Antiepileptic Drug Teratogenicity
-A Human and Laboratory Translational Study

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Abstract

Antiepileptic drug (AED) associated teratogenicity has been well documented in the literature. The risk of physical birth defects during the first trimester of pregnancy is increased threefold for most AEDs and over ten-fold for the most teratogenic AED, valproate. Despite this risk, women require long term treatment to stop or reduce the occurrence of seizures and the consequent harm to both mother and foetus, including the possibility of sudden unexpected death in epilepsy. The mechanism resulting in this teratogenicity, and in particular why some women are more susceptible to have children with AED induced birth defects is incompletely elucidated. In recent years there has been emerging evidence that AEDs may be interacting with genomic factors to result in birth defects. These genomic factors may be susceptibility alleles in the mother or father, de novo mutations in the child or epigenetic factors such as alterations in DNA methylation in the mother or child. The studies reported in this thesis aim to a) develop an animal model of valproate induced defects that closely mimics a human clinical setting and can be used to better understand the pathogenesis of AED induced defects, and b) identify genomic markers of AED induced defects using whole genome analysis of human samples and determining if having epilepsy is a contributing factor to the onset of these defects. For aim a) the development of the animal model entailed using an epileptic strain of rats, Genetic Absence Epilepsy Rats from Strasbourg, determining a dose at which dietary valproate is therapeutic, mating the rats and conducting a morphological assessment of both internal and external defects. For aim b) human samples were collected and subjected to whole genome analysis, including whole exome sequencing and DNA methylation scans. Additionally, birth defect rates for non-epileptic women in the Australian Pregnancy Register were also separately quantified. The human samples for investigations were collected from participants and their families enrolled in the Register. Using both human and animal models this study aimed to generate new knowledge, which could ultimately lead to a pharmacogenomic approach to the selection of AEDs for women who wish to become pregnant. This would allow women to make more informed decisions, reduce the risk of having a baby with a birth defect and potentially assist in the formation of new AEDs with lower teratogenic risk.
Declaration

I, Dana Jazayeri hereby declare that:

i) The thesis comprises only my original work towards the Doctor of Philosophy except where indicated in the accompanying Preface

ii) Due acknowledgement has been made in the text to all other materials used

iii) The thesis is fewer than 100 000 words in length exclusive of tables, maps, bibliographies and appendices.

Signature: 

Dana Jazayeri

Date: 7th May 2018
Preface

This dissertation was carried out at the Department of Medicine and Neuroscience, Royal Melbourne Hospital between July 2014 and May 2018 with the support of a scholarship from Prof. Terence O’Brien under the following NH&MRC program grants: #1059858 and #APP1091593. All human studies were undertaken under the supervision of: Prof. Terence O’Brien, Prof. Frank Vajda, Prof Patrick Kwan, Dr. Marian Todaro and Dr. Slave Petrovski. Animal studies were under the supervision of Assoc/Prof. Nigel Jones and Prof. Terence O’Brien. Assistance from additional people has been acknowledged below.

The animal work for this project was conducted at the Department of Medicine, Royal Melbourne Hospital, University of Melbourne Biological Research Facility. Genetic Absence Epilepsy Rats from Strasbourg (GAERs) rats and Non-Epileptic Controls (NECs) were obtained from The University of Melbourne Biological Research Facility and Wistar rats from the Animal Resource Center (ARC) in W.A. Valproate mixed diet was produced by Specialty Feeds, Glen Forrest, W.A. My percentage contribution for Chapter 3 was 70% with the assistance of Emma Braine involved in the EEG surgeries and tail vein blood samples and Andrew Naughton with the cardiac blood samples. My contribution to Chapter 4 was 90% in collaboration with Dr. Stuart McDonald who guided the internal spinal measurements at La Trobe University, provided the chemicals and microscope and assisted and changing solution. In addition, the University of Melbourne histology facility (Laura Leone and Tina Cardamone) assisted with the full pup histopathological analysis and H&E sectioning and staining. The valproate blood levels for both Chapters 3 and 4 were analysed at Melbourne Pathology, Royal Melbourne Hospital by Maria Bisignano.

Recruitment of participants for the human studies was done via The Australian Pregnancy Register of Antiepileptic Medication. The register was established in 1999 by Prof. Frank Vajda in collaboration with Prof. Terence O’Brien. The Australian Pregnancy Register is supported by the Epilepsy Society of Australia, The Royal Melbourne Hospital Neuroscience Foundation, Epilepsy Action, The National Health and Medical Research Council and the Pharmaceutical Industry, viz. Sci-Gen, UCB Pharma, Eisai, Genzyme and Sanofi. My contribution to Chapter 6 was 60% with the assistance of Alison Hitchcock, Janet Graham, Lucy Vivash involved in the recruitment process and Dr. Alison Anderson and Dr. Slave Petrovski with the sequencing analysis. Additionally, the students who started this project
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i) Genome wide association studies were conducted at: deCode genetics, Reykjavik Iceland with the assistance of Dr. Roland Krause.

ii) Whole exome sequencing was conducted at the Institute for Genomic Medicine, Columbia University, NY, USA

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Abbreviations

AED- antiepileptic drug
APR- Australian Pregnancy Register of Antiepileptic medication
AZ- acetazolamide
BD- birth defect
BPD- bipolar disorder
CBZ- carbamazepine
CLZ- clobazam
CM - congenital malformation
DNA- deoxyribonucleic acid
EURAP- European Pregnancy Register
FBM: felbamate
FHS- fetal hydantoin syndrome
FVS- fetal valproate syndrome
GAERS: genetic absence epilepsy rats from Strasbourg
GBP- gabapentin
GWAS- genome wide association studies
HDAC- histone deacetylase
ILAE- International League Against Epilepsy
LAC: lacosamide
LTG- lamotrigine
LEV- levetiracetam
MS- multiple sclerosis
NECs: non-epileptic controls
PB- phenobarbital
PG- pregabalin
PHT- phenytoin
RNA- ribonucleic acid
ROS- reactive oxygen species
SB- spina bifida
SNP- single nucleotide polymorphism
TGB: tiagabine
TPM- topiramate
VGB: vigabatrin
VPA- valproate
WES- whole exome sequencing
WWE: women with epilepsy
ZNS: zonisamide
Chapter 1

Introduction and Literature review

1.1 Epilepsy Overview

Epilepsy is a neurological disorder characterized by recurrent paroxysmal, self-limiting abnormal electrical activity in the brain. It was one of the first brain disorders to be detailed with the first records dating back as far as 3000 years ago (Magiorkinis, Sidiropoulou, & Diamantis, 2010; Russo, 2017). For many centuries the disorder was misunderstood with society viewing it as occurring due to “evil” forces. Treatments for the condition consequently were focused on supernatural or religious methods (Vatanoglu-Lutz, Ataman, & BİÇEr, 2016). It was not until the 18th and 19th centuries that the nature of epilepsy was beginning to be understood, a more scientific approach was taken and the first antiepileptic drugs (AEDs) were developed.

Epilepsy is the most common neurological disorder. It affects approximately 50 million people worldwide (Brodie et al., 1997). It is a condition which has been repeatedly classified into syndromes. These syndromes are defined by specific clinical features such as: type of seizure, age of seizure onset and part of the brain involved, and provide a way to help determine the cause and optimal modes of therapy (Engel, 2006). The principal manifestations of the condition of epilepsy and all syndromes are seizures. The three main classifications of seizures as per the International League Against Epilepsy 2017 are: generalized, focal and unknown onset (Fisher et al., 2017). Generalized seizures affect the whole brain and can be either motor or non-motor. Motor seizures are either tonic (muscles stiffening), clonic (muscles jerking) or tonic-clonic (muscles stiffening then jerking). Non-motor seizures are: absence, myoclonic or atonic.

Focal seizures only affect a portion of the brain in one particular area and are classified as focal-aware or focal-impaired aware. In addition to being generalized or focal some seizures are classified as “unknown onset” because they do not fit into either category. There are currently no truly curative or anti-epileptogenic drugs available and AED’s, which ablate seizures, but do not cure epilepsy therefore remain the most effective treatment for control of epileptic seizures.
1.2 Epilepsy Treatment

Antiepileptic drugs (AEDs) have been widely used since the early 19th century for treating epileptic seizures. The very first report was in 1812 when potassium bromide was found to treat seizures for “hysterical epilepsy connected with the menstrual period” in women (Brodie, 2010). Following this in 1912, phenobarbital (PB) was developed as an AED by chance after being administered to patients with tonic clonic seizures as a hypnotic and found to be effective (Hauptmann, 1912). Since then further research has produced numerous AEDs. The next major discovery on the basis of screening many compounds for anti-epileptic activity yielded phenytoin in 1940 (Merritt & Putnam, 1938) followed by the discovery of a number of drugs in 1950-1990 which are referred to as traditional AEDs. These included: troxidone in the 1940’s, ethosuximide in 1958 and carbamazepine (CBZ), valproate (VPA) and the benzodiazepines in the 1960’s (Brodie, 2010). At the end of the 1990’s a second generation of new drugs were developed for medical therapy most of which are available in Australia. These were: vigabatrin (VGB) and zonisamide (ZNS) in 1989, felbamate (FBM) and gabapentin (GBP) in 1993, lamotrigine (LTG) in 1994, topiramate (TPM) in 1996, tiagabine (TGB) in 1997, levetiracetam (LEV) in 1999, oxcarbazepine in 2000, pregabalain (PGB) in 2005, rufinamide (RFM) in 2007, lacosamide (LAC) in 2008 and eslicarbazepine acetate in 2009 (Arzimanoglou et al., 2010).

About 60-70% of patients have control of their seizures with AEDs (Kwan & Brodie, 2000). The reason for this is that patients can have a broad range of epilepsy types based on their seizure aetiology. The underlying cause of epilepsy varies according to the syndrome, which in turn may have a variety of largely genetic but also some environmental causes. The genetic abnormality may differ as to the chromosome where it is located, or a single or multiple genetic abnormality of different types. These variable aetiologies consequently require a variety of treatment types.

1.3 Antiepileptic drug use for non-epilepsy indications

Since the 1950’s, AEDs have been increasingly used to treat mood disorders. They are particularly-effective for bipolar disorder, VPA even becoming first line treatment (Lerer, Moore, Meyendorff, Cho, & Gershon, 1987). Most AEDs have more than one mechanism of action and therefore different therapeutic targets (Johannessen Landmark, 2008). The common pathophysiology between the diseases using AEDs for treatment is the modulation of neuronal excitability primarily in the central and peripheral nervous system and striated muscle (Johannessen Landmark, 2008). This neuronal excitability is modulated by
alterations in ion channels, receptors and intracellular pathways particularly those involved with and GABAergic and glutamatergic synapses.

The efficacy of AEDs in treating mood disorders such as bipolar disorder lead onto the research of AEDs for treatment of psychiatric conditions such as anxiety (Van Ameringen, Mancini, Pipe, & Bennett, 2004). Anxiety disorders can be categorised into many subcategories including generalised anxiety disorder, acute stress disorder, social phobia, post-traumatic stress disorder (PTSD), panic disorder, obsessive compulsive disorder (OCD) as well as mixed anxiety conditions ("Types of anxiety disorders," 2011). Anxiety disorders have been associated with abnormalities in the GABA and glutamate systems (similar to that seen in epileptic seizures) resulting in over excitability of the brain.

Non-epilepsy neurological conditions that are treated by AEDs include neuropathic pain, multiple sclerosis and migraine. Neuropathic pain is the result of disease or damage to the peripheral nervous system. It does not respond well to therapies commonly used to treat pain as well as to standard doses (Tremont-Lukats, Megeff, & Backonja, 2000). It occurs as a result of increased hyper excitability in either central or peripheral areas that are damaged (Tremont-Lukats et al., 2000). This hyper-excitability is again caused by modulation in sodium channels, receptors and GABAergic and glutamatergic systems, similar to what is seen is epileptic seizures (Spina & Perugi, 2004). Neuropathic pain is also a common symptom of patients with multiple sclerosis (MS) (Beiske, Holmoy, Beiske, Johannessen, & Johannessen Landmark, 2015). Multiple sclerosis (MS) is a progressive disease caused by damage to the myelin sheath of cells in the central nervous system. Women are 2-3 times more likely to develop MS than men and more than half of patients with MS develop their symptoms during their child bearing years (Buraga & Popovici, 2014). AEDs are also used to treat seizures in MS (Anderson & Rodriguez, 2011).

In recent years, there has been increasing speculation that neuronal hyper-excitability and alteration in GABA levels may also contribute to the onset of migraine (Spina & Perugi, 2004). Because of this, new agents such as AEDs have been assessed for their potential pharmacological therapy and began to be used in the 1980’s-2000’s (Bagnato & Good, 2016a). In addition to the conditions mentioned, AEDs are promising in the treatment of other conditions including Parkinson's disease, neonatal cerebral haemorrhage, myotonia, spasticity and amyotrophic lateral sclerosis (Beghi, 1999).
1.4 Antiepileptic drug teratogenicity

The first reports of AED induced birth defects were in the 1960’s and 1970’s (Speidel & Meadow, 1972) not long after the thalidomide catastrophe. It has since become well established that exposure to AEDs during the first trimester of pregnancy increases the risk of birth defects significantly, especially for the most widely documented teratogen VPA. It is most frequently impossible to cease taking the drug during this time due to the likely recurrence of seizures and consequent risk of harm to both the mother and the foetus. Although there are a number of different AEDs with different pharmacological properties for the treatment of different seizure type, there are no known AEDs that are established to be definitely non-teratogenic. Further research is required to establish the teratogenicity of the newer or second generation AEDs.

