Epilepsy is a chronic neurologic disorder characterized by persistent seizure activity with psychological, neurological, cognitive and social consequences (1). Seizures are symptoms of uncontrolled excitability of neurons. As per the estimation of world health organization a mean number of 8.93 epilepsy cases per 1000 people were reported, thus affecting approximately 50 million people worldwide (2). 2.4 million new cases occur globally and at least 50% of the cases begin at the childhood. In developed countries, annual new cases are between 40 to 70 per 100 000 people in the general population. In developing countries, this figure is often close to twice as high due to the higher risk of experiencing conditions that can lead to permanent brain damage. Close to 90% of epilepsy cases worldwide are found in developing regions. 70 % to 80% of the patients could lead normal life if treated properly with an early identification. In developing countries, 60% to 90% of people with epilepsy receive no treatment due to inadequacies in health care resources and delivery, and due to social stigma. (3, 4)

Epilepsy is one of the world's oldest recognized conditions. Fear, misunderstanding, discrimination and social stigma have surrounded epilepsy for centuries. Some of the stigma continues today in many countries
and can impact the quality of life for people with the disorder and their families. (4, 5)

1.1 Prevalence of epilepsy in India

Epilepsy is one of the growing neurological disorders in India. The prevalence rate stands at around 5/1000 populations and incidence rate varies from 38 to 49.3 per 100,000 populations per year. The significant risk factors include febrile seizures, family history of epilepsy and head trauma. The generalized seizures were the commonest seizures in the Indian population. Treatment gap estimated to be up to 73.7% to 78% in India (6). Another study noted seizure recurrence in 31% of patients (of all ages) during a follow-up period of 18 months. Longer duration of active epilepsy (relative risk 2.86, 95% CI 2.35–3.48) and a higher number of seizures before seizure control (1.50, 1.30–1.73) increased the risk of recurrence. Seizure-free duration before beginning drug withdrawal (2 years vs. 4 years) did not significantly influence this risk (7). Meta analysis based on twenty published studies shows that 3,207 persons under investigation had epilepsy in sample population of 598,910. This resulted in a crude prevalence of 5.35/1,000. The overall prevalence after the correction was per 1,000 (and its 95% CI) was 5.33 (4.25-6.41); with urban areas at 5.11 (3.49-6.73); rural areas, 5.47 (4.04-6.9); men, 5.88 (3.89-7.87); and women 5.51 (3.49-7.53). The analysis presented with a higher prevalence of epilepsy in urban men and women compared with rural ones, however the
difference was not statistically significant. Age-specific prevalence rates were higher in the younger age group, with the onset of epilepsy reported mostly in the first three decades of the sample population's lives. The treatment gap (i.e., the percentage of those with epilepsy who were receiving no or inadequate treatment) was more than 70% in the rural areas (8).

1.2 Symptomatology, Pathology and Treatment

Epilepsy comprises a large number of syndromes, which vary greatly with respect to their symptomatology, clinical course, treatment and prognosis. However, all these syndromes share a characteristic manifestation i.e. recurrent spontaneous seizure as a chronic condition. An epileptic seizure is a sudden, highly synchronized electrical discharge of neurons, which may occur in virtually every cortical area of the brain, disrupting normal brain functioning varying from few seconds to hours in the so called postictal phase (9). Recurring seizures have devastating behavioral, social and occupational consequences. They may additionally damage the brain and increase pre-existing neurological deficits. Seizures are transient signs and/or symptoms due to abnormal, excessive or synchronous neuronal activity in the brain. These electrical discharges could be associated with other pathological processes such as brain trauma, infection, neoplasm's or congenital disorders, which could produce abnormal wiring of neurons. But most of the cases do not have any demonstrable abnormality in the nervous system, other than the electrical discharges. Hence, demonstrating the
changes associated with electrical discharges and characterizing the seizure is important to classify and manage the epilepsy.

Unfortunately, the current anticonvulsant drugs and complementary therapeutic methods are not sufficient to control seizures in about a third of the epileptic patients. Moreover, some patients cannot tolerate the side effects of the drugs or develop pharmacological resistance. Thus, there is an urgent need for early identification of temporal lobe epilepsy leading to the better treatment of patients.

Temporal lobe epilepsy (TLE) is the most frequent type of epilepsy observed in adult patients with resistance to pharmacological treatment. In TLE, the origin of seizure activity typically involves the hippocampal formation, which displays major neuropathological features, described with the term hippocampal sclerosis (HS) (10). HS is the most frequent pathological substrate of refractory mesial temporal lobe epilepsy (11). Complex partial seizures (CPS) are the predominant seizure type associated with HS (12). CPS could be temporal or extra temporal in origin. CPS due to temporal could be of medial temporal or neocortical. Medial temporal lobe epilepsy (MTLE) is commonly due to mesial temporal sclerosis (MTS). CPS is characterized by three cardinal features aura, amnesia and automatism (3a).

