)</th>
<th>adj $p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-21-5p*</td>
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</tr>
<tr>
<td>miR-451a*</td>
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</tr>
<tr>
<td>miR-886-3p*</td>
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<tr>
<td>miR-9-3p</td>
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<tr>
<td>miR-17-5p</td>
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<td>miR-30c-5p</td>
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### CH and TLE samples

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<tr>
<td>miR-548a-3p</td>
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</table>

Key: CH: Control headache; FC: fold change; TLE: temporal lobe epilepsy; SE: Status epilepticus.

15 and 5 microRNAs were significantly differentially expressed when comparing CH CSF to SE and TLE CSF respectively. Key: (*) microRNAs selected for validation, CH: Control with headache, TLE: temporal lobe epilepsy, SE: Status epilepticus, FC: fold change.
5.2.2 Validation of the differentially expressed microRNAs in CSF from TLE and SE patients

To confirm the OA findings, three microRNAs with the highest fold change and lowest p-values (miR-21-5p, miR-451a and miR-886-3p) were selected for validation with individual RT-qPCR. In addition, miR-204-5p and miR-19b-3p were chosen for validation as they were significantly differentially expressed in both control/TLE and control/SE. Finally, as the significantly differentially expressed microRNAs between the control and TLE samples all had borderline significant p-value, and none of them had a log₂-fold change above 2, we chose miR-223-3p as it has been shown to be expressed in astrocytes and microglia (Jovicic et al., 2013).

A fourth group of CSF samples (CND) collected from patients with other neurological diseases was included in the validation step to further verify the sensitivity and specificity of the detected microRNAs. This included CSF from AD, MS and other diseases including primary and secondary brain tumours.

A major finding in the current study was the identification of microRNAs that were specific for SE. Levels of miR-451a were higher in SE compared to controls, TLE and other neurological disease samples (Figure 5.2A, Table 5.2). Likewise, Levels of miR-21-5p, were significantly higher in SE when compared to both CH and TLE (Fig. 5.2B, Table 5.2). A tendency toward miR-21-5p upregulation was also observed when comparing SE samples with CND group. The specific increase of these microRNAs in SE samples suggests the promising potential of miR-451a and miR-21-5p, either alone or in combination, as a specific biomarker for SE.

Despite showing the same trend during validation, the decrease in miR-886-3p level in SE compared to controls was found not to be significant. In general, miR-886-3p level was higher in both CH and TLE samples when compared to both SE and CND. The only significant difference was between TLE and CND (Fig. 5.2C, Table 5.2). However, a borderline significance was detected between CH and CND groups. This draws our attention to the possibility of other factors that might be responsible for this difference.
Figure 5.2: Validation phase of CSF microRNA.

Boxplots showing the distribution of normalized ct following TaqMan individual microRNA assays of (A) miR-19b-3p, (B) miR-21-5p, (C) miR-451a, (D) miR-204-5p, (E) miR-886-3p and (F) miR-223-3p in each of the four sets of samples: CH (N = 25), CND (N = 25), SE (N = 16) and TLE (N = 14). CH: controls with headache, TLE: temporal lobe epilepsy, SE: status epilepticus, CND: controls with other neurological diseases. Note: lower ct indicates higher expression.
Table 5.2: Validation of differentially expressed microRNAs in CSF of CH, TLE, SE and CND.

<table>
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<tr>
<th>A</th>
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Log₂ FC and Adj. p-value of (A) miR-19b-3p, (B) miR-21-5p, (C) miR-451a, (D) miR-886-3p, (E) miR-204-5p and (F) miR-223-3p. CH: controls with headache, TLE: temporal lobe epilepsy, SE: status epilepticus, CND: controls with other neurological diseases, FC: fold change.
Among the three microRNAs found to be differentially expressed between control and TLE during profiling (miR-204-5p, miR-19b-3p and miR-223-3p), levels of miR-19b-3p were confirmed as significantly differentially expressed (Figure 5.2D, Table 5.2). Levels of miR-19b-3p were significantly lower in TLE samples compared to controls, SE and samples from patients with other neurological diseases, indicating reduced CSF levels of miR-19b-3p may be a biomarker of TLE. No difference in the levels of miR-204-5p and miR-223-3p was detected between the study groups (Figure 5.2E, F and Table 5.2).

In general, the validation experiments confirmed the OA profiling data (with the exception of miR-19b-3p) and statistical analysis showed a number of the microRNA to be significantly differentially expressed between these four groups.

5.2.3 Influence of age on CSF levels of microRNAs

Since the SE and CND groups included, on average, older patients than TLE and control groups, the age-related influences on the expression of the validated microRNAs in CSF was checked. The relative expression of the four microRNAs in both control groups was plotted against age. While there was no influence of age on CSF levels of miR-451a (Figure 5.3A), miR-21-5p (Figure 5.3B) or miR-19b-3p (Figure 5.3C), there was a strong correlation for miR-886-3p (Figure 5.3D) indicting the dysregulation of miR-886-3p is likely age-related. We therefore dropped miR-886-3p from the remaining analyses.

5.2.4 Absolute quantification of miR-451a, miR-21-5p and miR-19b-3p in CSF samples

To further confirm the validation results, a set of three CSF samples from each group and for each microRNA were chosen to identify the absolute copy number of miR-451a, miR-21-5p and miR-19b-3p per microliter of CSF. Samples were chosen based on their ct values during the validation step; the samples with ct values close to the average ct for that specific microRNA in each group were included.
Figure 5.3: Correlation of level of differentially expressed microRNAs with age.

The normalized ct value of (A) miR-19b-3p, (B) miR-21-5p, (C) miR-451a and (D) miR-886-3p was plotted against age. No correlation was observed for miR-451a, miR-21-5p or miR-19b-3p. A strong correlation for miR-886-3p was detected suggesting an effect of age on the CSF level of this microRNA.
First, to identify the dynamic range of the dPCR in measuring microRNA copy number in CSF samples, cDNA was transcribed and diluted by two fold from 1:2 up to 1:32. The serially diluted samples were run on dPCR chips to obtain the absolute copy number of miR-451a, miR-21-5p and miR-19b-3p in each dilution series. An excellent linearity between the cDNA input and copy number values measured for each targeted microRNA was observed confirming the suitability of dPCR for detecting microRNA copy numbers in samples with very low RNA content (Figure 5.4 A-C).

The absolute microRNA copy number was then calculated in each set of samples. Figure 5.4D shows representative miR-451a chips from each study group. Results indicated that miR-451a had the highest copy number in CSF of SE patients with an average of 279.5 copies / µl CSF (range 25.9-745.4). In contrast, a very low expression of miR-451a was observed in samples from the other three groups. The average copy number of miR-451a in controls, TLE and CND samples was 7.7 (range 8.6-10.4), 12.2 (range 6.0-13.7) and 11.9 (range 11.0-13.1) respectively (Figure 5.4E) confirming both the OA and the validation data.

Similarly, the average copy number of miR-21-5p was higher in SE (75.9 copies/µl) followed by CND samples (59.0 copies/µl), further supporting OA and validation data. miR-21-5p content was lower in TLE and control samples (14.7 and 31.2 copies/µl respectively) (Figure 5.4F). On the other hand, the average copy number of miR-19b-3p in SE samples was 57.4 which is higher than the content in controls, TLE and CND groups (6.6, 24.3 and 27.1 copies/ µl respectively) (Figure 5.4G).
Figure 5.4: Absolute copy number of miR-451a, miR-21-5p and miR-19b-3p in CSF samples.

(A,B,C) Graphs depicting detection of miR-451a, miR-21-5p and miR-19b-3p per μl of dPCR reaction assay in serially diluted cDNA samples confirming suitability for measuring microRNA copy numbers in CSF. (D) Representative chips show the number of miR-451a positive wells in cDNA isolated from CSF samples for each group. Blue dots represent positive signal from amplified miR-451a. (E,F,G) Copy numbers for miR-451a, miR-21-5p and miR-19b-3p in representative CSF samples from the four groups (n = 3/group). CH: controls with headache, TLE: temporal lobe epilepsy, SE: status epilepticus, CND: controls with other neurological diseases.
5.2.5 miR-451a, miR-21-5p and miR-19b-3p as predictive biomarkers for TLE and SE

To evaluate the diagnostic value of the three identified microRNAs in discriminating TLE and SE samples from each other and from the two control groups, ROC analysis was performed and AUC was calculated as an index for the accuracy of the test. The closer the value of the AUC to 1 the better the sensitivity and specificity of the microRNA.