1.4.1 Types of major physical malformations

Women taking AEDs during the first trimester of pregnancy have an increased risk of having a child with: major physical malformations (MCM), cognitive deficits and growth delay (Kaneko et al., 1992; Lindhout & Schmidt, 1986). These malformations are dose related (Tomson et al., 2011; Vajda, 2012). AED associated malformations are not drug specific and can be present in numerous forms. MCM’s can be divided into body systems in humans, such as; cardiac, neurological, skeletal, renal and other. Cardiac anomalies associated with AED-induced teratogenicity include: atrial septal defect, ventricular septal defect, Tetralogy of Fallot, patent ductus arterioles, pulmonary stenosis and coarctation of the aorta (Pennell, 2008). These are the most common AED induced malformations followed by urogenital defects and cleft palate (D. S. Hill, Wlodarczyk, Palacios, & Finnell, 2010). Urogenital defects include glandular hypospadias and renal anomalies. Neural tube defects, associated particularly with VPA (discussed in section 1.4.6) include spina bifida of various degrees of severity and anencephaly (Pennell, 2008). There are two main types of neural tube defects (NTDs) cranial NTDs and spinal NTDs. Cranial NTDs include exencephaly/anencephaly a condition in which the neuroepithelium is not enclosed by the skull and is exposed from the developing brain. Spinal NTDs include: spina bifida occulta (incorrect formation of vertebrae under the skin), meningomyelocele (protrusion of meninges from spinal opening) and myelomeningocele (spinal cord is visible in the spinal opening and there is partial or complete paralysis below).
1.4.2 Neurocognitive impacts

In addition to major physical malformations, AEDs have been found to have an impact on neurocognitive function with reports since as early as the early 1970’s (Hanson, Myrianthopoulos, Harvey, & Smith, 1976; R. M. Hill, Verniaud, Horning, McCulley, & Morgan, 1974). Early studies found that children exposed to certain AEDs while in utero scored lower on psychological tests (Losche, Steinhausen, Koch, & Helge, 1994). More recently, Meador et al. (2009)- (2013) assessed neuro-cognition in a cohort of children at both 3 years and 6 years. There was a significant dose dependent reduction in IQ scores of children exposed to VPA as compared with other AEDs at 3 years of age. The next lowest was CBZ followed by PHT and then LTG. At 6 years, the results also showed reduced IQ scores in the children that had been exposed to VPA particularly in verbal and non-verbal ability, memory and executive function. Again, this was dose dependent (Meador et al., 2013). Further, Adab et al. (2004) assessed cognitive function of children over the age of 6 exposed to VPA prenatally and found IQ to also be significantly lower in VPA exposed group. Similar findings have been reported by a number of studies including the Australian Pregnancy Register (Nadebaum et al., 2011), as well as a recent study in a Georgia (Kasradze et al., 2017). A consistent finding across many studies is the effect of VPA on verbal IQ and language (Bromley, Baker, & Meador, 2009). VPA’s affect on cognition is also dose dependant (Meador et al., 2009).

Most recently there has also been an association reported between VPA exposure and autism (Rasalam et al., 2005; Williams et al., 2001). One of the largest studies showing this examined 655 615 children which comprised of 508 children that had been exposed to VPA in utero. VPA during pregnancy resulted in a significant increased risk of autism spectrum disorder and childhood autism in children (Christensen et al., 2013). The Australian Pregnancy Register also found that children exposed to AEDs had an increased Childhood Autism Rating Scale (CARS) with a particular association between high VPA doses and autism (Wood et al., 2015).

It should be noted that there are some discrepancies in results of studies looking at developmental delay and this is most likely due to confounding factors such as seizures during pregnancy, mothers socioeconomic status, intelligence, medical complications during pregnancy (Nicolai, Vles, & Aldenkamp, 2008). Mothers with a lower socioeconomic status, for example, are less likely to take folate (Relton, Hammal, Rankin, & Parker, 2005), more likely to drink and smoke (Jacobson, Chiodo, Sokol, & Jacobson, 2002) and may be
bias towards certain AEDs based on their price (Tettenborn, 2006) all of which may influence their pregnancy outcome. In addition, mothers who take VPA on average have a lower full scale, verbal and performance IQ as well as a lower education level (Eriksson et al., 2005). These factors can be difficult to separate from the affect of the AEDs.

1.4.3 Interaction of epilepsy and AEDs

Antiepileptic drug associated birth defects have been reported from retrospective data since as early as the 1960’s, but it has only recently been confirmed that it is the AEDs causing these birth defects and not the epilepsy or seizures. L. B. Holmes et al. (2001) screened 128,049 pregnant women at delivery and identified three groups: AED exposed, AED naïve but with a history of seizures and AED naïve and no history of seizures. L. B. Holmes et al. (2001) found that 5.7% of infants exposed to AEDs had a major malformation compared to 0% and 1.8% in AED naïve with seizure history and AED naïve but with no seizure history respectively. This study confirmed speculations that AEDs are the cause of birth defects and not epilepsy.

1.4.4 Individual antiepileptic drugs; detailed comments

Antiepileptic drugs are classified as either traditional AEDs (old) or second generation AEDs (new-developed in the 1990’s). There are currently 17 different AEDs available in Australia, with different drugs being used to treat different seizure types. Not enough is known about the teratogenicity of new AEDs due to a lack of prospective data, however it is thought that they are not more teratogenic than older AEDs (Vajda, 2014). As previously mentioned, VPA, a traditional AED, is the most significant teratogen increasing malformation rates over tenfold (Morrow et al., 2006; Tomson et al., 2011; Vajda et al., 2004). Although to a lesser extent, other AEDs have also been found to be teratogenic including: PB, PT, CBZ, LTG and TPM and this effect is dose related in each case. (Pennell, 2018; Tomson et al., 2018). Malformation rates from the prominent international pregnancy registers have been presented in **Table 1.** When reporting malformation rates it is important to consider sample size and confidence intervals. These have not been shown in the table as the data shown was pooled from different studies which reported their findings in different ways. In addition, while international pregnancy registers broadly follow similar definitions of what constitutes as a malformation these sometimes vary and this should be taken into consideration. The North American Register for example, mentioned they may have had lower malformation rates to other registers due to the “strict outcome inclusion criteria” (Hernandez-Diaz et al., 2012). Despite this, differences in outcome criteria have rarely been
a major subject of debate with consistent findings across international studies. The time of follow up may also have an effect on different reported malformation rates. After birth, the North American Register and UK Pregnancy register recorded malformations up to 3 months after birth (Hernandez-Diaz et al., 2012; Morrow et al., 2006) while EURAP and the Australian Pregnancy Registers follow up at around 12 months after birth (Vajda, Graham, et al., 2012, Tomson et al., 2018). While unlikely, it is possible that at 12 months after birth unnoticed malformations may have been detected.

Traditional (old) AEDs

Phenobarbital

Phenobarbital was the first AED developed, and has been used for over 100 years. It is a barbiturate and is widely used in developing countries because it controls many different seizure types, is inexpensive, and is easily accessible (Bhalla et al., 2015). Throughout the Western world there have been reductions in its use over the years (Nicholas, Ridsdale, Richardson, Ashworth, & Gulliford, 2012) and this may be partly due to concerns of its tolerability (Brodie & Kwan, 2012). Interestingly these tolerability issues, such as sedation, depression and behavioural problems are reported in developed countries with minimal reports in developing countries (Brodie & Kwan, 2004). The reasons for this discrepancy may be due to differences in the perception of intolerability or higher doses used in developed countries (Kwan & Brodie, 2004). Barbiturates are enzyme inducers and affect levels of other drugs.

PB is effective in treating both generalized and partial seizures. It is not widely used for treating non-epilepsy indications. Reports of PB as a potential teratogen began from 1963 (Müller-Küppers, 1963). A meta-analysis by Holmes et al., (2001) found a statistically significant increase of malformations associated with PB when taken during pregnancy. More recent studies from the North American AED pregnancy register found a malformation rate of 11/199 (5.5%) with prominent birth defects being cardiac anomalies (5/11), oral clefts (4/11) and hypospadias (1/11) (Hernandez-Diaz et al., 2012). A 2018 study by EURAP found a malformation rate of 19/294 (6.5%) (Tomson et al., 2018). Similarly, Eadie (2008) found from comparing several studies that malformation rate of PB is between 1.5 and 5.3%. Malformations that have been associated with PB include cleft lip and cardiac malformations.
Phenytoin

The next AED to be introduced was phenytoin in 1938 (Merritt & Putnam, 1938). It is used to treat both generalized and partial seizures as well as seizures that occur after neurosurgery (Goldenberg, 2010). It is not used to treat absence epilepsy. It has been shown to cause cognitive impairment, sedation and depression in some patients (Nadkarni & Devinsky, 2005) as well as chronic encephalopathy when exposed in the long-term (Ettinger, 2006). This chronic encephalopathy may have been caused by cerebral atrophy (Nadkarni & Devinsky, 2005). Other side effects include locomotor dysfunction, psychosis, hyperkinesia and liver disease (Iivanainen & Savolainen, 1983). Recently, there have even been fatal reactions in some patients with brain tumours (Faisal et al., 2017) raising the question as to whether or not it should be used in this sub group of patients. In addition it PHT has long been associated with gingival hypoplasia (Kimball, 1939).

Despite having been shown to have anxiolytic and mood stabilising properties (Birse, Derry, & Moore, 2012), PHT is not often used as a mood stabilising agent (Nadkarni & Devinsky, 2005). It does not show antidepressant properties (Grunze, 2010). Over half of patients exposed to PHT for pain have a reduction in initial symptoms with less than half continuing to experience continued pain relief (Spina & Perugi, 2004). There is evidence that PHT can be effective in treating trigeminal neuralgia however these studies were not controlled (Cheshire, 1997). Recently a phenytoin based cream has been developed for use in the treatment of neuropathic pain (Kopsky & Keppel Hesselink, 2017), however this has not yet been subject to randomized trials. Further studies are required to support phenytoin as effective in treating neuropathic pain.

PHT teratogenicity has been reported since 1972 (Speidel & Meadow, 1972). Other reports have followed elucidating that PHT does indeed result in an increase of birth defects and in particular fetal hydantoin syndrome (FHS). This entity is particularly associated with PHT and symptoms include: physical malformations such as craniofacial defects, limb defects, hypoplasia of the nails and digits as well as neurodevelopmental delay (Singh, Bhatia, Mohan, & Sharma, 2016). Van Dyke, Hodge, Heide, and Hill (1988) assessed foetal malformations in 52 exposed families and found 15/52 families to have at least one child w10:32 PM N=ith a birth defect. One study found there to be an association between cleft palate and PHT (Puho, Szunyogh, Metneki, & Czeizel, 2007).The UK pregnancy register found a malformation rate of 3/82 (3.7%) 1 of which was a facial cleft abnormality, 1 a car-
Carbamazepine was developed in 1953 and was put on the market to in 1962, first for trigeminal neuralgia, then for it an antiepileptic properties not long after (Shorvon, 2009). It is an important AED for the treatment of focal seizures, however it is also used to treat generalized seizures and mixed seizure types (Goldenberg, 2010). It poses risk of serious side effects in some populations especially in the Han Chinese and Thai populations, due to an allele described as the HLA-B* 1502 which may predispose to an acute dermatolysis and complications such as Steven Johnson Syndrome.

CBZ has been the drug of choice for trigeminal neuralgia for around 50 years. It is preferred over VPA and PHT because it is highly effective in small doses. It is considered first line treatment (Cheshire, 1997). The majority of patients with trigeminal neuralgia benefit when first administered CBZ but only half continue to have reduced symptoms after continued exposure (Spina & Perugi, 2004). In 2004, CBZ began to be used to treat mania, particularly in Japan where lithium was not yet available. It was the first anticonvulsant to be used for bipolar disorder (Ballenger & Post, 1978). Currently, in addition to seizure control, CBZ is used to treat: trigeminal neuralgia, neuropathic pain, bipolar disorder, mania, schizophrenia, PTSD, panic disorder and hyperekplexia.

