PHARMACOGENETIC STUDY TO EVALUATE THE TERATOGENIC EFFECTS OF ANTI-EPILEPTIC DRUGS

MANNA JOSE

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PHARMACOGENETIC STUDY TO EVALUATE THE TERATOGENIC EFFECTS OF ANTI-EPILEPTIC DRUGS

A THESIS PRESENTED BY

MANNA JOSE

To

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THIRUVANANTHAPURAM

In partial fulfilment of the requirements for the award of
DOCTOR OF PHILOSOPHY

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CERTIFICATE

I, **MANNA JOSE**, hereby certify that I had personally carried out the work depicted in the thesis entitled "**PHARMACOGENETIC STUDY TO EVALUATE THE TERATOGENIC EFFECTS OF ANTI-EPILEPTIC DRUGS".** No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

Manna Jose

MANNA JOSE

Date: 7/9/2011

DR. SANJEEV V. THOMAS

PROFESSOR

DEPARTMENT OF NEUROLOGY

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM

This is to certify that **MANNA JOSE** in the department /division of NEUROLOGY of this Institute has fulfilled the requirements Prescribed for the Ph.D degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum.

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Clearance was obtained from the Institutional Ethics Committee / Institutional Animal Ethics for carrying out the study.

Signature

Date: 6/9/2011

DR. MOINAK BANERJEE

SCIENTIST E –2
HUMAN MOLECULAR GENETICS LABORATORY
RAJIV GANDHI CENTRE FOR BIO TECHNOLOGY
POOJAPPURA
THIRUVANANTHAPURAM

This is to certify that **MANNA JOSE** in the department /division of HUMAN MOLECULAR GENETICS LABORATORY of this Institute has fulfilled the requirements Prescribed for the Ph.D degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum.

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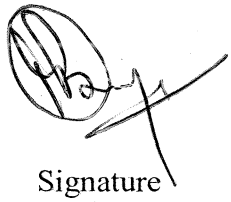

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is evaluated and approved by

DR. SANJEEV V. THOMAS

Name of Guide

Name of thesis examiner

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MANNA JOSE

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SYNOPSIS

PHARMACOGENETIC STUDY TO EVALUATE THE TERATOGENIC EFFECTS OF ANTIEPILEPTIC DRUGS

INTRODUCTION

Epilepsy is a neurological disorder that affects people in every country. With a prevalence of approximately 1%, it is estimated that 50 million persons worldwide have the disorder (Wang, et al., 2003). An estimated 7 million people are affected with epilepsy in India, of which 40% of them are women (Reddy, 2005). Malformations in fetuses of epileptic mothers are a major concern in the present context. Pregnancy in WWE is known to be associated with a higher risk of congenital malformations than is associated with pregnancy in non-epileptic women. The outcome of pregnancies of WWE is generally recognized to be two to three times poorer than that of the general population (Barrett et al., 2003). A number of factors have been proposed to explain the increasing risk of adverse pregnancy outcomes including a genetic predesposition to congenital abnormalities, maternal seizures and teratogenic effects of AEDs during pregnancy. The antiepileptic therapy rather than the maternal disease or convulsions is indicated as the major cause of malformations detected at birth (Holmes, et al., 2001). A large number of both epidemiological and experimental studies have provided strong evidence for teratogenic effects of AEDs and present efforts are now being directed toward elucidation of how AEDs might interfere with embryonic development

(Dansky, et al., 1992). The most commonly administered anti-epileptic drugs are as follows: Phenobarbital, Carbamazepine, Valproic acid, Phenytoin, Clonazepam, Lamotrigine and Topiramate. "Fetal Antiepileptic Drug Syndrome" (FADS) is a common term used to describe the overlap between the drug-specific syndromes for PB, PHT and CBZ (Finnell et al., 1997). Malformations are assumed to be caused by a common mechanism (Vorhees 1987; Finnell 1991; Danielsson 1997). The list of suggested mechanisms for AED teratogenicity is long and diverse. Genetic polymorphisms in CYP450 enzymes, methylene tetrahydrofolate reductase (MTHFR) enzyme and drug transporters play a major role in the teratogenicity of different AEDs. There has been little work carried out in the context of pharmacogenetic studies into AED teratogenicity in India so far.

The current work will help to study the influence of single nucleotide polymorphisms of drug transporters and drug metabolizing enzymes on the development of congenital malformations in offspring of women with epilepsy.

OBJECTIVES

1. To compare the MDR1, MTHFR, CYP2C9 and CYP2C19 polymorphisms of WWE whose offspring have malformations with that of WWE whose offspring are normal.
2. To ascertain any association between the pharmacogenetic status of women with epilepsy and type of malformations, polytherapy and monotherapy and good and poor control of seizures.

MATERIALS AND METHODS

The study was carried out at Kerala Registry of Epilepsy and Pregnancy (KREP), Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Trivandrum and at Rajiv Gandhi Centre for Biotechnology (RGCB), Trivandrum. The blood sample collection and clinical profiling of the participants in the study was done at KREP whereas the molecular profiling was carried out at RGCB. Women with epilepsy (WWE) were recruited from KREP and will be asked to complete a questionnaire on family history, medical history and life style. Women who have had two or more unprovoked seizures are eligible for inclusion. Women who had completed index pregnancy will be eligible. Classification of epilepsy was followed according to the International League Against Epilepsy. Patients were subjected to all routine examination mentioned in the registry. 10ml of blood sample was drawn from women with epilepsy. Patients were divided into one group whose children have malformations and into a second group whose children are normal. The first group will form the cases and the second group will form the controls. The healthy controls were selected from volunteers and staff from RGCB. 10ml of blood sample was also drawn from these controls.

DNA was extracted by the standard organic extraction protocol from all the samples (Sambrook et al, 1989) and subsequently further genotyping procedures are carried out to validate the polymorphisms in MDR1, MTHFR, CYP2C9 and CYP2C19 genes. The quality and quantity of DNA isolated is analysed in a spectrophotometer (BioSpec-1601, Shimadzu). The MDR1, MTHFR, CYP2C9

and CYP2C19 genotypes were identified by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analyses. The restriction digestion of the products after PCR amplification was carried out using the corresponding restriction enzymes. Gels were visualized using UV illumination and documented in a Flour STM multi imager system (BioRad).

STATISTICAL ANALYSIS

The allelic and genotypic distributions were tested by Chi Sqaure analysis. Haplotypic association of samples was performed using the COCAPHASE program in the UNPHASED v3.011 package (http://www.mrc-bsu.cam.ac.uk/personal/frank/ software/unphased/) with 10,000 permutations. To estimate the LD between pairs of loci in the patient and control populations, the standardised disequilibrium coefficient (D') and the squared correlation coefficient (r^2) were calculated using the Haploview 4.1 (http://www.broad.mit.edu/mpg/haploview/). LD blocks were defined in accordance with Gabriel's criteria (Gabriel 2002).

RESULTS

Study group consisted of WWE 244 WWE (Mean age-25 years) and 140 healthy controls (Mean age- 29years). Among 244 WWE patients (generalized epilepsy = 111, localization related epilepsy = 133), 121 patients had offsprings with congenital malformations. CBZ, PHT, VPA, PB are the most commonly used drugs. Among the malformation group, 78 were on monotherapy and 43 on polytherapy. 89 offspring of women with epilepsy had cardiac malformations

and 33 had non-cardiac malformations. Also, in this group, 90 patients had infrequent seizures (n<=3) during pregnancy and 12 months before pregnancy while others had frequent seizures (n>3).

Genotyping of MDR1 polymorphisms

The MDR1 polymorphisms studied include Pro T129C, Ex 02 -1G/A, Ex 06+139C/T, C1236T, Ex 18-76T/A, G2677T, C3435T. We observed that Ex 18-76T/A and G2677T polymorphisms are associated with epileptogenesis whereas Ex 06+139C/T strongly associated with malformations. When these MDR1 polymorphisms were screened for its role in type of pharmacotherapy, seizure control and type of malformation we did not see any specific association. Distinct difference in the LD patterns was observed between healthy controls, epileptic controls and malformation group. Variations in the pattern of LD could also be observed between malformation types, treatment regimen and seizure frequency.

Genotyping of MTHFR polymorphisms

MTHFR polymorphisms studied include C677T and A1298C. The allele and genotype comparisons were made to see if there was any association between the polymorphisms but none of the polymorphisms were observed to have any role in malformations or drug dosage, seizure control and type of malformation.

Genotyping of CYP2C9 polymorphisms

CYP2C9*2 and CY2PC9*3 polymorphisms were included in this study. CYP2C9*2 is formed by a C430T substitution on exon 3 which leads to Arg144Cys conversion resulting in the formation of an enzyme with decreased activity. CY2PC9*3 allele is due to a C1075T on exon 7 of CYP2C9, which results in an altered protein with an Ile359Leu substitution and exhibits further reduced enzyme activity than the CYP2C9*2 variant. CYP2C9*1*2 genotype was found to be associated with the healthy controls. CYP2C9*3 polymorphism was absent in the epileptic and healthy controls.

Genotyping of CYP2C19 polymorphisms

CYP2C19*2 and CY2PC19*3 polymorphisms were analysed in this study. CYP2C19*2, the most common variant allele of CYP2C19, is the result of a single base pair 681G>A mutation on exon 5, leading to an aberrant splice site. This change alters the reading frame of mRNA from amino acid 215, and produces a stop codon 20 bp downstream, leading to a truncated protein. This variant accounted for the poor metabolizer phenotype.

CYP2C19*3, consisting of a 636G>A on exon 4, leads to amino acid change Ile359Leu and confers poor metabolizer phenotype. CYP2C19*2 polymorphism was not associated with malformation status or epileptogenesis. CYP2C19*3 polymorphism was not identified among the healthy controls, epileptic controls and malformation group.

CONCLUSION

The offspring of women with epilepsy exposed to AEDs have an excess risk to develop congenital malformations when compared to offspring of women with epilepsy not on AEDs and normal controls. Well-recognized causes of differences among the fetuses who are normal and fetuses who develop malformations maybe due to variations in the genetic makeup of drug transporters, cytochrome P-450 (CYP), other drug-metabolizing enzymes. Seven MDR1 transporter polymorphisms (three exonic, three intronic and one promoter SNP) were genotyped in the malformation group, epileptic controls and the healthy controls. It was observed that Ex 18-76T/A and G2677T polymorphisms are associated with epileptogenesis. C allele of Ex07 +139C/T was also found to be strongly associated with malformation in haplotypic combination with its neighbouring SNPs. This haplotypic association was observed to extend upto four loci haplotypic combination more specifically with Pro 129T/C, Ex 03 -1G/A, Ex 07 +139C/T and Ex13 1236C/T. None of the MDR1 polymorphisms had any specific association with type of pharmacotherapy, seizure control and type of malformation. The MTHFR polymorphisms C677T and A1298C were not found to have any association with malformation, epileptogenesis, or type of pharmacotherapy. CYP2C9*3 was linked with the malformation cases when compared to healthy controls but CYP2C19*2 and CYP2C19*3 polymorphisms had no association with malformations and epileptogenesis.

The observations in this study supports the hypothesis that certain MDR1 polymorphisms play a potential role in the development of congenital malformations which is independent of the nature of malformations or seizure frequency.

Thus, pharmacogenetics is found to have an influence on AED teratogenicity in the present context. The outcome of the research will aid in better management of epilepsy in pregnant women, fine tuning the administration and dosage of antiepileptic drugs to WWE and reduce the risk of drug-induced malformation.

Chapter 1

INTRODUCTION

Epilepsy is a neurological disorder that affects people in every country. According to IBE and WHO, it is a common and serious neurological disorder and one of the world's most prevalent non communicable diseases. With a prevalence of approximately 1%, it is estimated that 50 million persons worldwide have the disorder (Wang, et al., 2003). Of these, approximately 400,000 are women of childbearing age. The prevalence of epilepsy among pregnant women has been estimated to be between 0.3 and 0.4% (Gaily et al., 1990, Dansky and Finnell 1991). An estimated 7 million people are affected with epilepsy in India, of which 40% of them are women (Reddy, 2005). The overall prevalence of epilepsy in India is estimated to be 0.53% (Sridharan et al., 1999). Nearly half of the two and half million women with epilepsy (WWE) in India belong to the childbearing age group (Thomas, 2002). 30% of the 75,000 WWE in Kerala come under the reproductive age group (Thomas, et al 2001).

Malformations in fetuses of epileptic mothers are a major concern in the present context. Pregnancy in WWE is known to be associated with a higher risk of congenital malformations than is associated with pregnancy in non-epileptic women. Each year 40000 infants are exposed to anticonvulsant drugs in utero worldwide, with the estimated birth of 1500-2000 infants having congenital

malformations as a consequence of intra-uterine exposure to antiepileptic drugs (AEDs) (Azarbayjani , 2001).

The outcome of pregnancies of WWE is generally recognized to be two to three times poorer than that of the general population (Barrett et al., 2003). Epidemiological studies have shown an increased risk of abnormalities in children of women with epilepsy including major and minor congenital malformations. Major congenital malformations (MCMs) include malformations of the spinal cord and spine (spina bifida), the heart (such as hole in the heart), the ribs, the bladder, the sexual organs and the fingers and toes (such as unseparated fingers). Babies born with MCMs may need surgery after their birth. Oro-facial clefts and heart lesions dominate among these malformations caused due to antiepileptic drugs. Skeletal anomalies are less frequent. Microcephaly, intestinal abnormalities and Neural Tube Defects (NTDs) also occur (Janz and Fuchs, 1964). Speidel and Meadow in 1972 reported that malformations were twice as common in infants exposed to AEDs in utero and abnormalities were not specific to the type of AED exposure. Impairment of behavioural development is also seen as a teratogenic effect of AEDs (Meador, et al., 2004).

Babies born to mothers who have epilepsy also have a slightly higher risk of having minor malformations. Minor malformations do not necessarily require medical treatment and may not be permanent. Examples of minor malformations include small fingers and toes with small nails, facial features such as wide set eyes, low set ears and short neck.

A number of factors have been proposed to explain the increasing risk of adverse pregnancy outcomes including a genetic predesposition to congenital abnormalities, maternal seizures and teratogenic effects of AEDs during pregnancy. The antiepileptic therapy rather than the maternal disease or convulsions is indicated as the major cause of malformations detected at birth (Holmes, et al., 2001).

A large number of both epidemiological and experimental studies have provided strong evidence for teratogenic effects of AEDs and present efforts are now being directed toward elucidation of how AEDs might interfere with embryonic development (Dansky, et al., 1992).

1.1 *Anti-epileptic drugs and teratogenic mechanisms*

The most commonly administered anti-epileptic drugs are as follows: *Phenobarbital, Carbamazepine, Valproic acid, Phenytoin, Clonazepam, Lamotrigine and Topiramate.*

There is a large overlap in the pattern of teratogenicity between phenytoin and phenobarbitone. Barbiturates have been associated with the same major and minor abnormalities and dysmorphic features as with PHT. These include congenital heart defects, facial clefts, craniofacial abnormalities and growth defi ciency (Seip 1976; Rating et al. 1982; Kallén et al.1989). A common term known as "Hydantoin-Barbiturate Embryopathy" was proposed by Majewski et al, 1981.

The main teratogenic effect of carbamazepine results in increased incidence of craniofacial and limb development in exposed children (Hiilesmaa et al., 1981; Bertollini et al., 1987, Jones et al., 1989). Buehler in 1987 reported a striking similarity between the malformation pattern in children exposed to carbamazepine and fetal hydantoin syndrome; thus they seem to share the same teratogenic mechanism.

A fetal valproate syndrome has been described which is characterized by posterior neural tube defects and a distinctive facial appearance featuring upward slanting palpebral fissures, epicanthic folds, and posteriorly rotated ears (DiLiberti et al., 1984).

"Fetal Antiepileptic Drug Syndrome" (FADS) is a common term used to describe the overlap between the drug-specific syndromes for PB, PHT and CBZ (Finnell et al., 1997). Malformations are assumed to be caused by a common mechanism (Vorhees 1987; Finnell 1991; Danielsson 1997).

There have been several postulates to explain the mechanism of teratogenesis (Table 1).

Table 1- Mechanisms of teratogenicity
1. Disturbances in folate metabolism
2. Bioactivation of antiepileptic drugs to reactive intermediates
3. Co oxidation of AEDs like phenytoin to free radical intermediates
4. Alterations in Vitamin K metabolism
5. Teratogenicity related to pharmacological action of AEDs

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1.2 *Genetic susceptibility to the teratogenic effects of AEDs- pharmacogenetic approach to AED teratogenicity*

The fact that not all infants of WWE undergoing AED therapy are born with CM emphasizes the importance of maternal and fetal genotype and its interaction with various environmental factors (Hansen and Hodes 1983). Genetic polymorphisms in CYP450 enzymes, methylene tetrahydrofolate reductase (MTHFR) enzyme and drug transporters play a major role in the teratogenicity of different AEDs.

CYP450 enzymes like CYP2C9, CYP2C19 and CYP3A4 which are involved in the metabolism of AEDs are polymorphic as a result of single nucleotide polymorphisms (SNPs), gene deletions and gene duplications. The variability in these enzymes has an impact on the pharmacokinetics (PK), metabolism, safety and efficacy of drugs, which in turn contribute to the teratogenicity of these drugs (Rodrigues et al., 2002). The genetic polymorphisms in the CYP450 drug metabolizing enzymes can affect the metabolism of AEDs. The harmful metabolites thus formed can pass from the mother to the fetus through the placenta, which can in turn act as teratogenic agents. Thus, the study of the genetic variation in these enzymes are important in the present context.

Genetic defects in folate metabolism include MTHFR gene defects like the C677T and A1298C SNPs and Methionine synthase gene defects like A2756G polymorphism which leads to decreased activity of the enzyme. Defective

MTHFR and methionine synthase enzymes results in decreased synthesis of methionine. This leads to impairment in cell function and defective neurulation. Epilepsy is also resistant to drug treatment in about one-third of the cases and over expression of the drug resistant proteins like multidrug resistant gene-1 P-glycoprotein (MDR1) and multi-drug associated protein 1 (MRP1) suggests one possible mechanism for drug resistance. (Sisodiya et al., 2002). This is also another mechanism that affects the drug efficacy and handling.

It has been hypothesized that overexpression of Pgp in the cerebrovascular endothelium in the region of the epileptic focus may lead to drug resistance in epilepsy. Recently it had been shown that some commonly used AEDs are substrates to the Pgp. Localisation of Pgp in the human placenta is important for protecting the fetus from unintended, harmful drug exposure, but also for limiting the access of drugs to the fetus after maternal AED intake (Wang, et al., 2004). P-gp is expressed by the placenta syncytiotrophoblast throughout gestation and at term it is localized to the microvillous maternal facing plasma membrane. Studies in Pgp knockout animals have shown that in its absence, fetal concentrations of substrate drugs are much higher than in controls (Smit 1999). Differences in expression of Pgp at the placenta may be significant in determining fetal susceptibility to AEDs.

1.3 *Significance of the study*

The possible teratogenic effects of AEDs on the developing fetus remains the most major concern today in the care of women with epilepsy during pregnancy. Well-recognized causes of differences among the fetuses who are

normal and fetuses who develop malformations maybe due to variations in the genetic makeup of cytochrome P-450 (CYP), other drug-metabolizing enzymes and drug transporters. Pharmacogenomics, which involves the study of these polymorphisms, through the use of an individual's genetic information, has the potential to assist clinicians with tailoring a drug regimen to that individual, aiming to optimize drug therapy and limit drug toxicity. It may also, in the near future, help with the development of dosing protocols and monitoring techniques to enhance the efficacy and safety of medication therapy.

There has been little work carried out in the context of pharmacogenetic studies into AED teratogenicity in India so far. The current work will help to study the influence of single nucleotide polymorphisms of drug transporters and drug metabolizing enzymes on the development of congenital malformations in offspring of women with epilepsy.

1.4 *Hypothesis*

The main hypothesis of this study is:

1. MDR1, MTHFR, CYP2C9 and CYP2C19 gene polymorphisms is comparable between the WWE whose offspring have malformation and WWE whose offspring are normal.

1.5 *Objectives*

1. To compare the MDR1, MTHFR, CYP2C9 and CYP2C19 polymorphisms of WWE whose offspring have malformations with that of WWE whose offspring are normal.
2. To ascertain any association between the pharmacogenetic status of women with epilepsy and type of malformations, polytherapy and monotherapy and good and poor control of seizures.

Chapter2

REVIEW OF LITERATURE

2.1 *Epilepsy- an overview*

Epilepsy is a common chronic neurological disorder characterized by recurrent unprovoked seizures. These seizures are transient signs and/or symptoms of abnormal, excessive or synchronous neuronal activity in the brain (Blume, et al., 2001). Epilepsy is derived from the Greek word (epilepsia/ epilambanein) which means "to be seized", "to be attacked". Hughlings Jackson in the 1860s viewed epilepsy as a vascular disorder. By 1873, in his paper "On the anatomical, physiological and pathological investigation of epilepsies" he proposed his famous definition: "Epilepsy is the name for occasional sudden excessive rapid and local discharges of grey matter."

In the past, epilepsy was associated with religious experiences and even demonic possession. In ancient times, epilepsy was known as the "Sacred Disease" because people thought that epileptic seizures were a form of attack by demons, or that the visions experienced by persons with epilepsy were sent by the gods. Epilepsy is not contagious and is not caused by mental illness or mental retardation. Epilepsy may be the oldest known neurological disorder. Hippocrates identified epilepsy as a disease of the brain in his work, *On The Sacred Disease,* in 400 B.C.

Epilepsy is not a specific disease or a single syndrome, but a broad category of symptom complexes arising from any number of disordered brain functions that themselves may be secondary to a variety of pathologic processes. Epilepsy can also be known by other names like convulsive disorder, seizure disorder and cerebral seizures. The International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE) has come with new definitions for epilepsy (Fisher et al., 2005). According to them, epileptic seizure is defined as a transient occurrence of signs and/or symptoms due to abnormal excessive and synchronous neuronal activity in the brain" and also as "a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures and by the neurobiologic, cognitive, psychological and social consequences of the condition".

Epilepsy is caused by a tendency for hyperexcitability to develop in one or another region of the central nervous system (CNS). The likelihood that epilepsy will develop after an epileptogenic insult depends on the area of brain damaged, the type of damage, the age at which the damage occurs and genetic predisposition (Figure 1).

The basic pathophysiological understanding of epileptic seizures was deduced by the 19th-century physician John Hughlings Jackson based solely on clinical observations. In 1861, J. Russell Reynolds labeled convulsions as associated with a structural disorder of the nervous system as symptomatic epilepsy and seizures associated with no structural abnormalities inside or outside the

nervous system as idiopathic, or true, epilepsy. In 1881, Sir William Gowers classified epilepsy as grand mal, petit mal and hysteroid.

The overall incidence of epilepsy (excluding febrile convulsions and single seizures) in developed societies has been found to be around 50 cases per 100,000 persons per year (Shorvon SD,et al 1983, 2000). The figures for developing countries are generally higher, in the range of 100-190/100,000/year (Shorvon SD et al., 1996). The overall prevalence of epilepsy in India is estimated to be 0.53% (Sridharan et al, 1999), 0.7% (Reddy, 2005). The usual prevalence figure is about 5-10 cases per 1000 persons, excluding febrile convulsions & single seizures; independent of location (Shorvon SD et al., 1983, 2000).

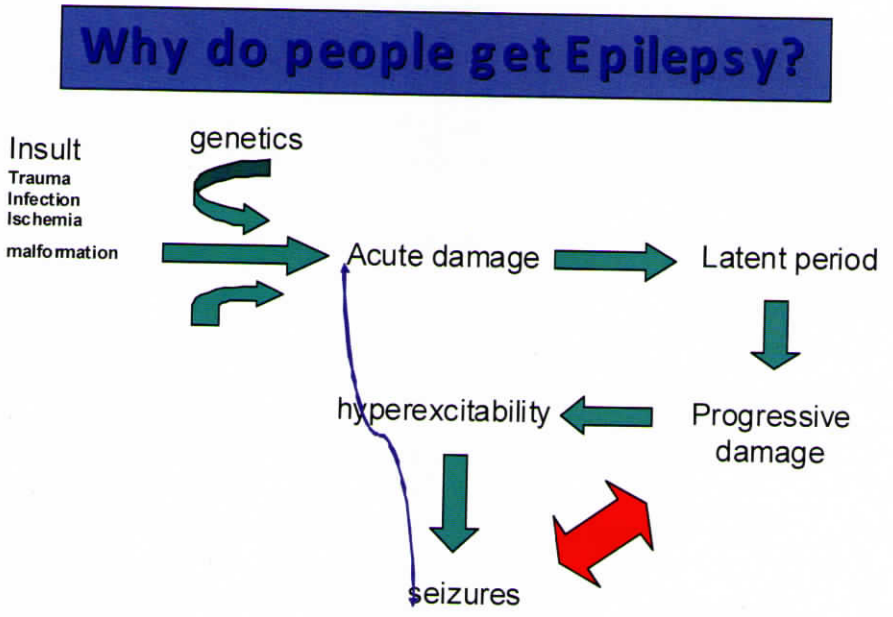


Figure 1- Different reasons as to why people get epilepsy

2.2 *Seizure and Epilepsy classification*

In the 19th century, epilepsy was classified into three broad categories, based upon presumed mechanisms and causes: idiopathic, symptomatic and sympathetic epilepsy.

A second approach to classification was based upon clinical manifestations of the epileptic seizure. Jackson (1931) divided seizures into attacks, which were generalized and attacks starting unilaterally. ILAE took up classification in 1960s based on descriptive scheme based upon the clinical and EEG manifestations of seizures.

Gastaut in 1970 proposed 6 criteria for classification: clinical form, interictal EEG, ictal EEG, anatomical substrate, age and etiology. Three main categories of seizure type were identified, based on clinical and EEG features:

1. Partial subdivided into simple and complex
2. Generalized into convulsive and non-convulsive
3. Unilateral- features confined to just one half of the body

2.2.1 Seizure classification

In 1981, the International League Against Epilepsy (ILAE) developed an international classification of epileptic seizures that divides seizures into 2 major classes: partial-onset seizures and generalized-onset seizures. Partial-onset seizures begin in a focal area of the cerebral cortex, whereas generalized-onset seizures have an onset recorded simultaneously in both cerebral

hemispheres. Some seizures are difficult to fit into a single class, and they are considered unclassified seizures.

Partial seizures are further classified as simple partial seizures, complex partial seizures, or secondarily generalized tonic-clonic seizures.