The best performing single microRNA in differentiating SE from the TLE, control and CND groups was miR-451a with AUC of 0.91, 0.8 and 0.74 respectively (Figure 5.5 A-C). Altogether, miR-451a was successful in differentiating SE samples from all other sample types with an AUC of 0.8 (Figure 5.5 G). Similarly, the best performance for miR-21-5p was in separating SE from control samples, with an AUC of 0.83. The AUC for miR-21-5p in separating SE from TLE and CND was 0.8 and 0.72 respectively (Figure 5.5 D-F). The ability of miR-21-5p to differentiate SE from all other samples gave an AUC of 0.78 (Figure 5.5H). This indicates that both microRNAs have a possible role in sensitive and specific separation of SE from other conditions. The combination of miR-451a and miR-21-5p data to discriminate SE samples from the three other groups generated an AUC of 0.85 which was as accurate as combining the three microRNAs (Figure 5.5 I, J).

Levels of miR-19b-3p showed the best separation between TLE and control, SE, CND and all groups combined with AUC of 0.73, 0.78, 0.76 and 0.75 respectively (Figure 5.6 A-D). To improve the sensitivity and specificity of miR-19b-3p, different combinations between this microRNA and other studied microRNAs were analysed. Combining miR-19b-3p with miR-21-5p or miR-451a increased the AUC to 0.78 and 0.82 respectively (Figure 5.5 E, F). However, the best discriminating potential was obtained by combining all three microRNAs (Figure 5.5 G). This increased the AUC to 0.83 which was more than combining any pair of microRNAs.
Figure 5.5: The diagnostic potential for miR-451a and miR-21-5p in SE.

ROC analysis was generated by blotting the sensitivity against specificity. AUC for miR-451a and miR-21-5p showed the separation of SE from TLE (A, D), CH (B, E), CND (C, F) and all samples combined (G, H). To improve sensitivity and specificity, binomial logistic regression for combination of miR-451a and miR-21-5p (I) and miR-451a, miR-21-5p and miR-19b-3p (J) increased the AUE for up to 0.85.
Figure 5.6: The diagnostic potential for miR-19b-3p in TLE.

Analysis of AUC indicated a good separation between TLE and CH (A), SE (B) and CND (C) and all samples combined (D). To improve sensitivity and specificity, binomial logistic regression for combination of miR-19b-3p and miR-21-5p (E) or miR-451a (F), or miR-21-5p and miR-19b-3p (G) increased the AUE for up to 0.83.
Collectively, these results suggest that CSF miR-19b-3p, miR-21-5p and miR-451a may be a novel combination of microRNAs which can discriminate TLE samples from controls, patients with other neurological diseases and SE. The results also show that the combination of two microRNAs (miR-21-5p and miR-451a) can distinguish SE samples from TLE, controls and other neurological diseases.

5.2.6 Extracellular transport mechanisms of microRNAs in CSF

Since unique pathophysiological aspects of neurological disease states such as the activation of astrocytes or microglia relative to neuronal injury and hyperexcitability may influence the release mechanisms and composition of extracellular microRNAs, an assessment of the form in which the three differentially expressed microRNAs are packaged and released into the CSF could provide additional discriminatory value. To investigate this, CSF samples from each group were pooled into 5 pools for CH, TLE and SE and 3 pools for AD and MS. The levels of miR-19b-3p, miR-21-5p and miR-451a complexed to Ago-2 or present in exosomes relative to the total microRNA content in CSF was analysed in each pool. A number of important findings were revealed (Fig. 5.7A-C). First, each specific microRNA has a unique pattern of distribution between exosomes and Ago-2. For miR-19b-3p, the best biomarker for TLE, the ratio between exosomal to Ago-2-bound form was roughly similar in CH, TLE and AD. However, majority of the microRNA was present in exosomes in MS and tended to be more Ago-2-bound in SE (Figure 5.7A). miR-451a, in general, was characterized by a very low expression in samples from CH, TLE, AD and MS. Despite that, a tendency towards exosomes rather than Ago-2 was evident in CH, SE and AD samples. The opposite was true for TLE samples with more microRNA bound to Ago-2 (Figure 5.6B). Likewise, miR-21-5p, was mainly exosomal in CH and MS samples, more of miR-21-5p was found to be bound to Ago-2 in SE, AD and TLE (Figure 5.7C).
Figure 5.7: Molecular carriage of CSF microRNAs.

Relative expression of Ago-2-bound versus exosomal microRNA fractions for miR-19b-3p (A), miR-451a (B) and miR-21-5p (C) in the five study groups. A unique release form was recognized for the three microRNAs in different disease conditions when compared to control. A microRNA that is mainly exosomal in controls was found to shift under pathological conditions to be more Ago-2 bound and vice versa, suggesting an influence of the normal/abnormal physiological state on CSF microRNA release forms. CH: controls with headache, TLE: temporal lobe epilepsy, SE: status epilepticus, MS: Multiple sclerosis, AD: Alzheimer’s disease.
Next, ROC analysis and calculation of the AUC was performed to investigate whether the form by which each microRNA circulates in CSF (Ago-2-bound or exosomal) could have a higher discriminatory value compared to total microRNA level for distinguishing SE and TLE samples from each other’s and from the two control groups. For separating SE from TLE, AD and MS the AUC for miR-451a, miR-21-5p and a combination of both was 0.58, 0.79 and 0.77 respectively when using the Ago-2-bound fraction of the microRNA (Figure 5.8 A). These values improved (0.8, 0.82 and 0.84 respectively) when using exosomal fraction (Figure 5.8 B). In regard to TLE, poor performance of both exosomal and Ago-2-bound fractions of miR-19b-3p was noted when separating TLE from other condition (AUC below 0.6) (Figure 5.8 C and D). This increased to more than 0.8 when combining exosomal part of miR-19b-3p to that of miR-21-5p and/or miR-451a (Figure 5.8 D). However, AUC failed to improve when using the Ago-2 bound form of microRNAs (Figure 5.8C). In general, all comparisons showed higher accuracy of the microRNA to separate SE and TLE from other conditions when the exosomal microRNA was used versus the Ago-2 microRNA implying that exosomal microRNA may in some way more accurately reflect the pathophysiology of SE or TLE.

5.2.7 Correlation of microRNA level in CSF and plasma

To determine whether levels of the same set of microRNAs in other forms of samples would reflect the CSF microRNA profiles, a correlation between miR-19b-3p, miR-21-5p and miR-451a levels in CSF and in plasma samples collected from the same patient and on the same day was performed in a set of TLE patients (n=7). This analysis is important because there would only be value in measuring CSF microRNAs if they provided diagnostic value above that which could be achieved simply by measuring their level in blood. There was no observed correlation for miR-19b-3p or miR-21-5p between CSF and plasma levels (Figure 5.9 A and B). The levels of miR-451a obtained from plasma showed a trend toward negative correlation with the levels in the same individual in CSF (Figure 5.9 C), although this did not reach statistical significance (p = -0.75, p = 0.066). These findings demonstrate that microRNAs in CSF provide distinct information not available from analysis of plasma.
Figure 5.8: ROC analysis and AUC for the exosomal and Ago-bound fractions of dysregulated microRNAs and their potential to separate SE and/or TLE from MS and AD.

In SE: (A) Ago-2 bound miR-21-5p, miR-451a and a combination of both produced an AUC of less than 0.8 for separating SE from other conditions, (B) exosomal fraction of miR-21-5p and miR-451a and a combination of both increased AUC for up to 0.85 for separating SE from other conditions. In TLE, (C) using Ago-2-bound form of miR-19b-3p alone or in combination with miR-21-5p and/or miR-451a produced an AUC less than 0.7, (D) exosomal form of miR-19b-3p performed poorly in differentiating TLE from SE, AD and MS, combining miR-19b-3p with miR-451a and/or miR21-5p improved AUC.
Figure 5.9: Correlation of microRNA level in CSF and plasma.