CBZ has been associated with an increased risk of major congenital abnormalities, primarily: neural tube defects, cardiovascular defects, cleft palate and urinary tract anomalies (Matalon, Schechtman, Goldzweig, & Ornoy, 2002). The UK pregnancy register found a malformation rate of 20/900 (2.2%), most of which were cardiac defects (6), followed by facial cleft (4), skeletal (3), gastrointestinal tract (2), hypospadias (2), neural tube defect (2) and other (1) (Morrow et al., 2006). The North American AED pregnancy register found a malformation rate of 31/1033 (3%) (Hernandez-Diaz et al., 2012). The Australian Pregnancy Register reported a 19/301 (6.3%) malformation rate. EURAP found a malformation rate of 6/152 (3.9%) (Tomson et al., 2018). When comparing several studies that had observed the malformation rates M. J. Eadie (2008) found that CBZ ranged from 0.8-6.1%.
Benzodiazepines

Benzodiazepines began to be used as anti-seizure drugs in 1960 over 30 years after they were first developed (Henriksen, 1998). They are first line treatment for status epilepticus as well as seizures occurring post anoxic insult (Riss, Cloyd, Gates, & Collins, 2008). Benzodiazepines used as AEDs are: diazepam and lorazepam - used primarily for seizure emergencies- clobazam (CBZ), clonazepam (CLZ), clorazepate, and midazolam- used for chronic management (Riss et al., 2008). Clonazepam has a longer half-life than the other benzodiazepines, it is mostly used for status epilepticus and is also used for Lennox Gastaut Syndrome (Ochoa & Kilgo, 2016). One of the advantages of using benzodiazepines is that they can be administered via different routes other than just orally (Riss et al., 2008). Many drugs in this class are commonly used to treat anxiety and it is therefore common for them to have side effects such as sedation. Out of the benzodiazepines, clobazam is better tolerated because it does not cause as many sedative side effects (Pernea & Sutcliffe, 2016). It is effective as adjunctive therapy for Lennox Gastaut syndrome (LGS) (Ochoa & Kilgo, 2016). Recent studies have suggested that it should be used more widely across epilepsy as adjunctive therapy (Shimizu et al., 2003).

One of the largest studies looking at BZ teratogenicity was conducted by the Swedish Birth Register and assessed 1979 infants that were exposed to BZ in utero (Wikner, Stiller, Bergman, Asker, & Kallen, 2007). The study found 105/1970 had a congenital malformation (5.3%) which was not that much higher than the background risk at the time of 4.7%. Notably, there were a higher number of pre-term births and low birth weight amongst the exposed group. Similarly, the Israeli Teratogen Information Service in 1998 found no differences in birth defect rate between benzodiazepine exposed (11/355) and control infants (10/382) (Ornoy, Arnon, Shechtman, Moerman, & Lukashova, 1998). They did, however report an increased incidence of both spontaneous and induced abortions in the benzodiazepine exposed group (106/460 compared with 42/424). The major international pregnancy registers have looked mostly at CLZ, out of the benzodiazepines. The Australian Pregnancy Register reported 0/24 malformations (Vajda, Graham, et al., 2012). The UK pregnancy register found no malformations in the 9 exposures (Morrow et al., 2006). North American Pregnancy Register 2/64 on CLZ had malformations (3.1%) (Hernandez-Diaz et al., 2012). A meta-analysis assessing 8 different studies found there to be no increase in teratogenic risk for benzodiazepines overall, but an increase in the risk of cleft palate (Enato, Moretti, & Koren, 2011).
Second generation (new) AEDs

Although there is not sufficient data to assess the teratogenicity of second generation AEDs, there have been early reports. These have been particularly discussed in a recent study by EURAP (Tomson et al., 2018).

Lamotrigine

Lamotrigine is categorized as a new AED, has had approval of the FDA since 1994 and is used for a broad range of seizure types. It was initially used as an add-on treatment. It is used for the treatment of: bipolar depression, schizophrenia, trigeminal neuralgia, PTSD and OCD. LTG reduces depressive symptoms in bipolar disorder in the most part for patients with severe depression (Geddes, Burgess, Hawton, Jamison, & Goodwin, 2004). Compared to lithium, LTG is better in preventing depressive episodes whereas lithium is more effective in treating manic episodes (Schaffer, Zuker, & Levitt, 2006).

There is limited evidence on LTG and schizophrenia however it has been found that it significantly reduces both positive and negative schizophrenia like symptoms, ketamine induced perceptual abnormalities as well as learning and memory impairment in healthy subjects (Anand et al., 2000). An early study found LTG to be an effective add on treatment for patients with trigeminal neuralgia (Zakrzewska, Chaudhry, Nurmikko, Patton, & Mullens, 1997). A more recent study by Goel, Tanwar, Singh, and Tripathi (2015) found LTG to be just as effective as CBZ in treating trigeminal neuralgia. Similarly, patients with OCD showed significant reductions in obsessive, compulsive and affective symptoms (Bruno et al., 2012).

LTG has the lowest risk of causing AED-associated birth defects and seems to be most commonly used during pregnancy (Emes et al., 2013). Despite having a reduced risk of birth defects compared to other teratogenic AEDs, some studies have reported LTG to be associated with malformations. In 2006, the UK pregnancy register conducted a study in which they evaluated 3607 cases (Morrow et al., 2006). 684 of these took LTG and 21 had birth defects (3.2%). The birth defect types in this study were hypospadias (6), cardiac (4), other (4), gastrointestinal tract (3), skeletal (2), facial cleft (1), neural tube defect (1). The North American AED/Pregnancy Register found a malformation rate of 31/1562 (2%) (Hernandez-Diaz et al., 2012). The Australian Pregnancy Register found malformation rates of 12/231 (5.2%) (Vajda, Graham, et al., 2012). A recent study by EURAP found a 74/2514 (2.9%) malformation rate (Tomson et al., 2018).
In addition, Campbell et al. (2014) evaluated pregnancy outcomes of 5206 women who were exposed to AEDs over 15 years. 2198 of these women were exposed to LTG and the malformation rate was 2.3%. Higher doses of LTG did not result in greater malformation rates than any dose of VPA. In addition, Holmes et al., (2008) found an association between cleft palate and LTG. Contrary to other studies, two studies from the International LTG Pregnancy Register found that LTG did not result in any birth defect increases (Cunnington et al., 2011). Their 2005 study had a small sample size, however, and noted that larger sample sizes would be required. This Register was company based and its paper cannot be accepted as an independent report.

**Topiramate**

Also a new AED, Topiramate (TPM) is used to treat generalised tonic-clonic seizures, focal seizures and seizures in Lennox Gastaut (Lyseng-Williamson & Yang, 2008). It is used to treat both children and adults and is often used as adjunctive therapy (Goldenberg, 2010). Some of the adverse effects of TPM can include: sedation, over breathing, tingling around lips and fingers, weight loss and renal calculi (Mervyn J. Eadie & Vajda, 2015).

In addition to treating epilepsy TPM is used for treatment of migraine, alcohol use disorders (AUD), neuropathic pain, post-traumatic stress disorder, OCD and social phobia. VPA is an FDA- approved first and TPM is an FDA- approved second drug for treating migraine. Ten studies have confirmed the efficacy of TPM in migraine prophylaxis, four of which are class 1 studies and 7 of which are class 2 studies (Bagnato & Good, 2016). In addition to these, many other studies have also confirmed the use of TPM in migraine (Krymchantowski & Jevoux, 2011; Mathew, Kailasam, & Meadors, 2002).

TPM has been assessed for use in bipolar disorder but has been found to have many side effects such as weight loss. In addition, TPM is used in the treatment of neuropathic pain such as trigeminal neuralgia. A meta-analysis of studies using CBZ and TPM found that there was no difference overall in the effectiveness of the two drugs for the treatment of trigeminal neuralgia, however TPM yielded better results after two months of treatment (Q.-P. Wang & Bai, 2011). Van Ameringen, Mancini, Pipe, Oakman, and Bennett (2004) assessed the effect of TPM on social phobia on 23 patients. The results showed an improvement of social phobia symptoms but not of depression or anxiety. Berlant and van Kammen (2002) found that TPM reduced nightmares in PTSD patients in 79% and reduced intrusions or flashbacks in 86%. Although the results of this study were promising there were limi-
tions and further studies must be conducted. Similarly, Van Ameringen et al (2004) found a significant improvement in TPM treated OCD patients however further studies are required to support this finding. It is also used for alcohol use disorders. Of 22 studies looking at the effect of TPM and alcohol, all but two showed improvements in alcohol symptoms (Guglielmo et al., 2015).

The Australian Pregnancy Register reported malformations in 1/31 monotherapy cases (3.2%) and 5/75 polytherapy (Vajda, Graham, et al., 2012). Morrow et al., (2006) reported malformations 2/28 (7.1%) of pregnancies compared with a malformation rate of 3.5% where no AEDs were taken. One of these individuals had cleft palate and one hypospadias. The North American pregnancy register found a malformation rate of 15/359 (4.17%). Similarly, Hunt et al. (2008) reported a 4.8% malformation rate in TPM monotherapy pregnancies. Most recently, TPM has been associated with increased cleft lip or palate abnormalities (Castilla-Puentes et al., 2014). In their 2014 study, Castilla-Puentes et al., (2014) evaluated fetal outcomes in pregnancies for women taking TPM for epilepsy and also for other indications and found that malformation rates were higher for women taking AEDs for epilepsy than women taking AEDs for other indications. A recent study by The Australian Pregnancy Register assessed foetal malformation rates in polytherapy combinations not including VPA and found there was an increase in malformations in combinations involving TPM as the dose increased (Vajda, O'Brien, Lander, Graham, & Eadie, 2016). The malformations types observed in these TPM polytherapy expose children were varied however there was a particular association between TPM and hypospadias. Most recently, a study by EURAP found a malformation rate of 6/152 (3.9%) (Tomson et al., 2018).

**Levetiracetam**

Levetiracetam is a new AED that was approved by the food and drug administration (FDA) in 1999 (Farooq et al., 2009). It was initially approved for add on treatment for focal, generalized epilepsy and juvenile myoclonic epilepsy and was later approved for monotherapy in the European union (but not in the United States) (Abou-Khalil, 2008). It has minimal side effects in children, but when reported were commonly associated with changes in behaviour and somnolence (Incecik, Herguner, & Altunbasak, 2012). In adults the most common side effects are headaches, dizziness, asthenia and also somnolence. (Dooley & Plosker, 2000).
In addition to epileptic seizures, LEV was found to be effective in reducing tics from La Tourette’s syndrome in children and teenagers in an open label study (Awaad, Michon, Minarik, & Rizk, 2009). It has also been found to be effective in treating bipolar disorder, however mostly in case reports and further studies are required (Goldberg & Burdick, 2002; Kaufman, 2004). The UK pregnancy register found that 0/22 pregnancies had a malformation (Morrow et al., 2006). The North American Pregnancy register found a malformation rate of 11/450 (2.4%). The Australian Pregnancy Register found a malformation rate of 0/22 (Vajda, Graham, et al., 2012). It may be that in the future a combination of LEV with LTG may be a preferred option as this is more effective against seizures and less teratogenic than other combinations (Vajda et al., Submitted for publication March, 2018).

1.4.5 Valproate (VPA)

Valproic acid (2 propyl-pentanoic acid) is a fatty acid and is used both in its acidic form (valproic acid), in its salt form (sodium valproate) and in its semi-salt form (valproate semi-sodium). It was first developed in 1882 however its antiepileptic properties were not discovered until by accident over a hundred years later by Pierre Emyrad and colleagues (Löscher, 1999). Emyrad had been testing a range of khelline derivatives for their potential use as anticonvulsants and was using VPA to dissolve these substances (as they were water insoluble) and found all derivatives were efficacious against seizures and that this was due to the VPA itself (Fischer, Ganellin, Ganesan, & Proudfoot, 2010). This lead to the first human trials and consequently the first approval of VPA was in France in 1967.

VPA is an effective AED that is used to reduce symptoms in epilepsy as well as numerous psychiatric and neurologic disorders. It is first line treatment for bipolar disorder and migraine and is also used to treat a number of anxiety disorders, schizophrenia, trigeminal neuralgia and neuropathic pain (Johannessen Landmark, 2008; Spina & Perugi, 2004). Some of its side effects include gastrointestinal disturbances, weight gain, pancreatitis, liver failure hyperammonemia and postural tremor (Perucca, 2002). It is prescribed to treat both generalised and focal seizures and can treat patients with all manifestations of genetic generalized epilepsy (Vajda, 2014). It works as an anticonvulsant by having an effect on GABA, as well as on a neuropeptide called NPY. There are also numerous mechanisms of action at a cellular level which may explain its effect in psychiatric disorders (Grunze, 2008).
Despite its efficacy, VPA is the most teratogenic AED with several studies finding that infants whose mothers were exposed to VPA had an increased rate of major malformations compared to general population (Vajda et al., 2004). The malformation rates in the Australian Pregnancy Register were: 35/215 (16.3%), the North American Pregnancy Register were: 30/323 (9.3%), the UK pregnancy register were: 44/715 (6.2%). A most recent study by EURAP found a malformation rate of 142/1381 (10.3%) (Tomson et al., 2018). Out of 10 major studies, 8 found that VPA caused major malformations in from 6-15% of patients, with 6 of these studies showing statistically significant increases (M. J. Eadie, 2008). A meta-analysis by Meador et al., (2008) analysed 65,533 pregnancies in women with epilepsy through 59 studies and found that 10% of women taking VPA had children with birth defects compared with 7.36% in PHT, 4.91% for PB, 4.62% with CBZ and 2.91% with LTG. These numbers are similar to what has been found in the previous studies listed and so it can therefore be concluded that VPA is the most significant teratogen, and that PB, PHT, CBZ, TPM and LTG are all teratogens of lesser effect.