Surgical removal of mesial temporal lobe tissue is a promising therapeutic option in many of these patients. In MTLE, the epileptic focus is located within a circumscribed area of the mesial temporal lobe, often involving the hippocampus, entorhinal cortex and amygdala. Several lines of evidence
support the crucial involvement of these structures in human MTLE: (13-15).

1.3 Classification of epilepsy

As per the scheme proposed by International League Against Epilepsy (ILAE) epileptic seizures described. As per this ILAE scheme seizures are divided into partial and generalized seizures. Partial seizures only involve a localized part of the brain, whereas generalized seizures involve the whole of both hemispheres. Further, partial seizures may be subdivided into simple and complex seizures. This describes the effect of such a seizure on consciousness.

**Simple partial seizures** - originates in one part of the brain and spread to involve other areas of the brain. However, simple seizures will not affect the behavior of the person as there is no loss of consciousness (1).

**Complex seizures** - Interrupt consciousness to varying degrees, thus affecting the behaviour of the person. But complex seizures usually begin in temporal lobe or frontal lobe and temporal lobe and spread to the opposite side in the same area but do not spread or involve in the whole brain. This in turn led to the loss of normal awareness of the environment but without a convulsion.
Secondarily generalized seizures – these are the seizures that begin with bilateral symmetry on both sides of the brain. Mostly, these seizures are inherits and they may be classified as convulsive or absence seizure.

Limbic epilepsy - A particularly difficult form of partial epilepsy is termed limbic epilepsy, because it arises from foci within the limbic system, particularly the temporal lobe (synonyms include complex partial epilepsy, temporal lobe epilepsy, psychomotor epilepsy). Limbic epilepsy is the single most common form of epilepsy, accounting for about 40% of all cases of adult epilepsy. Limbic seizures are often resistant to antiseizure drugs: 30% of adults experience recurrent limbic seizures despite state-of-the-art treatment (16). Limbic seizures induce impairment of consciousness, thereby severely limiting important activities of daily living (such as driving or maintaining employment) and leaving the individual susceptible to bodily injury.

Symptomatic and idiopathic epilepsies: The partial epilepsies are broadly divided into symptomatic and idiopathic epilepsies. The term symptomatic" implies that the epilepsy is a symptom or consequence of some underlying structural lesion of cerebral cortex. This inference is based on the association of lesions in specific regions of cortex with epilepsy, the temporal relation of the lesion to the emergence of epilepsy, and the cure of the epilepsy following excision of the lesion is some instances. Diverse structural lesions can cause limbic epilepsy; in adults, these most commonly arise as a consequence of head trauma or stroke. An increasingly
appreciated cause of symptomatic partial epilepsy is cortical dysgenesis, a
defect of cortical development with distorted neural migration. The term
idiopathic implies that no overt cause has been detected, except for genetic
causes (17, 18). The past 15 years has witnessed the discovery of an array of
genes associated with epilepsy genes (19, 20). Positional cloning of inherited
epilepsies in humans, mice, and flies has identified tens of responsible
mutant genes. The unexpected occurrence of an epileptic phenotype in
mutant mice in which a given gene has been deleted or overexpressed has
also revealed many epilepsy genes.

Approximately 50% of all forms of epilepsy arise in the absence of
neurological deficits or brain lesions and have no known or suspected
external cause. They are classified as idiopathic epilepsies and many of these
epilepsies have been shown to be of genetic origin. Acquired or symptomatic
epilepsies are epilepsy syndromes of known origin due to pre- and post-
natal acquired factors, such as brain infection, brain injury, neoplasm's or
stroke. Hippocampal sclerosis is another major lesion associated with the
medial (medial) temporal lobe epilepsy. As per an estimate, MTLE with
hippocampal sclerosis (HS) could account for 20% of the total epilepsy (21).