Correlation between CSF and plasma expression of (A) miR-19b-3p, (B) miR-21-5p and (C) miR-451a. No significant correlation was detected for miR-19b-3p or miR-21-5p and miR-451a levels between CSF and plasma.
5.2.8 Cellular localization of CSF microRNA biomarkers

To gain a deeper insight about the differentially expressed microRNAs and to establish whether the detected microRNA signal was specific i.e. due to release from one or more brain cell type or non-specific, for example due to blood cell contamination of CSF secondary to BBB disruption in response to seizure, the cellular distribution of the three microRNAs was investigated using fluorescent in situ hybridization. Positive signals for miR-21-5p and miR-451a were detected in tissue sections from the hippocampus of SE patients (Figure 5.10 A, E). Localization of microRNA signal was determined by co-staining of the sections with astrocytic marker (GFAP) (Figure 5.10 B, F). Expression of both microRNAs was observed in astrocyte cell bodies as well as astrocytic processes (for miR-451a) (Figure 5.10 C, G). In contrast, we were unable to detect miR-451a and miR-21-5p expression in tissue sections obtained from TLE patients (Figure 5.10 I, J). To assess and exclude the possibility of any nonspecific probe-microRNA binding that may generate a positive signal, two negative controls were included (a scrambles sequence probe and no probe staining) (Figure 5.10 K, L). Efforts to visualize miR-19b in all section types were unsuccessful.

5.2.9 Pathway links between CSF microRNAs and patho-mechanisms of TLE and SE

Several bioinformatics tools are available to investigate the biological function and the mechanistic role of potential microRNA biomarker candidates in disease conditions. The aberrant expression of the studied microRNAs indicates their possible role in regulating certain pathways with a well-known involvement in the pathophysiology of TLE or SE such as those relating to neuronal injury, neuroinflammatory or remodelling processes which are strongly activated in models and human studies (Pitkänen and Lukasiuk, 2011, Henshall et al., 2016).
Figure 5.10: Cellular localization of microRNA biomarkers of SE.

Panels show representative fluorescent *in situ* hybridization for miR-21-5p and miR-451a in human hippocampal tissue sections from SE and TLE patients. Positive miR-21-5p (A) and miR-451a (E) signals were detected in tissue sections of SE patients. Co-staining of tissue sections with the astrocytic marker GFAP (B, F) allowed the localization of microRNA cellular expression (C, G). Note astrocyte location of both microRNAs in SE tissue whereas expression is low-to-undetectable in TLE samples (I, J) and in the negative controls (Scr probe or microRNA probe omitted) (K, L). TLE: temporal lobe epilepsy, Scr: scrambled; SE: status epilepticus.
5.2.9.a miR-19b-3p, miR-21-5p and miR-451a target prediction and their association with epilepsy

The experimentally validated target genes of the three microRNAs were retrieved from miRTarBase (Chou et al., 2016). Only human targets with strong evidence were retained revealing, in total, 135 protein-coding mRNA predicted for being targets for any of the three microRNAs (Table 5.3). Many of the mRNA targets identified were found to encode proteins with known or implied roles in TLE or regulated in models of SE. miR-21-5p, miR-451a and miR-19b-3p was predicted to target 102, 15 and 24 mRNAs respectively. A number of the identified mRNA targets were found to be regulated by 2 of the three identified microRNA for example BCL-2 and PTEN and MYC (Figure 5.11).

5.2.9.b Identification of microRNA regulatory network and its association with other known epilepsy-related microRNAs

The possibility of a common targeting potential of miR-21-5p, miR-451a and miR-19b-3p (CSF-miRs) and other known epilepsy-related microRNAs (Epi-miRs) was then analysed. A very complex regulatory network was constructed which may describe the functional relevance of CSF microRNA set and model the microRNA-microRNA and microRNA-target interactions. For this purpose, the experimentally validated targets for miR-19b-3p, miR-21-5p and miR-451a were cross checked with these of other 15 microRNAs for which there is in vivo functional data showing effects on seizures and/or pathology in epilepsy models using antagonirs or mimics. These microRNAs are miR-22-3p, miR-23b-3p miR-34a-5p, miR-124-3p, miR-128-3p, miR132-3p, miR-134-5p, miR-146a-5p, miR-155-5p, miR-184-3p, miR-199a-5p, miR-203-3p, miR-210-3p miR-219a-5p and miR-324-5p (reviewed in (Henshall et al., 2016)).
Table 5.3: List of experimentally validated targets (with strong evidence) for each of the differentially expressed microRNAs, extracted using miRTarBase.

<table>
<thead>
<tr>
<th>miR-21-5p</th>
<th>miR-19b-3p</th>
<th>miR-451a</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT2</td>
<td>FMOD</td>
<td>TM9SF3</td>
</tr>
<tr>
<td>ANKRD46</td>
<td>GAS5</td>
<td>PCBP1</td>
</tr>
<tr>
<td>ANP32A</td>
<td>GDF5</td>
<td>PDCD4</td>
</tr>
<tr>
<td>APAF1</td>
<td>HIPK3</td>
<td>PELI1</td>
</tr>
<tr>
<td>BASP1</td>
<td>HNRNPK</td>
<td>Pias3</td>
</tr>
<tr>
<td>BCL-2</td>
<td>HPGD</td>
<td>PLAT</td>
</tr>
<tr>
<td>BCL-6</td>
<td>ICAM1</td>
<td>PLOD3</td>
</tr>
<tr>
<td>BMPR2</td>
<td>IGF1R</td>
<td>Ppara</td>
</tr>
<tr>
<td>BTG2</td>
<td>IL12A</td>
<td>PPIF</td>
</tr>
<tr>
<td>CCL20</td>
<td>IL1B</td>
<td>PTEN</td>
</tr>
<tr>
<td>CCR1</td>
<td>IRAK1</td>
<td>PTX3</td>
</tr>
<tr>
<td>CDC25A</td>
<td>ISCU</td>
<td>RASA1</td>
</tr>
<tr>
<td>CDK2AP1</td>
<td>JAG1</td>
<td>RASGRP1</td>
</tr>
<tr>
<td>CLU</td>
<td>JMY</td>
<td>RECK</td>
</tr>
<tr>
<td>COL4A1</td>
<td>LRRFIP1</td>
<td>REST</td>
</tr>
<tr>
<td>CXCL10</td>
<td>MAP2K3</td>
<td>RF1L</td>
</tr>
<tr>
<td>DAXX</td>
<td>MARCKS</td>
<td>RHO</td>
</tr>
<tr>
<td>DERL1</td>
<td>MEF2C</td>
<td>RHOB</td>
</tr>
<tr>
<td>DOCK4</td>
<td>MMP2</td>
<td>RMND5A</td>
</tr>
<tr>
<td>DOCK5</td>
<td>MMP9</td>
<td>RP57</td>
</tr>
<tr>
<td>DOCK7</td>
<td>MSH2</td>
<td>RTN4</td>
</tr>
<tr>
<td>DUSP10</td>
<td>MSH6</td>
<td>SASH1</td>
</tr>
<tr>
<td>E2F1</td>
<td>MTAP</td>
<td>SATB1</td>
</tr>
<tr>
<td>E2F2</td>
<td>MYC</td>
<td>SECISBP2L</td>
</tr>
<tr>
<td>EGFR</td>
<td>MYD88</td>
<td>SERPINB5</td>
</tr>
<tr>
<td>EIF4A2</td>
<td>NCAPG</td>
<td>SERPIN1</td>
</tr>
<tr>
<td>ELAVL4</td>
<td>NCOA3</td>
<td>SETD2</td>
</tr>
<tr>
<td>ERBB2</td>
<td>NFIA</td>
<td>SIRT2</td>
</tr>
<tr>
<td>FASLG</td>
<td>NFIB</td>
<td>SMAD7</td>
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</table>
Predicted target transcripts for miR-21-5p, miR-19b-3p and miR-451a for which there is strong evidence (reporter assay, western blot, qRT-PCR or qPCR). Retrieved from miRTarBase. Thicker lines represent stronger microRNA-target interaction as evidenced by more publications. Pink/red coloured targets are also targets of one or more of the functionally-validated epi-miRs.
Among the 650 target transcripts identified for the 15 Epi-miRs, 42 mRNA targets were also predicted to be regulated by at least one microRNA of the CSF-miR set (Table 5.4) indicating a possible simultaneous regulatory role of the identified microRNAs in regulating certain epilepsy pathophysiological processes. Two of the 15 Epi-miRs (miR-23b-5p and miR-324-5p) did not have any common target with any of the CSF-miRs.