Numerous studies have found that administration of high doses of VPA result in a high risk of malformations compared with low doses (K. J. Meador, 2008). Meador et al., (2008) calculated the percentage of increased risk when taking higher doses of VPA in 7 different studies and found 6 of these studies to be show significant increases. Meador (2008) calculated that in a study by Vajda et al., (2006) there were 34.5% malformations when doses above 1400mg were taken compared with 5.5% malformations when doses less than 1400mg were taken and in a study by Artama, Auvinen, Raudaskoski, Isojarvi, and Isojarvi (2005) there was a 23.8% risk of malformations with doses above 1500mg and 9.5% below 1500mg. The incidence of SB has also been shown to be dose dependent with lower doses producing less severe malformations (Vajda et al., 2004). Doses over 450mg are currently regarded as unacceptable (Tomson et al., 2011) however doses above 200mg are often not prescribed to women (Personal Communication, 2018). It is recommended that where possible, women do not take VPA if there is a possibility they may become pregnant. The mechanism in which it acts to cause teratogenicity is not known, however there are several hypotheses (detailed in section 1.5).

1.4.6 Valproate and neural tube defects

Valproate is associated with a 1.5-2.5% increased risk of spina bifida (1.1% poly-therapy, 2.5% monotherapy) compared to the 0.35% risk of most AEDs (Lindhout & Schmidt, 1986). Other studies have found a statistically significant association between
VPA and SB (Arpino et al., 2000) and an increased risk of neural tube defects as high as 3.8% compared to 1% with CBZ monotherapy and 0% compared to other AED monotherapy regimens when pooling data from a number of different studies (Samrén et al., 1997). The incidence of SB has also been shown to be dose dependent with lower doses producing less severe malformations. (Vajda, O'Brien, Graham, Lander, & Eadie, 2013). Following the established link between spina bifida and VPA, numerous animal studies have been conducted to further understand the underlying mechanisms. The first animal model for VPA-induced spina bifida was developed by Ehlers, Sturje, Merker, and Nau (1992b). This model used Han: NMRI mice and VPA was administered at different doses from 300mg/kg, 400mg/kg, 450mg/kg and 500mg/kg, three times daily at each dose. Spina bifida occulta was the most common malformation (95% at 500mg) with spina bifida aperta, exencephaly and tail malformations also observed. As in clinical studies, a dose effect was observed. A follow up study by Ehlers et al., (1992) developed a model for inducing spina bifida aperta at a greater extent. Here, Ehlers et al., (1992) found that administration of VPA on day 8 of gestation did not result in any spina bifida aperta, while if administered on day 9, spina bifida aperta could be induced. Similar studies have since been conducted: Briner and Lieske (1995) injected Long Evans rats with either 600mg/kg valproic acid once in the day or twice in a day(1200mg/kg in total). Of the 85 rats administered valproic acid once, 4 had spina bifida aperta and 1 had exencephaly. Of the 87 rats administered valproic acid twice in the day, three had spina bifida aperta. Mahabady, Varzi, Ranjbar, and Rahgazar (2011) found that upon AED administration to Wistar rats, cleft palate, spina bifida and exencephaly were present in 17.7, 20 and 20% respectively.

Table 1. Teratogenicity of AEDs as found by international pregnancy registers

<table>
<thead>
<tr>
<th>Pregnancy Register</th>
<th>PB</th>
<th>PHT</th>
<th>CBZ</th>
<th>LTG</th>
<th>TPM</th>
<th>LEV</th>
<th>VPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australian Pregnancy Register¹</td>
<td>-</td>
<td>1/35 (2.9%)</td>
<td>19/301 (6.3%)</td>
<td>12/231 (5.2%)</td>
<td>1/31 (3.2%)</td>
<td>0/22 (0%)</td>
<td>35/215 (16.3%)</td>
</tr>
<tr>
<td>North American AED pregnancy register²</td>
<td>11/199 (5.5%)</td>
<td>12/416 (2.9%)</td>
<td>31/1033 (3%)</td>
<td>31/1562 (2%)</td>
<td>15/359 (4.2%)</td>
<td>11/450 (2.4%)</td>
<td>30/323 (9%)</td>
</tr>
<tr>
<td>UK Epilepsy and Pregnancy Register³</td>
<td>-</td>
<td>3/82 (3.6%)</td>
<td>20/900 (2.2%)</td>
<td>21/684 (3.07%)</td>
<td>2/28 (7.14%)</td>
<td>0/22 (0%)</td>
<td>44/715 (6.15%)</td>
</tr>
<tr>
<td>International Registry of antiepileptic drugs and pregnancy (EURAP)⁴</td>
<td>19/294 (6.5%)</td>
<td>8/125 (6.4%)</td>
<td>107/1957 (5.5%)</td>
<td>74/2514 (2.9%)</td>
<td>6/152 (3.9%)</td>
<td>-</td>
<td>142/1381 (10.3%)</td>
</tr>
</tbody>
</table>

Table 2. Mechanism of action of AEDs*

(+++=principal target, +++= probable target, +=possible target. All CA2+ channels have been grouped and all effects on GABA have been grouped)

<table>
<thead>
<tr>
<th></th>
<th>Na2+ channels</th>
<th>Ca2+ channels</th>
<th>K+ channels</th>
<th>GABA</th>
<th>Glutamate</th>
<th>Synaptic vesicle protein 2A</th>
<th>Carbonic anhydrase</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB</td>
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<tr>
<td>PHT</td>
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<tr>
<td>LTG</td>
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<tr>
<td>TPM</td>
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<td>LEV</td>
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<td>VPA</td>
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</tr>
</tbody>
</table>

*Table adapted from Sills (2011)

1.4.7 Polytherapy

About 25% of epileptic women need to use more than one AED in order to control their epilepsy (Vajda, 2014). The number of AED combinations is high and therefore the teratogenicity of polytherapy combinations has not been extensively studied. Many previous studies have found that polytherapy results in an increased risk of birth defects compared with monotherapy (Morrow et al., 2006; Tanganelli & Regesta, 1992). A recent study by Vajda et al. (2010) found this to not be the case. Vajda et al (2010) found from patients in the Australian Pregnancy Register that out of 282 polytherapy pregnancies 5.32% had malformations, and out of 791 monotherapy pregnancies, 7.84% had malformations. For women exposed to VPA in polytherapy, the risks were lower than for women exposed in monotherapy, particularly when administered with LTG. This study suggested that the risk of AED polytherapy may be due to the degree of exposure to valproate rather than the combination of AEDs. This finding has been reported also from the UK Pregnancy Register and the North American Pregnancy Register (L. B. Holmes, Mittendorf, Shen, Smith, & Hernandez-Diaz, 2011; Tomson et al., 2015b). There has been an increase in polytherapy malformation rates since the increase in use of TPM and LEV (Vajda et al., 2016). In a recent study assessing polytherapy combinations where VPA was not one of the drugs taken it was found that there was a dose dependant increase in malformations with TPM (Vajda et al., 2016).

1.5 Proposed mechanisms of AED teratogenicity

The mechanisms resulting in AED teratogenicity are currently unknown however there are a number of hypotheses including: altered folate metabolism, DNA methylation,
DNA acetylation and increased *de novo* mutations through an effect on usual DNA-repair machinery. As a basis of translational research, a literature review of the mechanisms and postulated experiments and past finding are reviewed in the following section.

### 1.5.1 Folate metabolism

One possible way that AEDs may exert their teratogenicity may be through altered folate related mechanisms (Finnell et al., 2000). Folate (folic acid) is the inactive form of vitamin B9 and when metabolized it is essential for numerous biological processes (Lloyd, 2013). Folate is essential for all DNA and RNA synthesis as well as DNA methylation (Kalhan & Marczewski, 2012). Folate deficiency may lead to altered gene expression and raised oxidative stress (Pulikkunnel & Thomas, 2005). During pregnancy folate levels are already reduced and previous literature has found that antiepileptic drugs such as VPA, further reduce these levels (Wilfert, Altena, Tijink, van Gelder, & de Jong-van den Berg, 2011). VPA reduces levels by interfering with absorption of folate in the intestine while CBZ, PHT and PB reduce levels by inducing microsomal liver enzymes (Kini et al., 2007). Although previous studies have found that women taking AEDs have reduced folate levels (Dansky, Rosenblatt, & Andermann, 1992) some studies have found folate supplementation to be ineffective in reducing malformations. The role of folate therefore remains controversial and there are a number of studies that highlight this uncertainty. These include studies by Craig, Morrison, Morrow, and Patterson (1999), Duncan et al. (2001) and Vajda, Hitchcock, et al. (2006) all of which describe cases in which folate supplementation either did not work sufficiently in preventing malformations or did not work at all. Vajda et al., (2006) found that malformation rates for those exposed to folate compared to those not exposed were not statistically significantly different. Craig, Morrison, Morrow & Patterson (1999) described a case of a woman who gave birth to a child with a neural tube defect (as well as other malformations) in spite of taking a high dose of folic acid periconceptually in the first trimester. In addition, folate antagonism has been found to primarily result in an increased risk of neural tube defects and cardiovascular defects rather than the broad spectrum of birth defects resulting from AEDs (Matok et al., 2009). Despite this, it is recommended that women take at least 0.4mg daily before and during pregnancy (Harden et al., 2009)

### 1.5.2 DNA methylation

Another possible way AEDs may exert their teratogenic effects may be through alterations in DNA methylation signatures. DNA methylation is a process in which a methyl group (CH3) is added to cytosine or adenine nucleotides stopping expression of that particu-
lar segment of the gene by preventing the activator protein from starting transcription. This normally occurs at CpG islands, which are segments of DNA with a high number of C G repeats (the p denotes phosphate). These segments are typically 1000 to 2000 base pairs long. DNA methylation is an essential epigenetic regulator, as not all genes are expressed in every cell (Attwood, Yung, & Richardson, 2002). Alterations in DNA methylation processes during gestation could affect multiple organ systems. AEDs can affect multiple organ systems further supporting the hypothesis that altered DNA methylation levels may cause AED induced birth defects. Previous studies have looked at methylation patterns in children of mothers exposed to AEDs during pregnancy. In a study conducted by Smith (2009), the DNA of 201 neonates were analysed for average methylation levels across 27 578 CpG sites. DNA was extracted from the umbilical cord and it was found that the neonates who were exposed to AEDs for the longest time showed a decrease in average global methylation. DNA methylation in 14 genes specifically significantly reduced upon increased exposure to AEDs during pregnancy. For 19 of the neonates, placental tissue was also available. In this tissue, out of 14 CpG sites 3 were significantly reduced in DNA methylation. This study did not find any evidence that changes to DNA methylation levels were particular to any specific AEDs or that using more than one AED would cause more reduced methylation levels. It should be noted, however, that most of the women in this study were taking Lamotrigine (36) with only 3 women taking VPA AND LEV, 2 taking CBZ and 1 taking TPM, PHY, GABA. 6 women were taking multiple AEDs. Lamotrigine however is the one AED associated with the fewest birth defects

In addition to human studies, a number of studies have looked at the effect of DNA methylation on animal models, neurons and cultured cells. Dong et al., (Dong, Guidotti, Grayson, & Costa, 2007) found that VPA facilitates DNA de-methylation in neurons. Similarly, Detich, Bovenzi, and Szyf (2003) conducted a study in which they observed the effect of VPA on methylation in embryonal kidney 293 cells. They found that VPA results in active DNA de-methylation further supporting this theory. In a follow up study it was found that induction of a DNA methylation inhibitor could induce these genes, thus providing evidence that VPA can alter the expression of genes (Milutinovic, D'Alessio, Detich, & Szyf, 2007). In addition to human studies, there have also been animal studies to assess the effect of AEDs on methylation. Tung and Winn (2010) injected CD-1 mice with 400mg/kg VPA and then assessed for epigenetic alterations. No significant differences in global or CpG island DNA methylation was found, however there was histone modification observed at particular sites. Rat studies have also looked at specific genes, showing that valproate induces
DNA de-methylation in WNT1 and WNT2 resulting in increased protein expression of WNT1 and WNT2 (Z. Wang et al., 2010). The WNT/beta catenin pathway is an important pathway for controlling embryonic development and adult homeostasis (B. T. MacDonald, Tamai, & He, 2009). This study suggests that DNA de-methylation of these genes may be attributed to VPA exposure, however the authors suggest there may be other epigenetic modifications that increase mRNA expression of these genes and consequently increased protein expression upon VPA exposure. It should be noted that this study was assessing the potential epigenetic mechanisms resulting in autism spectrum disorder associated with VPA exposure, however the results are still relevant. In another study, Wistar rats treated with 300mg/kg VPA on gestation days 8, 9 and 10 VPA had reduced maternal methionine serum concentration, and in the foetuses exposed to VPA, methylation levels were reduced (Alonso-Aperte, Ubeda, Achon, Perez-Miguelsanz, & Varela-Moreiras, 1999). In this study, no neural tube defects were found however there were changes of the axial skeleton upon closer examination.