- **Simple Partial Seizure (SPS)**

Depending on the site of origin of the attack in the motor cortical representation area, the appropriate portion of the body will be involved in focal seizure activity (lasts from minutes to hours).

- **Complex Partial Seizure (CPS)**

Consciousness is impaired with associated automatisms. Coordinated adapted involuntary motor activity occurs during clouding of consciousness either in the course of or after a seizure and usually followed by amnesia. Eg. Eating automatisms.

- **Secondarily generalized seizures** often begin with an aura that evolves into a complex partial seizure and then into a generalized tonic-clonic seizure. However, a complex partial seizure may evolve into a generalized tonic-clonic seizure, or an aura may evolve into a generalized tonic-clonic seizure without an obvious complex partial seizure.

Generalized-onset seizures are classified into 6 major categories: (1) absence seizures, (2) tonic seizures, (3) clonic seizures, (4) myoclonic seizures, (5) primary generalized tonic-clonic seizures, and (6) atonic seizures.

.□ **Absence seizures** are brief episodes of impaired consciousness with no aura or postictal confusion. They typically last less than 20 seconds and are accompanied by few or no automatisms. Facial automatisms are most common, and repetitive blinking is the most common facial automatism. Hyperventilation or photic stimulation often precipitates these seizures which typically begin during childhood or adolescence, though they may persist into adulthood.

□ **Myoclonic seizures** consist of brief, arrhythmic, jerking, motor movements that last less than a second. Myoclonic seizures often cluster within a few minutes. If they evolve into rhythmic, jerking movements, they are classified as evolving into a clonic seizure.

□ **Clonic seizures** consist of rhythmic, motor, jerking movements with or without impairment of consciousness. Clonic seizures can have a focal origin with or without impaired consciousness. The focal seizures are classified as simple or complex partial seizures. The typical generalized clonic seizures simultaneously involve the upper and lower extremities.

□ **Tonic seizures** consist of sudden-onset tonic extension or flexion of the head, trunk, and/or extremities for several seconds. These seizures typically occur in relation to drowsiness, shortly after the person falls asleep, or just after he or she awakens. They are often associated with other neurologic abnormalities.

□ **Tonic-clonic seizures** are commonly referred to as grand mal seizures. They consist of several motor behaviors, including generalized tonic extension

of the extremities lasting for few seconds followed by clonic rhythmic movements and prolonged postictal confusion. On clinical evaluation, the only behavioral difference between these seizures and secondarily generalized tonic-clonic seizures is that these seizures lack an aura.

- **Atonic seizures** occur in people with clinically significant neurologic abnormalities. These seizures consist of brief loss of postural tone, often resulting in falls and injuries.

2.2.2 Epilepsy classification

Two systems of classification of epilepsies are in use today. The International League Against Epilepsy (ILAE) Classification of Epileptic Seizures 1981 divides seizures into three types, with subtypes of each:

- Partial (seizures involving only part of the brain)
- Generalized (seizures involving both sides of the brain)
- Unclassifiable

The advantage of this system is that seizures can be classified relatively easily and the choice of medication is dictated by seizure type.

A problem presented by this simple classification system is that the same patient may have more than one type of seizure, either together or in sequence. Many patients' seizures also change over the course of their illness. These problems paved the way for another system of classification-the ILAE Classification of

Epilepsies and Epileptic Syndromes 1989. According to this system, epilepsies are divided into four broad groups:

- Localization-related (involves one or more distinct parts of the brain)
- Generalized (involves both sides of the brain at the same time)
- Undetermined whether localized or generalized
- Special syndromes.

Within the localized and generalized groups, there are further subdivisions into idiopathic (unknown cause), symptomatic (identifiable cause), or cryptogenic (hidden cause).

ILAE in 1989 classified epilepsies also to incorporate anatomical, EEG, etiological, seizure type and precipitation and syndromic features (Table 2).

Table 2- The International League Against Epilepsy (ILAE) Classification of Epilepsies and Epileptic syndromes- 1989	
1.Generalized Epilepsies and syndromes	
Idiopathic	Benign neonatal familial convulsions Benign myoclonic epilepsy in infancy
	Childhood absence epilepsy
	Juvenile absence epilepsy
	Juvenile myoclonic epilepsy
	Epilepsy with generalized tonic clonic seizures on awakening.
Cryptogenic or symptomatic	West's syndrome
	Lennox-Gastaut syndrome
	Epilepsy with myoclonic-astatic seizures
Symptomatic	Early myoclonic encephalopathies
	Early infantile encephalopathy with burst suppression
2.Localization related Epilepsies and syndromes	
Idiopathic	Benign childhood epilepsy with centrotemporal spikes
	Childhood epilepsy with occipital paroxysms.
Symptomatic	Epilepsia partialis continua
Cryptogenic	Symptomatic focal epilepsy with unknown aetiology
	Special syndromes-situation-related syndromes
3.Epilepsies and syndromes undetermined whether focal or generalized	
	Neonatal seizures
	Severe myoclonic epilepsy in infancy
	Epilepsy with continuous spike-waves during slow wave sleep
	Acquired epileptic aphasia(Landau-Kleffner syndrome)
4.Special syndromes	
	Febrile convulsions
	Isolated seizures or isolated status epilpeticus
	Seizures occuring only when there is an acute metabolic or toxic event due to factors such as alcohol, drugs, eclampsia, non-ketotic hypergylcinaemia.

2.3 *Genetics of epilepsy*

Twelve out of forty individual epileptic syndromes described are considered familial like Childhood Absence Epilepsy, JME, Febrile Seizures and Progressive Myoclonic Epilepsy. Twin studies implicate strong genetic determinants in many types of seizures and seizure disorders esp. CAE and JME (Berkovic, et al.,1994, Lennox, et al., 1951). Concordance for epilepsy in monozygotic twins was 62% compared with 18% in dizygotic pairs which indicates a strong genetic liability for epilepsy. Hereditary disorders with epilepsy can be classified according to the mechanisms of inheritance as:

1 Mendelian disorders in which epilepsy forms a part of the phenotype

2 Idiopathic epilepsies with Mendelian inheritance

3 Epilepsies with complex inheritance

4 Idiopathic epilepsies associated with cytogenetic abnormalities

Specific combinations of epilepsy genes determine specific epilepsy phenotypes whereas some epilepsy genes individually, have the potential to contribute to different types of epilepsy. Mutations in single genes account for some rare epileptic syndromes and familial disease that cause epileptic seizures. Multiple genes must determine the various neuronal functions that alters seizure threshold and predisposes to development of symptomatic epilepsy.

2.4 *Mechanisms of epilepsy*

Most forms of epilepsy develop over a defined time period. That is, at some point of time, the brain functions normally till a specific developmental sequence or injury occurs. A new state develops in which the neuronal circuits become hyperexcitable, leading to spontaneous recurrent seizures. This process is called **Epileptogenesis.**

Two essential physiologic elements represent the net effect of many complex interacting processes. An abnormality of cellular excitability, termed 'neuronal deregulation', arises from mechanisms that affect membrane depolarisation and repolarisation. The second element is a network defect which derives from mechanisms underlying the abnormal synchronisation of neuronal populations and propagation of the epileptic discharge within neural pathways.

For partial epilepsy, areas of hyperexcitability are associated with synaptic reorganization that occurs after brain injury. Neurons within epileptic areas undergo synchronous and paroxysmal depolarisations and fire bursts of action potentials. These are followed by periods of inhibition. Discharges do not remain confined in either anatomic spaces; more neurons are recruited into hypersynchronous activity, both in local areas and via synaptic pathways.

In the case of primary generalised seizures, events appear to start in diffuse bilateral brain areas all at once and have not provided a focal target for detailed examination. Many of these seizures are the result of genetic alterations. One speculated mechanism for some forms of inherited epilepsy are mutations of the genes which code for sodium channel proteins; these defective sodium channels

stay open for too long thus making the neuron hyper-excitable. Glutamate, an excitatory neurotransmitter, may thereby be released from these neurons in large amounts which by binding with nearby glutaminergic neurons triggers excessive calcium ion release in these post-synaptic cells. Such excessive calcium release can be neurotoxic to the affected cell.

2.4.1 Voltage gated ion channels

These are membrane spanning proteins that form a pore and the opening and closing of the pore are regulated by the transmembrane voltage gradient. The channels are composed of different subunits coded by different families of genes. A voltage sensor regulates the opening of channel which is located within the membrane. The selectivity filter determines the type of ions that can pass through the channel. The different ion channels are tabulated in Table 3:

Table 3- Types of voltage gated ion channels
1. Sodium channels
2. Calcium channels
3. Potassium channels

Voltage gated channels regulate the membrane potential, influence the integrating properties of the dendrites and the discharge mode of a cell and are responsible for the generation and propagation of action potentials. Ionic

channels determine the excitability of neurons and mechanisms that lead to transmitter release from presynaptic terminals (Figure 2).

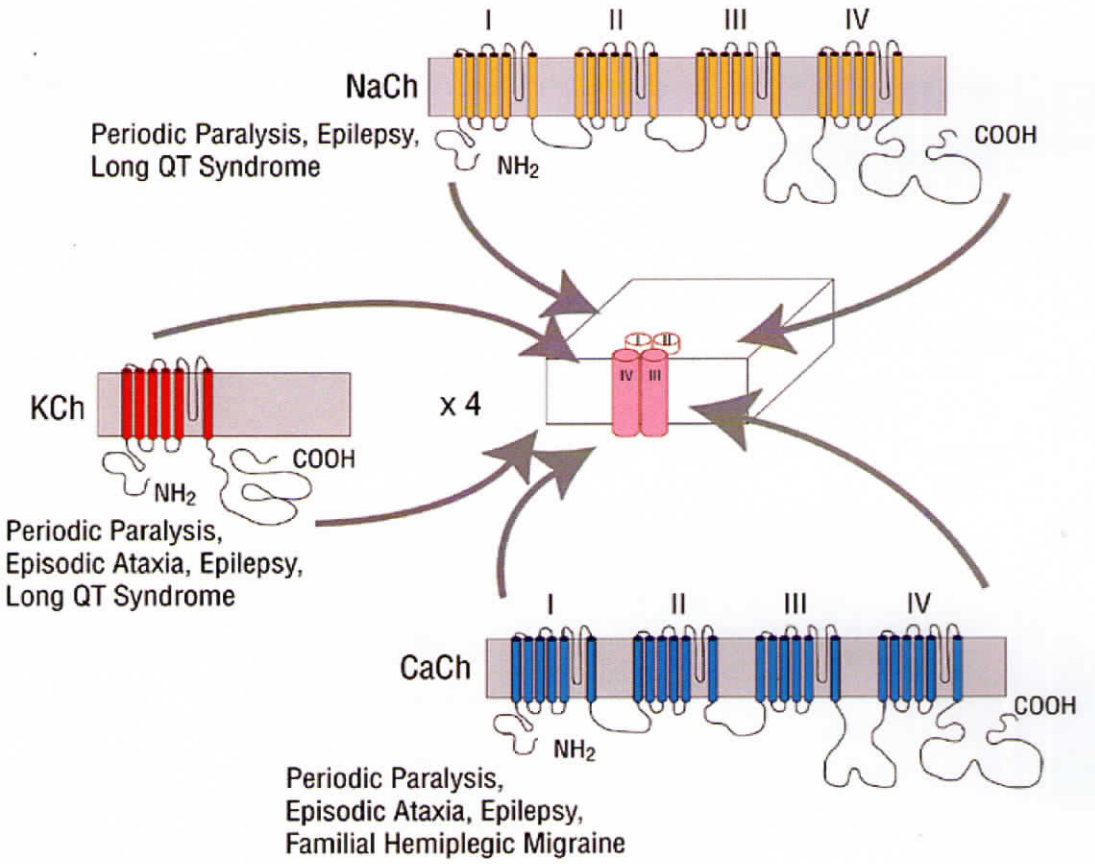
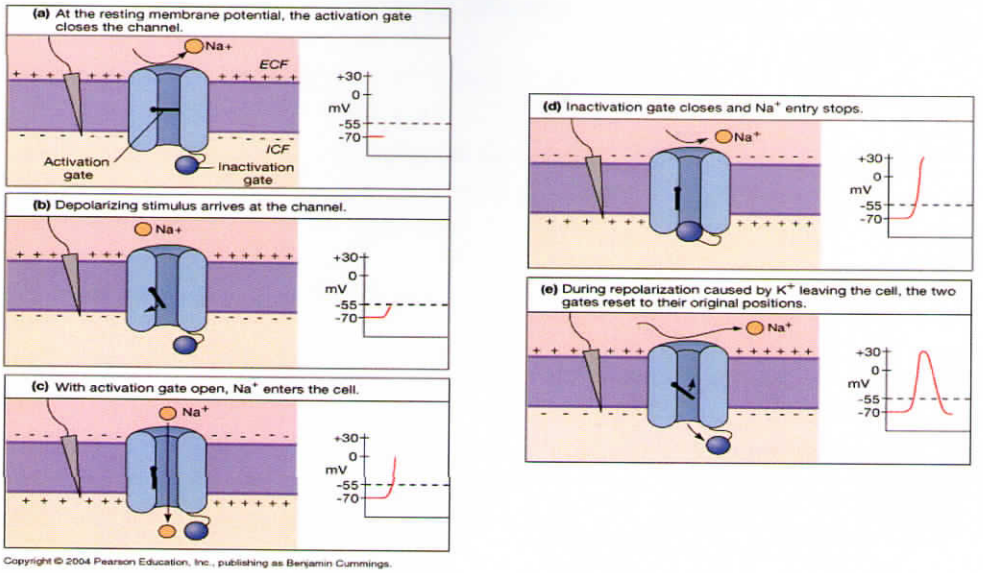


Figure 2- Voltage gated ion channels

2.4.2 Generation of membrane potentials

Under resting conditions, mostly K channels are open and hence K ions tend to leave the cells along their chemical gradient. This is prevented by the retaining force set up by negatively charged proteins. Channels permeable to Na and chloride ions are opened when a change in membrane potential occurs. During a seizure, resulting from activation of Na and K channels, Na ions accumulates within the neurons and K ions in the extracellular space. This leads to activation of the electrogenic Na pump and thence to a hyperpolarising drive. This drive contributes to the termination of seizures and is responsible for after hyperpolarization that follows a single seizure (Figure 3).



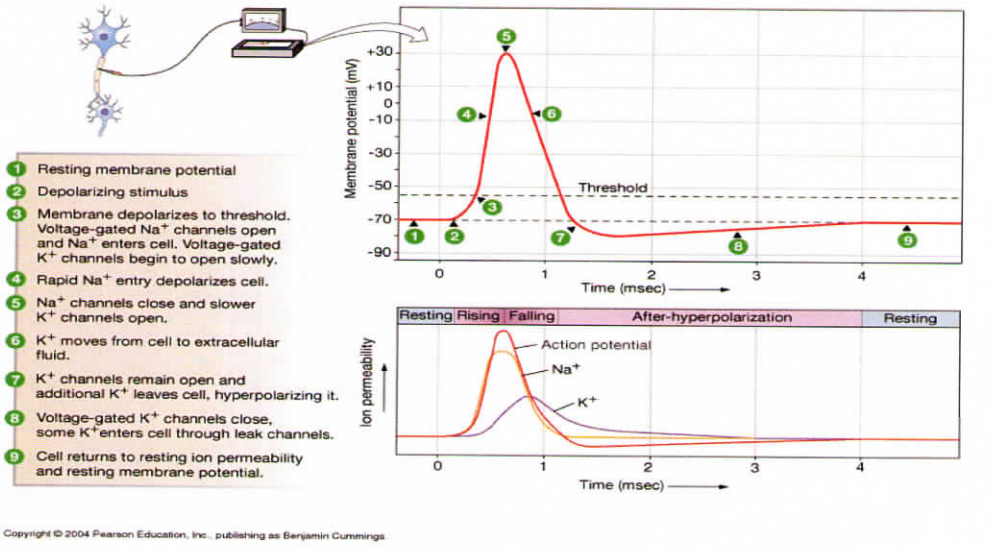


Figure 3- Membrane & Channel Changes during an Action Potential

Functional mechanism by which synaptic excitability can be transiently increased in local circuits includes:

Presynaptic factors involving accumulation of Ca in axon terminals during repetitive activation

Involves post synaptic factors such as increased activation of receptors

Inhibitory synapses tend to decrease in efficacy during the some kinds of repetitive activation

In LRE, abnormal increased excitability in the cortex occurs when a group of neurons in a localised area are activated simultaneously in an abnormally hypersynchronous manner, due to activation of recurrent excitatory circuits. The neurons undergo a large depolarising shift in membrane potential on top of which is a burst of action potentials. This is followed by a membrane potential during which the neurons are inhibited.

2.4.3 Excitatory and inhibitory neurotransmission

Signals released from one cell must find a way to transmit their message across the impermeable cell membrane. In ligand gated ionic channels, a specific receptor for the signal molecule opens in the membrane a pore that is permeable to a population of ions. This alters the intracellular concentration of ions such as Ca (NMDA type glutamate receptors) or chloride ions (GABA A R) and thereby alters cell excitability.

A receptor coupled to a G protein when changing shape, activates an enzyme on the inside surface of the membrane. This generates a small diffusible messenger- cAMP, cGMP or IP3. These will in turn act on enzymes such as protein kinases to modify the function of target molecules (receptors, ionic channels, other enzymes or binding proteins) to change cell excitability or gene expression.

2.4.3.1 *Ionotropic excitatory amino acid receptors*

3 classes of these receptors have been identified in the central nervous system based on the pharmacologic agonist that binds to specific receptor subtype and selectively opens the associated ion channel (Figure 4):

1 N-methyl D aspartate receptor

2 AMPA (alpha amino 3 hydroxy 5 methyl 4 isoxazole propionate) receptor

3 Kainic acid receptor

2.4.3.1.1 *NMDA receptors*

These receptors have two agonist binding sites- NMDA, glutamate, etc. They also require glycine as a coagonist. At hyperpolarised membrane potentials, activation does not result in current flow through the channel since extracellular Mg blocks the channel. At more depolarised potentials, Mg ions will be expelled from the channel and both monovalent and divalent cations can flow through the channel. The entry of Ca through this channel signals a biochemical cascade, resulting in the development of both long term potentiation at many synapses. Activation of this channel is slow.

2.4.3.1.2 ***AMPA receptors*** are responsible for the most rapid excitatory transmission within the vertebrate CNS. The associated channels are rapidly activated and inactivated. These receptors can be activated following the binding of L-glutamate but the most potent selective agonist is AMPA.

Following the binding of the agonist to both binding sites on the receptor, the protein conformation is altered, the ion channel opens and the cell depolarizes. The ion channel is permeable to monovalent cations, but both Na and K flow through the channel with varying degrees of permeability.

2.4.3.1.3 ***Kainate receptors***- AMPA and kainate can activate both AMPA and kainate receptors.

There are two agonist binding sites for the receptor and there is no agonist induced desensitization. Both are permeable to monovalent cations.

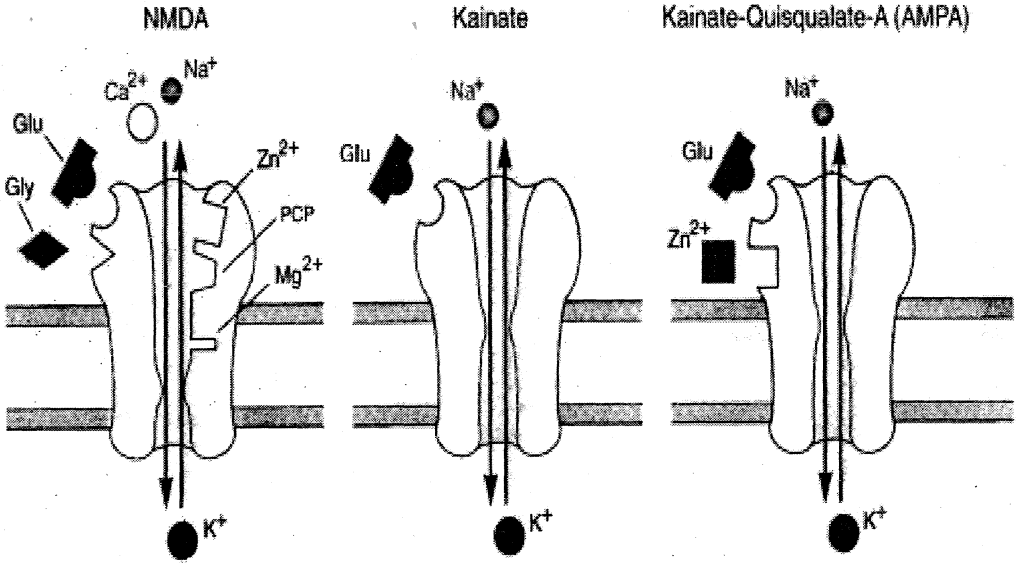


Figure 4- Three types of ionotropic excitatory amino acid receptors

2.4.3.2 ***Metabotropic excitatory amino acid receptors***

Excitatory amino acids are capable of activating a different group of receptors that are coupled via 2nd messenger systems to biochemical pathways and ion

channels. Based on coupling to different 2nd messenger systems and pharmacology, these receptors are classified as:

1 **Group I mGluRs**- includes the mGluR1 and mGluR5. Upon binding of ligand to the receptor, IP3 formation is increased, intracellular Ca is mobilised and arachidonic acid is released.

2 **Group II mGluRs**- include the mGluR2, mGluR3 and mGluR8 types.

3 **Group III mGluRs**- mGluR4, mGluR6, mGluR7. mGluRs on axon terminals mediate presynaptic modulation.

Activation of postsynaptic receptors may cause direct depolarization and modulation of intrinsic currents. When an agonist binds to mGluRs, activation of a variety of G protein occurs. This G protein coupled activation results in a diverse biochemical cascade which results in the modulation of a variety of cellular functions, such as current flow through voltage gated ion channels. These can directly influence activation of enzymes which leads to production of second messengers such as IP3, cyclic AMP, etc which activate protein kinases. These affect voltage and ligand gated channels in the membrane.

3 classes of neurotransmitters interact with these receptors. All fast neurotransmitters also act on these receptors including GABA, glutamate and Acetylcholine. The activation of these receptors leads to different cellular effects that can be either excitatory or inhibitory. This depends on the type of G protein activated, the intracellular second messenger and the target in a given cell as well as in a given neuronal network (Figure 5).

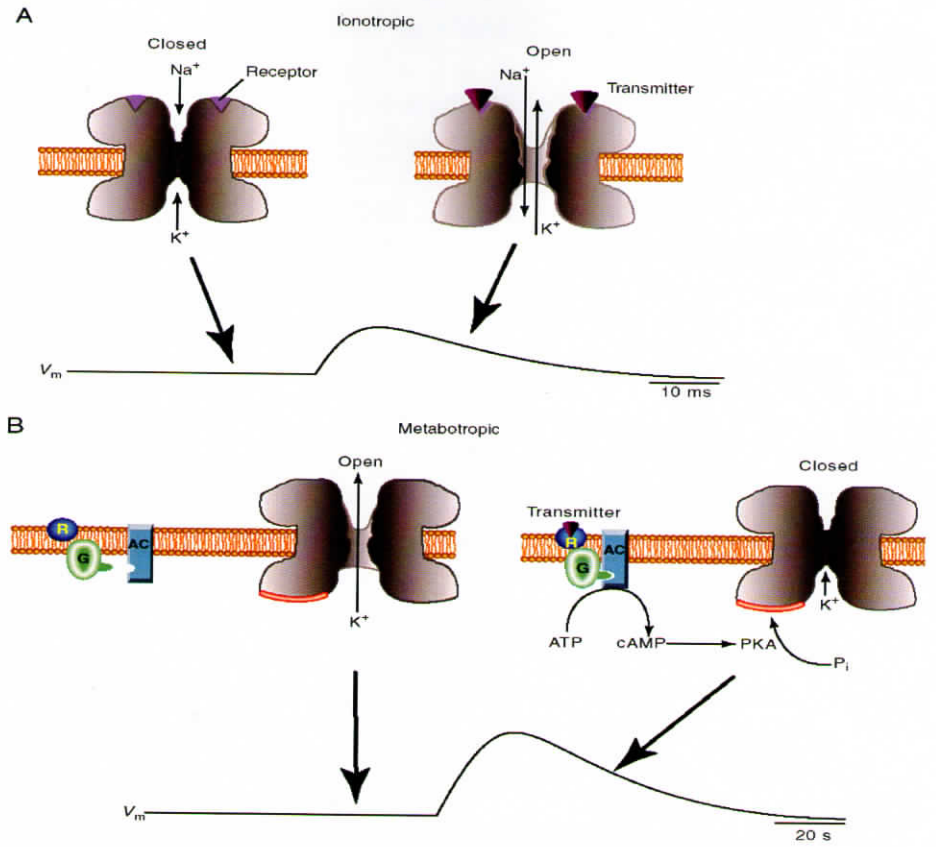


Figure 5- Mechanism of action of ionotropic and metabotropic receptors

2.4.3.3 *Basic neurophysiology of excitatory synaptic function*

The process of excitatory neurotransmission between cells begins with the generation of an action potential and the propagation down the axon. As the action potential invades the specialized presynaptic terminals of the axon, the membrane in this region becomes depolarized. This depolarisation results in the opening of ion channels permeable to Ca. As Ca rushes into the terminal, a biochemical cascade is set into motion.

When the neurotransmitter reaches the postsynaptic membrane via diffusion across the synaptic cleft, they bind to colocalised non-NMDA and NMDA synaptic receptors in the membrane of adjacent cell. Termination of the evoked EPSP has been attributed to processes like diffusion of glutamate away from the synapse and accounts for a good portion of the decay of the EPSP.

2.4.3.4 *Inhibitory synaptic transmission*

Inhibitory signals cause a wave of hyperpolarization along the membrane of a post-synaptic cell known as an inhibitory post-synaptic potential (IPSP). If enough EPSPs arrive at the axon terminal simultaneously, the membrane potential will rise above threshold, and an action potential will fire. IPSPs lower the membrane potential and if enough IPSPs have fired, these inhibitory signals will stop the neuron from firing. The duration of IPSPs is regulated by GABA uptake into presynaptic terminals and glia.