5.2.9.c Enrichment and gene ontology (GO) analysis for the differentially expressed microRNAs

To assess the biological impact of the differentially expressed microRNAs on different cellular processes and pathways, all the validated targets for miR-19b-3p, miR-21-5p and miR-451a were uploaded to Enrichr (gene set enrichment analysis web server) (Kuleshov et al., 2016). If the targets of CSF-miR set are enriched in a biological process or pathway, then it is reasonable to infer that the regulating microRNA is involved in that process. The top 5 biological processes enriched for CSF-miRs were tissue morphogenesis, apoptotic signalling pathway, intrinsic apoptotic signalling pathway, regulation of protein serine/threonine kinase activity and response to hypoxia. The validated targets for CSF-miRs were then uploaded to Enrichr (http://amp.pharm.mssm.edu/Enrichr/) to explore the gene ontology biological processes and molecular function. The significantly associated gene ontology terms (adjusted p-value < 0.05) were clustered based on their relatedness and any redundancy was removed. Visualization of these results showed that the largest clusters were formed from the gene ontology biological processes involving posttranscriptional regulation of gene expression (including regulation of neuron death), apoptotic signalling pathways, and tissue morphogenesis and remodelling, and the molecular functions involving transcription factor binding, P53 binding and histone deacetylase binding (Figure 5.12 A and B). Significantly enriched (adjusted p-value < 0.001) PANTHER pathways for targets of miR-19b-3p, miR-21-5p and miR-451a are shown in figure 5.12 C. A number of potential epilepsy-related pathways were identified including apoptosis signalling pathways, oxidative stress and toll receptor signalling.
Table 5.4: Number of common target transcripts between miR-21-5p, 451a and miR-19b-3p and a set of 15 microRNAs with an identified *in vivo* evidence of functional relation to TLE.

<table>
<thead>
<tr>
<th>microRNA</th>
<th>Number of common target transcripts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>miR-21-5p</td>
</tr>
<tr>
<td>hsa-miR-155-5p</td>
<td>10</td>
</tr>
<tr>
<td>hsa-miR-203a-3p</td>
<td>5</td>
</tr>
<tr>
<td>hsa-miR-34a-5p</td>
<td>7</td>
</tr>
<tr>
<td>hsa-miR-146a-5p</td>
<td>4</td>
</tr>
<tr>
<td>hsa-miR-22-3p</td>
<td>4</td>
</tr>
<tr>
<td>hsa-miR-124-3p</td>
<td>3</td>
</tr>
<tr>
<td>hsa-miR-199a-5p</td>
<td>3</td>
</tr>
<tr>
<td>hsa-miR-128-3p</td>
<td>3</td>
</tr>
<tr>
<td>hsa-miR-132-3p</td>
<td>2</td>
</tr>
<tr>
<td>hsa-miR-134-5p</td>
<td>2</td>
</tr>
<tr>
<td>hsa-miR-184</td>
<td>3</td>
</tr>
<tr>
<td>hsa-miR-210-3p</td>
<td>1</td>
</tr>
<tr>
<td>hsa-miR-219a-5p</td>
<td>1</td>
</tr>
<tr>
<td>hsa-miR-23b-5p</td>
<td>0</td>
</tr>
<tr>
<td>hsa-miR-324-5p</td>
<td>0</td>
</tr>
</tbody>
</table>

The microRNAs were selected based on the availability of *in vivo* functional data showing effects on seizures and/or pathology in epilepsy models using antagomirs or mimics (Henshall et al., 2016).
Figure 5.12: Enrichment and gene ontology analysis for the differentially expressed microRNAs.

(A,B) Visualization of the significantly associated GO molecular function (A) and biological processes (B) (adjusted \( p \)-value < 0.05) using REVIGO (Supek et al., 2011). The scatterplot shows the cluster in a two dimensional space. Bubble colour indicates the \( \log_{10} \) (adjusted \( p \)-value); bubble size indicates the frequency of the GO term in the underlying Homo sapiens database (bubbles of more general terms are larger). (C) Bar plot showing the \(-\log_{10} \) (adjusted \( p \)-value) of significantly enriched (adjusted \( p \)-value < 0.05) PANTHER pathways (Mi et al., 2016) for validated targets of miR-19b-3p, miR-21-5p and miR-451a. The numbers of targets in a given pathway are shown in parentheses.
5.3 Discussion

In the present chapter, the value of human CSF for the discovery of specific diagnostic biomarkers of both TLE and SE has been evaluated. The main hypothesis was that CSF, due to its intimate relation to brain tissue, would yield a unique microRNA signature that can reflect the underlying disease pathology. Results showed that the levels of miR-19b-3p, miR-21-5p and miR-451a were differentially expressed in CSF samples from TLE and SE patients compared with control subjects and other neurological diseases. Expression levels of these microRNAs in CSF was successfully combined using a logistic regression model to obtain a more specific and sensitive discrimination. Furthermore, models based on microRNA extraction from exosomes performed better in discriminating between samples from different groups when compared to models based on microRNA bound to Ago-2. Together, these findings support the potential of CSF microRNAs as diagnostic biomarkers which could provide assistance for differential diagnosis of epilepsy or SE from other neurological diseases.

Much effort in epilepsy biomarker studies, to date, has been directed towards identifying blood derived molecular biomarkers (Pitkanen and Lukasiuk, 2011a, Pitkanen et al., 2016). The fact that testing CSF is an invasive procedure which is not routinely requested in epilepsy patients hampered its use as a source of biomarkers. However, CSF is potentially more informative when compared to serum or plasma as there is closer contact between brain tissue and fluid. The complete isolation of CSF within the CNS is perfect to bypass any other confounding signals originating from other body organs. In addition, the volume of CSF into which microRNAs disperses is much smaller thus avoiding the dilutional effect of larger volumes (of blood) on aberrant microRNA signal.

The present study represents the first report on CSF microRNAs as potential biomarkers of TLE and SE. microRNA content of CSF was screened in the profiling phase using a qPCR-based platform. The presence and abundance of microRNAs in
CSF is still relatively poorly understood compared to plasma or serum and there is not yet consensus as to the number of microRNAs that can be detected under normal conditions. The number of microRNAs detected is also likely to be influenced by the clinical procedures, profiling methods and the analysis techniques used (i.e. the criteria used to declare microRNAs present or absent, filtering, etc.). Recent work suggests numbers of CSF microRNAs range from 50 or less in some studies (Ca et al., 2012) to over 400 (Denk et al., 2015). Other studies have reported detection in the range of 50 to 100 microRNAs on average per sample (Sala Frigerio et al., 2013, Holm et al., 2014, Sørensen et al., 2016) suggesting the number of microRNA identified in common-to-all CSF samples in a particular study is often much lower. In the present study, over 40 microRNAs were called present in at least half the samples. Accordingly, the OA platform performs within the expected range. Moreover, many microRNAs that were identified among the most abundant in the present study, have been reported as highly expressed in CSF using other detection platforms, for example RNA-seq (Burgos et al., 2013). None of the CSF microRNAs were unique compared to the previous findings in human plasma (chapter 3). However, the poor correlation between CSF and plasma or serum microRNA levels (Burgos et al., 2013, Freischmidt et al., 2013, Liu et al., 2014) suggests that there is potentially an independent origin of the microRNA in CSF compared to plasma/serum.