1.5.3 DNA acetylation

Valproate inhibits the action of histone de-acetylases (HDAC), an enzyme involved in the process of acetylation (Phiel et al., 2001). Acetylation is the process in which an acetyl group (CH3CO-) is added to the lysine residue of the histone core making the overall charge of the histone negative rather than positive. When this happens, DNA- also negatively charged- is repelled, dissociating and allowing transcription to take place. The addition of an acetyl group is initiated by the enzyme histone acetyl transferase (HAT) while the removal of this acetyl group is initiated by the enzyme histone de-acetylase (HDAC). In normal circumstances when HDAC removes this acetyl group, the overall charge of the histone remains positive and stays tightly bound to the negatively charged DNA preventing transcription from occurring. Valproate binds to the active center of HDAC inhibiting its action and thus inhibiting the removal of the acetyl group and consequently initiating further transcription. Thus, similarly to DNA methylation- histone hypoacetylation is associated with silent genes (Dobosy & Selker, 2001). It is hypothesized that this increased acetylation may also result in increasing the accessibility of DNA de-methylation to take place (Detich et al., 2003). In addition to VPA, TPM also directly inhibits HDACS while LEV has an indirect effect through its major carboxylic metabolite (Eyal et al., 2004).
1.5.4 Genetic variants and *de novo* mutations

Another hypothesis is that AEDs exert their teratogenic effect by interacting with genetic variants, or resulting in increased *de novo* mutations. Risk alleles may be present in the mother of children with AED-induced birth defects. Alternatively, it could be that these AEDs interfere with usual DNA-repair machinery, thus resulting in an increased rate of *de novo* mutations in children with AED-associated birth defects. *De novo* mutations are mutations that are occurring for the first time in the germ cells of the mother or father during gametogenesis and thus are only present in the genetic make-up of the child. Previous studies have found that two of the wide spectrum birth defects- autism spectrum disorder (Sanders et al., 2012) and cleft palate (Barbaro et al., 2012)- contain *de novo* risk factors. Germline DNA methylation also has the potential to alter nucleotide substitution rates at specific regions and consequently may result in *de novo* mutations.

1.6 Evidence for a Pharmacogenomic Influence

1.6.1 Human Studies

Women who have children with an AED-induced birth defects have a 39-55% increased chance of having a subsequent child with a birth defects compared with a 9.6% chance in women who had a healthy child (Moore et al., 2000). The risk is further increased for VPA, the Australian Pregnancy Register finding that women who had a child with a VPA induced birth defect had a higher incidence of consequent affected children (if continuing the drug) compared with mothers who had unaffected children (57.2% increased compared with 7%) (Vajda et al., 2013). In addition, these women were more likely to have a family history of birth defects indicating a genetic predisposition to birth defects. 41.8% of women with a child with a birth defects had a family history of birth defects in 1st and 2nd degree relatives compared to 8.6% in women with non-effected children (Vajda, O'Brien, et al., 2006). Other evidence includes increased concordance of twin birth defects (Hall et al., 1988). Although twins do not always have the same birth defect there is an increased likelihood that both will be affected. In addition, previously mentioned studies finding altered DNA methylation levels upon VPA exposure in neonates, rats, neurons and cells as well as *de novo* mutations as risk factors for known AED birth defects all provide further evidence that genetic factors may be the cause of AED-induced birth defects.
1.6.2 Animal Studies

Animal studies have also provided evidence for genetic susceptibility. Different rodent strains show differences in susceptibility to birth defects as well as differences in birth defect types when exposed to AEDs (Faiella et al., 2000; Finnell et al., 2000). They result in varying degrees of neural tube defects such as spina bifida and exencephaly, orofacial defects such as cleft palate, skeletal defects such as rib anomalies and cardiac defects depending on the dose, strain, and method of administration (Ehlers et al., 1992b; Mahabady et al., 2011; Vorhees, 1987). The main focus of AED teratogenicity studies in the literature are VPA and neural tube defects.

Finnell et al., (2000) compared the susceptibility to VPA induced neural tube defects for two different inbred mouse strains: SWV and LM/Bc. The mice were administered VPA on gestational day 8:12 and it was found that only 19.8% of the LM/Bc strain foetuses developed NTD’s while 35% of SWV foetuses developed NTD’s. Finnell et al., (2000) also assessed the expression of different genes in the neuroepithelium of developing embryos in the two strains. The genes that were evaluated were growth factor genes; folate pathway related genes as well as several cell-cycle checkpoint genes. They were evaluated 6 hours after VPA exposure (8:18). They found a significant reduction in folate binding proteins FBP-1 in VPA exposed SWV embryos. Despite this, there was significantly higher transcriptional activity in the gene in the SWV strain compared with Lm/Bc strain (however there was still significantly higher transcriptional activity in FBP-2 in the Lm/Bc strain). There was also a significantly higher expression of MTHFR in VPA exposed Lm/Bc embryos compared with controls and also compared with VPA exposed SWV embryos. In addition, there was an up-regulation of brain derived neurotrophic factor (BDNF), nerve growth factor (NGF) and its receptor (NGF-R) as well as bcl2 and p53 genes in VPA exposed Lm/BC embryos compared to controls as well as in SWV mice. For SWV mice, they observed no significant alterations of BDNF, NGF or NGF-R and a non-significant downregulation of bcl2 and p53. Genetic differences in susceptibility to birth defects to other AEDs such as PHT have also been found in several mouse strains (Finnell, Abbott, & Taylor, 1989). This important paper demonstrates that there may be several different genes differentially effected by teratogenic doses of VPA in mice. These are all genes that are essential for development and it is interesting that the strain of mice more susceptible to birth defects (SWV) have reduced expression of folate pathway genes and down regulated cell cycle check point genes. This indicates that there are a number of different changes that result in the teratogenic phenotype and provides valuable information for further studies in terms of candidate genes.
1.7 Potential genomic candidates

1.7.1 AED transport/metabolism

Previous human studies have also looked at potential candidate genes, however no genetic factor has yet been validated. Jose (2014) hypothesized that polymorphisms in genes involved in the transport (ABC1) and metabolism (CYP2C9, CYP2C19 and CYP3A4) of AEDs may be responsible for AED-induced birth defects. Their study screened for previously reported specific polymorphisms and found that in women taking antiepileptic drugs ABCB1 polymorphism Ex07 + 139/ct was overrepresented, and in gene CYP2C19 allele *2 and *2*2 were under-represented. This indicates the potential involvement of these genes, however further analysis is required to validate this.

1.7.2 MTHFR gene

In addition to the ABC1 and CYP2C19 genes, Dean et al., (1999) and Dean et al., (2007) conducted studies focusing on methylene tetrahydrofolate reductase gene (MTHFR). MTHFR is an enzyme involved in folate metabolism and has been known to be involved in the onset of congenital malformations (Botto & Yang, 2000). It provides the 5-methyl-tetrahydrofolate required by the MTR gene for the re-methylation of amino acid homocysteine. In their 1999 study Dean et al., found that the 677 C > T mutation frequency in the MTHFR gene was significantly higher in mothers taking AEDs than controls. The 677 C>T mutation is related to increased thermobility and decreased activity and it has previously been associated with neural tube defects. Although this study did find an association between a gene and AED exposure induced birth defects, it did have its limitations. Women counted as AED exposed mothers did not necessarily have a child with a congenital malformation (only18/52) because the main outcome measure was foetal anticonvulsant syndrome. Foetal anticonvulsant syndrome is non-specific and was diagnosed based on the criteria that the child had a specific facial appearance (and one of a) AED associated defect b) a medical disorder c) developmental delay. Thus, the findings are not entirely relevant to the aims of this study. In their study Dean et al., (2007) used malformation rates, neurodevelopment delay and foetal anticonvulsant syndrome as separate outcome measures. In addition, they looked at a broader range of genes. In this study, the primary finding was that women with an excess of MTHFR 677TT homozygotes were more likely to have a child with a birth defect than women with 677CC homozygotes.
A larger study following this found that although these genes may play a role in susceptibility, it is likely that this is not the primary mechanism (Kini et al., 2007). It was found that AED exposure in women who have a CC or TT genotype increases the risk of having a child with a birth defect, however the results were not statistically significant (Kini et al., 2007). There is therefore currently insufficient evidence to implicate these specific genes and further studies must be conducted.

1.8 Need and application of genetic biomarkers in clinical practice for AED teratogenicity

There are currently no disease modifying agents for epilepsy and AEDs therefore remain the most effective treatment. Many women on AEDs who have either planned or unplanned pregnancies have no choice but to continue AED therapy throughout pregnancy with no clear understanding of their individual risk. If women on AEDs were able to know the risk they faced they would be able to make more informed decisions. If these genetic biomarkers became known, new drugs may be able to be formulated for use by populations at risk.

Pharmacogenomics is the role of genes in drug response. It aims to personalize medicine based on individual genetic profiles. It is currently being used in a number of different areas including HIV treatment, cancer treatment and epilepsy treatment. Individuals can be tested for genetic biomarkers which if present, provide information about the individuals’ likely response to a particular drug. Identifying these factors would not only helpful in helping to make more informed decisions for doctor and patient, but also ultimately to find the pathophysiology of the defects. Identifying genetic risk factors associated with adverse drug reactions allows for genetic counselling prior to drug administration and in the future to potentially form new therapies.

1.9 Summary

Antiepileptic drugs are well established to increase the risk of birth defects in babies born to women taking these at the time of conception. Human and animal studies have provided evidence that genetic and/or epigenetic factors predispose some women to have children with birth defects when taking AEDs during pregnancy. Genetic and epigenetic factors have been explored in previous studies. However, no genetic or epigenetic factor has yet been validated. Although it is established that genomic factors predispose an individual to
having a child with an AED induced birth defect, the nature of these genetic factors remain elusive.
Chapter 2

Basic Design of Research

The research questions for this thesis have been explored concurrently through both human and animal studies. The dual aims were to a) develop an animal model of valproate (VPA) induced defects that closely replicates a human clinical setting and can be used to better understand the pathogenesis of AED induced defects b) identify genetic and epigenetic markers of AED induced defects using whole genome analysis of human samples and determining if having epilepsy is a contributing factor to the onset of these defects.

2.1 Research Questions

1. What are the genetic and epigenetic factors that could make some women more susceptible to having children with birth defects when taking AEDs during pregnancy and are these factors specific to women with epilepsy?

2. How can an animal model closely reflecting a clinical setting be developed and how can it be used to assist in identifying and contribute to further understanding these genetic and genomic mechanisms?

The chapters of this thesis have been separated into animal studies in chapters 3-4 and human studies in chapters 5-6. Both sections ultimately aimed to find genetic biomarkers associated with antiepileptic drug induced birth defects. Human studies aimed to do this through a direct genomic analysis of affected families and animal studies through the development of animal models that were designed to allow us to control variables and can ultimately help in assisting human analysis.
2.2 Animal study research design

The purpose of the animal study was to develop an animal model of VPA induced birth defects using an established epileptic strain of rats; viz Genetic Absence Epilepsy Rats from Strasbourg (GAERS), administering VPA chronically to the rats through the diet at a dose that suppresses seizures and results in potentially therapeutic blood levels. While other animal models for VPA teratogenicity do exist, they are often of limited value because they do not closely mimic clinical circumstances; the animals are non-epileptic, "therapeutic" doses are not used and drugs are administered via stressful methods at particular time points rather than chronically. The following two chapters outline the animal studies as follows:

Chapter 3 aimed to find which dose of VPA when administered to epileptic rats via the rat diet results in seizure suppression and therapeutic blood levels. This dose would then be able to be used in Chapter 4 which focuses on teratogenicity studies. The hypotheses’ for this chapter were that 1. Higher doses of VPA would result in greater seizure suppression 2. Higher doses would result in higher VPA blood levels close to human therapeutic levels.

Chapter 4 aimed to administer VPA at the dose determined from the pilot study in chapter 3 to both epileptic and non-epileptic strains of rats, mate the rats and assess for external and internal birth defects. The rats were assessed for malformations both externally, as well as internally for spinal defects and they underwent a full histopathological analysis. The hypothesis was that VPA- exposed pups would exhibit morphological differences compared with non-exposed pups.

Animal studies allowed for controlling of variables such as the nature of the drug, dose, animal strain, epilepsy, number of rats and duration of exposure. Ultimately the purpose of using animal models was to assist the human analysis in identifying causative mechanisms. After the successful development of this model, rat pup brains exposed to VPA underwent RNA sequencing. This is still in the process of completion. Human and animal data were accumulated at the same time, with the expectations that animal data may give us a direction towards the analysis of the human data.
2.3 Human study research design

The specific goals of the human studies were in two parts: a) to identify genomic markers of AED induced birth defects in families with an affected child and b) to determine the role of epilepsy in AED induced birth defects.

Chapter 5 aimed to identify genetic markers that are present in families with a child with an AED induced birth defect through whole genome analysis. Specifically, we were looking for differences in antiepileptic drug exposed vs non-exposed and affected vs unaffected mothers, children and trios. Subjects were recruited through the Australian Pregnancy Register as well as from private practitioners. There were three main hypotheses for this chapter:

1. There are highly penetrant protein coding variants capable of causing large effects that increase the risk of birth defects for pregnant women treated with AEDs, particularly valproate.
2. Children with AED-induced birth defects, particularly those exposed to valproate will have an increased load of functional de novo mutations.
3. Children with AED-induced birth defects, and/or their mothers will show alterations in DNA methylation levels compared with unaffected children and/or mothers.

Methods used to identify these genetic markers were:

i) whole exome sequencing
ii) DNA methylation scans.