2.4.3.4.1 *GABA A receptor*

These receptors are almost exclusively chloride selective and GABA ARs are macromolecular proteins that contain specific binding sites for GABA, barbiturates, benzodiazepines, etc. 2 molecules of GABA are required to bind to 2 independent sites on the GABA AR channel

2.4.3.4.2 *GABA B Receptor*

Seven transmembrane proteins are coupled to Ca or K ion channels via GTP binding proteins and are located on pre-synaptic and post synaptic membranes. These receptors are also been identified on presynaptic terminals of GABAergic neurons and thus produce autoinhibition of GABA release from GABA nerve terminal. When activated by GABA pre-synaptically, GABA B R reduces synaptic transmitter release by decreasing pre-synaptic Ca entry. And when activated post synaptically, GABA B R produces slow post synaptic inhibition by increased K conductance.

GABABRs are G-protein coupled receptors and coupled to the voltage gated Ca channel or K channel via a Gi or Go type G protein.

2.4.3.4.3 *Glycine*

Glycine is stored in small presynaptic vesicles and is released from glycinergic terminal on depolarisation and following Ca entry. Glycine receptors are expressed either as a homopentamer of a subunits, or a complex now thought to harbour 2a and 3b subunits that contain an intrinsic Cl- channel. Glycinergic inhibition mediates most of the fast inhibitory synaptic transmission in spinal cord and brain stem.

2.4.4 *Neuromodulators*

Neuropeptides may facilitate or inhibit the action of neurotransmitters. This can be the result of peptide action on neurotransmitter synthesis, on metabolizing enzymes or on receptors.

2.4.4.1 *Nitric oxide (NO)*

Nitric oxide is generated by oxidation of amino acid L-arginine, catalysed by nitric oxide synthase. It enhances release of several neurotransmitters.

2.4.4.2 *Cyclic AMP (cAMP)*

Neurotransmitters unable to cross the cell membrane binds to a specific extracellular site on the receptor protein and activates a G-protein, which in turn activates the enzyme adenylyl cyclase converting ATP to cyclic AMP.

2.4.4.3 *Inositol 3 phosphate-Diacylglycerol system (IP3-DAG)*

Many growth factors particularly of the neurotrophic family bind to receptors that activate the hydrolysis of membrane phospholipids. By turning on different subunits of phospholipase C, they break down phosphoinositide phosphates into 2 main components:

DAG and a family of soluble inositol phosphate messengers such as IP3. DAG activates protein kinase C, generating a wide variety of intracellular changes.

2.4.4.4 *Calcium*

During a seizure, extracellular Ca decreases; this precedes the onset of seizure. The opening of Na channels on the surface of presynaptic terminals leads to depolarization, which opens voltage sensitive Ca channels. Ca comes into the presynaptic terminal and activates a variety of enzymes (CamKII) which phosphorylates synapsin I, releasing it from vesicle which fuses to the synaptic membrane and releases neurotransmitter. Calcium plays a key role in type of neuronal injury induced by repetitive seizure.

Seizure opens both NMDA gated and voltage gated Ca channels and when they produce energy failure during SE, glutamate reuptake is impaired, leading to increased Ca influx thru NMDA and other channels.

2.5 *Antiepileptic drugs*

An antiepileptic drug is one which decreases the frequency and/or severity of seizures in people with epilepsy. AEDs treat the symptom of seizures and not the underlying epileptic condition. Modern treatment of seizures started in 1850 with the introduction of bromides but had many side effects. In 1912, phenobarbitone which then was used to induce sleep, was found to have antiseizure activity and became the drug of choice for many years, but it had no effect on absence seizures. A number of medications similar to phenobarbital were developed, including primidone. In 1940, phenytoin was discovered to be an effective drug for the treatment of epilepsy, and since then it has become a major first-line AED in the treatment of partial and secondarily generalized

seizures. In 1945, trimethadione was developed as the first drug for use in absence seizures. Between 1960 and 1974, only one new drug, diazepam was approved for use. Its major contribution was effectiveness in the treatment of status epilepticus. In 1968, carbamazepine was approved, initially for the treatment of trigeminal neuralgia; later in 1974, it was approved for partial seizures. Ethosuximide has been used since 1958 as a first choice drug for the treatment of absence seizures without generalized tonic-clonic seizures. Valproate was licensed in Europe in 1960 and in the United States in 1978, and now is widely available throughout the world. It became a drug of choice in primary generalized epilepsies and in the mid 1990s was approved for treatment of partial seizures. These anticonvulsants were the mainstays of seizure treatment until the 1990s, when newer AEDs with good efficacy, fewer toxic effects, better tolerability, and no need for blood level monitoring were developed. The new AEDs have been approved in the United States as add-on therapy only, with the exception of topiramate and oxcarbazepine; lamotrigine is approved for conversion to monotherapy.

2.5.1 Metabolism of AEDs

Most AEDs are metabolized in the liver by hydroxylation or conjugation. These metabolites are then excreted by the kidney. Some metabolites are themselves active (carbamazepine, oxcarbazepine, primidone). Gabapentin undergoes no metabolism and is excreted unchanged by the kidney. Most AEDs are metabolized by the P450 enzyme system in the liver.

Valproic acid is metabolized by a combination of conjugation by uridine glucuronate (UDP)-Glucuronyltranferase (UGT) via conjugation and by mitochondrial beta-oxidation.

A major player contributing to various forms of drug toxicity is the cytochrome P-450 (cyt P-450) family of enzymes. This system evolved to oxidize xenobiotics (foreign compounds, such as drug molecules) in order to prepare them for excretion after conjugation to a highly water-soluble moiety, such as glucuronic acid. In order to form the watersoluble glucuronide by the so-called Phase II reaction, the lipid-soluble drug molecule must have a functional group suitable for conjugation, such as a hydroxyl group, conferred on the drug by the Phase I reaction catalyzed by cytochrome P-450. The above reaction can be used to describe the metabolism involving the hydroxylation of phenytoin (PHT), carbamazepine (CBZ), and others before those drugs can be glucuronidated by a Phase II reaction involving UDP-glucuronyltransferase.

2.5.2 Mechanism of Action of all AEDs

The basic principal of AEDs is to control electrolytes and neurotransmitters across neuronal membrane. AEDs suppress neuronal activity just enough to prevent abnormal or repetitive firing.

Mechanisms of action of AEDs- can be divided into 3 categories (Table 4):

- Enhancement of GABAergic transmission
- Reducing excitatory transmission

- Stabilise membrane and prevent depolarisation by action on ion channels

Table 4- Classification of antiepileptic drugs		
Enhance GABA transmission	**Inhibit excitatory aminoacid transmission**	**Action on Ion Channels**
Benzodiazepines	**Felbamate**	**Na+:**
(diazepam, clonazepam)	**Topiramate**	**Phenytoin, Carbamazepine, Lamotrigine**
Barbiturates (phenobarbital)		**Topiramate**
Valproic acid		**Valproic acid**
Gabapentin		**Ca++:**
Vigabatrin		**Ethosuximide**
Topiramate		
Felbamate		
Most effective in myoclonic but also in tonic-clonic and partial		**Valproic acid**
		Na+:
Clonazepam: for Absence		**For general tonic-clonic and partial seizures**
		Ca++:
		For Absence seizures

2.5.2.1 *Enhancement of GABAergic transmission*

Gamma-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter. GABA transmission is associated with an influx of chloride ion. Influx of a chloride ion suppresses neuronal activity and increases the activity of GABA at the chloride-ionophore-GABA-receptor complex.

2.5.2.1.1 ***Drug Classes that potentiate GABA***

1 Barbiturates

2 Benzodiazepines

3 Other agents

2.5.2.1.1.1 ***Barbiturates***

2.5.2.1.1.1.1 ***Phenobarbital***

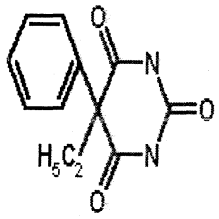


Figure 6- Structure of Phenobarbital

Phenobarbital is indicated for partial and generalised seizures and also status epilepticus (Figure 6). It binds at the allosteric site on GABA receptor and increases the duration of opening of chloride channel. Phenobarbital causes the calcium dependent release of neurotransmitters at high doses and depresses glutamate mediated excitation. It reduces sodium conductance and calcium influx.

Phenobarbital undergoes extensive biotransformation in the liver. P-hydroxy phenobarbital is the major metabolite excreted as the glucuronide conjugate. N-glucosidation is another important metabolic pathway.

2.5.2.1.1.1.2 *Primidone*

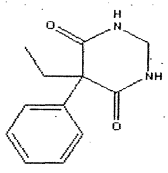


Figure 7- Structure of primidone

Primidone (Figure 7) is rapidly metabolized into phenobarbital which is the primary metabolite with the main epileptic action and into phenylethylmalonamide.

2.5.2.1.1.2 *Benzodiazepines*

Benzodiazepines bind to a site on the GABA receptor different from that of barbiturates. The other mechanism by which benzodiazepines may act is by increasing the frequency of opening of chloride channel.

2.5.2.1.1.2.1 *Clobazam*

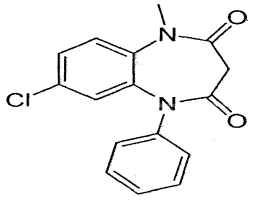


Figure 8- Structure of clobazam

Clobazam is indicated in refractory partial seizures and secondary generalised seizures, Lennox-Gastaut syndrome and status epilepticus. It also affects

voltage sensitive calcium ion conductance and sodium channel function (Figure 8).

Clobazam is absorbed rapidly and about 75-85% is bound to plasma proteins. It is extensively oxidized in the liver to N-desmethyl clobazam which possesses antiepileptic activity and is excreted in bile as glucuronate and in the urine as sulphate.

2.5.2.1.1.2.2 *Clonazepam*

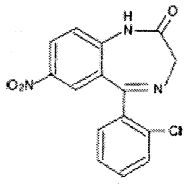


Figure 9- Structure of clonazepam

Clonazepam is administered for the long term treatment of some epilepsies, absence epilepsy, generalized tonic-clonic seizures, myoclonic epilepsy caused by primary generalised epilepsy and also symptomatic secondary generalised epilepsy (Figure 9). It acts as an agonist at GABA A receptor and also affects voltage sensitive calcium ion conductance and sodium channel function.

Clonazepam is metabolised in the liver first by acetylation and the acetylated compound is then reduced and nitrated. Metabolites have no significant antiepileptic action.

2.5.2.2 *Reducing excitatory transmission*

Targets for potential AEDs are the receptors for excitatory amino acids and antagonists acting on NMDA receptors, AMPA receptors (*phenobarbital, topiramate*) and metabotropic receptors.

2.5.2.2.1 *Topiramate*

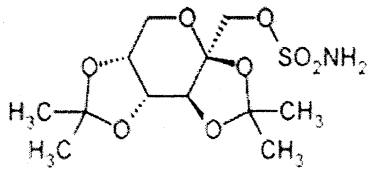


Figure 10- Structure of topiramate

Topiramate has multiple mechanisms of action: it blocks AMPA glutamate receptors, blocks voltage-sensitive sodium channels and augments GABA activation of GABA A receptor (Figure 10).

Topiramate is metabolized in the liver by the CYP2C19 enzyme. At least 8 metabolites have been identified formed by hydroxylation, hydrolysis or cleavage of sulphamate group. None of the metabolites have antiepileptic action.

2.5.2.3 *Modification of ionic conductance*

Several of the most important antiepileptic drugs affect membrane excitability by an action on voltage-dependent ionic channels. Phenytoin, carbamazepine,

lamotrigine, phenobarbital, valproate and topimarate act by prolongation of the inactive state and refractory period of the voltage-dependent sodium channel. Ethosuximide and trimethadione act by decreasing the T-type calcium current.

2.5.2.3.1 *Drugs that Suppress Sodium Influx*

These drugs dampen CNS activity by delaying an influx of sodium across neuronal membranes into intracellular spaces. There is no abuse potential or CNS depression.

2.5.2.3.1.1 *Phenytoin*

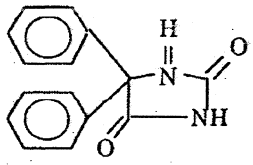


Figure 11- Structure of phenytoin

Phenytoin is indicated for partial and generalised seizures, status epilepticus and neonatal convulsions (Figure 11). It blocks ionic movements in the sodium channel during the depolarization process.

Phenytoin is extensively metabolised by the hepatic P450 mixed oxidase system (CYP2C9). The first step involves a zero-order kinetic reaction accounting for the non-linear dose: serum level relationship. The para-hydroxylation step is mainly followed by glucuronidation, although there are a range of minor metabolites. None of the metabolites have antiepileptic activity and all are excreted via the kidney (Figure 12).

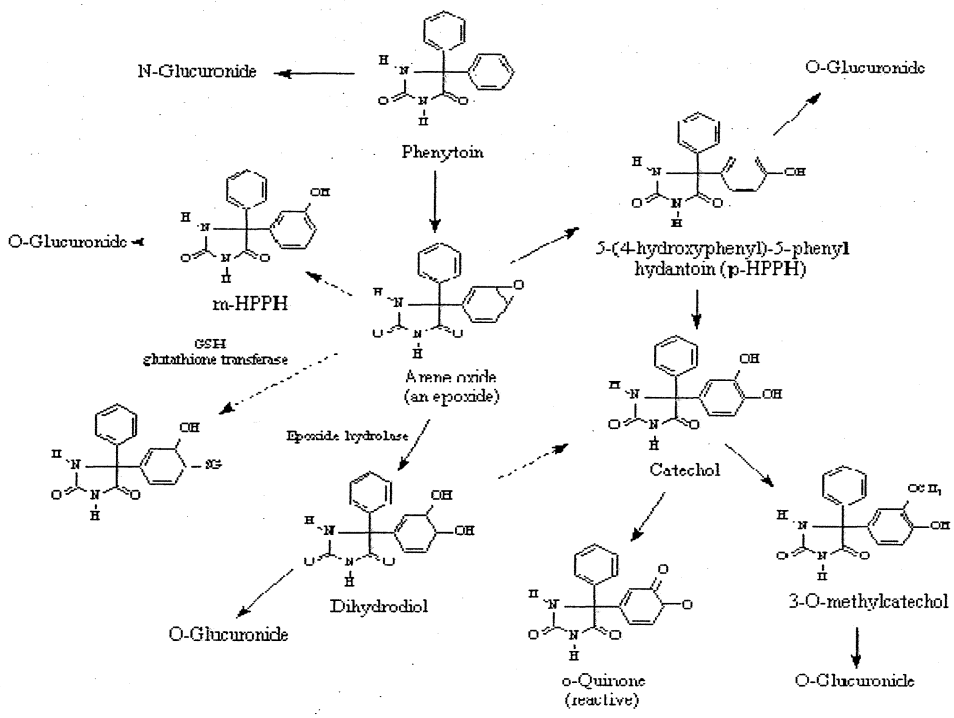


Figure 12- Phenytoin metabolism

2.5.2.3.1.2 *Carbamazepine*

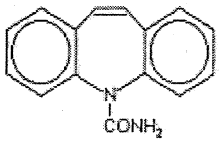


Figure 13- Structure of carbamazepine

Carbamazepine is indicated against partial seizures, generalized tonic clonic seizures, benign rolandic epilepsy, depression and pain syndromes (Figure 13). It acts by the use and frequency dependent blockade of sodium channels, blockade of NMDA receptor activated sodium and calcium flux.

Carbamazepine is extensively metabolized by the liver. Major pathway is first epoxidation to CBZ 10,11-epoxide (also has antiepileptic action) and then

hydrolysis to CBZ 10,11-trans-dihydrodiol. The pathway also includes other conjugated and unconjugated metabolites (Figure 14).

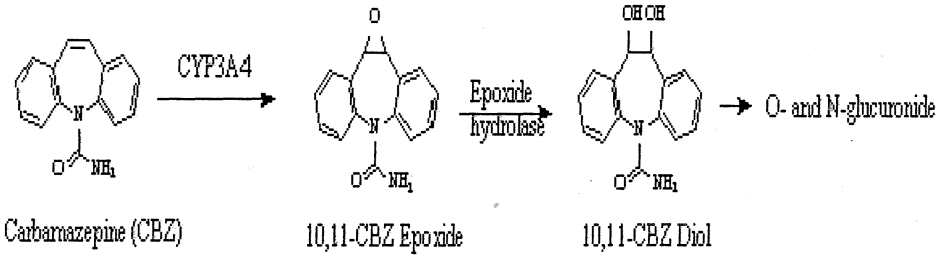


Figure 14- Carbamazepine metabolism

2.5.2.3.1.3 *Oxcarbazepine*

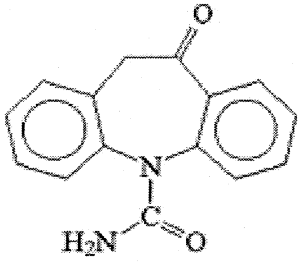


Figure 15- Structure of oxcarbazepine

The mechanism of action of oxcarbazepine (Figure 15) is similar to that of carbamazepine.

Oxcarbazepine is rapidly metabolized to the biologically active 10 monohydroxy metabolite (MHD)- 10, 11 dihydro 10 hydroxy 5H dibenzol azepine 5 carboxamide (which is responsible for the antiepileptic action). This is then conjugated to a glucuronide compound.

Hydroxylation is rapid and complete. The lack of epoxidation could be the reason for the better tolerability of the drug.

2.5.2.3.1.4 ***Valproate***

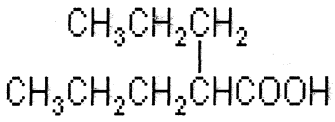


Figure 15- Structure of valproate

Valproate is administered for generalized tonic-clonic seizures, absence epilepsy, juvenile myoclonic epilepsy, photosensitive epilepsy, Lennox-Gastaut syndrome and neonatal convulsions (Figure 15). Valproate acts by blocking sodium and calcium channels, inhibits GABA transaminase and increases GABA synthesis.

Valproate is rapidly eliminated from the body by hepatic metabolism by a variety of pathways, the main one being hepatic microsomal oxidation followed by glucuronidation. At least 30 metabolites have been identified, the 4-ene metabolite being responsible for hepatic toxicity (Figure 16).

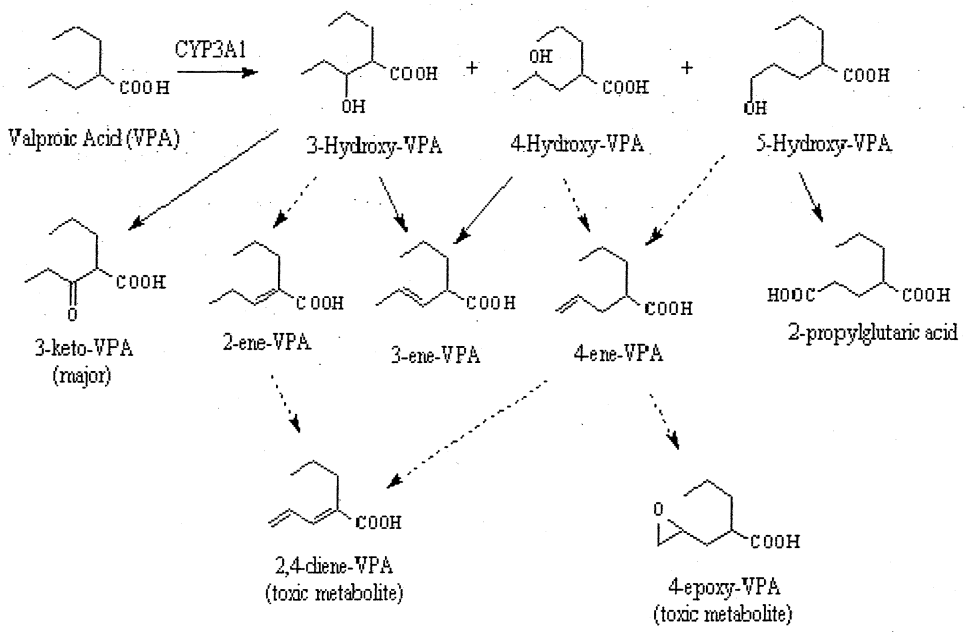


Figure 16- Valproate metabolism

2.5.2.3.1.5 *Lamotrigine*

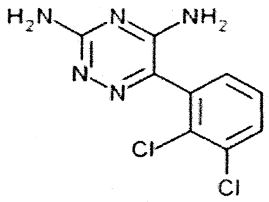


Figure 17- Structure of lamotrigine

Lamotrigine is administered against partial and generalised seizures viz. primary generalized tonic clonic seizures, typical and atypical absence, atonic and myoclonic sz, and Lennox-Gastaut syndrome (Figure 17). It acts by the stabilization of neuronal membranes by blocking voltage dependent sodium channel conductance and blocks calcium channels; also decreases the release of glutamate.

Lamotrigine undergoes extensive metabolism in the liver, largely to the inert glucuronide conjugate, most of which is renally excreted. A small percentage remains unchanged in urine.

2.5.2.3.2 *Drugs that Suppress Calcium Influx*

2.5.2.3.2.1 *Ethosuximide*

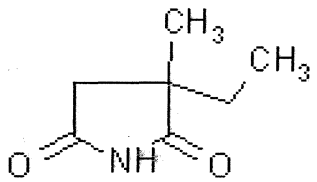


Figure 18- Structure of ethosuximide

Ethosuximide is indicated for generalised absence seizures and resistant idiopathic generalized epilepsy (Figure 18). It inhibits low threshold calcium currents in thalamus and also acts at the GABA A receptor.

Ethosuximide is extensively metabolized in the liver, first by oxidation and then conjunction by CYP3A enzyme; metabolites have no significant antiepileptic action.

2.5.2.4 *Newer antiepileptic drugs*

2.5.2.4.1 *Vigabatrin*

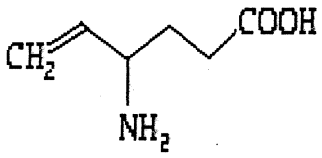


Figure 19- Structure of vigabatrin

Vigabatrin is indicated against CPS and JME, Lennox-Gastaut syndrome and infantile spasms (Figure 19). It irreversibly inhibits GABA transaminase, greatly raises the extracellular GABA concentration in the brain.

Vigabatrin is minimally metabolized (<5%) and is eliminated primarily by renal excretion of the unchanged drug.

2.5.2.4.2 *Felbamate*

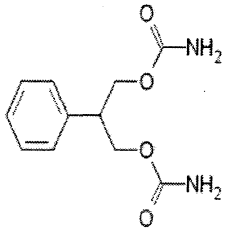


Figure 20- Structure of felbamate

Felbamate is indicated against partial seizures with or without generalization and Lennox Gastaut syndrome (Figure 20). It modulates sodium channel conductance and blocks the NMDA receptor.

Felbamate is extensively metabolized by liver via hydroxylation and conjugation and 40-49% of drug is recovered in urine as parent compound. Major metabolites are p-hydroxy-felbamate, 2-hydroxy-felbamate, a monocarbamate and 3-carbamoyloxy-2-phenylpropionic acid.

2.5.2.4.3 *Gabapentin*

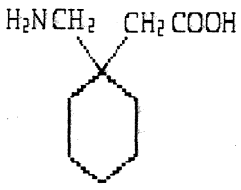


Figure 21- Structure of gabapentin

Gabapentin is used to control partial seizures and secondary generalized tonic clonic seizures (Figure 21). It is not metabolized and is entirely excreted in an unchanged form.

2.5.2.4.4 *Pregabalin*

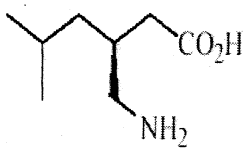


Figure 22- Structure of pregabalin

Pregabalin produces an increase in glutamic acid decarboxylase and also a decrease in neuronal calcium currents by binding of alpha 2 delta subunit of the voltage gated calcium channel (Figure 22). It undergoes negligible metabolism in humans.

2.5.2.4.5 *Tiagabine*

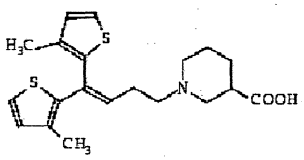


Figure 23- Structure of tiagabine

Tiagabine is used against partial seizures and secondarily generalised seizures and status epilepticus (Figure 23). It greatly increases cerebral GABA concentrations, via the inhibition of GABA transporter-1.

Tiagabine is extensively metabolized in the liver and the main enzymatic degradation is by CYP3A4. At least five major metabolites are found in plasma but none have antiepileptic action.

2.5.2.4.6 *Zonisamide*

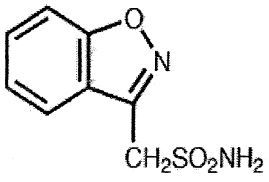


Figure 24- Structure of zonisamide

Zonisamide blocks voltage-dependent sodium channels and T-type calcium channels (Figure 24). It is used as an add-on therapy for partial and generalized seizures.

2.5.2.4.7 *Levetiracetam*

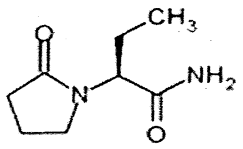


Figure 25- Structure of levetiracetam

Levetiracetam is used as an adjunctive therapy in the treatment of partial onset and generalised seizures (Figure 25).

The major metabolic pathway includes 3 inactive metabolites formed by hydrolysis of the acetamide group. The metabolism does not involve CYP450 enzymes.

2.6 *Congenital malformations and AED teratogenicity*

Congenital disorders include a broad category that includes a variety of conditions. Various subsets include:

1 Birth defect

2 Congenital physical anomaly

3 Congenital malformation is a deleterious physical anomaly, a structural defect perceived as a problem

4 Genetic diseases

5 Congenital metabolic disease- inborn errors of metabolism

Malformation- the fetus is genetically abnormal and programmed to develop abnormally. Malformation includes intrinsic defects that result in localised

abnormalities during the development of organs and body parts. Major malformations interfere with normal function. Minor malformations are not associated with severe health problems (Table 5).

Deformation- a genetically normal fetus develops aberrations in form, shape or position of body parts due to an abnormal uterine environment. Deformations essentially results from mechanical forces, intrauterine constraint and lack of fetal movement breech presentation. Eg. Limb reduction anomalies, craniostenosis, oligohydramnios

Disruption- genetically normal fetus suffers a morphologic defect resulting in disruption of normal development. Eg. Extrinsic forces, vascular insults- gastroschisis, vascular exchange of necrotic debris- rupture of the amnion results in band formation that adheres to the developing fetus.

Table 5- System malformations

Organ system	Examples
Central nervous system	Neural tube defects- spina bifida, meningocoele; anencephaly, microcephaly, holoprosencephaly
Cardiovascular system	Atrial septal defect, Ventricular septal defect, PDA, pulmonary stenosis
Gastrointestinal system	Cleft lip/palate, gastroschisis, diaphragmatic hernia
Genitourinary system	Hypospadias, renal atresia
Musculoskeletal system	Talipes equinovarus, achondroplasia

2.6.1 Malformations due to chromosomal aberrations

50% of embryonic deaths and 0.9% of live borns develop malformations due to chromosomal aberrations (Snijders, 1995).