1.4 Temporal lobe epilepsy

Temporal lobe epilepsy (TLE) is considered as one of the most common
partial epilepsy type TLE patients often exhibits pharmaco resistance to
antiepileptic drug. Severe cognitive dysfunction is one of the characteristic
feature of this group (22). Surgical intervention by temporal lobectomy is
effective option for the cases where drug-treatment fails. Patients with TLE
experience recurring episodes of spontaneous neuronal activity originating
from the temporal lobe. TLE is considered to be a multi factorial disease,
implying the involvement of multiple susceptibility genes and complex gene-
environment interactions. (23, 24)

TLE can be divided in two main categories, mesial TLE (MTLE) and lateral
TLE (LTLE). MTLE arises in the hippocampus, parahippocampal gyrus and
amygdala (mesial/limbic structures) while LTLE arises in the neocortex of
the temporal lobe of the brain. MTLE can further be characterised based on
the underlying lesions associated with it such as seizure group with
hippocampal sclerosis (HS) and a group without HS (nonHS). MTLE patients
with associated HS pathology often suffered from early life seizures, like
febrile seizures (FS) (12). There are reports that an estimated 36%
association of HS with temporal lobe epilepsy (11, 25), Brain infections like
meningitis and encephalitis are also common. NonHS MTLE is often
associated with specific lesions, such as vascular malformations, neoplasm
and dysplasia. HS is characterized by neuronal cell loss, astrogliosis, granule
cell dispersion and mossy fiber sprouting. The extent of this pathology varies
between patients and is graded by the system devised by Wyler (26). The
neuronal cell loss is not uniformly distributed in the hippocampus, but
mainly confined to the pyramidal cornus amonis (CA) 1, CA3 and CA4
neurons. Pyramidal CA2 neurons and granule cells are largely spared.
Granule cell dispersion is characterized by dispersed granule cells which
form a wider than normal granule cell layer (27-29). The mechanism of granule cell dispersion is ill understood but recent data suggests that displacement of mature neurons rather than altered neurogenesis underlies this dispersion (30). Mossy fiber sprouting is characterized by reorganization of the mossy fibers. Mossy fibers that normally innervate hilar neurons send collaterals to the inner third of the molecular layer of the dentate gyrus (31, 32). Such fibers are thought to form recurrent excitatory circuits and contribute to synchronous firing and epileptiform activity. Whether HS is the cause or consequence of seizures is still a matter of controversy. Presumably HS can be both a cause and an effect of seizures (33, 34).

A better understanding of molecular and functional mechanisms underlying increased excitability in MTLE may eventually result in novel and advanced strategies for the treatment of this chronic disorder. In contrast to idiopathic generalized epilepsies, MTLE does not appear to have a significant genetic component (35, 36). Its pathogenesis may rather involve a combination of developmental, metabolic and/or hypoxic changes. In contrast to its etiology, much more is known about histopathological and functional abnormalities in the chronic stage of MTLE. Several lines of evidence suggest that the hippocampal formation is critically involved in MTLE: (1) recordings from intracerebrally implanted electrodes often identify the onset of electrographic abnormalities within this structure (37). Surgical removal of the amygdala and hippocampal formation considerably diminishes or abolishes seizures in most TLE patients (38). In many TLE
patients, the hippocampal formation exhibits a characteristic and stereotypical pattern of neuropathological damage (39). For these reasons, research on the mechanisms of increased seizure susceptibility in TLE has focused on functional and morphological alterations in the hippocampus proper, its most important input and output structures and the seizure focus zones, i.e. the entorhinal cortex and the amygdala. Figure 1 shows the detailed drawing of the structures associated with the TLE. The same figure has used to mark the seizure intensities based of electrocorticogram.

1.5 Background of research questions

Despite considerable progress in the management and treatment of epilepsy patients, there are still many problems remaining unsolved. The most important include pharmacoresistance in around one third of patients, unknown mechanisms of epileptogenesis and heterogeneity of clinical outcomes in patients with chronic epilepsy. Reliable biomarkers are essential for identifying specific problems and quantitatively measuring our success in resolving them, not only for research purposes, but also for individual patient care. Surprisingly, the field of epilepsy has no reliable biomarkers, as yet (40). In parallel to structural neuroimaging or electrophysiological studies, there is an increasing interest in biochemical brain-specific surrogate markers. There is a need for sensitive in vivo assessment methods that are quantitative, reliable, reproducible, and safe. For repeated use over a period of years, they must be acceptable to patients and to a healthy control group. If large numbers of patients are to be evaluated, it is beneficial if the methods can be reliably applied in
multiple sites. Biochemical biomarkers specific for the CNS pathology and disease fulfill the above criteria. This paves the way for potential applications of biochemical biomarkers such as monitoring seizure related pathological processes, following the process of epileptogenesis after brain insult, and indentifying patients at risk of pharmacoresistance.