Chapter 6 assessed the role of epilepsy in AED induced birth defects from subjects in the Australian Pregnancy Register. Although it is known that it is primarily not the epilepsy causing AED induced birth defects, whether or not epilepsy is a contributing factor has not yet been elucidated. This is an important question to answer to not only be able to identify the exact risk of an important patient group but also to help narrow down potential genetic markers. This chapter assesses this risk in a cohort of women from the Australian Pregnancy Register taking antiepileptic drugs for non-epileptic indications. The data accumulated from these two studies provides an insight into potential genetic markers as well as the role of epilepsy.
Chapter 3

Seizure suppression in a model of genetic absence epilepsy by dietary valproate

3.1 Abstract

Background: The antiepileptic (AED) drug valproate (VPA) is a highly effective anticonvulsant used primarily for treatment of epileptic seizures. Animal models of epilepsy are valuable tools to study effects of new and existing AEDs, particularly with reference to drug efficacy and tolerability. Conventional methods of AED administration often involve acute injection protocols, and rarely allow for prolonged administration which is far more relevant to human circumstances. In addition, repeated injections can be stressful for the animal, consequently effecting seizure frequency, particularly if delivered intermittently, as well as providing potential risks to the animal and the investigator.

Methods: This study describes a novel method of VPA administration delivered through the rodent diet to an epileptic strain of rats, viz Genetic Absence Epilepsy Rats from Strasbourg (GAERs). We studied the efficacy, tolerability, and VPA plasma levels of this mode of delivery and a range of doses of VPA; 0g/kg, 5g/kg (low dose), 10g/kg (medium dose) and 20g/kg (high dose) (VPA per kg of food).

Results: The results showed that 20g/kg significantly suppressed seizures by 29.23% on average. This dose was well tolerated, with no apparent side effects. It corresponded to blood levels between 200-300 umol/L. Higher doses (40g/kg) were not tolerated, resulting in weight loss and dramatically reduced appetite, whereas lower doses did not influence seizures significantly. In addition, we found a linear relationship between dose of drug consumed and VPA plasma blood levels.

Conclusion: The results from this study show that administering VPA in the rodent diet is an effective way of chronically exposing rats to VPA with minimal stress on the animal and that doses below 20g/kg are not sufficient to achieve adequate seizure suppression levels.
3.2 Background

Valproate is a widely used antiepileptic drug (AED) used to treat a variety of seizure types including generalized absence seizures, and also mood disorders (Van Ameringen, Mancini, Pipe, & Bennett, 2004). It is an effective AED, but also has well-established teratogen, causing birth defects in between 6-17.4% of offspring exposed to VPA during pregnancy (M. J. Eadie, 2008). Animal models of epilepsy provide relevant platforms to assess drug efficacy, side effects and teratogenicity as well as potentially helping to further develop disease modifying therapies. Traditional preclinical methods of AED administration in rodent studies are intraperitoneal or subcutaneous injection and oral gavage. These methods are invasive and can be stressful for the animal and may therefore result in an increase in seizures, not accurately representing drug efficacy (Loscher, 2007). In addition, they pose a risk of injury to the animal, and to the investigator performing the procedure.

Other methods of drug administration include drug infusion through gastric tubes, osmotic mini pumps, implanted catheters or mixing the drug into drinking water. While such methods may be effective in providing prolonged administration, they also pose risks. One study delivered VPA to rats via osmotic mini pumps at doses of 175, 230, 600 and 1424mg/kg/day (Stout, Owens, Lindsey, Knight, & Nemeroff, 2001). Rats given 1424mg/kg died within 24 hours of being administered the drug. This method is likely not ideal when delivering high doses. Another study administered VPA mixed in with drinking water and found this was an effective method of drug delivery for low and medium doses of between 200 and 500 mg/kg, but when administered doses of 825mg/kg rats had an aversion to the water, drinking 5-10ml less (Frisch et al., 2009). When administering high doses, mixing in the water is unlikely to be an effective method. Implanted catheters can also cause negative side effects such as peritoneal irritation and even death (Bertram, 2005). New and improved methods of drug administration are required to ensure prolonged administration that mimics human drug exposure, while minimising animal stress levels and tolerability issues.

Maintaining effective drug levels throughout long periods of time is a challenge in developing an adequate animal model. The majority of AEDs have half-lives of between 8->24 hours in humans (Loscher, 2007) and therefore must be administered multiple times during the day. Drug half-lives are orders of magnitude lower in rodents. The half-life of VPA in rodents is 1-5 hours and so single administration is not effective in mimicking human exposure. Some studies administering antiepileptic drugs to rats give three IP injections for continued exposure to the drug, however only report seizure activity during the time they
injected- i.e. 8am, 2pm, 6pm (Leite & Cavalheiro, 1995; van Vliet et al., 2006). The rats are not exposed to the drug during the night and are therefore not chronically exposed. Despite this, Dedeurwaerdere et al. (2011) found that seizures were suppressed significantly in rats both exposed chronically and intermittently to VPA with no differences despite different methods of administration.

When assessing AEDs during single dose experiments, rats must be tested at the time of peak drug concentration. These kinds of experiments can provide information about immediate drug effects, however do not closely resemble drugs use in a clinical setting. The main reasons maintaining prolonged blood drug levels is important is to assess whether there is increased drug efficacy with prolonged treatment, decreased drug efficacy over time or drug dependence. In assessing AEDs in chronic epilepsy models it is essential that animals are chronically exposed (Grabenstatter, Clark, & Dudek, 2007).

Previous studies have administered AEDs in the rodent diet, but no study has administered VPA through this method and assessed seizure suppression and blood levels. One study administered carbamazepine (CBZ) via the diet to rats that previously experienced chemoconvulsant-induced status epilepticus (Ali, Dua, Constance, Franklin, & Dudek, 2012). It was found through seizure observation that CBZ significantly reduced seizures. Similarly, another study administered CBZ to Sprague Dawley rats via the rat diet in a kainite model of temporal lobe epilepsy and the rats exhibited a suppression in seizures (Grabenstatter et al., 2007). When comparing the efficacy of CBZ injections with CBZ mixed in the food it was found that CBZ mixed in food was found to be just as effective as injections. While fewer studies have looked at VPA in the diet, one study administered VPA in the rat diet in order to assess for the negative impact of VPA administration on bones (Senn et al., 2010). From this study, strains of mice that were resistant and others sensitive were identified, and the method of drug delivery was also found to be effective.

Genetic absence epilepsy rats from Strasbourg (GAERS) are a well-established rodent model for epilepsy. The phenotype is genetically determined, and the model exhibits many features of absence epilepsy, including primary generalized spike-wave discharges which have a pharmacoresponsive profile similar to humans, including response to VPA (Dedeurwaerdere et al., 2011). The model also displays anxiety related and depression-related behaviours which may be comparable to the psychiatric comorbidities experienced by many epilepsy patients (Jones et al., 2008). This study was conducted to assess the feasi-

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bility of delivering VPA to GAERS continuously via the diet. Our primary outcomes assessed included safety and tolerability, effects on seizures, and VPA blood levels.

3.3 Materials and Methods

3.3.1 Animals

Twenty-two GAERSMelb (Powell et al., 2014) rats were obtained from our breeding colonies at the Department of Medicine, Royal Melbourne Hospital, University of Melbourne Biological Research Facility. Thirteen were males weighing on average: 302.1g and nine were females weighing on average 174.5g. The rats were aged between 12-19 weeks. At this age, the seizure phenotype is well established in all animals (Jones et al., 2008). The facility was maintained on a light dark cycle of 12 hours light, 12 hours dark and a temperature range of 19 to 22 degrees. Animals were group-housed until surgery, where they were isolated for the remainder of the study. Ethics approval for this study was granted by Florey Institute Animal Ethics Committee (15-001-UM) and all experiments were carried out in accordance with this approved application.

3.3.2 Electrodes

At age 12-22 weeks EEG electrodes were surgically implanted in all rats to facilitate visualisation of EEG, and measurement of seizures, as previously described (Tringham et al., 2012). Briefly, the rats’ skull surface was exposed and six electrodes (E363/20/SPC Plastics One, Roanoke, VA, USA) were placed approximately 3.0mm anterior to the bregma, 2.0mm left and right of midline; 2.0 and 6.0mm posterior to the bregma, 2mm left and right of midline. The electrodes were inserted into a pedestal, and the entire assembly stabilized with cyanoacrylate. All animals were administered subcutaneously with 4mg/kg carprofen (Rimadyl, Sydney, Australia) both during surgery and post-operatively for pain relief, and the animals were then left for a week to recover.

3.3.3 Diet

Rats were administered a standard rodent diet as listed by Senn et al. (2010) for one week before the first week of EEG recordings, then given a diet premixed with either 0, 5 (low dose), 10 (medium dose) or 20g/kg (high dose) of VPA (Sigma Aldrich, St Louis, MO, USA) for one week before the second week of recordings. These doses were chosen based on the average amount of food eaten per day and the average body weight of the rats. Each group had either 5 or 6 rats receiving VPA 0g/kg (n=6), 5g/kg (n=5), 10g/kg (n=6), 20g/kg
On average, the rats ate 15 g of food per day with an average body weight of 160 g, which meant that from the amount of drug premixed in the food they received was estimated to be around either: 0, 500, 900 or 1900 mg/kg of VPA.

3.3.4 Study Timeline

For all rats, 24-hour EEG recordings took place twice per week. This entailed connecting the animal’s headpiece to an amplifier via a cable (Plastics One), and then to a computer. The EEG was visualized using Compumedics Profusion software. 2 x 24 hour EEG was recorded one week prior to treatment initiation to confirm all animals exhibited seizures and in order to obtain a baseline number of seizures. The following week rats were treated with either 0, 5, 10 or 20 g/kg of VPA. After one week of drug treatments another 2 x 24 hour recordings were conducted. 6 hours of each 24 hour recording were analysed (3 hours during the day, 3 during night). EEG recordings were analysed using SpikeWave Complex Finder (SWC, PLC van den Broek, Nijmegen, Netherlands) by an investigator blinded to treatment.

3.3.5 Blood serum levels

VPA levels in blood serum were assessed on the early morning of the final day of treatment. Animals were sacrificed by carbon dioxide (CO$_2$) euthanasia and a cardiac puncture was performed to sample the blood. The blood was left at room temperature for approximately 20 minutes and then spun at 2000 rpm in the centrifuge. Serum was stored at -80 degrees, before VPA levels were measured using AxSYM Valproic acid assay based on fluorescence assay immunoassay technology (performed at Melbourne Pathology, Royal Melbourne Hospital).

3.3.6 Statistical Analysis

Paired t-tests were performed to investigate whether there was a significant difference between the number of seizures before and after consumption of VPA at each dose (Figure 2.). Rat mean spike wave discharge frequency and percentage of time in seizure was then compared between groups i.e. 0, 5g/kg, 10g/kg and 20g/kg doses using a one-way ANOVA test.
3.4 Results

3.4.1 Food consumption
Food intake was not significantly different between the four groups of rats. Based on rat weight and the amount the rats ate per day, the average dose each rat received per day at 5, 10 and 20g/kg was: 308, 651, 1180 mg per day respectively. The data was normalised, based on starting rat weight (food intake/rat weight). It is illustrated in Figure 1.

![Graph showing normalised daily food intake for different doses of VPA](image)

**Figure 1.** The average amount of food eaten each day for each dose (normalised based on rat weight). The presence of VPA in food does not influence the feeding patterns of rats. Values represent mean ± SEM. 2 rats were not included in this analysis (1 control, 1 5g/kg exposed).

3.4.2 Seizure Suppression
When comparing the average number of baseline seizures and VPA exposed seizures at each dose, there was only a significant reduction of seizures at 20g/kg (Figure 2) \(P=0.0201\). When comparing the number of seizures between the doses the number of seizures were significantly lower for the 20g/kg group \(P=0.0395\) (Figure 3 a). The percentage of time in seizure was not significantly reduced for any of the groups.
3.4.3 Number of seizures per hour

**Figure 2.** Mean seizure frequency per hour in baseline recordings compared with VPA exposed recordings. a) Baseline compared with 0g/kg b) Baseline compared with 5g/kg c) Baseline compared with 10g/kg d) Baseline compared with 20g/kg. Each is represented as an average of the 2 x 24 hour recordings. Values represent mean ± SEM.

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**Figure 2.**

- **a)** Baseline compared with 0g/kg
- **b)** Baseline compared with 5g/kg
- **c)** Baseline compared with 10g/kg
- **d)** Baseline compared with 20g/kg

Values represent mean ± SEM.
Figure 3. Mean seizure frequency in terms of number of seizures per hour (Figure a) and percentage of time in seizure (Figure b) was similar between the 0, 5 and 10g/kg VPA doses but significantly reduced in the 20g/kg dose. Values represent mean + SEM.

3.4.4 Valproate blood levels

The animals not administered any VPA had no VPA in their blood. The cardiac blood serum concentrations increased linearly with the increase in dose (see Figure 4). Three cardiac samples were no able to be assayed due to haemolysis. The tail vein samples were only able to be analysed for three of the samples due to haemolysis. The three tail vein samples that were able to be analysed and these were compared to values derived from cardiac samples: 10g/kg: cardiac was: 117.88umol/L tail vein was: 138.68umol/l, 20g/kg cardiac was: 104.01umol/L tail vein was 55.47umol/L and 20g/kg cardiac was: 277.37umol/L tail vein was 284.305umol/L.