Despite the relatively low numbers of microRNAs in CSF those that were differentially expressed in TLE or SE was proportionally high. This supports CSF as a good source of biomarkers relative to blood in which the fraction of differentially expressed microRNAs was much smaller relative to those detected (chapter 4 and (Wang et al., 2015b, Wang et al., 2015a)). An obvious reason for the higher relative detection of differentially-expressed microRNAs in CSF is proximity to the area of principle pathology. Differences in microRNA levels between neurological diseases may be diluted within the systemic circulation whereas the volume and circulation of CSF may favour biomarker detection. Another strength of the present study was
to analyse individual samples during profiling rather than pooling patient samples which may mask important differences.

Clinically, the most interesting comparisons are between TLE or SE and the controls and other neurological diseases. Subtypes of each condition should ideally be possible to differentiate using a biomarker. Here, a set of three microRNAs, miR-19b-3p, miR-21-5p and miR-451a, were identified as differentially expressed in TLE or in SE compared to controls and other neurological diseases. ROC analysis supported good diagnostic accuracy of these microRNAs. A unique strength of the present study was the inclusion of CSF from patients with other neurological diseases to further confirm the sensitivity and specificity of these microRNAs in diagnosing TLE and/or SE. The discriminatory value of the microRNAs for TLE samples compared to controls, other neurological diseases and SE samples was further enhanced when using logistic regression models that combine all three microRNAs. In addition, a combination of miR-21-5p and miR-451a have successfully discriminate SE from TLE, controls and other neurological diseases. Thus, CSF microRNAs, individually or in combination offer potential biomarkers that successfully discriminate TLE and SE from each other’s and from other neurological diseases.

The present study included the first use of dPCR to estimate microRNA copy number in CSF. All previous studies in CSF were performed through obtaining microRNA relative expression using RT-qPCR. Having information about the microRNA copy number in CSF both in healthy controls and in diseased patients is very valuable in the context of biomarker studies as it provides direct information about the change in microRNA expression in health and disease. In addition to eliminating the need for reference samples and reference microRNAs, dPCR has the advantage of being more robust and accurate (Hindson et al., 2013). The QuantStudio® 3D Digital PCR Chip enables accurate quantification of microRNAs through partitioning the sample across 20,000 nanoscale reaction wells (Bellingham
et al., 2017). In the present study, the suitability of the dPCR to test the microRNA copy number in CSF samples was checked first. The excellent linear relation between the copy number and input amount of cDNA indicated an optimum efficiency, precision and sensitivity of dPCR in detecting CSF microRNA signal.

Second, in order to confirm the profiling and validation data, miR-451a, miR-21-5p and miR-19b-3p copy numbers were quantified in a set of three controls, TLE, SE and CND samples. As expected, the average copy number of miR-451a and miR-21-5p was much higher in SE samples confirming their role as biomarkers for this condition. miR-451a was only increased in CSF of SE patients with an average copy number of 279.5 copies/µl compared to less than 15 copies/µl in CSF of the three other groups. The observed wide dynamic range of miR-451a copy number in SE CSF (25.9-745.4 copies/µl) can be explained by many factors such as the semiology of SE, response to treatment, timing to lumber puncture and others. Levels of miR-21-5p were higher in both SE and CND patients also confirming validation results. The average copy number of miR-19b-3p determined by dPCR did not follow as closely with the profiling and validation, although levels in TLE samples were lower than in the SE samples as expected.

The present study attempted to gain insight as to the cellular origin of the differentially expressed microRNAs. CSF microRNAs may be intrathecal in origin (Ahlbrecht et al., 2016), or may originate from brain cells, surrounding brain tissues, or extracranial tissues due to blood- or cerebrospinal-brain barrier disruption associated with seizures (Teplyuk et al., 2012, Freischmidt et al., 2013). While miR-451a has been reported to be upregulated in brain tissue after seizures and trauma (Truettner et al., 2011, Freischmidt et al., 2013, Lee et al., 2016), it is known to be particularly abundant in blood cells (Azzouzi et al., 2015). It was important, therefore, to explore the cell types expressing this microRNA. In the present study, miR-451a and miR-21-5p were found to colocalize with GFAP positive astrocytes in SE patients. Given we did not find higher levels of other blood cell-abundant
microRNAs in SE samples the increase may reflect upregulation of miR-451a within this cell type or result from uptake from the local environment. Notably, direct brain exposure to blood components secondary to BBB leakage after SE was associated with albumin uptake into astrocytes which contributes to promoting a hyperexcitable state (Ivens et al., 2006). The same may be true for miR-451a over expression in astrocytes. However, additional experiments will be needed to resolve these possibilities and any pathophysiological significance for increased miR-451a expression on astrocyte function.

A novel approach in the present study was to investigate the physical form in which microRNAs circulate in the CSF as it is likely to differ between neurological diseases. In SE, microRNA release may be viewed as a consequence of acute neuronal injury (i.e. lytic release) or reactive gliosis (Henshall, 2013). In TLE where the impact of seizures is less harmful there may be a more controlled release of microRNAs from a mixed population of neurons and reactive glia. MicroRNAs can be released via either passive or active mechanisms (Turchinovich et al., 2016). Assessing the relative amounts of microRNAs between these systems offers an additional diagnostic sensitivity since it is likely that the relative glial and/or neuronal injury contributions to disease pathology differ between neurological diseases due to ischemic components versus degenerative versus inflammatory (Liou et al., 2003, Pitkanen et al., 2015). Neurons and glia may differ in their capacity to produce exosomes or release Ago-2-bound microRNAs and this may vary across diseases. In the present study, a higher amounts of exosomal miR-19b-3p in CSF from patients with MS was detected, a disease that is characterized by demyelination and glia-mediated neuroinflammatory mechanisms (Mishra and Yong, 2016). In contrast, miR-19b-3p was found to be mostly Ago-2-bound in SE samples, suggesting a more passive release after cell death. The amount of miR-451a and miR-21-5p that are released in the Ago-2 bound form was more in TLE and SE when compared to CH. Differences were found as well for miR-451a in MS and miR-21-5p in AD. Thus, the underlying pathophysiology may influence the physical form in which extracellular
microRNAs circulate and this can be captured to enhance diagnostic insights. The molecular mechanisms that underlie microRNA sorting into macrovesicles such as exosomes and their trafficking and receipt by target cells are increasingly understood and thought to represent forms of paracrine signalling. Indeed, the microRNA cargo within these vesicles has been demonstrated to influence gene expression in recipient cells (Valadi et al., 2007). Whether exosomal microRNAs in CSF have signalling roles or rather reflect the distribution of material from brain tissue in a more indirect way requires further investigation.

Additional findings have supported the presence of microRNAs in CSF as being directly related to disease pathophysiology. Over half of the microRNAs found to be differentially expressed in the profiling phase have been detected in at least one neural cell type, or brain region, in studies of rodent brains (Lagos-Quintana et al., 2002, Jovicic et al., 2013, Butovsky et al., 2014), and a quarter of them are differentially expressed in profiling studies of hippocampal tissue resected from TLE patients (Kan et al., 2012, Kaalund et al., 2014, Miller-Delaney et al., 2015). Over 80% of them can be found in EpimiRBase, a database of microRNA-epilepsy associations (http://www.epimirbase.eu/) (Mooney et al., 2016) implying that they have been identified as regulated in at least one epilepsy study in human or rodent. Indeed, the microRNAs that were identified here as potential CSF biomarkers (miR-19b-3p, miR-21-5p and miR-451a) have all been previously shown to be upregulated after SE in brain profiling studies of both rat (Bot et al., 2013, Peng et al., 2013, Risbud and Porter, 2013, Gorter et al., 2014, Roncon et al., 2015) and mouse (Jimenez-Mateos et al., 2011, Kretschmann et al., 2015).

miR-21-5p is one of the microRNAs that are widely dysregulated in many pathological conditions. Its dysregulation in various neoplastic, cardiovascular and neurological diseases reflects its diverse mechanisms of actions. miR-21-5p upregulation after SE and in chronic epilepsy was documented in animal models. An increased level of this microRNA has been reported in different hippocampal
regions only 4 hours after the onset of pilocarpine induced SE and continue for up to 3 weeks (Gorter et al., 2014, Risbud et al., 2011). In addition, glial (and neuronal) overexpression of miR-21-5p was reported in rat model of TBI. miR-21-5p upregulation was associated with more favourable neuronal function, less severe brain oedema and decrease in lesion volume after injury, suggesting a protective potential of this microRNA (Ge et al., 2014). The protective effect was also reported after ischemic stroke where an overexpression of miR-21-5p with downregulation of its predicted targets Faslg (a member of the TNF-α family) played a critical role in reducing ischemic cell death by targeting important cell death pathways (Buller et al., 2010).