There is general consensus that status epilepticus has deleterious effects on brain tissue, but whether brief recurrent seizures are also destructive to neurons is discussed controversial. The epilepsies form a heterogeneous group of conditions in which overt seizures are only one manifestation. Apparently, similar seizure may cause cerebral damage in the context of one form of epilepsy but not in another. Subclinical seizures and interictal epileptiform activity might also cause cerebral damage. Currently available methods to assess the degree of progression of neurodegeneration in epilepsy patients include neuropsychological assessment, neuroimaging or pathological studies of resected in vivo or postmortem brain tissue. However, they have many limitations and published data gave many contradicting results, as they were influenced by different confounding factors (41). Serial EEG studies have not been shown to be a sensitive indicator in this regard (42). Besides, there are examples of postmortem studies that demonstrated the poorly controlled generalized seizures including episodes of status epilepticus were not inevitably associated with neuronal damage and hippocampal sclerosis. This clearly indicates that other factors may play a role in modulation of the cascade of pathological events leading to cell death. In addition,
genetic factors may predispose some individuals to be at greater risk than others. Neuron specific enolase (NSE) and S100B protein demonstrated to be raised in CSF and serum of patients recovering from status epilepticus or recurrent seizures, although the results differed from study to study depending on the population characteristics and especially seizure type (43-46). This may at least in part be related to the fact that NSE and S100B are of limited cellular specificity as they are also associated with multiple sclerosis (47). Thus, there is still need for searching more highly specific and sensitive biomarkers for detecting in vivo damage to neurons following seizures. Epileptogenesis is defined as the process of developing epilepsy—a disorder characterized by recurrent seizures—following an initial insult. Seizure incidence during the human lifespan is at its highest in infancy and childhood. Animal models of epilepsy and human tissue studies suggest that epileptogenesis involves a cascade of molecular, cellular and neuronal network alterations. Within minutes to days following the initial insult, there are acute early changes in neuronal networks, which include rapid alterations to ion channel kinetics as a result of membrane depolarization, posttranslational modifications to existing functional proteins, and activation of immediate early genes. Sub acute changes occur over hours to weeks, and include transcriptional events, neuronal death and activation of inflammatory cascades. The chronic changes that follow over weeks to months include anatomical changes, such as neurogenesis, mossy fiber sprouting, network reorganization, and gliosis. At present there are only few studies identifying biochemical biomarkers specific for above processes. Similarly,
our knowledge on the mechanisms of the pharmacoresistance is very limited, what hampers biomarker research. One hypothesis assumes that refractory epilepsy is associated with a localized over-expression of drug transporter proteins such as P-glycoprotein (Pgp) in the region of the epileptic focus, which actively extrudes antiepileptic drugs (AEDs) from their intended site of action (48, 49). However, although this hypothesis has biological plausibility, there is no clinical evidence to support the assertion that AEDs are sufficiently strong substrates for transporter-mediated extrusion from the brain. The quantitative assessment of the expression of Pgp under in vivo conditions might provide evidence whether Pgp or other efflux transporters are involved in AED resistance. In conclusion, further extensive studies are needed in this new research area in epileptology. A reliable epilepsy biomarker could accelerate the diagnosis and treatment, as well as prevention, and eventual cure, of epilepsy. To identify the potential subset of molecules that could probably use as biomarkers for temporal lobe epilepsy we employed genomic as well as quantitative approaches as given in figure 2 and figure 8.
1.6 Genomics methods for molecular profiling

1.6.1 DNA microarrays:

Advantages in the field of genomics and proteomics enable to characterize the genes associated with the diseases. Molecular profiling studies which provide broad-spectrum genomic and proteomic data that could prove useful for the discovery of new drugs and biomarkers. DNA and mRNA contain the instructions that code for the function supplied by proteins and protein networks. New classes of diagnostic markers are being developed based on patterns of genomic information correlated with disease states. Although gene-related information is of great importance, DNA and mRNA are several layers of abstraction away from the physiologic events that determine health or disease because they are the information storage form of proteins. DNA microarrays provide readout of the transcriptional activity of genes but do not provide data on protein expression or post-translational modifications of proteins in the samples. Proteomic approaches, especially those involving mass spectrometry, provide data on protein expression as well as post-translational modifications in different disease conditions, which could lead to the discovery of biomarkers (50).

Most of the molecular profiling studies relying on the high throughput techniques that could survey and analyse thousands of molecules either in transcriptomics or in proteomics levels. Microarray technique that widely use the knowledge from the human genome sequencing uses oligonucleotide probes in order to identify the transcriptome. This technique surveys the expression of thousands of genes in a single experiment to map the changes
in the human genetic blueprint associated with disease. There are a limited number of studies (51-57) that have investigated temporal lobe epilepsy using DNA microarrays.