![VPA blood levels at 0, 5, 10 and 20g/kg](image)

Figure 4. VPA blood serum levels taken from cardiac samples on final day of exposure for 0, 5, 10 and 20g/kg VPA. VPA serum levels increased linearly with each increase in dose.

3.5 Discussion

This valproate dose/response study produced two main findings: administering VPA via the rat diet at doses between 5g/kg and 20g/kg is an effective method of chronic delivery which results in dose-dependent increases in plasma blood levels. Secondly, doses of 20g/kg, leading to plasma levels of 104-284umol/L significantly reduced seizures. These findings can be used for future studies aiming to administer continuous VPA in a minimally invasive fashion to assess for drug efficacy, side effects and teratogenicity. Further, we pro-
vide a methodological template which can be extrapolated for chronic treatment of other drugs in rodents.

Previous studies administering acute VPA to GAERS have observed a reduction in seizures (Marescaux, Vergnes, & Depaulis, 1992). This has been demonstrated regardless of the method of administration. One study found a similar reduction in seizures in GAERS rats both from both intermittent (intravenously via jugular vein cannula) and chronic (EEG/infusion cable) administration (Dedeurwaerdere et al., 2011). Similarly, VPA was found to reduce pilocarpine (PILO) induced seizures at doses of 600mg/kg (Leite & Cavalheiro, 1995) and cause a significant reduction in seizures when administered via osmotic mini-pumps for 5 days after administration. In that particular study, this method did result in tolerance to the drug after despite consistent VPA levels. Although seizure frequency at different time points was not assessed in this study, the two 24-hour EEG recordings were conducted on week 2 of drug administration and the number of seizures per hour is an average of 3 hours during the day and 3 hours during the night in each 24-hour time period.

VPA is used to treat both a multitude of seizure types: generalized, partial, convulsive and non-convulsive, as well as a variety of non-seizure related diseases. It works through numerous mechanisms in the central nervous system (Cecilie U. Johannessen, 2000). Although its exact mechanism has not yet been elucidated, there is sufficient evidence that one of its primary mechanisms is reducing excitability in the brain by potentiating the most abundant inhibitory neurotransmitter, GABA (R. L. Macdonald & Bergey, 1979). It is postulated that it does this by inhibiting enzymes that normally degrade GABA; GABA transaminase and succinic semialdehyde dehydrogenase (Harvey, Bradford, & Davison, 1975), increasing the synthesis of GABA and increasing GABA turn over (Loscher, 2002). Some studies have contradicted this, finding that GABA did not increase in the occipital lobe of patients exposed to VPA (Petroff, Rothman, Behar, Hyder, & Mattson, 1999) and that GABA only increased at high doses in rodents (Morre, Keane, Vernieres, Simiand, & Roncucci, 1984) however it is possible that these findings are specific to the regions studied and do not explain GABA levels across the whole brain (Loscher, 2002).

Other speculative mechanisms of the action of VPA include: reducing neuronal firing through voltage gated sodium channels (McLean & Macdonald, 1986), suppressing NMDA induced excitation and glutamate responses (Martin & Pozo, 2004; Zeise, Kasparow, & Zieglgansberger, 1991), reducing aspartate in the brain (Morland, Nordengen,
& Gundersen, 2012), receptor induced M- current suppression (Kay, Greene, Kang, Kosenko, & Hoshi, 2015) and alteration of other monoamine levels such as serotonin and dopamine (Baf, Subhash, Lakshmana, & Rao, 1994). Although these mechanisms may explain why VPA is effective in suppressing generalized and partial convulsive seizures they do not specifically explain the mechanism behind non-convulsive absence seizures (Kelly, Gross, & Macdonald, 1990), the focus of this study.

Absence seizures are generalized non-convulsive epileptic disruptions of neuronal activity. During an absence seizure spike wave discharges (SWD) occur. These SWD’s consist of two transient stages; the spike which is associated with action potential firing and the wave which is a secondary neuronal silence (Steriade, Contreras, & Amzica, 1994). Other drugs that reduce absence seizures such as ethosuximide have been found to block T-type calcium channels in thalamic neurons, however VPA was not found to do this (Coulter, Huguenard, & Prince, 1989). VPA was found to have this affect, however, in nodose ganglion neurons at a therapeutic concentration and it may therefore be that it is reduction of T-current in specific regions, and not globally, that is a contributing factor to absence seizure suppression (Kelly et al., 1990). Another possible way VPA may suppress absence seizures is by inhibiting γ-hydroxibutrate (GHB), a neurotransmitter that has been shown to elicit seizures in animal models of absence epilepsy (Sned, 1988). Further studies are required to better understand the mechanism behind this VPA induced absence seizure suppression.

Absence seizures were chosen as the seizure type to assess for this study because the GAERS model is a well-established animal model that allows to easily quantify seizure frequency. It more closely resembles a clinical setting in that the rats are exhibiting seizures constantly and allows for an effective way to monitor seizure suppression over long time periods. In GAERS rats, SWDs occur in the somatosensory cortex, and begin in layers 5 and 6 (Polack et al., 2007). This is not dissimilar to humans where absence seizures are initiated in cortical rather than thalamic regions (M. D. Holmes, Brown, & Tucker, 2004; Petsche, 1962; Sadleir, Farrell, Smith, Connolly, & Scheffer, 2006), another reason why this model is clinically relevant.

A particularly important property of VPA is that it is a potent teratogen increasing the risk of birth defects. This teratogenicity has been studied in animal models administering VPA to female rodents on between days 8-10 of pregnancy, and assessing pups for birth defects (Briner & Lieske, 1995; Ceylan, Duru, & Ceylan, 2001; Ehlers, Sturje, Merker, & Nau,
1992a; Ehlers et al., 1992b; Mahabady et al., 2011). All such studies administer VPA via IP injection. This route of administration during and prior to pregnancy can have stress related effects on the mother and pups that may cause abnormal pregnancy outcomes not related to the drug. Further, administering VPA between days 8-10- the time of neural tube formation—does not closely mimic human circumstances. An animal model where rats are administered the drug via their diet consistently before and during pregnancy does not entail these risks and would be a valuable tool to assess VPA induced teratogenicity.

3.6 Conclusion

This is the first study to assess the seizure suppression effects in GAERS of VPA administered VPA through the rat diet. It has confirmed the efficacy of mixing VPA in the food as an inexpensive, non-invasive, effective way of administering VPA chronically to epileptic rats. This approach will facilitate further research in the effects of chronic VPA administration including of teratogenicity in rats that are impregnated while receiving VPA. Future studies may further validate VPA blood levels by assessing them across different time points and assessing the efficacy of higher doses as well as other AEDs when administered via the rat diet.
Chapter 4

Effects of gestational sodium valproate on rodent offspring anatomy

4.1 Abstract

**Background:** Valproate (VPA) is a potent human teratogen increasing the risk of a range of congenital malformations including neural tube, skeletal, cardiac, urogenital and orofacial defects. The mechanism underlying this teratogenicity is not known. This study aimed to develop an animal model that replicates a human clinical setting by using both epileptic and non-epileptic strains of rats; therapeutic doses (determined in chapter 3) and chronic administration of VPA via the rat diet.

**Methods:** Three strains of rats were used in this study: An inbred epileptic strain of rats: Genetic Absence Epilepsy Rats from Strasbourg (GAERS), an inbred non-epileptic strain: non-epileptic controls (NEC) and an outbred non-epileptic strain: Wistar. Females in all three strains were administered either control diet or VPA (20g/kg) mixed diet for approximately two weeks prior to conception and for the duration of the pregnancy. Pups were extracted via C-section on day 21 of pregnancy and assessed for birth defects via: visual external assessment, internal spinal assessment, H&E staining of neural tube closure and whole pup histopathological analysis.

**Results:** Through external visual assessments it was found that there was significant reduction in weight, length and visual external assessment scores in VPA exposed pups compared with non-exposed pups in all three strains ($P<0.0001$). Internal spinal assessments revealed missing vertebral arches between T11 and C2 predominantly in VPA exposed pups in GAERS, NEC and Wistars (100%, 95% and 80% respectively) compared with controls (9.09%, 13.3%, 19.04% respectively).

**Conclusion:** This animal model is the first to administer therapeutic doses of VPA to epileptic rats chronically via the rat diet and assess pregnancy outcomes. Clear morphological dif-
ferences were found between VPA exposed compared with unexposed pups. This model may be used in future studies to reveal mechanisms involved in the pathogenesis of AED induced birth defects.

4.2 Background

Valproate (VPA) is a well-established human teratogen increasing the risk of birth defects by up to 17-fold (Vajda, O’Brien, Hitchcock, Graham, & Lander, 2003). Animal models of VPA induced birth defects have been used to assess teratogenicity since the 1980’s. These include rat, mouse, rabbit, monkey, xenopus and zebrafish models both in vivo and in vitro (Gurvich et al., 2005; Hendrickx et al., 1988; Menegola et al., 1996). From such animal models it is evident that birth defect presentation changes depending on the dose, time point of administration during gestation and method of administration (D. S. Hill et al., 2010). In human studies, VPA monotherapy is associated with a significantly increased risk of 6 main malformation types; spina bifida, atrial septal defect, cleft palate, hypospadias, polydactyly and craniosynostosis (Jentink et al., 2010). These defects are reflected to an extent in current animal models with the most common malformations in rat and mouse studies being defects of the ribs, vertebrae, craniofacial bones and digits (D. S. Hill et al., 2010).

Previous rat and mouse studies have assessed VPA teratogenicity using a broad range of doses and strains resulting in malformations of varying degrees of severity. Sprague-Dawley rats administered VPA at 400mg/kg/day exhibited cardiovascular, vertebral, rib, hypoplastic bladder, ectodactyly and hydronephrosis while higher doses resulted in embryo lethality and lower doses resulted in less severe malformations (Vorhees, 1987). Wistar rats administered VPA at doses of 300mg/kg on the 8-9th day of pregnancy resulted in spina bifida as well as cleft palate and exencephaly (Mahabady et al., 2011) while Wistar rats administered doses four times higher resulted in spina bifida occulta in 100% of exposed rats with 3% also having exencephaly (Ceylan et al., 2001). Similarly, mouse studies have found variable results depending on the strain with some strains exhibiting more fused ribs while others exhibiting less (Faiella et al., 2000). In mice, administration of high doses of VPA via IP injection on day 9 resulted in higher rates of spina bifida aperta compared with spina bifida occulta while low doses resulted in higher rates of spina bifida occulta than spina bifida aperta (Ehlers et al., 1992b). In a study comparing mice to rats, mice were found to be more sensitive to the teratogenic effects of VPA with 84% of mice exhibiting defects compared with 43% of rats (Menegola et al., 1996).
Other animal systems used to assess VPA teratogenicity include monkey, rabbit and zebrafish models. Rhesus monkeys administered VPA 20-600mg/kg presented a dose related increase in fetal/embryonic death, growth inhibition and skeletal and craniofacial malformations (Hendrickx et al., 1988). Rabbits administered VPA at 350mg/kg exhibited malformed and extra vertebrae and ribs as well as missing pollices (innermost digit of forelimb) (Petrere, Anderson, Sakowski, Fitzgerald, & de la Iglesia, 1986). Zebra fish models show that zebrafish embryos exposed to VPA exhibit altered locomotor activity and retinotectal projection area in optic tectum (Cowden et al., 2012). While these models replicate the clinical observation that VPA exerts teratogenic effects, they do not use doses that would necessarily be therapeutically effective and most often do not expose to the drug chronically to account for the short half-life of VPA in animal species.

The importance of having a clinically relevant model has been addressed early on in the literature by Nau, Zierer, Spielmann, Neubert, and Gansau (1981). The authors highlighted the limitations of conventional animal models and the fact that these models should reflect a human therapeutic setting. In their study, they assessed VPA teratogenicity in NMRI mice and used osmotic mini-pumps for a constant release of VPA at various doses in one group and injected doses of VPA in another group. They found that there were vast differences in the results; multiple dosing resulted in many malformations with the most severe being exencephaly. The constant rate application of VPA via mini-pumps resulted in less severe malformations and no exencephaly. Both groups showed weight loss. Although they have drawn attention to the importance of clinical relevance, their model and those preceding it do not have the advantage of using animals that exhibit seizures and that are therefore exposed to VPA specifically to suppress their seizures. Having this group as well as non-epileptic groups not only allows to better understand the effect of the epilepsy but also to replicate the specific biological effect of VPA in humans.

Although previous studies have assessed VPA teratogenicity in animal models, no study to our knowledge has used epileptic rats, nor doses that result in seizures suppression and therapeutic blood levels and administration via the rat diet. This study aims to assess the phenotype of epileptic as well non-epileptic rat pups exposed to VPA chronically under these circumstances.
4.2 Materials and Methods

4.2.1 Animals

Female and male GAERS_{MELB} (Powell et al., 2014) (n=10) and inbred non-epileptic controls (NEC) (n=14) rats were obtained from our breeding colonies at the Department of Medicine, Royal Melbourne Hospital, University of Melbourne Biological Research Facility. Outbred Wistar rats (n=10) were obtained from the Animal Resource Center (ARC), Western Australia. All rats were obtained at approximately 9 weeks of age and were kept under a 12-hour light-dark cycles using artificial lighting at room temperature (22-24 degrees). Ethics approval was obtained from Florey Animal Ethics committee. All experiments were carried out according to these guidelines.