In contrast to miR-21-5p, little is known about miR-451a dysregulation in TLE and SE conditions. miR-451a was found to be upregulated in cortex and hippocampus of chronic epileptic mice and in human TLE tissues (McKiernan et al., 2012a, Lee et al., 2016). miR-451a upregulation was also reported in animal models of TBI (Truettner et al., 2011). Although there have been no functional studies in CNS, miR-451a has been identified for having an important role in cellular protection against oxidative stress damage. This was reported in RBCs, macrophages, cardiac muscles and other tissues (Cho et al., 2015, Wang et al., 2012b), were accumulation of reactive oxygen species has been reported in miR-451a/− cells after exposure to H2O2. Accordingly, the upregulation of miR-21-5p and miR-451a can be viewed as a measure to alleviate the burden of continuous or repeated seizures on neuronal cells and thereby reduce neuronal damage.

Further insight regarding microRNA function was gained from examining the target transcripts of these microRNAs. Among targets with strong correlation to epilepsy are BCL2L11 (BCL-2-like protein 11) and TP53 (Cellular tumour antigen p53). Both are targets of miR-19b-3p and were previously reported to be upregulated in experimental and human TLE (Shinoda et al., 2004, Engel et al., 2007). Upregulation of these targets might be secondary to the identified downregulation of miR-19b-
Another interesting target for miR-19b-3p is DNA (cytosine-5)-methyltransferase1 (DNMT1) which regulates DNA methylation, a process that is altered in human epilepsy (Miller-Delaney et al., 2015).

Targets of miR-21-5p that have been implicated in epilepsy include apoptotic protease-activating factor 1 (APAF1) that contributes to caspase activation during SE (Henshall et al., 2001a), IL-1β and myeloid differentiation primary response protein 88 (MYD88), both involved in inflammatory signalling in epilepsy (Maroso et al., 2010).

Other interesting targets include the ABCB1/MDR1 (Multidrug resistance protein 1) which is targeted by miR-451a and is associated with pharmaco-resistance to antiepileptic drugs (Aronica et al., 2003). Other targets of miR-451a with biological relevance to epilepsy include the anti-apoptotic AKT1 (Henshall et al., 2002) and BCL-2 (Henshall et al., 2000).

MicroRNAs usually fine tune gene expression through combinatory and cooperative approach. The identification of multiple target sites for different microRNAs in the 3’UTR of a specific mRNA was reported to have a synergistic effect on the degree of downregulation (Doench et al., 2003). Thus by identifying the regulatory relationship and the potential of common targeting between miR-21-5p, miR-19b-3p, miR-451a and other epilepsy related microRNAs a further support to the mechanistic involvement of these microRNAs in epilepsy might be provided. An example of this common targeting relationship is phosphatase and tensin homolog gene (PTEN) gene that is a predicted target of miR-21-5p and miR-19b-3p. PTEN was also predicted to be targeted by two other Epi-miRs (miR-22-3p and miR-128-3p). This gene has recently been linked to excitotoxic neuronal damage. Targeting this gene and inhibiting its function was found to rescue neuronal death after systemic administration of kainate and decrease the reactive astrogliosis in the CA3 area of the hippocampus (Saxena and Nadkarni, 2011). Similarly, BCL-2, a pro-apoptotic gene was predicted to be targeted both miR-21-5p and miR-451a. In addition, BCL-2
is negatively regulated by two other epilepsy related microRNAs, miR-34a-5p and miR-184.

Other gene that was found to be regulated by miR-21-5p and miR-451a is V-Myc Avian Myelocytomatosis Viral Oncogene Homolog (MYC). MYC is a transcription factor that orchestrates more than 15% of all cellular genes. An altered MYC expression has been reported in many tumours (Dang et al., 2005). MYC over expression increased sensitization of cells to apoptosis secondary to inhibition of the anti-apoptotic and activation of the proapoptotic members of BCL-2 family (Hoffman and Liebermann, 2008). Predicted targeting of this gene by miR-21-5p and miR-451a might be an important mechanism to reduce seizure burden in SE and to promote neuroprotection. Data obtained in the present study showed that MYC is also predicted to be targeted by 3 other Epi-miRs which are miR-155-5p, miR-184 and miR-34a-5p, suggesting a possible role in epilepsy and seizure mechanisms.

Furthermore, the significantly associated biological process and pathways of the validated targets of the microRNA were reflective of known processes and pathways in TLE and SE such as tissue morphogenesis, remodelling and cell differentiation, p53 and apoptosis signalling and TLR and mTOR pathways. This provides encouraging mechanistic support into their biomarker potential despite the fact that microRNA-target predictions are heavily biased by the bulk of the validation work being performed in cancer and cell biology fields (Godard and van Eyll, 2015, Bleazard et al., 2015).

In conclusion, the present study identifies CSF microRNAs as potential biomarkers of TLE and SE. Analyses of the physical form in which the microRNAs circulate provide further diagnostic insight and the targets of the microRNAs and the pathways in which they operate suggest plausible mechanistic links to known pathomechanisms. These findings should be confirmed in other patient cohorts.
6. Final discussion, future studies and concluding remarks
6.1 Discussion

Up till now, TLE continues to be a major burden on the global health system. Both the high rates of misdiagnosis along with the high rate of pharmaco-resistance, in addition to a lack of specific measures to predict prognosis and treatment response have increased the need for a sensitive and specific biomarker for this disease. The search for a practical and a more cost-effective tool for diagnosing seizures and epilepsy and for differentiating them from other episodic phenomena involving neuronal functions continues to be a real challenge. Epilepsy diagnosis is often a complex, multilevel process. The first step in this process is confirming the epileptic nature of the episode and differentiating it from other seizure imitators. Next, specifying the type of seizure and type of epilepsy and finally (if applicable) diagnosing the specific epilepsy syndrome (Fisher et al., 2017b). The need for biomarkers is clearly evident at each diagnostic level and further extends to having a prognostic and theranostic benefits such as identifying at risk people, selecting the best therapeutic options and predicting the course of disease and comorbidities within epilepsy patients.

Currently, a definitive diagnosis of epilepsy can only be achieved after long-term video EEG monitoring. Advances in understanding the molecular basis of epilepsy and the relative ease in profiling the human genome, transcriptome and proteome in these patients has led to the identification of certain molecules with biomarker potential. These molecular biomarkers could be used in isolation or in parallel with neuroimaging and EEG studies to confirm TLE diagnosis. Ideally, the identified biomarker should be sensitive and specific for TLE. Furthermore, it should be quantitative, reproducible, and safe to measure. MicroRNA biomarkers fulfil the above criteria and this paves the way for potential applications of microRNAs in monitoring seizures and diagnosing TLE.
Despite their recent discovery, microRNAs are among the most intensively studied molecules in human and experimental models of epilepsy. The identified link between microRNAs and the development of TLE has nourished the hopes for better understanding of TLE pathogenesis, for developing a new class of sensitive and accurate diagnostic and prognostic biomarkers and, possibly, the development of new therapeutics.