2.6.1.1 ***Numerical alterations*** (aneuploidy)- Aneuploidy is the second major category of chromosome mutations in which chromosome number is abnormal. An aneuploid is an individual organism whose chromosome number differs from the wild type by part of a chromosome set. Generally, the aneuploid chromosome set differs from wild type by only one or a small number of chromosomes.

2.6.1.2 ***Structural chromosomal aberrations***- Structural chromosome abnormalities occur when there is a change in the structure or components of a chromosome. These abnormalities occur when part of a chromosome is missing, a part of a chromosome is extra, or a part has switched places with another part. Ultimately, this leads to having too much or too little genetic material, which is a cause of some birth defects.

2.6.2 Malformations due to genetic defects

2.6.2.1 ***Single gene defects***- A single gene disorder is the result of a single mutated gene. There are estimated to be over 4000 human diseases caused by single gene defects.

2.6.2.2 ***Multiple gene defects***

2.6.2.2.1 ***Autosomal dominant disorders-*** Only one mutated copy of the gene will be necessary for a person to be affected by an autosomal dominant disorder. Each affected person usually has one affected parent. There is a 50% chance that a child will inherit the mutated gene. Eg. Huntington's disease, Neurofibromatosis 1, Marfan Syndrome.

2.6.2.2.2 ***Autosomal recessive disorders-*** Two copies of the gene must be mutated for a person to be affected by an autosomal recessive disorder. An affected person usually has unaffected parents who each carry a single copy of the mutated gene. Eg. cystic fibrosis, sickle-cell disease

2.6.2.2.3 ***X-linked disorders-*** X-linked diseases are single gene disorders that reflect the presence of defective genes on the X chromosome. This chromosome is present as two copies in females but only as one copy in males. Like autosomal single gene disorders, X-linked diseases can be either recessive or dominant.

2.6.3 Mitochondrial disorders

Mitochondrial diseases comprise those disorders that in one way or another affect the function of the mitochondria or are due to mitochondrial DNA. Mitochondrial diseases take on unique characteristics both because of the way the diseases are often inherited and because mitochondria are so critical to cell function.

2.6.4 Multifactorial inheritance- Isolated birth defects such as club foot, cleft lip, cardiac abnormalities and NTDs are inherited through a combination of genetic and environmental factors. While the genetic cause and unknown factors share the major chunk of congenital malformations, identifiable teratogens are responsible for these in less than 1% **(Boyle 1994)**.

2.6.5 Teratogens

An agent is considered a teratogen if its administration to the pregnant mother causes, directly or indirectly, structural or functional abnormalities in the fetus, or in the child after birth, which may not be apparent until later life.

A teratogen can induce chromosomal abnormalities, prevent implantation of the conceptus, and cause abortion of the early embryo, late fetal death, congenital malformations, or intrauterine growth retardation. In the neonate there may be functional impairment, e.g. deafness. Behavioural abnormalities and mental retardation may also occur.

Susceptibility to teratogens is genetically determined. Teratogenic agents may be beneficial or harmless to the mother but harmful or lethal to the embryo or the fetus. Most drugs and chemicals will cross the placenta.

The timing of the exposure is critically important and determines the type of fetal toxicity that might occur, e.g. malformation or functional impairment. If susceptibility to teratogens occurs between the second and eighth weeks when body parts and organs start to develop, the condition is known as embryopathy. If major effects are caused after 8 weeks, the condition is known as fetopathy.

Teratogenicity is usually dose-dependent, and there is evidence that the teratogenicity of a drug may be enhanced by co-administration of a second drug.

A wide range of different chemicals and environmental factors are suspected or are known to be teratogenic in humans and in animals:

Drugs and environmental chemicals: alcohol, aminopterin, androgenic hormones, chlorobiphenyls (PCBs), diethylstilbestrol, diphenylhydantoin (Phenytoin, Dilantin, Epanutin), ethanol, ethidium bromide,

organic mercury, penicillamine, tetracyclines, thalidomide, trimethadione, uranium, valproic acid.

Ionizing radiation: atomic weapons, radioiodine, radiation therapy

Infections: cytomegalovirus, herpes virus, parvovirus B-19, rubella virus (German measles), syphilis, toxoplasmosis, Venezuelan equine encephalitis virus

Metabolic imbalance: alcoholism, endemic cretinism, diabetes, folic acid deficiency, iodine deficiency, hyperthermia, phenylketonuria, rheumatic disease and congenital heart block, virilizing tumors

Tobacco, Alcohol, and Caffeine: Smoking, Alcohol use, and consumption of caffeine

2.6.6 Antiepileptic drug teratogenicity

Most studies indicate that the antiepileptic drug therapy rather than the maternal epilepsy is the major cause of malformations in offspring of WWE. Janz in 1964 explained the various types of malformations that are caused by antiepileptic drugs (Janz and Fuchs, 1964). Olafsson in 1998 compared WWE taking AEDs with untreated controls and found a 2.7 fold increase of major malformations in the treated cohort. Infants of WWE not taking AEDs during pregnancy had no increase in anomalies or cognitive dysfunction rate (Holmes 2001).

Poorly controlled epilepsy during pregnancy may carry risks for complications and adverse obstetric outcome (Viinikainen 2006).

Several AEDs such as PB, PHT, TMD, CBZ and VPA have been shown to have teratogenic potential in studies in humans (Finnell and Dansky 1991). Due to a great deal of overlap between the drug-specific syndromes for PB, PHT, TMD, DMD and CBZ, a term "fetal antiepileptic drug syndrome" has been proposed (Finnell et al. 1997).

2.6.6.1 *Antiepileptic drug syndrome*

2.6.6.1.1 *Phenytoin*

Fetal hydantoin syndrome is the specific syndrome seen in offspring of WWE on phenytoin. Minor anomalies such as midfacial hypoplasia, broad nasal bridge, ocular hypertelorism and an arched upper lip, and hypoplasia of distal

phalanges and nails and major anomalies such as CLP and cardiac anomalies and pre and postnatal GR (microcephaly and MR) are specific features of this syndrome (Hanson 1986; Dansky and Finnell 1991)

2.6.6.1.2 *Carbamazepine*

An increased incidence of craniofacial and limb development in seen in children exposed to carbamazepine during gestational period (Hiilesmaa et al. 1981; Bertollini et al. 1987 Jones et al. 1989). There is a similarity between the malformation pattern in children exposed to CBZ and those with fetal hydantoin syndrome (Buehler, 1987). This means both phenytoin and carbamazepine share the same teratogenic mechanism.

2.6.6.1.3 *Valproic acid*

Fetal valproate syndrome is the term used to explain the teratogenic characteristics in children exposed to valproate. This syndrome is characterized by neural tube defects. Dysmorphic features like distinctive facial appearance featuring upward slanting palpebral fissures, epicanthic folds, and posteriorly rotated ears are also seen in these children (DiLiberti et al. 1984, Vorhees 1987).

2.6.6.1.4 *Barbiturates*

Barbiturates cause the same major and minor abnormalities and dysmorphic features as that of phenytoin. Cardiovascular diseases, facial clefts, craniofacial

abnormalities and growth deficiency are the major malformations caused by barbiturates (Seip 1976; Rating et al. 1982; Kallén et al.1989). There is a large overlap in the pattern of teratogenicity between phenytoin and phenobarbitone and hence a common term known as "Hydantoin-Barbiturate Embryopathy" was coined by Majewski in 1981.

2.6.6.2 *Mechanisms of AED induced congenital malformations*

A similar pattern of malformations caused by different antiepileptic drugs suggests that they are caused by a common mechanism. (Vorhees 1987b; Finnell 1991; Danielsson 1997). The malformation pattern of VPA is markedly different from that of other AEDs which suggests a different mechanism of teratogenicity. (Dansky 1991; Lindhout 1992).

The different mechanisms of teratogenicity are listed below:

2.6.6.2.1 *Disturbances in folate metabolism*

Several investigators have examined the relation between maternal folic acid ingestion and NTDs in offspring through the use of a case-control design, a retrospective method in which cases (in this instance NTD infants) are compared with controls (infants without NTDs born to otherwise identical women) for the exposure of interest (ie, folic acid supplements in early gestation).

Muilnare, et al., in 1988 studied the association between multivitamin use during the periconceptional period and the occurrence of neural tube defects

using data from the Atlanta Birth Defects Case-Control Study. They found an overall apparent protective effect of periconceptional multivitamin use on the occurrence of neural tube defects, with a crude estimated relative risk of 0.40 (95% confidence interval, 0.25 to 0.63).

A controlled trial conducted in 1993 has established that use of a 4-mg folic acid supplement before and during early pregnancy reduces the risk of recurrent neural tube defects (NTDs) by 72% (Werler, et al., 1993).

With a case-control study, Shaw, et al., in 1995 investigated whether periconceptional intake of supplemental or dietary folate reduced the risk of having a neural tube defect (NTD)-affected pregnancy. Shaw observed a decreasing risk with increasing folate intake from combined dietary sources and vitamin supplements.

A decreased risk of NTD is associated with both increased folate intake and higher red blood cell folate concentrations (greater than 906 nmol/L); though the experimental evidence is stronger for increased folate intake and NTD risk reduction (IOM, 1998).

Wals, et al., in 2008 assessed changes in the prevalence of neural-tube defects in Canada before and after food fortification with folic acid was implemented. They found that food fortification with folic acid was associated with a significant reduction in the rate of neural-tube defects in Canada. The decrease was greatest in areas in which the baseline rate was high.

Shaw, et al., in 2009 focused on 118 SNPs involved in folate transport and metabolism. The observations do not implicate a particular folate transport or

metabolism gene to be strongly associated with risks for spina bifida or conotruncal defects.

Scanlon, et al., in 1998 compared cases with cardiac outflow tract defects (N = 126) with controls representative of the same birth cohort (N = 679). Infants with clinically recognized syndromes were excluded. Daily total maternal folate intake of > or =245 microg was inversely related to risk of cardiac outflow tract defects among those with transposition (odds ratio estimates: 0.65, 0.78, and 0.76 with increasing quartile of daily folate intake), but positively related among those with normally related vessels (corresponding odds ratio estimates: 1.18, 1.59, and 1.68). This difference disappeared when maternal intake of supplemental folic acid of > or =400 microg compared with <400 microg was considered, excluding dietary intake [odds ratio (OR) = 1.04; 95% confidence interval (CI) = 0.5-2.2 for infants with transposition, and OR = 0.91; 95% CI = 0.5-1.8 for those without transposition of the great arteries].

Research findings to date suggest that periconceptional use of multivitamins containing folic acid is associated with a reduction in the occurrence of congenital heart defects and a possible reduced occurrence of orofacial clefts, although the data are less convincing for orofacial clefts (Botto, et al., 2003, 2004).

Neurology practice parameter (2009) recommends routine supplementation of folic acid 0.4mg to 4mg/day for women with childbearing potential receiving AEDs. The USPSTF recommends that "All women capable of pregnancy

should take a daily vitamin supplement that contains 0.4 to 0.8 mg (400 to 800 μg) of folic acid." (U.S. Preventive Services Task Force recommendation statement, 2009.

An abnormal pattern of folate metabolism would result in a decreased rate of DNA synthesis and gene methylation with deleterious effects on the developing embryo (Finnell et al. 1991). Patients on AED therapy usually develop folate deficiency (Reynolds 1974) and this has been related to impaired development of offspring (Smithells et al. 1976).

Similarity in malformation pattern caused by exposure to PHT and to folate antagonists aminopterin (Goetsch 1962) and methotrexate (Milunsky 1968) have attributed teratogenesis to disturbed folate metabolism.

Several studies have attributed to the development of neural tube defects in humans to low blood folate levels (Smithells 1976).

2.6.6.2.1.1 *Genetic defects in folate metabolism*

Methylenetetrahydrofolate reductase (MTHFR) is an important enzyme involved in the folate metabolism pathway in humans, encoded by the *MTHFR* gene. MTHFR irreversibly reduces 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. 5-Methyltetrahydrofolate is used to convert homocysteine (a potentially-toxic amino acid) to methionine by the enzyme methionine synthase. There are DNA sequence variants associated with this gene. In 2000 a report brought the number of polymorphisms up to 24 (Sibani,

et al., 2000). Two of the most investigated are C677T (rs1801133) and A1298C (rs1801131) single nucleotide polymorphisms.

Individual with two copies of 677C (677CC) have the "normal" or "wildtype" genotype. 677TT individuals (homozygous) are said to have mild MTHFR deficiency. 677CT individuals (heterozygotes) are almost the same as normal individuals because the normal MTHFR can make up for the thermolabile MTHFR. Low folate intake affects individuals with the 677TT genotype to a greater extent than those with the 677CC/CT genotypes. 677TT (but not 677CC/CT) individuals with lower plasma folate levels are at risk for elevated plasma homocysteine levels (Jacques, et al., 1996).

At nucleotide 1298 of the MTHFR, there are two possibilities: A or C. 1298A (leading to a Glu at amino acid 429) is the most common while 1298C (leading to an Ala substitution at amino acid 429) is less common. 1298AA is the "normal" homozygous, 1298AC the heterozygous, and 1298CC the homozygous for the "variant".

Wenstrom, et al., in 2001 determined whether the cytosine-to-thymine mutation at base 677 of the gene for methylenetetrahydrofolate reductase (C677T MTHFR), which has been associated with neural tube defects, is also associated with congenital cardiac malformations. Fifty percent of these isolated congenital cardiac defects were associated with either the C677T MTHFR mutation or elevated amniotic fluid homocysteine levels, or both.

A common mutation on the *MTHFR* gene, A1298C, has also been described as a risk mutation. In a case-control study conducted by de Marco, et al. in 2002

the frequencies of these C677T and A1298C polymorphisms were studied in 203 Italian probands with non-syndromic NTDs. This study shows that the *MTHFR* A1298C polymorphism is a genetic determinant for NTD risk in Italy.

Van Beynum, et al., in 2006 studied the association between MTHFR 677C>T variants and CHD risk and found that the maternal MTHFR 677C>T variants are a risk factor for CHD in offspring, confined to conotruncal heart defects.

The malformations found in fetal anticonvulsant syndromes (FACS) are associated with folic acid deficiency and methylene-tetrahydrofolate reductase (MTHFR) polymorphisms in the general population. To investigate a possible association between FACS and MTHFR genotype, Dean, et al., in 2007 recruited 200 mothers who had taken anti-epileptic drugs in pregnancy, and delivered at Aberdeen Maternity Hospital over a 26-year period. The risk of having a child with congenital malformation or FACS was three to four times higher for mothers who were MTHFR 677TT homozygotes compared with MTHFR 677CC homozygotes.

Folate deficiency and the presence of the 677C > T (CT) polymorphism in the methylenetetrahydrofolate reductase (*MTHFR*) gene have been implicated in the causation of malformations in the fetus (particularly cleft lip and palate and neural tube defects).

There have been contradictory results also regarding the relation between MTHFR genotype and the risk of malformations. Kini, et al., in 2007 studied the *MTHFR* genotype and rate of major malformations (MM) in 187 mother-

child pairs where the mothers had epilepsy and in 236 matched control pairs. There was no association between the child's *MTHFR* genotype and the rate of MM. This study indicates that although the maternal *MTHFR* genotype may confer susceptibility to the teratogenic effect of AEDs, particularly VPA, it is likely that the main teratogenic effects are mediated through other mechanisms.

2.6.6.2.2 Bioactivation to reactive intermediates

Many drugs are converted to epoxide intermediates by reactions catalysed by the microsomal monooxygenase system. Arene oxides are unstable epoxides in the metabolism of aromatic compounds to transhydrodiols. Toxic epoxides elicit carcinogenic and mutagenic effects by covalent binding to vital cell molecules. Epoxide hydrolase (seen in the microsomal fractions of adult and fetal human hepatocytes) detoxifies epoxides by their conversion to dihydrodiols. Genetic defect in arene oxide detoxification seems to increase the risk of congenital malformations (Spielberg 1981).

2.6.6.2.3 Free radical intermediates of AEDs and teratogenicity

Phenytoin is metabolized or bioactivated by cooxidation during prostaglandin synthetase and hydroperoxidase catalysed synthesis of prostaglandins (Wells et al, 1989). These form free radical intermediates that initiate oxidative stress and bind to proteins and nucleic acids to produce cytotoxicity.

2.6.6.2.4 *Alterations in vitamin K metabolism*

The reasons as to why the antiepileptic drugs carbamazepine, phenytoin and phenobarbital cause vitamin K deficiency in the fetus are unknown to date. These drugs induce CYP450 enzymes in maternal and fetal liver (by crossing the placenta), which results in the increased oxidative degradation of vitamin K, leading to vitamin K deficiency in both the adult and fetus, causing neonatal hemorrhage (Howe 1999).

2.6.6.2.5 Teratogenicity related to pharmacological action of AEDs

Watkinson and Miłlicovsky (1983) reported that cultured rodent embryos treated with PHT develop marked maternal bradycardia. Phenytoin teratogenicity might be related to the induced maternal hypoxia. Danielsson (1992) showed that exposure of pregnant rabbits to a teratogenic dose of PHT induced digital reduction defects and orofacial cleft preceded by vascular disruption. Exposure of cultured rodent embryos to PB, CBZ, TMD and DMD on gestational day 10 *in vitro* resulted in concentration dependent severe bradycardia, arrhythmia or cardiac arrest.

Adverse fetal effects after episodes of ischemia/hypoxia are caused by reperfusion/reoxygenation damage and generation of reactive oxygen species (ROS) (Fantel et al, 1992). Generation of ROS is associated with vascular disruption in the distal part of embryo limbs.

2.6.6.2.6 Genetic susceptibility to the teratogenic effects of AEDs

The fact that not all infants of WWE undergoing AED therapy are born with CM emphasizes the importance of fetal genotype and its interaction with various environmental factors. Inbred strains of mice were found to differ in their response to the embryopathic effects of phenytoin. A/J animals, the most susceptible strain, were mated to C57BL/6J mice, the most resistant strain. The susceptibility of the F1 hybrid offspring was determined by the susceptibility of the mother. B6A(F1) offspring were as resistant as C57BL/6J parental mice, and AB6F1 hybrids were as susceptible as A/J mice. This finding was more validated as far as orofacial anomalies were concerned. (B6A)F2 offspring (second generation) were as resistant as their C57BL/6J grandparents (Hansen and Hodes, 1983).

2.6.6.2.6.1 Genetic polymorphisms of CYP450 enzymes

Genetic polymorphisms of CYP450 enzymes is one of the mechanisms which contributes to the teratogenicity of different AEDs. Nucleotide changes in the maternal CYP450 enzymes can cause defective metabolism of AEDs leading to the formation of toxic metabolites or unchanged AED substrates, which are in turn transported by the drug transporters in the placenta to the fetus. CYP2D6, CYP2C9 and CYP2C19 are polymorphic as a result of SNPs, gene deletions and gene duplications. These polymorphisms have an impact on the pharmacokinetics, metabolism, safety and efficacy of drugs (Rodrigues, et al. 2002).

The role of cyt P-450 in teratogenicity of AEDs is suggested by the classic human pregnancy experience that has resulted in guidelines suggesting monotherapy over polytherapy in women with epilepsy in the reproductive age group. Kaneko, et al., in 1998 found a malformation rate of 6.5 for pregnancies managed with monotherapy vs 15.6 for those managed with multiple AEDs.

The study by Lindhout et al., in 1992 and by Kaneko, et al., in 1999 of patients from Japan, Canada, and Italy further supported that view that monotherapy is associated with a better pregnancy outcome. An American study conducted in Boston by Holmes et al., in 2001 found a malformation rate of 20.6% for monotherapy vs 28% for those treated with two or more AEDs. The classic data reflect the fact that most classic AEDs were powerful inducers of CYP 450.

The immediate product of CYP450 reaction is a highly activated intermediate arene oxide. This oxide can react with a protein and form a hapten that elicits an immunological reaction. This interaction occurs with nucleic acids and results in mutagenicity (Sankar, et al., 2007).

Activity of reactive intermediate-scavenging systems such as glutathione reductase, superoxide dissmutase and catalase may play a role in teratogenesis (Sankar, 2007).

Genetic susceptibility to the PHT teratogenicity has been demonstrated in different strains of mice (Hansen and Hodes 1983). Some mice were susceptible to PHT-induced cleft lip/palate, whereas others were relatively resistant. Genetic predisposition is susceptibility of the embryonic heart to the arrhythmogenic effects of PHT is one suggested explanation (Danielsson et al,

1992). Genetic predisposition might also be related to the content and potency of antioxidant enzymes in capturing and detoxifying the harmful ROS.

2.6.6.2.6.2 Genetic polymorphisms of drug transporters

Differences in expression of drug transporters at the placenta may be significant in determining fetal susceptibility to AEDs. P-glycoprotein (Pgp), encoded by multidrug resistance gene (MDR1) is expressed by the placenta syncytiotrophoblast throughout gestation and at term it is localized to the microvillous maternal facing plasma membrane. Studies in Pgp knockout animals have shown that in its absence, fetal concentrations of substrate drugs are much higher than in controls (Smit 1999). In the placenta, multidrug resistant protein 1 (MRP1) is expressed throughout gestation and localized to the fetal blood vessel endothelial cells. Effect of single nucleotide polymorphisms on placental expression in these MDR transporters will contribute to fetal susceptibility. Few studies to date have looked at the effect of SNPs on placental P-gp and multidrug resistant protein 2 (MRP2) expression. Tanabe in 2001 reports reduced expression of MDR1 in individuals expressing the G2677(A,T) and /or T-129C alleles and 3435T allele. Meyer in 2005 demonstrated that the G1249A polymorphism resulted in a significant decrease in placental MRP2 expression in preterm placentas.

2.6.6.2.7 Pharmacogenetics and its influence on AED teratogenicity

Arno Motulsky founded the branch of pharmacogenetics in 1957. Pharmacogenetics is defined as the study of the genetic factors that underlie differences among individuals in their response to drugs. The main aim of pharmacogenetics is to elucidate the mechanism of drug effects and enhance clinical care by identifying moderators of adverse or therapeutic effects. The main challenge is to find the gene variants responsible for the variation in drug response and to understand how they interact with one other and the environment. Thus, by understanding the gene variants, it is possible to adjust the therapy accordingly in each patient.

Pharmacogenetics studies the individual variation in drug metabolism and distribution and it involves the study of a single gene. Pharmacogenomics includes both pharmacogenetics plus variation among individuals in drug targets and disease mechanism. It involves the study of multiple genes.

The observed prominent variability in individual response to pharmacotherapy, in part, depends on well-known factors easily assessable, like: age, sex, weight, liver and renal function, co-medication, heterogeneity in the disease, nutritional state or smoking. Furthermore, inherited variants in drug metabolizing enzymes, transporters, receptors and molecules of signal transduction cascades may have a major impact on drug response.

Genes of pharmacogenetic interest will cover four major categories of responses according to: genes encoding a subcategory of the disease, genes that encode the drug target can be polymorphic, drug efficacy and genes for proteins

involved in drug metabolism, absorption and distribution associated with toxicity.

2.6.6.2.7.1 Genomic variation

Genome variations are differences in the sequence of DNA from one person to the next. Variations are found all throughout the genome, on every one of the 46 human chromosomes. But this variation is by no means distributed evenly. Instead, some parts of the genome are "hot spots" of variability, with hundreds of possible variations of a sequence. Other parts of the genome, meanwhile, don't vary much at all between individuals- they are said to be stable.

There are different kinds of genomic variations and they include:

2.6.6.2.7.1.1 ***Single nucleotide polymorphisms (SNPs)-*** A single-nucleotide polymorphism is a DNA sequence variation occurring when a single nucleotide — A, T, C, or G — in the genome (or other shared sequence) differs between members of a species (or between paired chromosomes in an individual) (Figure 26). SNPs occur when there is a single nucleotide exchange with more than 1% frequency in the population.

SNP are the most common type of genetic variation among people and is the molecular basis for most phenotypic differences between individuals.

Within a population, SNPs can be assigned a minor allele frequency— the lowest allele frequency at a locus that is observed in a particular population. This is simply the lesser of the two allele frequencies for single-nucleotide polymorphisms. There are variations between human populations, so a SNP

allele that is common in one geographical or ethnic group may be much rarer in another.

Single nucleotide polymorphisms may fall within coding sequences of genes, non-coding regions of genes, or in the intergenic regions between genes. SNPs within a coding sequence will not necessarily change the amino acid sequence of the protein that is produced, due to degeneracy of the genetic code. A SNP in which both forms lead to the same polypeptide sequence is termed synonymous (sometimes called a silent mutation) — if a different polypeptide sequence is produced they are *nonsynonymous*. A nonsynonymous change may either be missense or nonsense, where a missense change results in a different amino acid, while a nonsense change results in a premature stop codon. SNPs that are not in protein-coding regions may still have consequences for gene plicing, transcription factor binding, or the sequence of non-coding RNA.

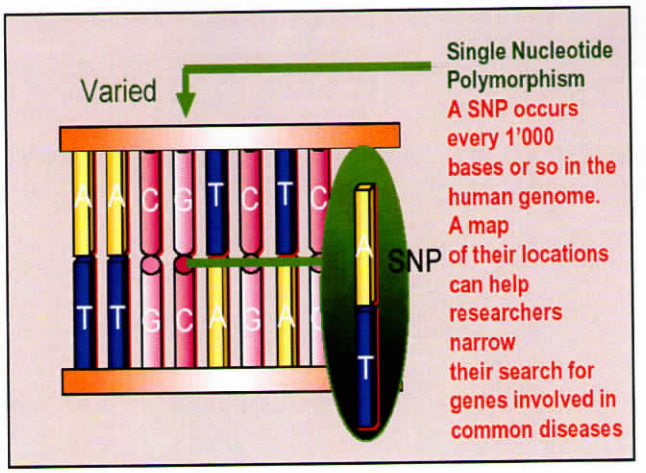


Figure 26- Single nucleotide polymorphism

2.6.6.2.7.1.2 Small insertions / deletions

An insertion mutation is the addition of one or more nucleotide base pairs into a DNA sequence. This can often happen in microsatellite regions due to the DNA polymerase slipping. Insertions can be anywhere in size from one base pair incorrectly inserted into a DNA sequence to a section of one chromosome inserted into another. Insertions can be particularly hazardous if they occur in an exon (Figure 27).