1.6.2 Literature update on gene expression profile on epilepsy

Emerging evidence supports the role of inflammation and neurogenesis in TLE (58). Increased expression of NF-kB in the astrocytes of sclerotic hippocampus, suggests the activation of this pathway in this disease but further detailed study are needed to delineate further the role of inflammatory cytokine-induced molecular changes in astrocytes of sclerotic hippocampus may unlock hitherto undiscovered secrets of the pathophysiology of MTLE (59). Ozbas-Gerceker et al used SAGE (serial analysis of gene expression) to get a global view of the gene profile in human hippocampus in epileptic patients with HS. SAGE libraries were generated from control hippocampus obtained by autopsy and from hippocampal surgical specimens of patients with MTLE with HS. The comparative analysis showed 143 transcripts that were differentially expressed, including genes involved in basic metabolism, transcription, protein synthesis, signal transduction, and synaptic plasticity (60). Microarray is a comprehensive and hypothesis-free analysis method which is helping scientists discover and understand expression pattern of genes and disease pathways Gene expression arrays can be used to explore
molecular signatures, which is not only important to understand the gene expression profile of disease but can be used as candidate biomarkers for detection, progression and pharmacoresistance. Microarray based genome scale gene expression profiling provides an opportunity to study the molecular changes underlying the development of epilepsy (61). Microarray studies (animal model based as well) have revealed that thousands of genes belong to specific classes altered after Status Epilepticus, and many of them were never reported/linked to epilepsy or seizures (51, 62). Becker et al performed oligonucleotide microarrays with 8799 probes, to investigate subregional gene expression profiles in rats subjected to pilocarpine-induced epilepsy. The analysis identified differentially expressed genes which are associated with the different stages of epilepsy, that include genes associated with mechanisms of cellular stress, injury, transcription factors and genes linked to cytoskeletal and synaptic reorganization. A number of genes (n=18) differentially expressed during the chronic epileptic stage showed corresponding expression patterns in hippocampal subfields of patients with pharmacoresistant temporal lobe epilepsy (n = 5 temporal lobe epilepsy patients; U133A microarrays, Affymetrix; covering 22284 human sequences). Comparative analysis of gene profiles with the pharmaco resistant TLE patients showed similar expression patterns of the animal experiments (51).
Table 1: Summary of Microarray Studies on Human Temporal Lobe Epilepsy

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Clinical Diagnosis</th>
<th>Microarrays platform</th>
<th>Differentially expressed genes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Hippocampus with or without sclerosis</td>
<td>Human Array-Ready oligo set (version 2.0, Operon Biotechnologies)</td>
<td>618</td>
<td>van Gassen et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>Hippocampi from medically intractable TLE subjects</td>
<td>U133A (Affymetrix) with 14,500 genes</td>
<td>138</td>
<td>Lee et al. (2007)</td>
</tr>
<tr>
<td>2.</td>
<td>Drug-refractory TLE</td>
<td>MICROMAX PE, With 2,400 genes</td>
<td>6</td>
<td>Jamali et al. (2006)</td>
</tr>
<tr>
<td>3.</td>
<td>Surgical samples of temporal cortex (ECoG spiking areas)</td>
<td>U133A (Affymetrix)</td>
<td>76</td>
<td>Arion et al. (2006)</td>
</tr>
<tr>
<td>4.</td>
<td>Surgical samples of dentate gyrus and CA1 regions of hippocampus with Ammon’s horn sclerosis</td>
<td>U133A (Affymetrix)</td>
<td>18</td>
<td>Becker et al. (2003)</td>
</tr>
</tbody>
</table>

Microarray analysis of sclerotic hippocampus from MTLE patients revealed changes in several molecular signaling pathways, which included the increased expression of genes associated with astrocytes structure (Glial fibrillary acidic protein, ezrin-moesin-radixin, palladin), calcium regulation (S100 calcium binding protein beta, C-X-C motif receptor 4) and blood brain barrier function and inflammatory responses (63). The gene expression level in human AHS versus control/lesion-associated hippocampus was determined by (52). 21 differentially expressed genes were related to gene transcription control, calcium homeostasis, and neuronal signaling. Induced
expression of ataxin-3 and GFAP (in astrocytes), as well as reduced transcription of calmodulin in AHS was confirmed/validated using RT-PCR. This profiling outcome was compared with results of qPCR expression analysis at the cellular level (after laser capture microdissection), which provided higher cellular resolution for selected signals. The induction of ataxin-3 (in neurons) and GFAP (in astrocytes) was confirmed at cellular level but no significantly different expression was found at cellular level for calmodulin. These results suggested a possibility of function of loss of calmodulin expressing neurons in AHS hippocampus (52). The recent study by Jamali et al. using microarray in MTLE patients, reported the genes encoding for serotonin receptor (HTR2A), a neuropeptide Y receptor, a protein (FHL2) associated with the KCNE1 (minK) potassium channel subunit and with presenilin-2 and three immune system related proteins, were described as consistently down or up-regulated in the endothelial cortex of MTLE patients compared with non epilepsy autopsy control (56).