The main focus of the present work was to assess the potential of microRNAs as TLE and/or seizure biomarkers. Although each chapter has a somewhat distinct focus, the overall purpose was to identify a unique extracellular microRNA signature in TLE. In addition to investigating the effect of certain biological and experimental factors on plasma microRNA expression in healthy controls, the present study represents the first effort to extensively characterize the biomarker role of plasma microRNAs in TLE. The present study has the advantage of recruiting patients from different geographic origins which will help in identifying a common diagnostic test that can be applied to different populations. Moreover, combining results from two profiling platforms (used for the initial microRNA profiling) had increased the chances of identifying disease specific microRNA signature as well as has allowed, for the first time, the identification of a microRNA dysregulation signature of epilepsy with minimal confounding bias of the platform used. The obtained results showed that microRNA signatures are affected by the study population demographics indicating the genetic background and other factors influence on the extracellular microRNA profiles. A substantial effect of the microRNA profiling technique was also observed in line with other findings indicating that, it is very important to include two or more methods while designing microRNA biomarker discovery phase (phase one). Despite this, a set of microRNAs that have a common dysregulation pattern irrespective of both factors was identified. This dysregulation was revealed by profiling and confirmed in a proof-of-concept validation using individual qPCR. Together, this provides favourable support for a role of these molecules as diagnostic biomarkers for both TLE and/or epileptic seizures.
The key outcome of the plasma microRNA biomarker study was the identification of a set of dysregulated microRNAs with a value in discriminating TLE from healthy controls. A receiver operating characteristic analysis of the highest achieving microRNA in this regard gave an AUC up to 0.86 (for miR-654-3p). This microRNA performed better than any other microRNA for TLE diagnosis, miR-654-3p is of particular interest since there has been little to no previous work linking the microRNA to epilepsy. A unique post-ictal dysregulation pattern was also observed for the first time in TLE patients for several microRNAs (especially miR-328-3p and 27a-3p) in comparison to its baseline level in TLE patients and/or to its level in healthy volunteers. The existence of a microRNA dysregulation signature after a seizure occurs has an exceptional diagnostic value within the context of differentiating seizures from other similar episodes. Moreover, such a microRNA signature could, in addition, provide support for determining the frequency of seizures and in monitoring treatment response within the TLE patients. Most of the identified microRNAs have been previously linked to TLE pathophysiology either using human or experimental models, thus, increasing confidence in their relevance as TLE biomarkers.

Importantly, the scope of the present study was not limited to plasma microRNA analysis but was extended to examine microRNA dysregulation in other biofluids and their potential for TLE diagnosis. Within this context, CSF represents the most promising type of biofluid that can be utilized in neurological disease biomarker studies. The proximity of CSF to the area of the primary pathology and its continuity with the brain extracellular space, make it a perfect media to discover neurological biomarkers. Studies reported here analysed, for the first time, the microRNA signature in CSF of TLE patients. The search for CSF biomarkers was also extended to involve other important neurological conditions including one that shares a similar clinical presentations and patho-physiological mechanisms to epilepsy; SE. An important finding was that a number of microRNAs were found to be dysregulated in the CSF of TLE and/or SE patients. The value of these microRNAs
especially in differentiating SE from other conditions (TLE, AD, and MS) was very
evident with a high sensitivity and specificity. In addition, comparing the plasma and
CSF levels of the studied microRNAs proved the superiority of CSF for detecting a
differential microRNA signature especially in SE patients. Calculation of the
absolute copy number of several key microRNAs in CSF samples provided further
evidence for their biomarker role. As expected, microRNAs were found in a very low
copy numbers in control CSF. However, levels of miR-451a and miR-21-5p were
found to be substantially increased in patients with SE. Moreover, investigating the
form and mechanism of microRNA release into biofluid has provided an insight
about the role of these microRNAs in the pathophysiology of each condition and
offered ways to further enhance biomarker potential. Exosomal trafficking may
indicate a more controlled form of release compared to Ago-2 bound release which
may be indicative of necrotic release. The form may also reflect cell-of-origin
mechanisms of microRNA release. The identification of a shift in the microRNA
release form, from the normal conditions, alongside recognition of the cellular
origin of these microRNAs provided a further confirmation of specific links to
pathomechanisms of TLE and SE.

Another useful contribution of the present study was the confirmation of microRNA
stability between different times of the day for sampling and between baseline
microRNA signatures in men and women. These parameters were specifically
chosen to be analysed due to their direct relevance to study design. That is, if
microRNA profiles varied substantially according to time of day or displayed strong
sex differences then this would have to be incorporated into any planned sampling
times in biomarker studies. The studied cohort of TLE patients involved both sexes
with blood collection at different times of the day depending on seizure occurrence.
Moreover, the collection of more than one blood sample from each participant,
over different intervals of time (few hours to few days) necessitates confirming the
morning-afternoon and day-to-day stability of microRNA expression profiles. The
obtained results, alongside other previously published data, confirmed this stability
and hence, provided good support for the suitability of microRNAs as disease biomarkers. Effects of different experimental variables on microRNA expression were also analysed. Investigating the effect of sample type, blood collection method, RNA extraction method and others were necessary not only to gain experience in each of them but also to decide the best protocol that will be applied to the whole study.

6.2 Limitations, challenges:

For the plasma biomarker study, inconsistent results obtained after different microRNA profiling methods represented a significant challenge when moving ahead in identifying microRNAs that will accurately identify TLE and/or seizure. It should be noted that differences in microRNA profiling methodologies (qPCR versus RNA-seq), different analytic approaches (including different normalization strategies), population types and others, were among the challenges faced in the present study. Decisions on the parameters to be used for filtering and analysing the OA results were the most challenging. The approach chosen was conservative (in terms of criteria for calling present) but necessary in order to increase the confidence in the data obtained and to lower the incidence of false positive results. However, this have come at the expense of lower microRNA detection call rates. Choosing the appropriate normalization method was another challenge. No single method has been universally accepted for this type of study. The Delta ct method was chosen for normalization as it reduces the technical variability between the samples based on the level of expression of several stable microRNAs within each sample and across the study cohort. Another advantage of this method is the ability to use the same set of microRNAs as normalisers for the subsequent validation of results, thus simplifying study design while improving the reproducibility of data.

The main limitation of the plasma study was the low number of samples used for validation of results. This limits the confidence with which these biomarkers can be judged as representative of all TLE patients. A number of additional analyses, given
larger sample numbers can be envisioned. These, for example, are (1) Increasing the number of TLE patients as well as healthy controls. (2) Including patients with different other epilepsy syndromes as well as PNEAs. (3) Correlating the level of expression of the validated microRNAs with several clinical parameters related either to seizure (such as semiology, severity, duration, etc.), to TLE (such as presence of HS, drug responsiveness, other comorbid conditions, etc.) or to other neuroimaging and EEG findings. These measures will help in verifying the diagnostic performance of these microRNAs.

Another factor to consider is that the present study was conducted in near-ideal collection circumstances where all the factors that may cause a difference in microRNA expression have been reasonably accounted for. For example, the presence of haemolysis, blood collection technique, processing method, etc. However, this is unlikely to be the situation in the clinical setting. It is likely that under real-world conditions it will often be difficult to obtain blood samples with high quality RNA and minimal haemolysis and therefore, a more thorough investigation of sampling factors in addition to other biological factors such as age of the patient, time of blood collection and sex differences should be conducted.

For the CSF study, similar challenges were faced as with the plasma study in relation to data filtering and analysis. The main obstacle in the CSF study, however, was in obtaining sufficient samples. CSF is not routinely obtained from TLE patients as it currently does not have an accepted diagnostic use (except in case of suspicion of autoimmune aetiology). Due to this reason the study was conducted on only 15 TLE and 18 SE patients. Moreover, different centres have widely differing practices with regards to collecting and storing CSF. While for some centres this is routine (e.g. Magdeburg), this biofluid was rarely obtained at others (e.g. Marburg). It will be of great benefit to further validate the currently obtained CSF results in a larger cohort of patients. As with the plasma study, including other epilepsy syndromes, PNEAs patients and correlating the findings with the clinical data for both TLE and SE
patients will enhance the value of these biomarkers. Perhaps of most value would be to compare CSF (or plasma) profiles in NCSE to another condition with similar clinical presentation such as vasovagal syncope or coma.