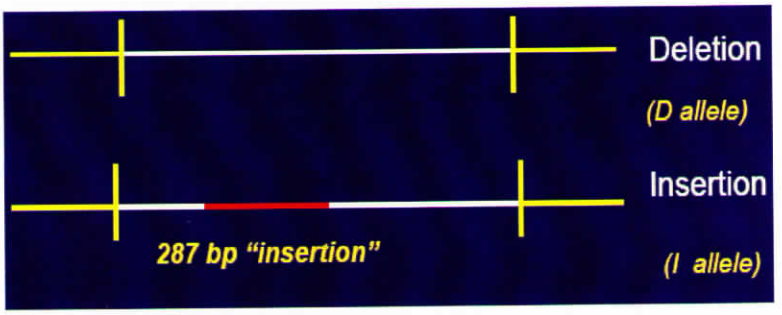


Figure 27- Angiotensin Converting Enzyme Gene D/I polymorphism- Intron 16

A deletion mutation is a mutation in which a part of a chromosome or a sequence of DNA is missing. Deletion is the loss of genetic material. Any number of nucleotides can be deleted, from a single base to an entire piece of chromosome. Deletions can be caused by errors in chromosomal crossover during meiosis.

2.6.6.2.7.1.3 Variable number tandem repeats (VNTR)

A Variable Number Tandem Repeat (VNTR) is a location in a genome where a short nucleotide sequence is organized as a tandem repeat. These can be found on many chromosomes, and often show variations in length between individuals.

*5'-CATGTACC**TATATATATA**GGGCCG-3' TA=5*

*3'-GTACATGG**ATATATATAT**CCCGGC-5'*

*5'-CATGTACC**TATATATA**GGGCCG-3' TA=4*

*3'-GTACATGG**ATATATAT**CCCGGC-5'*

2.6.6.2.7.2 Haplotype

Haplotype is a set of SNPs on a single chromatid that is statistically associated. It is thought that these associations, and the identification of a few alleles of a haplotype block, can unambiguously identify all other polymorphic sites in its region. Haplotype effect is assumed when allelic variants often influence drug response that does not directly lead to an altered protein. Here, the non-functional variant is linked to other variants, exerting functional effects for eg. by influencing regulation of gene expression.

2.6.6.2.7.3 Polymorphisms in drug metabolizing enzymes

Intersubject variability in metabolic rate results in the different drug metabolism phenotypes. Interindividual differences in drug concentration and pharmacodynamic effects have been explained by genetic variants of drug

metabolizing enzymes (DMEs) since 50 years ago. Functional consequences of genetic polymorphisms have been examined for most DMEs.

2.6.6.2.7.3.1 Polymorphic DMEs

CYP450s- CYP2D6, CYP2C9, CYP2C19, CYP3A4- CYP450 enzymes act on endogenous substrates, xenobiotics including plant and fungal products, pollution, chemicals and drugs. They are expressed mainly in liver (Figure 28).

Other enzymes- NAT, FMO, GST, EpH

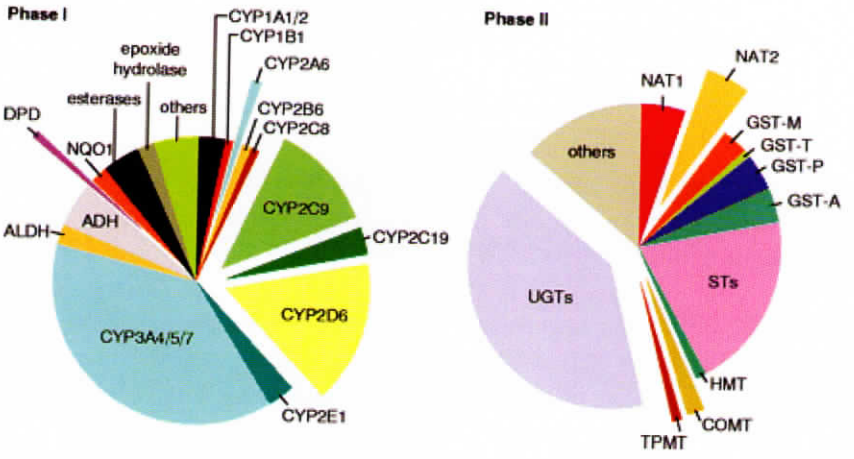


Figure 28- Cytochrome P450 enzymes and other enzymes Most Commonly Involved in Drug Metabolism

2.6.6.2.7.3.1.1 Cytochrome 450 group of enzymes

Cytochrome P450 (abbreviated **CYP**, **P450**, infrequently **CYP450)** is a very large and diverse superfamily of hemoproteins found in all domains of life. Cytochromes P450 use a plethora of both exogenous and endogenous compounds as substrates in enzymatic reactions. Usually they form part of multi-component electron transfer chains, called P450-containing systems.

Human CYPs are primarily membrane-associated proteins, located either in the inner membrane of mitochondria or in the endoplasmic reticulum of cells. CYPs metabolize thousands of endogenous and exogenous compounds. Most CYPs can metabolize multiple substrates, and many can catalyze multiple reactions, which accounts for their central importance in metabolizing the extremely large number of endogenous and exogenous molecules. In the liver, these substrates include drugs and toxic compounds as well as metabolic products such as bilirubin (a breakdown product of hemoglobin). Cytochrome P450 enzymes are present in most other tissues of the body, and play important roles in hormone synthesis and breakdown (including estrogen and testosterone synthesis and metabolism), cholesterol synthesis, and vitamin D metabolism. Hepatic cytochromes P450 are the most widely studied.

All drugs are detoxified and eventually excreted from the body, and many require bioactivation to form the active compound. CYPs are the major enzymes involved in drug metabolism and bioactivation, accounting for 75% of the total metabolism.

Many drugs may increase or decrease the activity of various CYP isozymes by either inducing the biosynthesis of an isozyme (enzyme induction) or by directly inhibiting the activity of the CYP (enzyme inhibition). This is a major source of adverse drug interactions, since changes in CYP enzyme activity may affect the metabolism and clearance of various drugs. For example, if one drug inhibits the CYP-mediated metabolism of another drug, the second drug may accumulate within the body to toxic levels, possibly causing an overdose. Hence, these drug interactions may necessitate dosage adjustments or choosing drugs which do not interact with the CYP system. Such drug interactions are especially important to take into account when using drugs of vital importance to the patient, drugs with important side effects and drugs with small therapeutic windows, but any drug may be subject to an altered plasma concentration due to altered drug metabolism.

A classical example includes anti-epileptic drugs. Phenytoin, for example, induces CYP1A2, CYP2C9, CYP2C19 and CYP3A4. Substrates for the latter may be drugs with critical dosage, like amiodarone or carbamazepine, whose blood plasma concentration may decrease because of enzyme induction.

2.6.6.2.7.3.1.1.1 *CYP2D6 polymorphisms*

CYP2D6 is localised to chromosome 22. CYP2D6 is characterized by a high interindividual variability in catalytic activity caused by genetic polymorphisms (Bertilsson 2002). The presence of two, one or no functional CYP2D6-gene copies results in respectively, extensive metabolisers (EM), intermediate

metabolisers (IM) and poor metabolisers (PM) (Zanger 2001). Inheritance of 3 or more functional alleles by gene amplification determines the ultra-rapid metaboliser (UM) phenotype showing higher than average activity.

2.6.6.2.7.3.1.1.1.1 *Relation to AEDs*

Borlak et al in 1994 studied CYP2D6 polymorphisms and found a significantly greater proportion of those with epilepsy to be poor drug metabolisers compared to normal controls; this explains the CYP2D6 polymorphisms have a susceptibility to epilepsy. Patients with epilepsy not responsive to AEDs may metabolise the drug faster than do responders. A slow metaboliser could potentially be nonresponsive as side effects could limit the use of AEDs.

2.6.6.2.7.3.1.1.2 *CYP2C9 polymorphisms*

CYP2C9 is the most important member of the CYP2C subfamily and is the largest contributor among the 4 isoforms in this subfamily. 40% of Caucasian populations possess partially defective functional forms. More than 100 SNPs are described in the regulatory and coding regions of the CYP2C9 gene have been reported to date. CYP2C9*2 (Arg144/Cys) and CYP2C9*3 (Ile359/Leu) are the 2 most common variant alleles in Caucasian subjects. These variants lead to impaired metabolic activity. These polymorphisms are less prevalent in African Americans and Asians. CYP2C9*5, CYP2C9*6, CYP2C9*8 and CYP2C9*11 were detected at a low frequency in only African-derived populations.

Adithan et al., in 2003 observed that CYP2C9*3 is the most frequent mutant allele found in the Tamilian population. The distribution of this mutant allele in the Tamilian population was found to be lesser than in Caucasians but higher than in Chinese.

CYP2C9*6 contains an adenine base pair deletion at nucleotide 818, which results in a premature stop codon and a truncated inactive protein.

2.6.6.2.7.3.1.1.2.1 ***Relation to AEDs***

Van der et al in 2001 showed in a group of 60 persons with epilepsy on PHT therapy, the mean dose required to achieve a therapeutic serum concentration was 37% lower in those with at least one mutant allele (CYP2C9*3 or *2) than the wild type. Extreme cases of PHT toxicity also have been associated with CYP2C9 gene mutations in the coding region and also in the 5' flanking regions. CYP2C9*4 has been reported in one Japanese patient who had an adverse drug reaction to PHT (Omari 2007). Odani et al in 1997 showed that in a small number of patients with epilepsy heterozygous for the 3 allele, the maximal PHT elimination rate was significantly lower than in wild type but average daily PHT dose was not.

Mamiya in 1998 also showed lower average doses in heterozygous patients (CYP2C9*1*3) than in wild type. Both studies were conducted in Japan, and because the 2C9 polymorphisms are uncommon in Asian populations, the number of individuals with *2 and *3 allele was very small. No patients were found homozygous for the *2 or *3 allele, so the impact of complete absence of

active enzyme was not observed. This suggests that the optimal dosing of PHT might be affected by the 2C9 genotype. In the White population where there is a high frequency of *2 and *3 alleles, 2C9 genotyping could be a useful tool in predicting the appropriate PHT dose.

2.6.6.2.7.3.1.1.3 *CYP2C19 polymorphisms*

CYP2C19 polymorphism has been reported to be caused by point mutations of G to A in exon 5(CYP2C19*2) and G to A in exon 4 (CYP2C19*3). Genetic mutations (CYP2C19*2/*2, 3/3, or 2/3) leads to a truncated and completely inactive enzyme and impairs the metabolism of these drugs. CYP2C19*2, the most common variant allele of CYP2C19, is the result of a single base pair 681G>A mutation on exon 5, leading to an aberrant splice site. This change alters the reading frame of mRNA from amino acid 215, and produces a stop codon 20 bp downstream, leading to a truncated protein. This variant accounted for the poor metabolizer phenotype.

CYP2C19*3, consisting of a 636G>A on exon 4, leads to amino acid change Ile359Leu and confers poor metabolizer phenotype.

2.6.6.2.7.3.1.1.3.1 *Relation to AEDs*

CYP2C19*7 and CYP2C19*8 were isolated by Ibeanu and group in 1999, which contributes to the PM phenotype in Caucasians. CYP2C198 protein exhibited a dramatic reduction in metabolism of S-mephenytoin and

tolbutamide, respectively, when compared with the wild-type CYP2C191B protein.

Variations in PHT metabolism were reported for allelic variants of CYP2C19 in a Japanese population (Watanabe 1998). Mamiya et al in 2000 genotyped CYP2C19 in 784 patients receiving PB. PB total plasma clearance was only 19% less in patients with CYP2C19*2/*2 and *2/*3 than in those with *1/*1. Fraction of PB eliminated by CYP2C9/CYP2C19 dependent oxidation is significantly smaller than for PHT. Approximately 3% and 20% of the Caucasians and Asian population respectively are poor metabolizers; there is no ultrarapid metaboliser phenotype known.

*CYP2C9*2* and **3* mutant alleles caused decreased hydroxylation of phenytoin *in vivo*, whereas the mutant alleles of *CYP2C19* played only a minor role in the metabolism of phenytoin in a study conducted on healthy individuals in South India (Rosemary, et al., 2006).

2.6.6.2.7.3.1.1.4 *CYP3A4 polymorphisms*

CYP3A4 is abundant in the liver and intestine and it accounts for nearly 50% of CYP450. 5 common non-synonymous mutations are noted in the coding region of CYP3A4; CYP3A4*14 (T44C); *15 (G14387A); *10 (G14422C); *16 (C15721G); and *12 (C22002T). Extensive population differences were observed in the frequencies of various CYP3A4 alleles (Lamba 2002).

2.6.6.2.7.3.1.1.4.1 *Relation to AEDs*

CBZ is metabolised by CYP3A4; but there has been no evidence reported of variability in CBZ metabolism due to genotype. Wandel and Sata in 2000 studied that none of the reported genetic polymorphisms in 3A4 has clearly shown to have a clinical phenotype.

2.6.6.2.7.3.1.1.5 *N-acetyl transferase polymorphism*

Of the new AEDs, only zonisamide is eliminated by a polymorphic metabolic pathway, mediated by NAT2. Approximately 50% of Whites and 10% of Asians or blacks are poor acetylators. Only 15% of ZNS is metabolised by NAT2- acetylation pathway and will effect only a fraction of total elimination of ZNS.

2.6.6.2.7.3.1.1.6 *Epoxide hydrolase polymorphism*

Epoxide hydrolase can detoxify epoxides by converting them to dihydrodiols. Data document amino acid variations for epoxide hydrolase; these polymorphisms may alter enzyme function, possibly affecting protein stability (Hassett et al., 1994). Lower concentrations of epoxide hydrolase have been found in women who have children with fetal hydantoin syndrome (Buehler et al., 1990; Raymond et al., 1995). The metabolic conversion of CBZ involves an epoxide. Carbamazepine is acted upon by microsomal epoxide hydrolase (mEH) which is known to exhibit polymorphisms in the human (Hassett 1997). PHT

along with an inhibitor of mEH, showed enhanced incidence of cleft lip and palate (Martz, 1977).

2.6.6.2.7.3.1.1.7 ***Folate pathway genes polymorphisms***

Different genetic polymorphisms mainly in the genes involved in the folate pathway genes could explain why majority of women exposed to VPA produce offspring without NTDs.

In humans, an increased prevalence of C677T mutation in methylene tetrahydrofolate reductase (MTHFR) gene correlates with the incidence of neural tube defects (NTDs) (Botto et al, 1999). A meta-analysis carried out by van der Put in 1997 combined all known Dutch control groups, a total of 1273 individuals and found a prevalence of the 677C-T mutation of 8.4%. The A1298C mutation is also associated with decreased enzymatic activity. Combined heterozygosity for C677T and A1298C mutation might be another genetic risk factor for NTDs (van der Put, 1998).

In 1991, Steegers-Theunissen et al. suggested that maternal hyperhomocysteinemia **was** a risk factor for NTDs. Subsequent studies demonstrated increased tHcy in mothers of children with NTDs even in the absence of low circulating folate, suggesting a direct adverse effect of homocysteine on the developing fetus (van der Put, et al., 2001).

The influence of AEDs and/or common polymorphisms (677C-T, 1298A-C) of the MTHFR gene on the recurrence time of hyper-total-homocysteinemia was investigated in 59 hyper-homocysteinemic patients. Out of the 73% patients

who exhibited hyper- total-homocysteinemia, the greater proportion belonged to the MTHFR677TT/1298AA genotype (Belcastro et al., 2007).

2.6.6.2.7.4 *Polymorphisms in drug transporters*

P-glycoprotein (Pgp) is a well-characterized ABC-transporter of the MDR subfamily. P-gp is also called ABCB1, ATP-binding cassette sub-family B member 1 and MDR1 and is coded by the MDR1 gene. The membrane-associated protein encoded by this gene is a member of the superfamily of ATP-binding cassette (ABC) transporters. Members of the MDR subfamily are involved in multidrug resistance. The protein encoded by this gene is an ATP-dependent drug efflux pump for xenobiotic compounds with broad substrate specificity. Human MDR1 is located on chromosomal region 7q21. It consists of a core promoter region and 29 exons.

Several isoforms of P-glycoprotein have been found in numerous species, including insects, fish, amphibians, reptiles, birds and mammals. Three isoforms of this protein have been identified in rodents: mdr1a, mdr1b and mdr2 and two in humans: Class I-MDR1 and Class II- MDR2 (Ng, et al., 1989).

The role of MDR proteins in the protection against toxic agents is also expressed by their strategic distribution to soecific tissues. They are expressed in important pharmacological barriers, such as the brush border membrane of intestinal cells, the biliary canalicular membrane of hepatocytes, and the luminal membrane in proximal tubules of the kidney. MDR proteins are also present in

the endothelial cell of the brain capillaries and in the epithelial cells in the choroid plexus, both contributing to the blood-brain barrier (BBB).

ABC proteins bind ATP and use that energy to drive out the various molecules across the cell membranes. Most of the known functions involve the shuttling of hydrophobic compounds either within the cell as part of a metabolic process or to outside the cell for transport to other organs of for secretion from the body (Bates, et al., 2001). The major physiological role of multidrug transporters, especially Pgp, is the protection of cells and tissues from xenobiotics (Bodo, et al., 2003).

Pgp transports large hydrophobic, uncharged or slightly positively charged compounds. It has been hypothesized that overexpression of Pgp and other efflux transporters in the cerebrovascular endothelium in the region of the epileptic focus may lead to drug resistance in epilepsy. This hypothesis is supported by several findings. In patients with drug resistant epilepsy there is increased expression of efflux transporters within the epileptic foci. In animal models seizures have been found to induce increased expression of Pgp. Recently it had been shown that some commonly used AEDs are substrates to Pgp (Wang, et al., 2004).

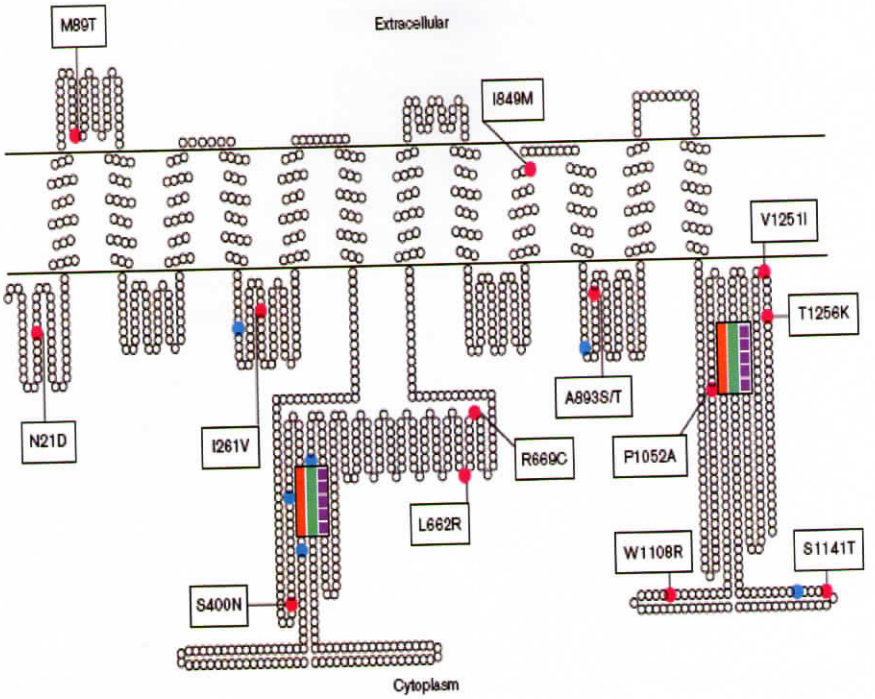


Figure 29- Polymorphic Sites in ABCB1. (Kroetz, D.L., 2003. Sequence diversity and haplotype structure in ABCB1)

One of the most widely recognised SNPs in MDR1 gene is C to T transformation at position 3435 of exon 26 and this polymorphism is synonymous. 65 to 83% of Africans express the TT genotype but only around 25% of Whites do so, Asians lying in between. Individuals with the TT genotype have reduced Pgp activity and higher plasma concentration of drugs after oral administration (Sills, 2006). Although this polymorphism is synonymous, it can result in a defective gene product and thus higher plasma concentration of the drug substrate in haplotypic association with other polymorphisms within the same gene (Figure 29).

2.6.6.2.7.4.1 *Relation to AEDs and epilepsy*

After the potential association between Pgp overexpression in the brain and refractory epilepsy described by Tishler et al. (1995), several reports illustrated high levels of Pgp and MRPs expression in epileptogenic brain specimens from patients with refractory epilepsy. In these studies, Pgp was highly expressed not only in vascular endothelium cells, but also in brain parenchymal cells (D'Giano et al., 1997; Lazarowski et al., 1999; Sisodiya et al., 1999; Dombrowski et al., 2001; Sisodiya et al., 2002).

Hung et al., in 2005 showed that the three loci, C1236T, G2677T and C3435T, jointly influenced the treatment response for epileptic patients. Haplotypic analysis demonstrated that patients with the CGC, TGC, and TTT haplotypes were more likely to be drug resistant.

Seo et al, in 2006 investigated the effect of ABCB1 polymorphisms on AED responsiveness and on the pharmacokinetics of CBZ in epileptic patients with the indication for CBZ therapy. Drug-resistant patients were more likely to have the T allele and the TT genotype at C3435T and the TT genotype at G2677T/A. The frequency of the T-T-T haplotype at C1236T, G2677T/A and C3435T was significantly higher and the CC-GG-CC diplotype was lower in the drug-resistant patients than in the drug-responsive patients.

Higher frequency of the CC genotype and a lower frequency of the TT genotype of C3435T polymorphism among patients with drug resistant epilepsy are compared with those with drug responsive epilepsy (Siddiqui, 2003).

Zimprich et al, in 2004 studied patients with temporal lobe epilepsy and found that a common haplotype (including the 3 SNPs at C1236T, C3435T and G2677T/A) was associated with drug resistance.

The *ABCB1* 3435C→T polymorphism and three-SNP haplotype, plus a comprehensive set of tag SNPs across ABCB1 and adjacent ABCB4, were genotyped in a cohort of 503 epilepsy patients with prospectively measured seizure and drug response outcomes. There was no evidence that *ABCB1* common variation influences either seizure or drug withdrawal outcomes after initiation of antiepileptic drug therapy (Leschziner, et al., 2006).

Many studies have reported a lack of association between MDR1 polymorphisms and response to AED treatment. Sills et al., in 2005 failed to corroborate a previously reported association between the C3435T polymorphism in the human MDR1 gene and pharmacoresistant epilepsy.

An analysis of multiple SNPs in an Irish population showed that there was no original association of drug-resistant epilepsy with C3435T, nor any association with other functional variants at SNP or haplotype level in the ABCB1 gene. (Shahwan et al., 2007). The effect of the C1236T, G2677T/A, and C3435T single-nucleotide polymorphisms of MDR1 on drug resistance was studied in north Indian patients with epilepsy. Genotype and haplotype frequencies of these polymorphisms did not differ between drug-resistant and drug responsive patients (Lakhan et al., 2009). Brain ABCB1 mRNA and protein expression is not substantially influenced by major ABCB1 genetic variants. (Mosyagin et al., 2008).

There was no statistically significant difference between allele and genotype frequencies of refractory and drug responsive epilepsy patients in a South Indian population. The predicted haplotype frequencies of the three polymorphisms (C1236T, G2677T/A, and C3435T) did not show significant difference between cases and controls (Vahab, et al., 2009).

Human MRP family consists of 7 members. Stephen et al in 2001 demonstrated that expression of Pgp1, cMRP/MRP2, MRP5 is elevated in tissue from drug resistant epilepsy patients. Differences in expression at the placenta may be significant in determining fetal susceptibility to AEDs. Few studies to date have looked at the effect of SNPs on placental P-gp and MRP2 expression. Tanabe in 2001 reports reduced expression of MDR1 in individuals expressing the G2677(A,T) and /or T-129C alleles and 3435T allele.

2.6.6.2.7.5 ***Target polymorphisms***

Autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) is related to mutations in the gene for nicotinic acetylcholine (Ach) receptors that leads to loss of some or all receptor activity. Cellular systems with Ach receptors genes with the loss of function mutations responded more strongly to CBZ than did cells with wild type NACR genes. This correlates to the general responsiveness of individuals with ADNFLE to CBZ.

The SCN1A IVS5–91 G>A intronic polymorphism shows significant association with maximum doses of CBZ and PHT. The polymorphism disrupts the consensus sequence of the 5′ splice donor site of a highly conserved

alternative exon and affects the proportions of the alternative transcripts in individuals with epilepsy. This is the first polymorphism to be reported in a drug target associated with the use of an AED (Tate, 2005).

2.6.6.2.7.6 *Asssociation studies*

When a particular genetic variant confers susceptibility to the disease, it would be expected to occur with higher frequency in affected individuals compared to normal individuals. This is referred to as association. Association provides evidence for etiological involvement, thus for therapeutic potential, but it does not indicate, by itself, the relevant therapeutic modulation (i.e. inhibition or activation); used as a diagnostic marker. Determination of whether a candidate gene is truly involved in drug response is typically carried out by association studies. Alleles may be considered to be causative when:

- SNP in the allele generates a STOP codon and disrupts the gene
- SNP in the allele changes the sequence of the encoded protein
- SNP in the allele causes over-expression of the gene
- SNP in the allele causes under-expression of the gene

2.6.6.2.7.7 *SNP genotyping methods*

The increase in interest in SNPs has been reflected by the furious development of a diverse range of SNP genotyping methods.

2.6.6.2.7.7.1 *Hybridization-based methods*

We can interrogate SNPs by hybridizing complementary DNA probes to the SNP site. The challenge of this approach is to reduce cross-hybridization between the allele-specific probes.

2.6.6.2.7.7.1.1 *DASH- Dynamic allele specific hybridisation*

This method takes advantage of the differences in the melting temperature in DNA that results from the instability of mismatched base pairs. A genomic segment is amplified and attached to a bead through a PCR reaction using a biotinylated primer. The amplified product is attached to a streptavidin column and washed with NaOH to remove the unbiotinylated strand. An allele specific oligonucleotide is then added in the presence of a molecule that fluoresces when bound to double stranded DNA. The intensity is then measured as temperature is increased until the melting temperature (Tm) can be determined. An SNP will result in a lower than expected Tm (Howell et al., 1999).

2.6.6.2.7.7.1.2 *Molecular Beacons*

A molecular beacon is a specifically engineered single-stranded oligonucleotide probe. It comprises of complementary regions at each end and a probe sequence located in between the two ends (Figure 30).