Van Gassen et al performed Microarray analysis on hippocampus specimen from TLE patients with and without hippocampus sclerosis and from autopsy controls. The analysis identified 618 differentially expressed genes, which are functionally associated with immunity and defense, such as the chemokines CCL3 and CCL4 were highly (>10-fold) up regulated. Other highly affected gene classes include neuropeptides, chaperonins (protein protection), and the ubiquitin/proteasome system (protein degradation) (57).
Genome-wide gene expression in lymphoblastoid cell lines (LCLs) was determined using microarrays derived from five discordant and four concordant monozygotic (MZ) twin pairs with idiopathic absence epilepsies and five unaffected MZ twin pairs. The expression profiling showed differential regulation of EGRI (an immediate early gene) and RCN2 (coding for the calcium-binding protein Reticulocalbin 2) (64).

Fassunke et al investigated the gene expression pattern of gangliogliomas associated with pharmacoresistant focal epilepsies (65). The experiments were performed in comparison of micro dissected ganglioglioma and adjacent control brain tissue obtained from the neurosurgical access to the tumor of identical patients. The differentially regulated genes identified include, those, which are related to intra- and intercellular signaling including protein kinase C and its target NELL2.
1.7 Proteomics methods for molecular profiling

1.7.1. Mass spectrometry:

Mass spectrometry-based quantitative proteomics has emerged as a powerful approach that has the potential to accelerate biomarker discovery, both for diagnostic as well as therapeutic purposes. Proteomics has traditionally been synonymous with 2D gels but is increasingly shifting to the use of gel-free systems and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Quantitative proteomic approaches have already been applied to investigate various neurological disorders, especially in the context of identifying biomarkers from cerebrospinal fluid and serum.

Different mass spectrometric methods are available for proteomic profiling and identification of biomarkers. One of the platforms is surface-enhanced laser desorption-ionization (SELDI), which has been used to obtain disease specific proteomic patterns (66). However, in this approach, only mass spectrometry peak patterns are obtained and the exact identity of the peaks are not determined (i.e. the proteins are not identified in this type of mass spectrometry) (67). Other platforms such as tandem mass spectrometry permit actual identification of amino acid sequences of peptides and are preferable to SELDI for detecting biomarkers.
1.7.2. Quantitative proteomics:

There are several labeling approaches for performing mass spectrometry-based quantitative proteomics analysis. These include labeling methods such as stable isotope labeling with amino acids in cell culture (SILAC) (68) and isobaric tags for relative and absolute quantitation (iTRAQ) (69) cysteine labeling using isotope-coded affinity tags, (70) labeling with isotopically labeled acrylamide and C-terminal labeling using $^{18}$O-labeled water (71). iTRAQ reagents are a set of isobaric tags that bind to primary amines by covalent bond leading to the labeling of peptides. Measuring the relative intensity of these reporter ions in MS/MS spectra allows the relative quantitation of the proteins in samples. Choe et al. used 8-plex iTRAQ quantitation of proteins in cerebrospinal fluid of the patients with Alzheimer's disease (72).

There are a few mass spectrometry based approaches to study the proteome associated with epilepsy. However, most of them are on animal models (73, 74). The molecular profiling approach will accelerate our understanding of the molecular basis of temporal lobe epilepsy and will lead to new insights for the treatment, detection, and prevention of these diseases. Our approaches could potentially provide molecular associations of pathobiological process that are associated with TLE, which include Ammons Horn Sclerosis, pharmaco resistance and sprouting of mossy fibers. The discoveries of molecular profiling could be utilized for identification of
candidate biomarkers associated with the disease as these molecules could be specifically perturbed in a disease specific manner.

Although successful biomarkers have been developed to date, advances in genetics and proteomics promise to usher in a new era of abundant, informative biomarkers that could transform the application of molecular biology to human disease. Scenarios for the use of biomarker-based diagnostics for TLE include the following: risk assessment, noninvasive screening for early-stage disease, detection and localization, disease stratification and prognosis, response to therapy.

1.8. Tools for investigating epileptogenesis:

Most of the current knowledge is available from the studies carried out in various epilepsy models such as status epilepticus models developed in animals like as mouse, rat and subhuman primates (75).