6.3 The path forward:

Turning now to practical issues with translating these microRNA biomarker discoveries into a clinical tool. A formal structure to guide the process of biomarker development was proposed by Pepe and colleagues (Pepe et al., 2001) and adopted by the National Cancer Institute Early Detection Research Network (EDRN). Despite being mainly formulated for discovering cancer biomarkers, this framework was proved applicable for all other diseases (Füzéry et al., 2013). The five phases of biomarker development include (1) preclinical exploratory studies with the main aim being to identify one or more potentially useful biomarkers which can then be further developed in subsequent stages of the pipeline; (2) clinical assay validation to prove the sensitivity and specificity of the test; (3) retrospective longitudinal repository studies to evaluate the capacity of the biomarker to detect preclinical disease; (4) prospective screening studies to determine the detection rate of the biomarker and this involves screening people for the sake of disease diagnosis and treatment and the final phase is; (5) the disease control studies with its main aim is to estimate the reduction in disease mortality afforded by the screening test (Pepe et al., 2001, Füzéry et al., 2013).

The present study represents a successful phase I biomarker study. Moving forward from here is a long and complex process that requires a breadth of data, expertise, and scientific consensus. This is best achieved by collaboration with pharmaceutical and diagnostics companies with the main aim is to create an effective plan for phase II clinical trial for biomarker discovery.

Step one in this plan larger validation studies designed to support the present preliminary findings. These studies must report microRNA levels in plasma collected
from several hundred patients and healthy individuals representing both sexes, all ethnicities and age groups so that appropriate conclusions can be drawn. This will clinically validate the test and determine its sensitivity and specificity. Here, a 2-stage phase II clinical trial can be designed where in the first stage, the sensitivity of the biomarker in diagnosing TLE can be assessed and in the second stage, the response of the biomarker to treatment and its behaviour in drug responsive versus drug refractory patients can be assessed. In addition to controls and TLE patients, a subgroup of PNEAS patients should be included as a second control group as differentiating these patients from TLE patients is as important as differentiating them from controls.

If these additional phases are successful, the next step is to assess how to move forward. This ultimately comes down to a choice of partnering with an existing company. There are a number of diagnostic companies working on microRNA biomarkers of diseases, including CNS diseases. Partnering with one of these companies would enable the next phases of translation to be successful.

A final and important factor in the ultimate success of a diagnostic would be how microRNA biomarkers link to the pathophysiology in TLE is still very limited. It will be important to compare the expression of the plasma and CSF microRNA biomarkers in hippocampal tissues of epilepsy patients as well as experimental animals and to characterize any effect of silencing or augmenting the microRNA on both epilepsy and epileptogenesis process. A first attempt at this was the identification of miR-451a in astrocytes exclusively in SE patients. Several studies have reported altered seizure thresholds or epileptic phenotypes in experimental animals upon altering the level of certain microRNAs in the brain. However, none of the microRNAs we identified as potential biomarkers (with the exception of miR-199a, where upregulation of the 5p strand was found to be associated with an epileptic phenotype (Wang et al., 2016)) were among those reported to date, thus they may represent excellent choices for functional manipulations.
Moreover, the issue of developing the ability to directly detect ultralow concentration of microRNAs in blood samples without the need for further processing steps is very appealing for diagnostic companies. Recently, collaboration between RCSI and the national centre for sensor research (DCU) has led to the generation of an electrochemical, microfluidic sensor that was successful in accurately determining microRNAs concentration in unprocessed plasma and CSF samples. In a study that was recently published, McArdle et al. (2017) described a theranostic one-step RNA detector (“TORNADO”) for the direct detection of miR-134 in plasma and CSF samples of healthy controls and of patients who experienced seizures (TLE and/or SE). The device is equipped with platinum nanoparticles that are functionalized with probe strand complementary to a particular region of the target microRNA. microRNA-probe hybridization leads to exposure of the electrocatalytic particles to the electrode surface and ultimately an electrochemical detection of the target microRNA. Using this device allowed for obtaining specific microRNA signals without the requirement to extract the RNA, reverse transcribe microRNA target, and run microRNA qPCR for signal detection, leading to a much simpler and quicker detection method that is very suitable for clinical utility. Testing this device in a clinical setting is the next step in biomarker study. This could be trialed in a suitable setting such as a neurological intensive care unit or video-EEG monitoring unit which would establish its usefulness in a hospital setting. All these experiments will pave the way to successfully translate these microRNAs into a clinically valid test.

6.4 Future prospective for microRNA biomarker studies

The transition of microRNA applications from bench to bedside (both as a biomarker and as therapeutic agents) necessitates addressing several challenges through further investigations. As biomarkers, various issues regarding circulatory microRNAs measurement and quantification need refining. First, a better understanding of the exact mechanisms by which microRNAs are released into the
circulation is essential. Second, up to now, there is no consensus on the most appropriate endogenous control for systemic microRNA analysis. In order to obtain reliable and reproducible results, there is a need to determine suitable normalization methods for blood-based microRNAs investigations. Furthermore, as additional short non-coding RNAs are continuously identified through biomarker discovery programmes, the available profiling technologies must adapt their platforms to incorporate newer potentially relevant targets. Functional validation of all microRNAs reported to be dysregulated in a disease state, and the identification of their target genes and pathways is also important.

With regard to therapeutics, whilst progress in this field is rapid and laudable, many obstacles must be overcome for microRNA based therapies to become a reality in management of different diseases. A significant amount of functional work remains to be done to fully elucidate the mechanisms by which microRNAs contribute to disease pathophysiology, and establish a better understanding of the complexity of gene expression regulation by microRNAs. Pharmacological difficulties include developing safe, effective, site specific delivery mechanisms for microRNA directed therapies. Despite these challenges, the remarkable potential of microRNAs as biomarkers and therapeutics cannot be under-estimated (Reschke et al., 2017).

Although microRNAs are the best known and the most frequently assessed non-coding RNA molecule for disease biomarkers, other non-coding RNA species offer future potential in this regard. Examples of these molecules are PIWI-interacting RNAs (piRNAs), small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs) and long non-coding RNAs (lncRNAs). PiRNAs are small non-coding RNAs of 24–31 nt length. In contrast to microRNAs, their biogenesis is Dicer-independent and they were found to interact with the PIWI subfamily of Argonaute proteins. Similar to microRNAs, piRNAs are involved in gene regulation through RNA degradation (Klattenhoff and Theurkauf, 2008). Despite their reported association with germline development, piRNAs have been identified as potential biomarkers for bladder (Chu
et al., 2015), breast (Zhang et al., 2013), and gastric (Cui et al., 2011) cancers. SnoRNAs are key components of the small ribonucleoproteins (snoRNPs) which are responsible for sequence-specific methylation of ribosomal RNA (Kiss-Laszlo et al., 1996). SnoRNAs have been shown to participate in post-transcriptional regulation of rRNA by targeting snoRNPs in the nucleus (King et al., 2003). These molecules have been proposed as potential biomarkers for several forms of human cancers (Thorenoor and Slaby, 2015). These non-coding RNAs represent an important reservoir for biomarker studies. With the exception of IncRNAs, none of the other ncRNA types were investigated within the context of neurological diseases and epilepsy. IncRNAs comprise a heterogeneous group of RNAs larger than 200 nt. They have gained a lot of attention recently as potential biomarkers for several malignant conditions (Yarmishyn and Kurochkin, 2015). IncRNAs have been shown to be involved in diverse tasks such as chromatin modulation, post-transcriptional and post-translational regulation, protein complex organization, and cell-to-cell signalling (Geisler and Coller, 2013). Recently, Lee et al. (2015) analysed a total of 4622 IncRNAs in two mouse models of epilepsy. Hundreds of them were identified as being dysregulated in each model and 118 were commonly dysregulated in both.

6.5 Conclusions

Overall this project has contributed significantly to our evolving knowledge of the role of microRNAs as biomarkers of seizure, TLE and SE. If the current momentum in microRNA translational research can be maintained, this will bring an exciting new dimension to the field of diagnostics and therapeutics for these diseases, and has the potential to transform current practice to the ideal of individualized care for patients in the near future.
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