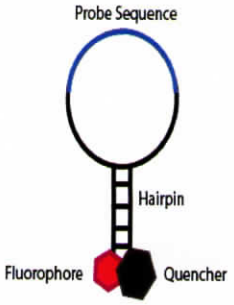


Figure 30- Molecular beacon

If the probe sequence encounters its target genomic DNA during the assay, it will anneal and hybridize (Abravaya et al., 2003). The beacon assumes a hairpin, or stem-loop, structure in its natural, isolated state. A flurophore is attached to one end of the probe and a fluorescence quencher is attached to the other end. If the fluorophore is in close proximity to the quencher, no fluorescence is emitted.

A molecular beacon can be designed to match a wild type allele and another to match a mutant of the allele and the two can be used to identify the genotype of an individual. If only the first probe's fluorophore wavelength is detected during the assay then the individual is homozygous to the wild type. If only the second probe's wavelength is detected then the individual is homozygous to the mutant allele.

2.6.6.2.7.7.1.3 *SNP microarrays*

In high density oligonucleotide SNP arrays, hundreds of thousands of probes are arrayed on a small chip, allowing for many SNPs to be interrogated simultaneously (Rapley & Harbron 2004). Probes used in microarrays are

designed to have the SNP site in several different locations as well as containing mismatches to the SNP allele. Specific homozygous and heterozygous alleles can be determined by comparing the differential amount of hybridization of the target DNA to each of these redundant probes. Approximately, 50,000 SNPs can be genotyped and analysed using microarrays.

2.6.6.2.7.7.2 *Enzyme-based methods*

2.6.6.2.7.7.2.1 *Restriction fragment length polymorphism (RFLP)*

This is the simplest and earliest method to detect SNPs. Restriction endonucleases and their high affinity to unique and specific restriction sites are made use of in the RFLP method. This method involves digestion on a genomic sample and determining fragment lengths through a gel assay. A failure to cut the genomic sample results in a larger than expected fragment implying that there is a mutation at the restriction site.

2.6.6.2.7.7.2.2 *Flap endonuclease- Invader assay*

This endonuclease catalyzes structure-specific cleavage. Cleavage is highly sensitive to mismatches and can be used to interrogate SNPs with a high degree of specificity (Olivier 2005).

The Invader assay involves the use of two specific oligonucleotide probes, together with the target DNA, which can form a tripartite structure recognized by cleavase. The first probe, Invader oligonucleotide is complementary to the 3' end of the target DNA. The last base overlaps the SNP nucleotide in the target

DNA. The second probe contains a base complementary to the SNP nucleotide, but also extends past the 3' side of the SNP nucleotide.

2.6.6.2.7.7.2.3 *Primer extension*

The extension method first involves the hybridization of a probe to the bases immediately upstream of the SNP nucleotide. Then follows a mini-sequencing reaction, in which DNA polymerase extends the hybridized primer by adding a base that is complementary to the SNP nucleotide. This incorporated base is detected and determines the SNP allele (Syvanen, 2001). With incorporation of ddNTPs, probes hybridize to the target DNA immediately upstream of SNP nucleotide, and a single ddNTP complementary to the SNP allele is added to the 3' end of the probe, which stops reaction. Each ddNTP is labeled with a different fluorescent signal allowing for the detection of all four alleles in the same reaction.

2.6.6.2.7.7.3 Other post-amplification methods based on physical properties of DNA

The characteristic DNA properties of melting temperature and single stranded conformation have been used in several applications to distinguish SNP alleles.

2.6.6.2.7.7.3.1 Single strand conformation polymorphism

This method is based on the principle that single stranded DNA (ssDNA) folds into a tertiary structure. Most single base pair mutations will alter the shape of

the structure of DNA. Firstly, the target DNA is PCR amplified. The double stranded PCR products are denatured using heat and formaldehyde to produce ssDNA. This DNA is then applied to a non-denaturing electrophoresis gel and allowed to fold into a tertiary structure. Differences in DNA sequence will alter the tertiary conformation and be detected as a difference in the ssDNA strand mobility (Costabile et al. 2006).

2.6.6.2.7.7.3.2 *Temperature gradient gel electrophoresis*

This method is based on the principle that partially denatured DNA is more restricted and travels slower in a gel. This property allows for the separation of DNA by melting temperature.

Chapter 3

MATERIALS AND METHODS

3.1 *Settings of the study*

The study was carried out at Kerala Registry of Epilepsy and Pregnancy (KREP), Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Trivandrum and at Rajiv Gandhi Centre for Biotechnology (RGCB), Trivandrum. The blood sample collection and clinical profiling of the participants in the study was done at KREP whereas the molecular profiling was carried out at RGCB.

The Kerala Registry of Epilepsy and Pregnancy was set up in 1998 at SCTIMST to examine the diverse reproductive problems encountered in persons with epilepsy, particularly those related to congenital malformations (Thomas, et al. 2001). This registry is first of its kind in India and also in other developing countries.

The two main aims of KREP are: firstly, to provide comprehensive medical services to WWE during preconception period, pregnancy and thereafter; secondly, to collect scientific data in a systematic manner on all aspects of the reproductive function of such persons and evolve a practical guideline that ensures optimal care.

Counseling for patients enrolled in KREP is also carried out to provide precise information on various aspects of epilepsy and pregnancy and helps patients to

make judicious decisions. Clinical and laboratory investigations were carried out at predetermined intervals as laid down in the protocol.

3.2 *Sample collection*

3.2.1 Women with epilepsy- WWE were recruited from KREP and were asked to complete a questionnaire on family history, medical history and life style. Patients were subjected to all routine examination mentioned in the registry. 10ml of blood sample was drawn from women with epilepsy. Patients were divided into one group whose children have malformations and into a second group whose children are normal. The first group will form the cases and the second group will form the controls.

3.2.1.1 ***Clinical Inclusion Criteria*:** Women who have had two or more unprovoked seizures are eligible for inclusion. Women who had completed index pregnancy will be eligible. Classification of epilepsy was followed according to the International League Against Epilepsy (ILAE). Women of age 18 years and above were considered for the study. Malformations in infants born to mothers with epilepsy were stratified according to the fetal outcome.

3.2.1.2 ***Exclusion criteria*:** Women who had seizures due to acute medical conditions were excluded from the study. Women who had febrile convulsions or provoked convulsions were also excluded.

3.2.2 Healthy controls- The healthy controls were selected from volunteers and staff from RGCB. 10ml of blood sample was also drawn from these controls. Informed consent was obtained from all participants.

3.3 *Methods*

All women with epilepsy were personally interviewed to collect details on medical history, family history and life style using a standardised proforma.

3.3.1 Blood sampling

10 ml random blood samples were drawn by venous puncture from all subjects.

3.3.2 Experimental methodology

3.3.2.1 *DNA isolation*

DNA was extracted by the standard organic extraction protocol from all the samples (Sambrook et al, 1989) and subsequently further genotyping procedures are carried out to validate the polymorphisms in MDR1, MTHFR, CYP2C9 and CYP2C19 genes.

3.3.2.1.1 *Reagents and solutions*

Stock solutions

1. *Tris buffer* (1M)

121.14g Tris in 1000ml (ph 8 with conc. HCl)

2. *EDTA* (0.5M)

93.06g EDTA in 500ml (pH 8 with NaOH pellets)

3. *NaCl* (5M)

29.5g NaCl in 500ml

4. 20% *SDS*

Add 20g SDS in 90ml distilled water, heat to 65°C, adjust pH 7.2 with conc. HCl and make upto 100ml.

5. *Tris saturated phenol*- pH 8

6. *Chloroform-isoamyl alcohol* (24:1)

7. 3M Sodium acetate (MW= 82.03)- 408.3g in 1000ml, pH adjusted to 5.2 with dil. Acetic acid

8. *70% Ethanol*

9. *Proteinase K*- 20mg/ml

Working solution- 150µl

10. *RBC lysis buffer- Buffer I*

TEN- 30:5:50mM

1M Tris- 7.5ml

0.5M EDTA- 2.5ml

5M NaCl- 25ml

Distilled water- 250ml

11. *WBC lysis buffer- Buffer II*

NE- 75:2mM

0.5M EDTA- 1ml

5M NaCl- 3.75ml

Distilled water- 250ml

12. *TE Buffer* (10:1mM)

1M Tris- 1ml

0.5M EDTA- 0.2ml

Distilled water- 100ml

13. *TBE buffer* (pH 8)

Tris base- 54g

Boric Acid- 27.5g

0.5M EDTA- 20ml

Distilled water- 100ml

3.3.2.1.2 *Technique*

1. 10ml of blood sample is drawn from the subjects.
2. Equal volume of lysis buffer I is added to the sample.
3. The sample is then maintained at -70°C for one day.
4. The sample is thawed by placing the sample in a water bath at 65°C.
5. After thawing, the sample is centrifuged at 10,000rpm for 10 min at 4°C.
6. The pellet obtained after centrifugation is homogenized with 10ml of lysis buffer II.
7. 1ml SDS (20%) and 75µl of proteinase K (150 µg/ml) is added to the mixture.
8. The mixture is incubated at 37°C overnight.

9. After overnight incubation, equal volume of Tris phenol is added to the mixture.
10. The mixture is shaken gently for 10 min and then centrifuged at 10,000rpm for 10 min at 4°C.
11. 5ml of Tris phenol is added to the aqueous layer after centrifugation.
12. The mixture is shaken gently for 2 min. 5ml of chloroform-isoamyl alcohol to the mixture.
13. The mixture is again shaken gently for 10 min and then centrifuged at 10,000rpm for 10 min at 4°C.
14. Equal volume of chloroform-isoamyl alcohol is added to the aqueous layer after centrifugation.
15. The mixture is shaken gently for 10min and then centrifuged at 10,000rpm at 4°C.
16. The aqueous layer is taken. $1/10^{th}$ volume of 3M sodium acetate (pH 5.2) and double volume of chilled absolute alcohol is added to the aqueous layer.
17. The mixture is shaken gently and the lump of DNA is separated from the solution.
18. The lump of DNA is washed twice with 70% ethanol and once with 100% ethanol (250µl, 5000rpm for 5min at 4°C).
19. The pellet is air dried.
20. After the pellet has dried, it is suspended in TE buffer.
21. The solution is stored in a cool room.

3.3.2.2 *DNA quantification*

The quality and quantity of DNA isolated is analysed in a spectrophotometer (BioSpec-1601, Shimadzu). The ratio of absorbance at 260nm and 280nm (A_{260}/A_{280}) is used to estimate the purity of DNA. A ratio between 1.7-1.9 is considered as good quality DNA without protein contamination. The absorption of 1OD (A_{280}) is equal to approximately 50µl of dsDNA. The concentration of DNA in 1µl of the DNA sample was calculated using following equation:

$$\frac{50 \times A_{260} \times \text{Dilution factor}}{1000}$$

3.3.2.3 *PCR*

The MDR1, MTHFR, CYP2C9 and CYP2C19 genotypes were identified by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analyses. The details regarding the primers and restriction enzymes of MDR1, MTHFR, CYP2C9 and CYP2C19 genotypes are given in **Tables 6, 7, 8 and 9** respectively. PCR was carried out in 10µl reaction mixtures consisting of 50 ng of template DNA, 10 pmols of each primer, 0.2 mM dNTP, PCR buffer containing 2 mM $MgCl_2$ and 0.5 U Taq DNA polymerase. The PCR was carried out in MJ Research Thermal Cycler with heated lid. PCR amplification consisted of an initial denaturation for 5 min at 95^0C, followed by 35 cycles of denaturation at 94^0C for 30s and extension at 72^0C for 30s. The terminal

extension was done at 72^{0}C for 10 min. The annealing temperature for each SNP is mentioned against each SNP in the corresponding tables.

Table 6- Primer sequences used to amplify PCR fragments, including the single nucleotide polymorphisms and restriction enzymes of the MDR1 genotypes

SNP	Primer Sequence	Restriction enzyme	Annealing temperature
Pro T129C	5'- TCAGCATTCAGTCAATCCGG-3' 5'- TTTGCGTGCCCCTACCTC –3'	MspA1I	62.2
Ex02-1G/A	5'- TCTTACTGCTCTCTGGCTTCG-3' 5'- CATTTATTTCAGAGCTGGAGGC-3'	FokI	60.0
Ex07+139C/T	5'-AGGTTTCATTTTGGTGCCTG- 3' 5'-GAACAAAAGGATGCACACGACA-3'	SspI	61.5
Ex 13 C1236T	5'-TACCTGTGTCTGTGAATTGCC-3' 5'-CCTGACTCACCACACCAATG-3'	HaeIII	59.3
Ex 18-76T/A	5'-TTTGTCAACATTTTTTTGAAGC-3' 5'-TATTATTGCAAATGCTGGTTGC-3'	ApoI	69.2
Ex 22 G2677T/A	5'-TGCAGGCTATAGGTTCCAGG-3' 5'- TTTAGTTTGACTCACCTTCCC-3'	BanI	67.9
Ex27 C3435T	5'-TGTTTCAGCTGCTTGATGG-3' 5'-AAGGCATGTATGTTGGCCTC-3'	Sau3AI	59.4

Table 7- Primer sequences used to amplify PCR fragments, including the single nucleotide polymorphisms and restriction enzymes of the MTHFR genotypes

SNP	Primer Sequence	Restriction enzyme	Annealing temperature
C677T	5'- TGAAGGAGAAGGTGTCTGCGGGA3' 5'- AGGACGGTGCGGTGAGAGTGG -3'	HinfI	68
A1298C	5' CTGGGcATGTGGTGGCACTGC 3' 5' CGCAGCCTGGCCTGCAGCTGG 3'	MboII	66

Table 8- Primer sequences used to amplify PCR fragments, including the single nucleotide polymorphisms and restriction enzymes of the CYP2C9 genotypes

SNP	Primer Sequence	Restriction enzyme	Annealing temperature
C432T	5' CACTGGCTGAAAGAGCTAACAGAG 3' 5' GTGATATGGAGTAGGGTCACCCAC 3'	Sau96I	60
A1077T	5' AGGAAGAGATTGAACGTGTGA 3' 5'GGCAGGCTGGTGGGGAGAAGGCCAA3'	StyI	60

Table 9- Primer sequences used to amplify PCR fragments, including the single nucleotide polymorphisms and restriction enzymes of the CYP2C19 genotypes

SNP	Primer Sequence	Restriction enzyme	Annealing temperature
G681A	5′-AATTACAACCAGAGCTTGGC-3′ 5′-TATCACTTTCCATAAAAGCAAG-3′	SmaI	57
G636A	5′ TATTATTATCTGTTAAC TAATATGA3′ 5′ACT TCAGGGCTTGGTCAATA 3′	BamHI	58

3.3.2.4 *Genotyping of MDR1 gene*

The DNA samples were subjected to quality check and quantified spectrophotometrically. The DNA samples were subjected to PCR using the primers designed specifically to amplify the above mentioned SNPs from (Genosys, Sigma). PCR was carried out in 25µl reaction mixtures consisting of 100 ng of template DNA, 5 pmols of each primer, 0.2 mM dNTP, PCR buffer containing 2 mM $MgCl_2$ and 0.5 U Taq DNA polymerase (Finnzyme). The PCR was carried out in MJ Research Thermal Cycler (PTC 200) with heated lid. PCR amplification consisted of an initial denaturation for 5 min at 95^0C, followed by 35 cycles of denaturation at 94^0C for 30s, annealing at 60^0C for 30s and extension at 72^0C for 30s. The terminal extension was done at 72^0C for 10 min. The amplification products were analyzed for SNPs by RFLP in the case of Ex02 -1G/A, Ex06 +139C/T, C1236T, Ex17 -76T/A, G2677A, G2677T and C3435T. The digestion was carried out in 20µl reaction mixtures according to

the manufacturer's protocol (NEB). The digestion products were resolved in 3.5% NuSieve agarose gel (3:1) and visualized by ethidium bromide staining, in a UV transilluminator. The PCR-RFLP results were confirmed by sequencing of the respective PCR products. In the case of SNPs Pro T129C, the PCR products were directly sequenced to detect the polymorphism. Sequencing was done using Big Dye Terminator kit (ver 2.0). and electrophoresed in ABI 3730 Genetic Analyzer. Sequences were analyzed using Genemapper software (Applied Biosystems).

3.3.2.5 Genotyping of MTHFR gene

MTHFR primer specific to C677T (exn 4-int5) were used to amplify 198bp of the genomic DNA containing the polymorphism by PCR. PCR was performed in a 10µl volume with 50ng DNA, 150µm dNTPS, 10pmol of each primer, 1.5mM MgCl2, 1xPCR buffer (NEB), and 1 U Taq polymerase (NEB). Amplification was performed on an automated MJ Thermocycler. PCR condition was 4 minutes for initial denaturation at 95°C, 35 cycles at 95°C, 30s for denaturation, 30s at 64°C for annealing and 45s at 72°C for extension, followed by 7min at 72°C for final extension.

The resulting PCR product was subjected to overnight restriction digestion at using 10U HinfI(NEB). The digested products were resolved at 120V for 30 minutes on a 2% agarose gel containing 0.5 µg/ml ethidium bromide. A 100bp DNA ladder (NEB) was used as a size standard for each gel lane. The gel was visualized under UV light using the Flour S multi imager (Bio-Rad). C allele

was represented by the uncut 198bp PCR product, and T allele consisted of two fragments at 178bp and 23 bp.

MTHFR Primer specific to A1298C (Intrn-7 Intron8) were used to amplify 321bp of the genomic DNA containing the polymorphism by PCR. PCR was performed in a 10µl volume with 50ng DNA, 150µm dNTPS, 10pmol of each primer, 1.5mM MgCl2, 1xPCR buffer (NEB), and 1 U Taq polymerase (Neb). Amplification was performed on an automated MJ Thermocycler. PCR condition was 4 minutes for initial denaturation at 95°C, 35 cycles at 95°C, 30s for denaturation, 30s at 66°C for annealing and 45s at 72°C for extension, followed by 7min at 72°C for final extension.

The resulting PCR product was subjected to overnight restriction digestion at using 10U Mbo I (NEB). The digested products were resolved at 120V for 30 minutes on a 3% agarose gel containing 0.5µg/ml ethidium bromide. A 100bp DNA ladder (NEB) was used as a size standard for each gel lane. The gel was visualized under UV light using the Flour S multi imager (Bio-Rad).A allele was represented by the 184bp, 31bp, 30bp,18bp PCR product, and C allele consisted of 156bp,31bp,30bp,28bp,18bp fragments.

3.3.2.6 Genotyping of CYP2C9 and CYP2C19 gene

All the PCR's were carried out with 50 ng of template DNA, 10 pmols of each primer, 0.2 mM dNTP, PCR buffer containing 2 mM $MgCl_2$ and 0.5 U Taq DNA polymerase. The PCR amplifications consisted of an initial denaturation for 5 min at 95^0C, followed by 35 cycles of denaturation at 94^0C for 30s and extension at 72^0C for 30s. The terminal extension was done at 72^0C for 10 min.

3.3.2.7 *RFLP*

The restriction digestion of the products after PCR amplification was carried out using the corresponding restriction enzymes. The digestion was carried out in 10µl reaction. BSA was included in the digestion mixture wherever necessary. Digestion products were resolved on agarose gels containing 0.5µg/ml EtBr after addition of 5µl of loading buffer consisting of 0.25% Orange G, 30% v/v glycerol and 0.5X TBE buffer. 100bp marker was also loaded. Gels were visualized using UV illumination and documented in a Flour STM multi imager system (BioRad).

3.3.2.8 Statistical analysis

The allelic and genotypic distributions were tested for association between the different groups using Chi Sqaure analysis. A p-value of less than 0.05 was considered to be statistically significant. The deviations from Hardy-Weinberg equilibrium (HWE) for genotype distributions were examined using the Pearson's chi-square test and exact test implemented in the FINETTI program. Haplotypic association of samples was performed using the COCAPHASE program in the UNPHASED v3.011 package with 10,000 permutations. To estimate the linkage disequilibrium (LD) between pairs of loci in the patient and control populations, the standardised disequilibrium coefficient (D') and the squared correlation coefficient (r^2) were calculated using the Haploview 4.1. LD blocks were defined in accordance with Gabriel's criteria (Gabriel, et al., 2002).

Chapter 4

RESULTS

4.1 *Descriptives*

The study group consisted of WWE 244 (Mean age-25 years; SD- 3.9) and 140 healthy controls (Mean age- 29years; SD- 3.5) (Table 10). Among 244 WWE patients (generalized epilepsy = 111, localization related epilepsy = 133), 121 patients had offsprings with congenital malformations. 89 offspring of women with epilepsy had cardiac malformations and 33 had non-cardiac malformations (Table 11). CBZ, PHT, VPA, PB are the most commonly used drugs (Table 12). Among the malformation group, 78 were on monotherapy and 43 on polytherapy. 52 WWE in the malformation group were seizure free during this period. Also, in this group, 90 patients had infrequent seizures (n<=3) during pregnancy and 12 months before pregnancy. Out of the 90 WWE, 20 had one seizure each, 14 had two seizures and 4 had three seizures each. 31 patients had frequent seizures (n>3). 73 patients in the malformation group had no seizures during the pre-pregnancy period whereas 48 had seizures. 72 had no seizures in the pregnancy period while 49 patients had seizures during this period. We made this arbitrary division (3 seizures) in order to divide the cohort in to balanced groups, so that the number of patients with seizures was almost equally divided above and below the three seizure mark.

Table 10- Clinical characteristics of the groups under study

	Non-malformation Group	Malformation Group
No	123	121
Age Mean ± SD	25±3.5	25±3.9
Epilepsy classification		
GE	53	58
LRE	70	63
AED usage		
Monotherapy	80	78
Polytherapy	43	43
Seizure freq Mean	10.6	13.1
No seizure	55	52
<=3	31	38
>3	37	31

Table 11- Number of offspring of patients with epilepsy classified based on type of malformations

Type of malformation	Number of offspring
CVS	**85**
CNS	**7**
GIT	**6**
GUT	**7**
Skeletal	**3**
CLP	**2**
CVS and GIT	**2**
CVS and GUT	**2**
GIT and GUT	**2**
CNS and Skeletal	**1**
Others	**4**

Table 12- Classification of cases based on the drug therapy

Antiepileptic drug	Number of patients
CBZ	33
VPA	28
PB	8
PHT	4
OXB	3
LTG	2
PHT,PB	9
CBZ,CLB	7
CBZ,PB	4
VPA,CLZ	3
CBZ,PRM	2
LTG,VPA	1
CBZ,TPM	1
PB,CLB	1
VPA,TPM	1
VPA,CLB	1
VPA,PB	1
OXB,PB	1
CBZ,PB,CLB	2
PHT,PB,VPA	2
PHT,VPA,CLZ	1
CBZ,CLB,VPA	1
PHT,PB,CLZ	1
CBZ,PB,VPA	1
CBZ,PHT,VPA	1
CBZ,CLZ,PB,VPA	1
VPA,PHT,PB,CBZ	1

4.1.1 MDR1 polymorphisms

With regard to MDR1 gene we have screened one functional polymorphisms (G2677T/A), five non-functional polymorphisms (Ex02 -1G/A, Ex06 +139C/T, C1236T, Ex17 -76T/A and C3435T) and T129C promoter polymorphism in the study population.

The allele and genotype frequencies of the 129T/C, Ex03 -1G/A, Ex07 +139C/T, Ex13 1236C/T, Ex18 -76T/A, Ex22 2677G/T and Ex27 3435C/T

variants were compared between WWE who have offspring with malformations and WWE whose offspring are normal (**Table 13**). The HWE was tested in the latter group and deviations were observed in ProT129C and Ex27 C3435T (**Table 14**). The distribution of alleles and genotypes in most of the SNP locus was similar between the two groups with exception to Ex07 +139C/T. With regard to Ex07 +139C/T SNP, the patients in the malformation group were significantly more likely to have the C allele than the T allele when compared with the persons in the control group. Patients in the malformation group were more likely to have the CC genotype than the TT genotype at Ex07 +139C/T in comparison with the patients with normal offspring ($p = 0.0002$). The C allele of Ex07 +139C/T was also found to be strongly associated with malformation in haplotypic combination with its neighbouring SNPs (**Table 15**). This haplotypic association was observed to extend to four loci haplotypic combination more specifically with Ex 03 -1G/A, Ex 07 +139C/T and Ex13C1236T. The LD plot between the EM and EC groups displayed shifts in the extent of LD between Ex 07 +139C/T, Ex13 1236C/T and Ex 17-76T/A SNPs (**Figure 31**).