**Status epilepticus models:** Status epilepticus refers to a state of continuous seizure activity. Status epilepticus can be produced experimentally by chemical convulsants or by virtually continuous electrical stimulations administered through intracerebral electrodes, the common feature being continuous limbic and motor seizures manifest by tonic (the initial sustained contraction) and clonic (brief, rhythmic contractions) contractions of limb and facial muscles that persist for hours (76). In these experimental models,
a transient episode of status epilepticus is followed by a seizure-free “latent period” of one to several weeks, after which spontaneous seizures emerge (77, 78). This mimics the human condition in which an otherwise normal individual undergoes an episode of status epilepticus and develops recurrent seizures following recovery. However, most of the studies associated with the functional basis of epilepsy-associated genes were conducted on different neuronal cell lines that include primary neuronal lines (79, 80). Various in vivo models of epilepsy have been established in both flies and mammals, including mice, rats, and subhuman primates. Models of epilepsy have also been developed in several in vitro systems, including freshly isolated rat hippocampal slices, primary cultures of dissociated embryonic rat cortical neurons, and intact hippocampus that include the commissural connections. An in vitro model that mimics status epilepticus also developed by incubating primary neurons briefly in magnesium free medium. The magnesium free period develops a synchronous epileptiform activity many neurons.

Kindling model of epilepsy has discovered about 35 years ago (81), kindling is an in vivo model of epileptogenesis in which a brief, low-intensity electrical stimulation is periodically administered to an experimental animal through stereotaxically implanted intra cerebral electrodes. Initially these low intensity stimulations induce no change in the EEG recorded at the site of stimulation and no change in behavior. Eventually they lower the local seizure threshold and evoke brief focal seizures that can be detected by EEG but are accompanied by no overt change in behavior. However, a continuous
administration of electrical stimulation results in the spontaneous seizures. Even though the studies conducted in these models could identify genes associated with epilepsy, it failed to provide a better understanding for the pharmacoresistance and the cause of underlying lesions such as Ammons horn sclerosis. Hence it is essential to integrate these studies with the human epilepsy cases where we indeed in need of a biomarker that could potentially indicate the form of epilepsy so that better clinical management will be possible.

Investigation of human brain tissues samples from epilepsy cases remains sparse. Table 1 describes expression analysis of human brain tissues. A subset of patients with intractable epilepsy has the potential for a surgical cure, most commonly those with hippocampal sclerosis (HS) (41). Surgical samples from patients with intractable epilepsy provide a unique opportunity to directly analyze the human epileptic focus in order to determine the probable molecular association and causes of pharmacoresistance of epilepsy. This could also provide knowledge about the lesions such as AHS associated with MTLE. More than 50% of medial temporal lobe epilepsy patients have acute unilateral atrophy of the hippocampus (25). Histopathologically, the hippocampus of these patients reveals a stereotypical pattern of damage with segmental neuronal cell loss in CA1 and CA4, whereas CA2 and dentate granule cells are more resistant. Also AHS cases show neuronal loss. But little progress has made with respect to the AHS association with epileptogenesis.
Another important feature of hippocampal sclerosis is a dense fibrillary astrogliosis in all segments with prominent neuronal cell loss, resulting in shrinkage and hardening of the tissue. This macroscopic aspect has been first described in 1880 and since then classified as Ammon’s horn sclerosis (AHS) (82). Several quantitative studies have confirmed neuronal cell loss in all segments of the hippocampus formation, although the sectors CA1 and CA4 are most severely affected (83). In addition, most AHS patients show neuronal cell loss in other mesial temporal areas such as the entorhinal cortex layer III (82) and the lateral nucleus of the amygdala (82, 84, 85). Together with the neuropathological observation of mesial temporal atrophy, many laboratories use the term ‘mesial temporal sclerosis’. It should be emphasized, however, that the association between AHS and damage of other mesial temporal areas has to be verified by histopathological evaluation of the entire neurosurgical specimen (39). Hence, the term AHS unless histopathological analysis confirmed additional neuronal damage within the amygdala and entorhinal cortex. Although human hippocampus resection tissue is available for research and many molecular changes have been described in the HS and nonHS hippocampus, little progress has been made in explaining epileptogenesis in human MTLE. Hence it is necessary to study the human brain tissues, which are associated with epilepsy. These focal points of the tissues could be identified by electrocorticogram and as a part of the standard treatment the surgery of the epileptic zones are performed, leading to a cure of most of the cases.
1.9. Objectives

1. Gene expression profiling and identification of differentially expressed genes in medial temporal lobe epilepsy

Rationale

Investigated differential expression profile of spiking zones against non-spiking zones in MTLE by employing whole human genome DNA microarrays, which have high sensitivity, specificity and show good reproducibility, thus allowing profiling gene expression, which could be a signature to temporal lobe epilepsy. We catalogued the differentially expressed mRNAs associated with MTLE. This approach helped to identify novel genes pertaining to MTLE as this was one of the first studies that investigated expression profiles of seizure spikes associated with TLE using whole human genome microarrays.