Table 13:*MDR1* Allelic and genotypic associations with Malformation and No malformation

Pro T129C		**Malformation**	**No malformation**	**X^2**	**P value**
Genotype Frequency	TT	96.22	92.0	0.06	0.8065
	TC	3.77	8.0		
	CC	0	0		
Allele Frequency	T	97.22	94.44	0.7356	0.3911
	C	2.77	5.55		
Ex 02 -1G/A		**Malformation**	**No malformation**	**X^2**	**P value**
Genotype Frequency	GG	94.33	96.1	0.04	0.8415
	GA	5.66	3.84		
	AA	0	0		
Allele Frequency	G	97.16	98.07	0.1617	0.6876
	A	2.83	1.92		
Ex 07+139C/T		**Malformation**	**No malformation**	**X^2**	**P value**
Genotype Frequency	CC	67.27	19.23	8.94	0.0028
	CT	18.18	50.0		
	TT	14.54	30.76		
Allele Frequency	C	76.36	44.23	18.04	**2.169e-005**
	T	23.63	55.76		
C1236T		**Malformation**	**No malformation**	**X^2**	**P value**
Genotype Frequency	CC	7.84	7.69	0.09	0.7642
	CT	43.13	61.53		
	TT	49.09	30.76		
Allele Frequency	C	29.41	39.29	1.578	0.2091
	T	70.58	60.73		
Ex 17-76T/A		**Malformation**	**No malformation**	**X^2**	**P value**
Genotype Frequency	GG	67.92	73.91	0.08	0.7773
	GA	28.30	21.73		
	AA	3.77	4.34		
Allele Frequency	G	82.07	85.42	0.2686	0.6043
	A	17.92	14.58		
G2677T		**Malformation**	**No malformation**	**X^2**	**P value**
Genotype Frequency	GG	13.79	7.14	0.503	0.7776
	GT	39.65	39.28		
	TT	46.55	53.57		
Allele Frequency	G	34.75	25.0	1.792	0.1807
	T	62.25	75.0		
C3435T		**Malformation**	**No malformation**	**X^2**	**P value**
Genotype Frequency	CC	3.33	7.69	0.06	0.8065
	CT	43.33	42.3		
	TT	53.33	50.0		
Allele Frequency	C	25.0	26.79	0.0636	0.8008
	T	75.0	73.21		

Table 14: Hardy Weinbergs Equilibrium in WWE with normal offspring

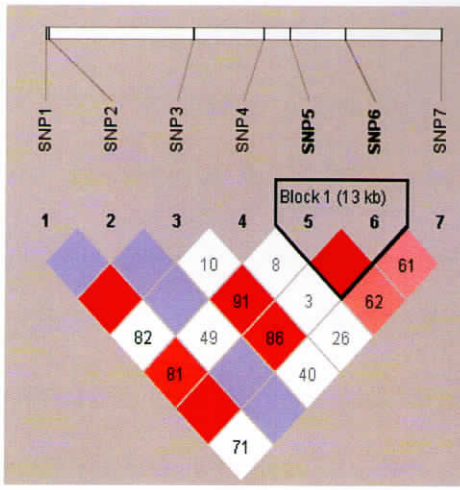
Loci	Genotype	No malformation	HWE
Pro T129C	TT	116 (115.13)	0.012665
	TC	6 (7.74)	
	CC	1 (0.13)	
Ex02-1G/A	GG	113 (113.13)	0.706866
	GA	8 (7.74)	
	AA	0 (0.13)	
Ex 07 +139 C/T	CC	19 (21.15)	0.425243
	CT	64 (59.71)	
	TT	40 (42.15)	
C1236T	CC	16(11.62)	0.062744
	CT	43(51.76)	
	TT	62(57.62)	
Ex17-76T/A	GG	73 (71.48)	0.154493
	GA	40(43.04)	
	AA	8(6.48)	
G2677T	GG	16 (14.34)	0.506024
	GT	52(55.32)	
	TT	55(53.34)	
C3435	CC	4 (10.25)	0.006089
	CT	63(50.51)	
	TT	56(62.25)	

Table 15- *MDR1* Haplotypes of Malformation and WWE with normal offspring

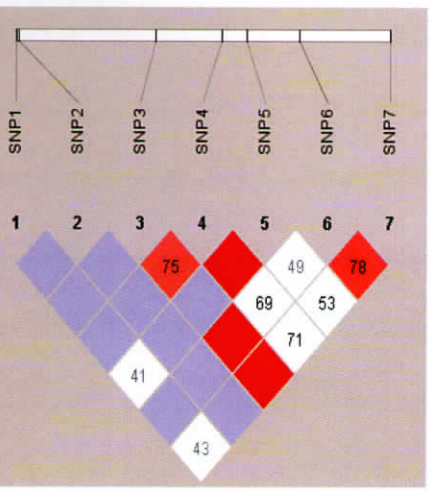
Marker	Haplotype	Malformation	No malformation	P value
Ex 02-1G/A - +139C/T	G-C	0.5006	0.4087	0.049
Ex 02-1G/A - +139C/T – C1236T	G-C-T	0.31	0.114	2.633e-007
T129C - Ex 02-1G/A - +139C/T -C1236T	T-G-C-T	0.308	0.11	1.027e-006

Figure 31- LD PLOT of MDR1 SNPs in (A) **Healthy controls**, (B) **No malformation**, (C) **Malformation(cases)**

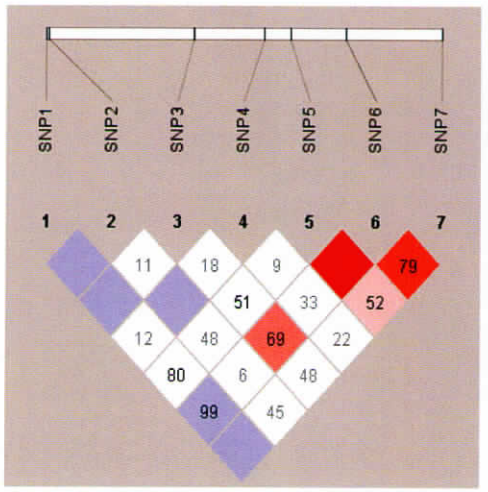
A



B



C



SNP1- **Pro T129C** SNP2- **Ex 02 -1G/A** SNP3- **Ex 06+139C/T**

SNP4- **C1236T** SNP5- **Ex 17-76T/A** SNP6- **G2677T**

SNP7- **C3435T**

The allele and genotype frequencies of the seven MDR1 variants were also compared between WWE who have offspring with malformations and healthy controls (**Table 16**). With regard to Ex18 -76T/A SNP, the patients in the malformation group were significantly more likely to have the T allele than the A allele when compared with the persons in the healthy control group. Patients in the malformation group were more likely to have the TT genotype than the AA genotype at Ex18 -76T/A in comparison with the healthy controls ($p = 0.013$). Also, with regard to G2677T SNP, the patients in the malformation group were significantly more likely to have the T allele than the G allele when compared with the persons in the healthy control group. Patients in the malformation group were more likely to have the TT genotype than the GG genotype at G2677T in comparison with the healthy controls ($p = 0.0067$).

Table 16: *MDR1* Allelic and genotypic associations with Malformation and healthy controls

Pro T129C		Malformation	Healthy controls	X^2	P value
Genotype Frequency	TT	96.22	88.57	0.96	0.3272
	TC	3.77	10.0		
	CC	0	1.42		
Allele Frequency	T	96.43	93.57	246.7	**1.335e-055**
	C	13.571	6.42		
Ex 02 -1G/A		**Malformation**	**Healthy controls**	**X^2**	**P value**
Genotype Frequency	GG	94.33	94.28	0.14	0.7083
	GA	5.66	5.71		
	AA	0	0		
Allele Frequency	G	97.16	97.14	0.001402	0.9701
	A	2.83	2.85		
Ex 07+139C/T		**Malformation**	**Healthy controls**	**X^2**	**P value**
Genotype Frequency	CC	67.27	49.27	0.26	0.6101
	CT	18.18	20.28		
	TT	14.54	30.43		
Allele Frequency	C	74.56	59.42	6.492	0.01084
	T	25.44	40.57		
C1236T		**Malformation**	**Healthy controls**	**X^2**	**P value**
Genotype Frequency	CC	7.84	9.23	0.04	0.8415
	CT	43.13	36.72		
	TT	49.09	53.84		
Allele Frequency	C	30.19	27.69	0.1771	0.6738
	T	69.81	72.30		
Ex 17-76T/A		**Malformation**	**Healthy controls**	**X^2**	**P value**
Genotype Frequency	GG	67.92	52.17	2.36	0.1245
	GA	28.30	43.47		
	AA	3.77	4.34		
Allele Frequency	G	82.07	73.91	2.559	0.1096
	A	17.92	26.08		
G2677T		**Malformation**	**Healthy controls**	**X^2**	**P value**
Genotype Frequency	GG	13.79	21.73	0.04	0.8415
	GT	39.65	43.47		
	TT	46.55	34.78		
Allele Frequency	G	33.62	43.47	2.667	0.1024
	T	66.37	56.52		
C3435T		**Malformation**	**Healthy controls**	**X^2**	**P value**
Genotype Frequency	CC	3.33	0	0.19	0.6629
	CT	43.33	70.76		
	TT	53.33	29.23		
Allele Frequency	C	24.19	35.38	3.814	0.05082
	T	75.81	64.61		

4.1.1.1 *Ascertainment of association between MDR1 SNPs and type of malformations/seizure frequency/treatment pattern*

In the next step we ascertained the association of these SNPs and type of malformation (cardiac vs non cardiac), or seizure frequency(low vs. high frequency) or treatment regimen(monotherapy vs polytherapy) within the malformation group. When the allele and genotype comparisons were made within malformation group for cardio-vascular malformations (n=89) and other malformations (n=32) no significant association was observed with any malformation (**Table 17**). When the relationship of ABCB1 SNPs were monitored with respect to treatment regimen we observed that the distribution of all the alleles and genotypes in the malformation group were similar among the patients with monotherapy (n=78) and with polytherapy (n=43) drug administration (**Table 18**). Thus, there was no association between the drug therapy regimen and drug transporter profile. Subsequently we evaluated the relationship of ABCB1 SNPs with seizure occurrence classified as infrequent seizures (n<=3; i.e n=38) and frequent seizures (n>3; i.e n=31) within the malformation group. In these groups too, we did not observe any significant difference in allele and genotype distribution when compared to the seizures frequency (**Table 19**). However, variations in the pattern of LD could be observed between malformation types, treatment regimen and seizure frequency (**Figure 32, 33 and 34**). These differences in the LD patterns would reflect in genotype association only with larger sample sizes in different malformation types, different treatment regimen and different seizure frequency groups.

Table 17- *MDR1* Allelic and genotypic associations in patients whose offspring have cardiovascular malformations and other types of malformations

Pro T129C		**CVDs**	**Others**	**X2**	**P value**
Genotype Frequency	TT	95.4	93.93	0.77	0.67
	TC	3.44	6.06		
	CC	1.14	0		
Allele Frequency	T	97.72	96.96	0.004	0.71
	C	2.87	3.03		
Ex 02 -1G/A		**CVDs**	**Others**	**X2**	**P value**
Genotype Frequency	GG	91.09	90.9	0.114	0.736
	GA	8.98	9.09		
	AA	0	0		
Allele Frequency	G	95.50	95.45	0.086	0.76
	A	4.49	4.54		
Ex 06+139C/T		**CVDs**	**Others**	**X2**	**P value**
Genotype Frequency	CC	35.95	33.33	0.814	0.665
	CT	28.08	36.36		
	TT	35.95	30.30		
Allele Frequency	C	50.0	51.51	0.044	0.833
	T	50.0	48.48		
C1236T		**CVDs**	**Others**	**X2**	**P value**
Genotype Frequency	CC	10.11	6.06	3.54	0.16
	CT	30.33	48.48		
	TT	59.55	45.48		
Allele Frequency	C	25.28	30.3	0.621	0.43
	T	74.71	69.7		
Ex 17-76T/A		**CVDs**	**Others**	**X2**	**P value**
Genotype Frequency	GG	71.76	63.63	4.712	0.094
	GA	21.17	36.4		
	AA	7.05	0		
Allele Frequency	G	82.35	81.81	0.009	0.92
	A	17.64	18.2		
G2677T		**CVDs**	**Others**	**X2**	**P value**
Genotype Frequency	GG	10.34	9.09	0.98	0.61
	GT	35.63	45.5		
	TT	54.03	45.5		
Allele Frequency	G	28.16	31.81	0.31	0.577
	T	71.83	68.2		
C3435T		**CVDs**	**Others**	**X2**	**P value**
Genotype Frequency	CC	2.24	0	2.26	0.328
	CT	47.19	60.6		
	TT	50.56	39.4		
Allele Frequency	C	25.84	30.3	0.485	0.486
	T	74.15	69.7		

Table 18:*MDR1* Allelic and genotypic associations in patients with polytherapy and monotherapy

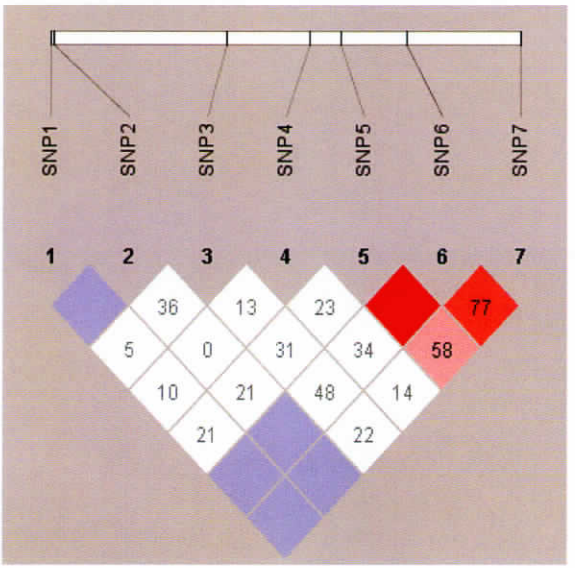
Pro T129C		**Polytherapy**	**Monotherapy**	**X^2**	**P value**
Genotype Frequency	TT	93.75	92.30	0.15	0.6985
	TC	6.25	7.69		
	CC	0	0		
Allele Frequency	T	96.87	96.15	0.03466	0.8523
	C	3.12	3.84		
Ex 02 -1G/A		**Polytherapy**	**Monotherapy**	**X^2**	**P value**
Genotype Frequency	GG	**93.33**	**94.73**	0.21	0.6468
	GA	6.66	5.26		
	AA	0	0		
Allele Frequency	G	96.66	97.36	0.03731	0.8468
	A	3.33	2.63		
Ex 06+139C/T		**Polytherapy**	**Monotherapy**	**X^2**	**P value**
Genotype Frequency	CC	66.66	65.78	0.03	0.8625
	CT	22.22	18.42		
	TT	11.11	15.78		
Allele Frequency	C	77.77	75.0	0.1041	0.747
	T	22.22	25.0		
C1236T		**Polytherapy**	**Monotherapy**	**X^2**	**P value**
Genotype Frequency	CC	5.88	8.33	0.01	0.9203
	CT	70.58	33.56		
	TT	23.52	58.33		
Allele Frequency	C	41.17	25.0	2.794	0.0945
	T	58.83	75.0		
Ex 17-76T/A		**Polytherapy**	**Monotherapy**	**X^2**	**P value**
Genotype Frequency	GG	**70.58**	**66.66**	0.01	0.9203
	GA	23.53	30.55		
	AA	5.82	2.77		
Allele Frequency	G	82.35	81.94	0.002625	0.9591
	A	17.64	18.05		
G2677T		**Polytherapy**	**Monotherapy**	**X^2**	**P value**
Genotype Frequency	GG	15.78	14.63	0.12	0.729
	GT	47.36	34.14		
	TT	36.84	51.21		
Allele Frequency	G	39.47	31.70	0.6885	0.4067
	T	60.52	68.29		
C3435T		**Polytherapy**	**Monotherapy**	**X^2**	**P value**
Genotype Frequency	CC	5.26	2.32	0.77	0.77
	CT	47.36	39.53		
	TT	47.36	58.13		
Allele Frequency	C	28.94	22.09	0.667	0.416
	T	71.05	77.90		

Table 19:*MDR1* Allelic and genotypic associations with Seizures and Non-Seizures (Epileptic controls)

Pro T129C		Seizure	Non-Seizure	X^2	P value
Genotype Frequency	TT	89.47	95.65	0.03	0.8625
	TC	10.52	4.34		
	CC	0	0		
Allele Frequency	T	94.73	97.82	0.5121	0.4742
	C	5.26	2.17		
Ex 02 -1G/A		**Seizure**	**Non-Seizure**	X^2	**P value**
Genotype Frequency	GG	**94.73**	90.9	0.34	0.5598
	GA	5.263	9.09		
	AA	0	0		
Allele Frequency	G	98.39	95.45	0.7933	0.3731
	A	1.61	4.54		
Ex 06+139C/T		**Seizure**	**Non-Seizure**	X^2	**P value**
Genotype Frequency	CC	76.47	54.54	0.23	0.6315
	CT	17.64	27.27		
	TT	5.88	18.18		
Allele Frequency	C	80.88	68.18	2.317	0.128
	T	19.12	31.81		
C1236T		**Seizure**	**Non-Seizure**	X^2	**P value**
Genotype Frequency	CC	10.0	7.69	0.32	0.5716
	CT	30.0	46.15		
	TT	60.0	46.15		
Allele Frequency	C	29.63	30.76	0.0162	0.8983
	T	70.37	69.23		
Ex 17-76T/A		**Seizure**	**Non-Seizure**	X^2	**P value**
Genotype Frequency	GG	64.70	**71.42**	0.3	0.5839
	GA	35.29	19.04		
	AA	0	9.52		
Allele Frequency	G	82.35	80.95	0.05933	0.8076
	A	17.64	19.04		
G2677T		**Seizure**	**Non-Seizure**	X^2	**P value**
Genotype Frequency	GG	11.76	11.53	0.13	0.7184
	GT	47.05	23.07		
	TT	41.17	65.38		
Allele Frequency	G	42.65	23.07	5.139	**0.02339**
	T	57.35	76.92		
C3435T		**Seizure**	**Non-Seizure**	X^2	**P value**
Genotype Frequency	CC	0	7.69	0.8	0.3711
	CT	21.05	30.76		
	TT	79.94	61.53		
Allele Frequency	C	25.0	23.07	0.06107	0.8048
	T	75.0	76.92		

Figure 32- LD PLOT of MDR1 SNPs in Patients with (A) **Monotherapy**, (B) **Polytherapy**

A



B

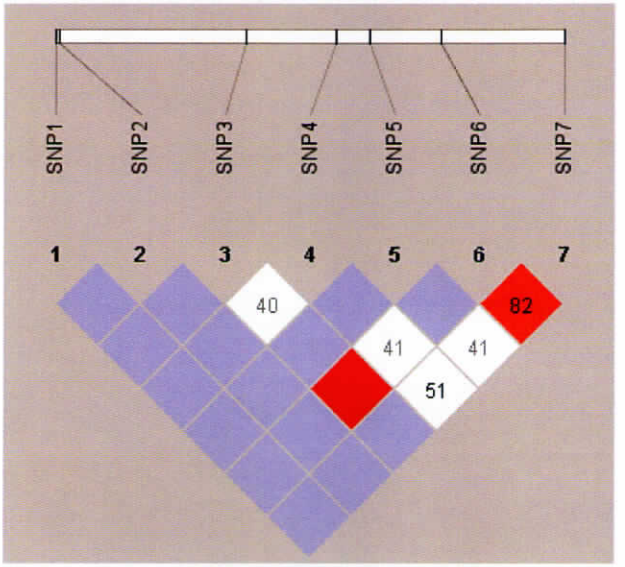
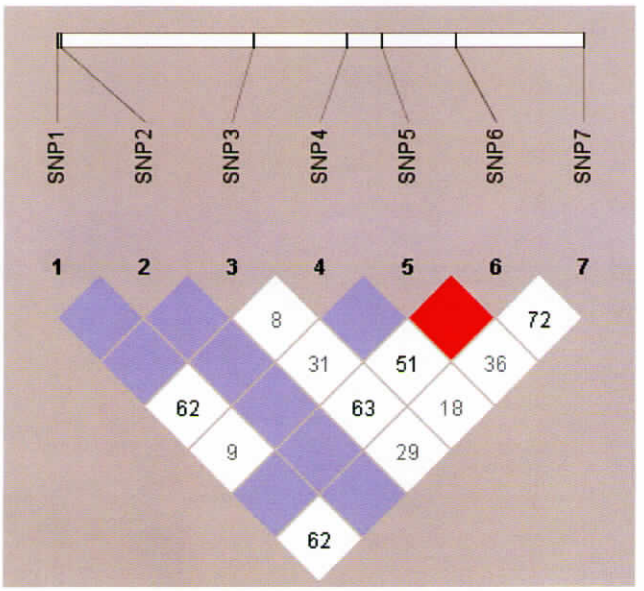


Figure 33- LD PLOT of MDR1 SNPs in Patients with (A) **Seizure**, (B) **Non seizure**

A



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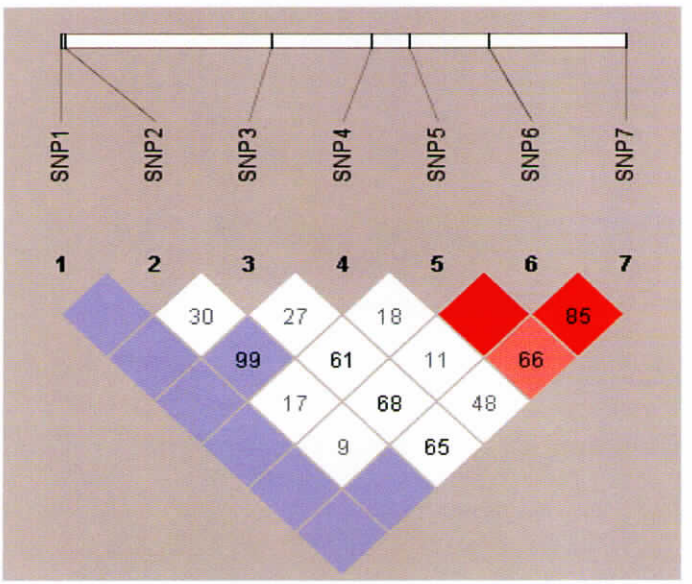
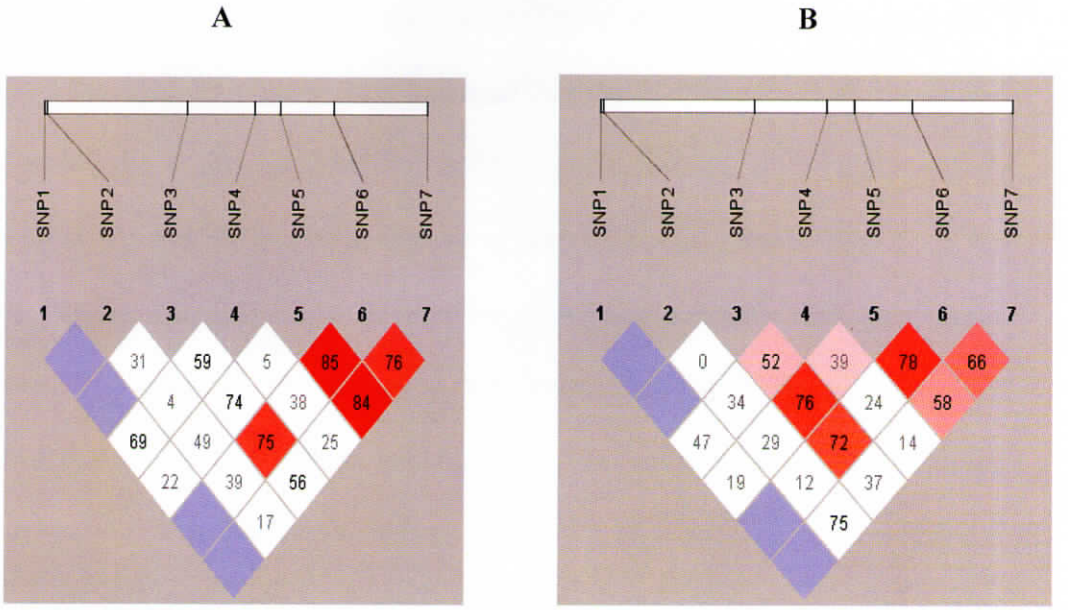


Figure 34- LD PLOT of MDR1 SNPs in Patients with offspring with (A) **Cardiovascular malformations** (B) **Other malformation types**



4.2 *MTHFR polymorphisms*

With regard to MTHFR gene we have screened two polymorphisms, C677T and A1298C in the study population.

The allele and genotype comparisons were made to see if there was any association between the polymorphisms but none of the polymorphisms were observed to have any role in malformations or drug dosage, seizure control and type of malformation. We ascertained the association of these SNPs between the malformation group and normal offspring, type of malformation (cardiac vs non cardiac), or seizure frequency (low vs. high frequency) or treatment regimen (monotherapy vs polytherapy) within the malformation group. There was no significant association between the SNPs and the presence of malformations

(Table 20 and 21). When the allele and genotype comparisons were made within malformation group for cardio-vascular malformations and other malformations no significant association was observed with any malformation **(Table 22)**. When the relationship of MTHFR SNPs were monitored with respect to treatment regimen we observed that the distribution of all the alleles and genotypes in the malformation group were similar among the patients with monotherapy and with polytherapy drug administration **(Table 23)**. Thus the drug therapy was also found to have no association with the drug transporter profile. Subsequently we evaluated the relationship of ABCB1 SNPs with seizure occurrence classified as infrequent seizures (n<=3) and frequent seizures (n>3) within the malformation group. In these groups too, we did not observe any significant difference in allele and genotype distribution when compared to the seizures frequency **(Table 24)**. Variations in the pattern of LD could be observed between malformation types, treatment regimen and seizure frequency **(Figure 35, 36, 37, 38)**.

Table 20- *MTHFR* Allelic and genotypic associations with Malformation and healthy controls

C677T		Malformation	Healthy Control	X^2	P value
Genotype Frequency	CC	85.24	89.23	0.16	0.6892
	CT	14.30	12.5		
	TT	0	0		
Allele Frequency	C	92.62	93.94	0.1758	0.675
	T	7.377	6.061		
A1298C		**Malformation**	**Healthy Control**	**X^2**	**P value**
Genotype Frequency	AA	21.42	15.87	0.04	0.8415
	AC	60.71	47.61		
	CC	17.85	36.50		
Allele Frequency	A	51.79	39.68	3.51	0.06099
	C	48.21	60.32		

Table 21- *MTHFR* Allelic and genotypic associations with Malformation and No-malformation

C677T		Malformation	No malformation	X^2	P value
Genotype Frequency	CC	86.44	92.3	0.267	0.6053
	CT	13.55	7.69		
	TT	0	0		
Allele Frequency	C	93.22	96.15	0.782	0.3765
	T	6.7	3.8		
A1298C		**Malformation**	**No malformation**	**X^2**	**P value**
Genotype Frequency	AA	20.37	10.71	0.99	0.6095
	AC	61.11	60.71		
	CC	18.51	28.57		
Allele Frequency	A	50.92	41.07	1.442	0.2298
	C	49.07	58.92		

Table 22- MTHFR allelic and genotypic associations with Cardio Vascular Defects and other malformations

C677T		*CVDs*	*Others*	*X2*	*P value*
Genotype Frequency	*CC*	*79.77*	*90.9*	*3.523*	*0.171*
	CT	*19.10*	*6.06*		
	TT	*1.12*	*3.03*		
Allele Frequency	*C*	*89.32*	*93.93*	*1.2*	*0.273*
	T	*10.67*	*6.06*		
A1298C		***CVDs***	***Others***	*X2*	*P value*
Genotype Frequency	*AA*	*21.34*	*30.30*	*1.386*	*0.50*
	AC	*49.43*	*48.5*		
	CC	*29.21*	*21.2*		
Allele Frequency	*A*	*46.06*	*54.54*	*1.386*	*0.239*
	C	*53.93*	*45.5*		

Table 23- *MTHFR* Allelic and genotypic associations in patients with polytherapy and monotherapy

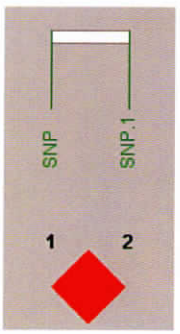
C677T		Polytherapy	Monotherapy	X^2	P value
Genotype Frequency	CC	89	83	0.056	0.8129
	CT	11	17		
	TT	0	0		
Allele Frequency	C	95	92	0.3824	0.5363
	T	5	8		
A1298C		**Polytherapy**	**Monotherapy**	**X^2**	**P value**
Genotype Frequency	AA	26	19	0.214	0.898
	AC	53	65		
	CC	21	16		
Allele Frequency	A	53	51	0.4135	0.5202
	C	47	49		

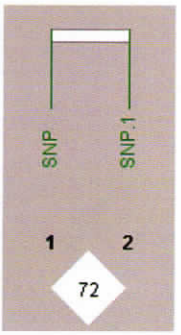
Table 24- *MTHFR* Allelic and genotypic associations in patients with seizures and No seizure

C677T		Seizure	Non Seizure	X^2	P value
Genotype Frequency	CC	91	77	1.476	0.22441
	CT	9	23		
	TT	0	0		
Allele Frequency	C	96	88	2.279	0.1311
	T	4	12		
A1298C		**Seizure**	**Non Seizure**	**X^2**	**P value**
Genotype Frequency	AA	18	27	0.295	0.8628
	AC	62	59		
	CC	21	14		
Allele Frequency	A	49	57	0.736	0.39
	C	51	43		

Figure 35- LD PLOT of MTHFR SNPs in (A) **Malformation** (B) **No malformation** (C) **Healthy controls**

(A) Malformation/Case **(B) No malformation** **(C) Healthy controls**





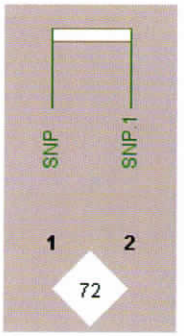


Figure 36- LD PLOT of MTHFR SNPs in patients on (A) **Polytherapy** (B) **Monotherapy**

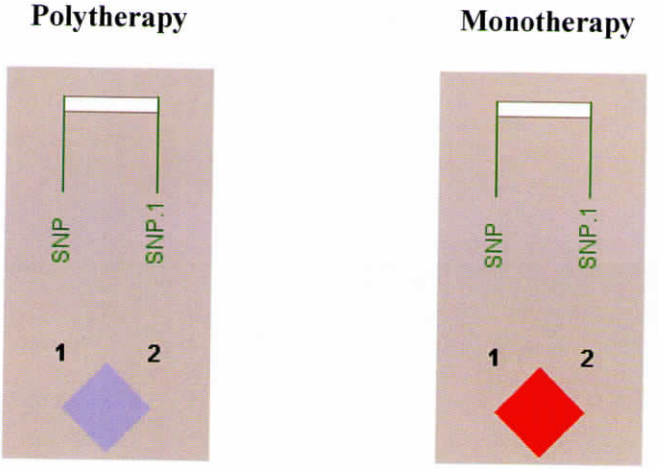


Figure 37- LD PLOT of MTHFR SNPs in patients with (A) **Seizure** (B) **No seizure**

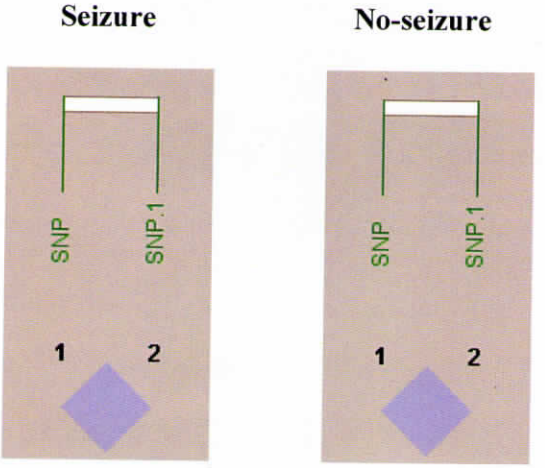
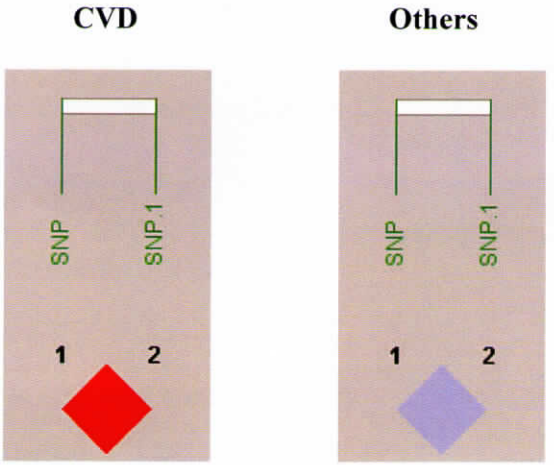


Figure 38- LD PLOT of MTHFR SNPs in patients with offspring with (A) **Cardiovascular malformations** (B) **Other malformation types**



4.3 *Genotyping of CYP2C9 polymorphisms*

CYP2C9*2 is formed by a C430T substitution on exon 3 which leads to Arg144Cys conversion resulting in the formation of an enzyme with decreased activity. CY2PC9*3 allele is due to a C1075T on exon 7 of CYP2C9, which results in an altered protein with an Ile359Leu substitution and exhibits further reduced enzyme activity than the CYP2C9*2 variant. CYP2C9*3 polymorphism, although less frequent in the population, was present in the malformation group. CYP2C9*3 polymorphism was absent in the non-malformation group and healthy controls. (**Tables 25, 26 and 27**).

While converting the Cyp2C9 genotype into their metaboliser phenotype we observe that poor metaboliser genotype is significantly represented in malformation group but interestingly this phenotype is associated with seizure control in malformation group. *2 and *3 are associated with poor metaboliser phenotype. Therefore, the *2 & *3 has been merged to *2. As a result the analysis was done based on *1 and wild type phenotype and *2 &*3 as poor metaboliser phenotype (**Tables 28, 29, 30 and 31**).

Table 25- *CYP2C9* Allelic and genotypic associations with Malformation and no-malformation group

Malformation Case: 121

No malformation group: 123

*1*1	*2*2	*1*2	*1*3	X^2	p-value	*1	*2	*3	X^2	p-value
0.92	0.00	0.60	0.02	4.61	**0.0999**	0.96	0.60	1.00	4.53	**0.1040**
0.97	0.00	1.00	0.00			0.98	1.00	0.00		

Table 26- *CYP2C9* Allelic and genotypic associations with Malformation and healthy controls

Malformation Case: 121

Healthy Controls: 140

*1*1	*2*2	*1*2	*1*3	X^2	p-value	*1	*2	*3	X^2	p-value
0.92	0.00	0.60	0.02	6.37	**0.0415**	0.96	0.60	1.00	6.24	**0.0443**
0.98	0.00	1.00	0.00			0.99	1.00	0.00		

Table 27- *CYP2C9* Allelic and genotypic associations with No malformation group and healthy controls

Epileptic controls: 123

No malformation group: 140

*1*1	*2*2	*1*2	*1*3	X^2	p-value	*1	*2	*3	X^2	p-value
0.97	0.00	1.00	0.00	0.32	**0.5704**	0.98	1.00	0.00	0.32	**0.5734**
0.98	0.00	1.00	0.00			0.99	1.00	0.00		

Table 28- *CYP2C9* Allelic and genotypic associations of Malformation and no-malformation group based on the metaboliser phenotype (*2 and *3 merged to *2 phenotype)

Cyp2C9	*1*1	*2*2	*1*2	p-value	*1	*2	p-value
Malformation	0.89	0.00	0.11		0.95	0.05	
Epilepsy Control	0.96	0.01	0.03	0.05	0.98	0.02	0.10

Table 29- *CYP2C9* Allelic and genotypic associations of polytherapy and montherapy based on the metaboliser phenotype (*2 and *3 merged to *2 phenotype)

Cyp2C9	*1*1	*2*2	*1*2	p-value	*1	*2	p-value
Polytherapy	0.88	0.00	0.12	0.80	0.94	0.06	0.80
Monotherapy	0.90	0.00	0.10		0.95	0.05	

Table 30- *CYP2C9* Allelic and genotypic associations of cardiovascular malformations and other types of malformations based on the metaboliser phenotype (*2 and *3 merged to *2 phenotype)

Cyp2C9	*1*1	*2*2	*1*2	p-value	*1	*2	p-value
CVDs	0.88	0.00	0.12		0.94	0.06	
Others	0.94	0.00	0.06	0.33	0.97	0.03	0.35

Table 31- *CYP2C9* Allelic and genotypic associations of seizure and non-seizure based on the metaboliser phenotype (*2 and *3 merged to *2 phenotype)

Cyp2C9	*1*1	*2*2	*1*2	p-value	*1	*2	p-value
Seizure	0.94	0.00	0.06	**0.04**	0.97	0.03	
No Seizure	0.83	0.00	0.17		0.91	0.09	**0.05**

4.4 *CYP2C19 polymorphisms*

CYP2C19*2 and CY2PC19*3 polymorphisms were analysed in this study. CYP2C19*2 polymorphism was not associated with malformation status or epileptogenesis. CYP2C19*3 polymorphism was not identified among the healthy controls, epileptic controls and malformation group **(Table 32, 33 and 34)**.

While converting the Cyp2C19 genotype into their metaboliser phenotype we observe that there is no significance of poor metaboliser genotype in relation to either the malformation group or the epilepsy control group or when the malformation group was split based on the therapy profile, type of malformations or the seizure/no-seizure group **(Tables 35, 36, 37 and 38)**.

Table 32- *CYP2C19* Allelic and genotypic associations with Malformation and No malformation group

Malformation Case: 121

No malformation group: 123

*1*1	*2*2	*1*2	*1*3	X^2	p-value	*1	*2	*3	X^2	p-value
0.37	0.22	1.00	0.00	1.32	**0.7237**	0.61	1.00	0.00	1.12	**0.5721**
0.37	0.18	0.98	0.01			0.63	0.99	1.00		

Table 33- *CYP2C19* Allelic and genotypic associations with Malformation and healthy controls

Malformation Case: 121

Healthy Controls: 140

*1*1	*2*2	*1*2	*1*3	X^2	p-value	*1	*2	*3	X^2	p-value
0.37	0.22	1.00	0.00	2.07	**0.3561**	0.61	1.00	0.00	0.88	**0.3482**
0.35	0.32	1.00	0.00			0.57	1.00	0.00		

Table 34- *CYP2C19* Allelic and genotypic associations with No malformation group and healthy controls

No malformation group: 123

Healthy Controls: 140

*1*1	*2*2	*1*2	*1*3	X^2	p-value	*1	*2	*3	X^2	p-value
0.37	0.18	0.98	0.01	5.15	**0.1611**	0.63	0.99	1.00	2.81	**0.2451**
0.35	0.32	1.00	0.00			0.57	1.00	0.00		

Table 35- *CYP2C19* Allelic and genotypic associations of malformation and epilepsy control based on the metaboliser phenotype (*2 and *3 merged to *2 phenotype)

Cyp 2C19	*1*1	*2*2	*1*2	p-value	*1	*2	p-value
Malformation	0.36	0.14	0.50	0.31	0.61	0.39	0.20
Epilepsy Control	0.41	0.08	0.50		0.67	0.33	

Table 36- *CYP2C19* Allelic and genotypic associations of polytherapy and monotherapy based on the metaboliser phenotype (*2 and *3 merged to *2 phenotype)

Cyp 2C19	*1*1	*2*2	*1*2	p-value	*1	*2	p-value
Polytherapy	0.44	0.14	0.42	0.31	0.65	0.35	0.34
Monotherapy	0.32	0.14	0.54		0.59	0.41	

Table 37- *CYP2C19* Allelic and genotypic associations of cardiovascular malformations and other malformations based on the metaboliser phenotype (*2 and *3 merged to *2 phenotype)

Cyp 2C19	*1*1	*2*2	*1*2	p-value	*1	*2	p-value
CVDs	0.34	0.15	0.51	0.43	0.59	0.41	0.21
Others	0.45	0.09	0.45		0.68	0.32	

Table 38- *CYP2C19* Allelic and genotypic associations of seizure and non-seizure based on the metaboliser phenotype (*2 and *3 merged to *2 phenotype)

Cyp 2C19	*1*1	*2*2	*1*2	p-value	*1	*2	p-value
Seizure	0.31	0.17	0.51	0.32	0.57	0.43	0.14
No Seizure	0.42	0.10	0.48		0.66	0.34	

Chapter 5

DISCUSSION

5.1 *MDR polymorphisms and teratogenicity*

Several studies have reported malformation risks pertaining to AEDs in pregnancy (Morrow et al., 2006; Atkinson et al., 2007). Nevertheless, there has been little attempt to investigate the underlying pharmacogenetic mechanism for teratogenicity of AEDs. Most antiepileptic drugs are planar lipophilic molecules and are potential substrates for the ATP-binding cassette subfamily B member 1 (ABCB1) transporter (also known as *MDR1* and P glycoprotein 170). At least four AEDs (carbamazepine, phenytoin, lamotrigine and valproate) are known to be transported by *ABCB1* transporter. Current evidence indicates that congenital malformations are attributable to fetal exposure to antiepileptic drugs. Fetal exposure to AEDs may be influenced by drug transporting proteins in the placenta, including P-glycoprotein (P-gp) that control the net transfer of AEDs from maternal to the fetal circulation. Genetic variations in the expression and activity of these transport proteins may influence fetal exposure to AEDs and thus the risk of teratogenicity (Atkinson et al., 2007). Thus, the *ABCB1* is an important candidate gene that can potentially influence the teratogenic effects of antiepileptic drugs. Most of the earlier studies have evaluated the involvement of *ABCB1* gene in refractory epilepsies pertaining to drug resistance in epilepsy. The present study is possibly the first one to evaluate the role of *ABCB1* in assessing the teratogenicity of AEDs in pregnant mothers with epilepsy.

ABCB1 overexpression can result from the effects of disease or drug treatment or from *ABCB1* polymorphisms (Kwan et al., 2005). The most convincing evidence for an association between *ABCB1* genotype and Pgp expression, function and therapeutic drug response came from a prospective study on 3435C/T polymorphism and its role on brain uptake of PB in patients with generalized epilepsy (Basic et al., 2008). CC genotype of C3435T polymorphism was noted to have two-fold higher expression when compared with that in individuals with the TT genotype (Hoffmeyer, et al., 2000). Since C3435T is a silent mutation, the effect of C3435T polymorphism on Pgp-mediated transport may result from linkage to other polymorphic sites in *MDR1* gene affecting amino acid residue or altered conformation. The synonymous 3435C>T polymorphism has been reported to be in linkage disequilibrium with a synonymous SNP in exon 13 (1236C>T) and a nonsynonymous SNP in exon 22 (2677G>TA), suggesting that the observed functional differences in Pgp, initially attributed to the exon 27 synonymous SNP, may be the result of the associated nonsynonymous polymorphism in exon 22, which results in amino acid exchanges (Ala893Ser or Ala893Thr) (Marzolini et al., 2004). As a result majority of the pharmacogenetic evaluation on AED response in epilepsy had been restricted to these three polymorphisms. However, many studies contradicted these observations (Marzolini et al., 2004, Shahwan et al., 2007). Therefore, in our study, in addition to these three polymorphisms we included four additional polymorphisms. In the present study, we analyzed 7 SNPs, which include 3 exonic SNPs, 3 intronic SNPs and 1 promoter SNP. The

2677G/T SNP, which results in Ala 893 Ser amino acid substitution is the only functional polymorphism in this series. The rest of the polymorphisms are either intronic or silent.

In our present study we report a strong allelic, genotypic and haplotypic association of Ex07 +139C/T in the mothers with malformations in their infants. We observed that the patients with infants having malformations were more likely to have the C allele and CC genotype of Ex07 +139C/T when compared to women with normal offspring. C allele of Ex07 +139C/T was also found to be strongly associated with malformation in haplotypic combination with its neighbouring SNPs. This haplotypic association was observed to extend upto four loci haplotypic combination more specifically with Pro 129T/C, Ex 03 -1G/A, Ex 07 +139C/T and Ex13 1236C/T. This indicated that the allelic or genotypic association of Ex07 +139C/T could be indicative of teratogenicity that is independent of the malformation type. These observations are significant as none of the earlier studies have investigated this polymorphism which is upstream to the most widely studied 3435C/T, 2677G/TA and 1236C/T polymorphisms. Ex07 +139C/T is an intronic polymorphism which is in strong haplotypic combination with a synonymous polymorphism at 1236C/T and regulatory region of Ex 03 -1G/A.

Most of the AEDs that are currently in clinical use, are known to induce major congenital malformations such as cardiac malformations, coarctation of aorta, spina bifida and other neural tube defects, hypospadias, cleft lip or palate and limb reduction defects (Morrow et al., 2006). Because of the limited number of

subjects under each category of malformation, we broadly divided the malformations in to cardiac or other malformations. In this series, we could not find any relation between *MDR1* polymorphisms and these two broad categories of malformations (cardiac versus non cardiac malformations). *MDR1* polymorphisms are also known to influence the plasma concentrations of these AEDs and therapeutic response in epileptic patients (Basic et al., 2008, Lazarowski et al., 2004, 2007, Simon et al., 2007). It has been reported that phenytoin and carbamazepine dose requirements were influenced by the genotype in position 3435C/T and 2677G/T of the *ABCB1* gene (Simon et al., 2007). Clinical data indicates that frequency of major malformations is more with polytherapy than with monotherapy (Morrow et al., 2006). Patients often end up on polytherapy when the seizures are difficult to control, although this may not be true in every situation. Earlier, it has been reported that CC genotype of C3435T is correlated with increased seizure frequency (Basic et al., 2008). Nevertheless, we could not identify the association between of *MDR1* polymorphisms and polytherapy or frequent seizures.

This study is possibly one of the first observations that indicate a role of *MDR1* gene with malformation due to AED usage during pregnancy. Our study supports the hypothesis that certain *MDR1* polymorphisms play a potential role in the development of congenital malformations which is independent of the nature of malformations or seizure frequency.

5.2 *MTHFR polymorphisms*

Extensive research has proved the relation between maternal folic acid ingestion and neural tube defects (NTDs) in their offspring. An abnormal pattern of folate metabolism results in a decreased rate of DNA synthesis and gene methylation with deleterious effects on the developing embryo (Finnell et al. 1991). Patients on AED therapy usually develop folate deficiency (Reynolds 1974) and this has been related to impaired development of offspring (Smithells et al. 1976). Methylenetetrahydrofolate reductase (MTHFR) is an important enzyme involved in the folate metabolism pathway in humans, encoded by the *MTHFR* gene. The two most extensively investigated polymorphisms in MTHFR gene namely C677T (rs1801133) and A1298C (rs1801131) has been associated with neural tube defects, is also associated with congenital cardiac malformations.

The allele and genotype comparisons of MTHFR were made to see if there was any association between the polymorphisms but none of the polymorphisms were observed to have any role in malformations or drug dosage, seizure control and type of malformation. Earlier studies have also reported an absence of any association between MTHFR genotype and the risk of malformations. Shotelersuk, et al, in 2003 found no association between the MTHFR polymorphisms C677T/A1298C as a risk factor for cleft lip. Kini, et al., in 2007 studied the *MTHFR* C677T genotype and rate of major malformations (MM) in 187 mother-child pairs where the mothers had epilepsy and in 236 matched control pairs. Kini and colleagues concluded that there was no association between the child's *MTHFR* genotype and the rate of MM but they indicated

that although the maternal *MTHFR* genotype may confer susceptibility to the teratogenic effect of AEDs, particularly VPA, it is likely that the main teratogenic effects are mediated through other mechanisms.

Nurk et al., in 2009 studied whether two polymorphisms in the MTHFR gene (677C→T and 1298A→C) are associated with pregnancy complications, adverse outcomes, and birth defects, as a part of the Hordaland Homocysteine Study. Although the polymorphisms were associated with increased risk of pregnancy complications like placental abruption, no significant associations were found between MTHFR polymorphisms and birth defects.

MTHFR C677T and A1298C polymorphisms are associated with complex congenital malformations, but whether these polymorphisms are associated with CHDs is not clear. Van Driel, et al., in 2008 checked for the association of these polymorphisms with CHDs and found that they are not strong risk factors for CHDs.

5.3 *CYP2C9 and CYP2C19 polymorphisms*

Epilepsy drug-resistance may depend on the metabolism of antiepileptic drugs (AEDs), transport to the epileptic focus and/or target sensitivity. Drug response also depends on multiple characteristics of the patient, the epilepsy, and the antiepileptic drugs used. Responses among patients to antiepileptic drugs (AEDs) may be highly variable, with respect to both drug efficacy and safety. Sanchez MB, et al., in 2010 investigated the association between polymorphisms related to antiepileptic drug metabolism (CYP2C9, CYP2C19,

and UGT), transport (ABCB1), and targets (SCN1A) both in a crude analysis and after adjusting by clinical factors associated with drug-resistance, and stratifying by patient age or aetiology of epilepsy. No significant association between polymorphisms and drug-resistance was found either in the crude analysis or in the adjusted analysis. However, adults with the ABCB1_3435TT or 2677TT genotypes had a lower risk of drug-resistance than those with the CC or the GG genotypes.

Anderson in 2008, has commented that of all the AEDs, only phenytoin undergoes significant metabolism by cytochrome P450 isozymes with significant genetic polymorphisms (CYP2C9, CYP2C19).

Klotz, U in 2007 reviewed the evidence as to whether polymorphic CYP2C9 and CYP2C19 affect the clinical action of AEDs. In the case of mephenytoin, defects in its metabolism may be attributable to >10 mutated alleles (designated as *2, *3 and others) of the gene expressing CYP2C19. Frequencies of poor metabolisers (PMs) and extensive metabolisers (EMs) vary among ethnic populations. CYP2C19 contributes to the metabolism of diazepam and phenytoin, the latter drug also representing a substrate of CYP2C9, with its predominant variants being defined as *2 and *3. There seems to be only a minor contribution of CYP2C19 to the metabolism of phenobarbital. In rare cases, valproic acid can be metabolised to the reactive (hepatotoxic) metabolite, 4-ene-valproic acid. It is not yet clear whether genetic variants of the involved enzyme (CYP2C9) are responsible for this problem.

Tate, SK, et al., in 2005 assessed whether variation in CYP2C9 gene associates with the clinical use of carbamazepine and phenytoin in cohorts of 425 and 281 patients, respectively. As per the study, CYP2C9*3 is highly associated with the maximum dose of the antiepileptic drug, phenytoin (P = 0.0066). This could implicate that persons with epilepsy with CYP2C9*3 are poor metabolisers of phenytoin, when it is administered as a treatment regimen.

Azzato, EM, et al., in 2010 assessed the effect of maternal genotype of functional polymorphisms in two genes involved in phenytoin metabolism, CYP2C9 (R144C, I395L) and EPHX1 (Y113H, H139R), on the presence of major craniofacial abnormalities (CFAs) in the child. They used data from the Collaborative Perinatal Project (1959-1974), a study involving 42 000 mothers and 55 000 children to assess the effect of maternal genotype. 174 pregnancies in 155 women were studied who used phenytoin throughout their pregnancy, gave birth to a live child and had available stored blood specimens suitable for DNA extraction. The maternal EPHX1 Y113/H139 (common) haplotype showed a significant protective association with CFAs in the child (OR: 0.29, 95% CI: 0.12-0.68, P=0.004), when compared to other haplotypes. CYP2C9 genotype was not related to fetal endpoints.

Grover, S, et al., in 2010 studied that functional variants from genes encoding CYP2C19, EPHX1, ABCB1 and SCN1A were highly polymorphic in North Indian epilepsy patients, and might account for differential drug response to first-line antiepileptic drugs.

Kesavan, R. et al., in 2010 showed that CYP2C9 genetic polymorphisms (particularly the *3 allele) were associated with high risk of epileptic patients developing PHT-induced neurological toxicity.

In the present study, CYP2C9*3 polymorphism, although less frequent in the population, was present in the malformation group. CYP2C9*3 polymorphism was absent in the non-malformation group and healthy controls. While converting the Cyp2C9 genotype into their metaboliser phenotype we observed that poor metaboliser genotype (2 & *3 merged to *2) is significantly represented in malformation group but interestingly this phenotype is associated with seizure control in malformation group. This might explain that when AEDs are administered as a treatment regimen and if the persons with epilepsy are poor metabolisers with either the CYP2C9*2 or *3 genotype, the AEDs will not be metabolized normally like the wild type genotype and hence this will lead to the poor seizure control in persons suffering from epilepsy.

CYP2C19*2 polymorphism was not associated with malformation status or epileptogenesis. CYP2C19*3 polymorphism was not identified among the healthy controls, epileptic controls and malformation group.While converting the Cyp2C19 genotype into their metaboliser phenotype, we observed that there is no significance of poor metaboliser genotype in relation to either the malformation group or the epilepsy control group or when the malformation group was split based on the therapy profile, type of malformations or the seizure/no-seizure group.

Chapter 6

SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

In the present study, with regard to the MDR gene, it was observed that a strong allelic, genotypic and haplotypic association of Ex07 +139C/T SNP is present in the mothers with malformations in their infants. We observed that the patients with infants having malformations were more likely to have the C allele and CC genotype of Ex07 +139C/T when compared to women with normal offspring. This C allele is also strongly associated with malformation in haplotypic combination with its neighbouring SNPs, extending upto four loci haplotypic combination more specifically with Pro 129T/C, Ex 03 -1G/A, Ex 07 +139C/T and Ex13 1236C/T. This indicated that the allelic or genotypic association of Ex07 +139C/T could be indicative of teratogenicity, independent of the malformation type. Ex07 +139C/T is in strong haplotypic combination with a synonymous polymorphism at 1236C/T and regulatory region of Ex 03 -1G/A. The functional relevance of these polymorphisms is presently unclear. Future prospects could include the investigation into the role of these polymorphisms with respect to MDR expression.

We could not associate any relation between *MDR1* polymorphisms and the two broad categories of malformations (cardiac versus non cardiac malformations)

and also no association was observed between *MDR1* polymorphisms and polytherapy or frequent seizures.

Novel allelic, genotypic and haplotypic association with Ex +139C/T MDR polymorphism opens up a crucial role of *MDR1* which needs to be investigated further in identifying the role of *MDR1* with AED response as this polymorphism is upstream to most of the earlier reported *MDR1* associations.

None of the polymorphisms of MTHFR were observed to have any role in malformations or drug dosage, seizure control and type of malformation.

The poor metaboliser genotype of CYP2C9 was significantly represented in malformation group and interestingly this phenotype is associated with seizure control in malformation group. Further studies are required to ascertain how CYP2C9*2 and *3 affect the AED metabolism and hence, the seizure control.

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