2. Validation of the microarray results by immunohistochemistry.

Rationale

The candidate genes identified by the microarray studies were validated by immunohistochemistry. The selection of these molecules for IHC was based on their biological / functional importance, site of localization and novelty. This provided cellular localization, expression pattern in different cell populations such as astrocytes and neurons. IHC also provided the concordance of expression with expression profiling. Though DNA microarrays are a preferred high throughput method for expression
profiling, it suffers from some of the shortcomings such as the amount of mRNA present in the cell does not correspond to the amount of protein translated, which can be assessed in IHC, as staining is a result of direct amount of protein in cells. Immunohistochemical staining were also carried out in an independent set of samples prove the potential use of identified molecules as biomarker.

3. Quantitative proteomic analysis using iTRAQ labeling and validation of candidate biomarkers by IHC.

Rationale

Quantitative proteomics approach by iTRAQ labeling is a high throughput approach to analyze proteins. iTRAQ labeling coupled with fractionation and mass spectrometry could identify differentially regulated proteins. We have used the same samples that had been employed for carrying out DNA microarrays. We carried out iTRAQ labeling, an *in vitro* method that uses NHS ester derivative to modify primary aminogroups by linking a mass balance group (carbonyl group) and a reporter group (based on N-Methyl Piperazine) to proteolytic peptides by the formation of an amide bond. The labeled peptides were fractionated by strong cation chromatography to reduce the complexity of the sample, thus to identify more proteins, especially the low abundant proteins. The quantitative proteomic analysis extends an advantage over DNA microarrays as mRNA amounts sometimes do not correspond to the amount of protein present in the cell. Thus, it is essential to quantitatively identify the proteins, which could be of a potential
biomarker for MTLE. Immunohistochemistry studies were carried out in the
selected candidate molecule in an independent set of MTLE samples.

1.10. Plan of Thesis

The present thesis is a compilation of the research that was performed on
the topic of "Molecular profiling of human temporal lobe epilepsy". This
research that was performed were described in four chapters, which include
introduction to the problem, which is human temporal lobe epilepsy, the
current understanding and analysis of the problem by reviewing the
literature and the approach that we adopted to investigate the human
temporal lobe epilepsy. Then, the chapters describe the results obtained
from the study along with the discussion. In the discussion section the
research summarized with future perspectives.

The structure of thesis organization is as follows:

Chapter 1.

Introduction
This chapter describes the prevalence, symptoms, classification, and
pathology of the epilepsy. The chapter also includes review of the literature
about the different models of epilepsy, methods employed for the profiling
of different epilepsy models as well has human cases. Data from the various
studies involving epilepsy has provided in a table format (Table 1). This chapter also describes the reasons behind the selection of the study and identifying the specific problem and research questions associated with human temporal lobe epilepsy.
Chapter 2.

Methods

This chapter describes details of the different methods that employed for the study. The approaches for the investigation of the research problem include genomics, proteomics as well as bioinformatics methods. These protocols include

1. Oligonucleotide microarrays
2. Microarray analysis.
3. Protein extraction from brain tissues.
4. Quantitative proteomics approach by labeling using iTRAQ.
5. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)
7. Bioinformatic analysis
   a. Gene Ontology classification
   b. Network analysis
8. Immunohistochemistry

Chapter 3.

Results and analysis

This chapter arranged in two parts – one describing the transcriptomics approach and another describing proteomic approach
This part describes the finding of differentially expressed genes derived from analysis of oligonucleotide microarrays analysis, which include novel genes associated with the epilepsy, known genes associated with epilepsy. Classification of the genes was also provided based on the literature of the epilepsy. The bioinformatics based network analysis was also described in order to identify the significant networks associated with the differentially regulated genes. Validation using immunohistochemical labeling (IHC) for STK31 and SMARCA4 were performed and described in detail in this chapter.

**Identification of potential biomarkers by iTRAQ labeling of TLE**

This part describes the quantitative proteomics methods employed to identify the proteomic biomarkers for temporal lobe epilepsy. Selected molecules also validated by immunohistochemistry such as CAMK2A.

**Chapter 4. Discussion**

A discussion of our findings with respect to the current knowledge in the field of TLE and the technologies employed. This also includes future perspectives of the study.