4.2.2 Diet and Drug Treatments

Animals in each of the three strains were fed a standard rodent diet as listed by Senn et al. (2010) pre-mixed with either a 0 dose (GAERS n=5, NEC n=8, Wistar N=4) or 20g/kg of VPA (GAERS n=5, NEC n=6, Wistar n=6) at approximately 9 weeks (2 weeks before the animals were mated). After 2 weeks, female rats were mated with males of the same strain in mesh cages over night for as many nights as it took to mate. Males were exposed to the drug only for the duration of mating. The presence of a copulatory plug indicated day 0 of pregnancy. Once the plug was present, females were again separated from the males for the course of pregnancy. Drug treated females were continued to be administered VPA mixed diet throughout the rest of the pregnancy.

4.2.3 VPA blood serum levels

A cardiac puncture was performed on all mothers on the day of the C-section to sample the blood. The blood was left at room temperature for approximately 20 minutes and then spun at 2000rpm in the centrifuge. Serum was stored at -80 degrees, before VPA levels were measured using AxSYM Valproic acid assay based on fluorescence assay immunoassay technology (performed at Melbourne Pathology, Royal Melbourne Hospital).

4.2.4 Teratology studies

The foetuses were extracted from the uterus on day 21, 1 day before expected birth. The dams were sacrificed via carbon dioxide (CO2) euthanasia, a cardiac blood sample taken (to assess for VPA blood levels) and the pups, placenta, liver, brain, ovaries and heart were removed and stored for possible future analysis. Pups were sacrificed by freezing. Pup
implantation sites were recorded, foetuses were photographed, weighed and crown-rump length recorded. Each animal was assessed for external malformations such as external spina bifida, exencephaly and any signs abnormal development. For approximately half of the litters, brains were removed and stored for subsequent mRNA analysis. For the other half of the litters, whole pups were stored in 4% PFA followed by 70% ethanol to be used for histopathological analysis, micro CT scans and H&E stains. The fathers were also sacrificed on the day of C-section, a cardiac sample taken, liver, brain and heart removed and stored.

4.2.5 Double staining studies
To assess for spina bifida occulta, cartilage and bone of foetuses were stained using the method described by Wassersug (1976). Foetuses were put in 10% formalin solution with a buffer of magnesium carbonate for 2 days and then skinned and eviscerated. The pups were then washed several times and stained with alcian blue and alizarin red. The staining process was carried out La Trobe University. Initial pups were stained using the process described by Wassersug (1976) but the protocol was then tweaked in an attempt to obtain better images. The following was the altered protocol: Stain for 72h with 0.01% alcian blue. Gradual rinse in reducing concentrations of ethanol for ~6h (70%, 40%, 15%, water for 2h each). 48h in 2% KOH. Put in PBS for 24 hrs. 0.001% alizarin red in 1% KOH check after 4h and 8h, possibly incubate for 24h. 3 rinses in 1% KOH for 2h. 24h graded glycerol in dH20 (20% glycerol, 50%, glycerol, 80% glycerol). The foetuses were imaged under stereomicroscope using Leica IM50, Version 5 Leica Microsystems AG. The distance between the cartilaginous ends of the vertebral arch were measured using LeicaQWin Standard V3.5.1, Leica Microsystems (Switzerland).

4.2.6 Histopathology
Histopathological analysis was carried out at The University of Melbourne, Department of Medicine for 6 GAERS control rats and 6 GAERS VPA exposed rats. Evaluation was carried out for thoracic and abdominal organs, skeletal tissue, nasal/oral region, brain, eyes and auditory/vestibular apparatus. Sections were taken from the midline, laterally through the body. Rostral sections were taken through the head. Sections were 5um thick and at 200um intervals. Dr John W. Finnie, senior veterinary pathologist at the University of Adelaide also provided a supplementary pathology report.
4.2.7 Haematoxylin and Eosin (H&E) staining

H&E staining was done at The University of Melbourne, Biomedical Sciences Histology Facility. Pups were embedded in paraffin and transverse serial sections of 5 microns were taken of the lower part of the spine. A test pup was sectioned and images were assessed by a collaborator and La Trobe University to determine where to start sectioning in order to assess the lower part of the spine. Sections were imaged using Leica IM50, Version 5 Leica Microsystems AG microscope and were scored as either being “open” or “closed”. For each strain, 8 VPA and 8 control pups were analysed (A total of 48 pups). These came from 12 litters (2 litters in each group).

4.2.8 Statistical Analysis

Mann Whitney Tests were used to compare the control groups with the VPA exposed groups in all three strains for the food intake, visual assessment scores, pup weight and length. Two-Way Anova was used to compare the effect of strain on weight and length and demonstrated using profile plots. Mann Whitney tests were also used to compare the number of vertebral arches in the lumbar and sacral regions.

4.3 Results

Results for each section have been presented for which rats/pups the data was available. Differences in n numbers for different data sets in the same group can be attributed to unavailability of data.

4.3.1 Food consumption

Food intake of females was measured to ensure there were no major changes in intake. There was no difference in GAERS regardless of which diet they were exposed to ($P=0.4857$). However, the NEC and Wistar rats fed the VPA diet consumed less food that their control diet counterparts. This difference reached statistical significance for NEC rats ($P=0.0025$) and was approaching significance for the Wistars ($P=0.0571$). The n number for the Wistar rats was small (n=3) and may explain why the difference was not significant. This data has been normalised based on rat weight (food intake/rat weight) and is illustrated in Figure 1.
Figure 1. Average daily food intake of female a) GAERS (control n=4, VPA n=4), b) NEC (control n=7, VPA n=5) and c) Wistars (control n=4, VPA n=3) prior to pregnancy. A Mann Whitney Test was used to test for significance. Data is presented as median+range.

4.3.2 VPA blood serum levels

A cardiac blood sample was taken on the day of the Caesarean-section immediately after the female was sacrificed. The blood sample was used to confirm how much VPA was in the blood of each animal and to confirm there was no VPA in the control animal blood. The range for GAERS rats was 109.56umol/L-376.53umol/L for NECs was 251.02umol/L-467.37umol/L and for Wistars rats was 216.35umol/L -398.03 umol/l. There was no VPA in the blood of controls. This data is illustrated in Figure 2.

Figure 2. VPA blood levels obtained from a cardiac sample for the a) GAERS (control n=5, VPA n=5) b) NEC (control n=5, VPA n=6) c) Wistars (control n=4, VPA n=6).
Figure 3. Images were taken at the time of C-sections and include all pups in each litter. a) GAERS control litter, b) GAERS VPA exposed litter c) NEC control litter d) NEC VPA exposed litter e) Wistar control litter f) Wistar VPA exposed litter.
4.3.4 Pup visual external assessment scores

A morphological scoring system (Figure 6) was developed to determine the overall development of each pup. The scoring system was modified from one used for toxicology screening in zebrafish (Augustine, 2011). Pups with a score of 1 were a small structure-less attachment to placenta or a clear reabsorption. It was difficult to distinguish between a full reabsorption and something more but any attachment with no structure was given a score of 1. The average pup scores are presented in Figure 7.

**MORPHOLOGICAL SCORING SYSTEM:**

4 = structure is entirely normal and pup are fully developed
3 = structure is normal but pups look unhealthy and underdeveloped
2 = some structure is evident but pups are severely underdeveloped
1 = no structure is evident, the pups is only small attachment to placenta or a reabsorption

![Images of pups with scores 4, 3, 2, and 1]

**Figure 4.** The scoring system used to score all pups extracted in the study. Adapted from (Augustine, 2011).

![Graphs showing average morphological scores for GAERS, NEC, and WISTAR]

**Figure 5.** Pup visual external assessment scores for a) GAERS (control n=5, VPA treated: n=5), b) NEC (control n=8, VPA treated n=6) and c) Wistars (control n=4, VPA treated: n=6). Mann Whitney Test was used to test for significance. Data is presented as median+range.
4.3.5 Average pup weight and length

Pup weight and length was measured on the day of the C-section. There was a significant reduction in birth weight \( (P<0.0001) \) and length \( (P<0.0001) \) in VPA exposed pups compared with controls in all three strains. This data is presented in Figure 8 (weights) and Figure 9 (lengths). A Two-Way Anova was conducted to compare the interaction effect of strain and treatment on the weight and length of control groups compared with VPA exposed groups in all three strains.

Figure 6. The average pup weight on the day of C-section for a) GAERS (control \( n=4 \), VPA treated \( n=4 \)), b) NEC (control \( n=5 \), VPA treated \( n=6 \)) and c) Wistars (control \( n=3 \), VPA treated \( n=6 \)). Pups with an external assessment score of 1 or below were not weighed. Mann Whitney Test was used to test for statistical significance. Data is presented as median + range.

Figure 7. Average crown-rump length for a) GAERS (control \( n=2 \), VPA treated \( n=2 \)) b) NEC (control \( n=4 \), VPA treated \( n=5 \)) and c) Wistars (control \( n=2 \), VPA: \( n=4 \)). Similar to for the birth weight, a Mann Whitney Test was used to test for significance. Data is presented as median+range
Figure 8. Profile plots from a two-way Anova comparing the interaction effect of strain and treatment on the weight in g (figure a) and length in mm (figure b) of control groups compared with VPA exposed groups in all three strains. Both plots show that the effect of VPA for weight and length is the same for Wistars and NECs (red and green) but less for the GAERS (blue). On average the VPA exposed pup lengths were reduced by 4.2659, 6.3367 and 7.0064 mm in GAERS, NEC and Wistars respectively. On average the VPA exposed pup weights were reduced by 0.9675, 1.5366 and 1.8064 g respectively.

4.3.6 Distances between the vertebral arches

The average horizontal distance between the vertebral arches was measured. Spinal measurements were included for all vertebral arches where information was available. If arches were missing or information was not available for certain arches all other measurements in that spine were still included. A Mann Whitney test was used to compare the average distance between VPA exposed and controls and each vertebral arch, a method used in previous studies (Ceylan et al., 2001; Ehlers et al., 1992b). Differences may have been attributable to differences in pup size.
**Figure 9.** The average horizontal distances between the ends of the vertebral arches for a) GAERS control (n=16 pups from 3 litters) GAERS VPA (n=17 pups from 3 litters), b) NEC control (n=41 pups from 6 litters) NEC VPA (n=26 pups from 4 litters) and c) Wistar control (n=24 pups from 3 litters), Wistar VPA (n=27 pups from 4 litters). Data is presented as mean distance between each vertebral arch + SEM. Black lines indicate control pups and coloured lines VPA exposed. *P*-Values were calculated at each in individual vertebrae. There was a significant difference between vertebral arch distances for control pups compared with VPA exposed pups for the GAERS rats at: T12 (**P=0.0292) T13 (**P=0.0048), L5 (*P=0.0189), S1 (*P=0.0235) and S2 (**P=0.0004). NEC rats between: T11 (****P<0.0001), T12 (P=0.0001), T13 (****P=0.0002), L1 (****P<0.0001), L2 (****P=0.0002), L3 (*P=0.0175), S2 (**P=0.0015) Wistars between: T11 (****P=0.0004), T12 (****P=0.0001), T13 (P=0.0007), L1 (P=0.0188), L2 (*P=0.0161), L3 (**P=0.0018), L4 (P=0.0003), L5 (*P=0.0383).

### 4.3.5 Missing vertebral arches

When measuring the distances between the vertebral arches it became clear that a number of pups were missing vertebral arches. In the lumbar region, there was a significant reduction in the number of vertebral arches in the VPA exposed pups compared with the controls for the NECs (P=0.0425) and Wistars (P=0.0107) however there was no significant difference for the GAERS (P=0.4848). This data is shown in **Figure 10.** In the sacral region, there was a significant difference between VPA exposed and controls in GAERS, NEC and Wistars (P=0.0035, P<0.0001, P=0.0016 respectively). The data is shown in **Figure 11.** Overall, between T11 and C2 there was a higher number of VPA exposed GAERS, NEC and Wistar pups missing vertebral arches (100%, 95% and 80% respectively) compared with controls (9.09%, 13.3% and 19.04% respectively) (**Table 1**). Only pups with full spinal data were included in the analysis. Full spinal data was not always available due to the quality of the images. Pups with no obvious missing vertebral arches but with no full spinal data available were excluded from the total number (to prevent inclusion of pups missing vertebral arches that were not able to be identified). Some of the pups were missing only caudal vertebrae (C1 and or C2); 4 GAERS VPA, 3 NEC controls, 2 NEC VPA, 4 Wistar controls and 3 Wistar VPA. In addition to the total number, another 3 GAERS VPA, 2 NEC control, 3 NEC VPA and 4 Wistar VPA were missing vertebral arches but were amongst the group that did not have full information on their spines available. Examples of pups with missing vertebral arches in each strain are shown in **Figure 11.**
Figure 10. The number of vertebral arches missing in the lumbar region in a) GAERS control (n=16 pups from 3 litters), GAERS VPA (n=17 pups from 3 litters), b) NEC control (n=41 pups from 6 litters), NEC VPA (n=23 pups from 4 litters) and c) Wistar control (n=24 pups from 3 litters), Wistar VPA (n=27 pups from 4 litters). Mann Whitney Test was used to test for significance. Data are presented as median+range.

Figure 11. The number of vertebrae in the sacral region of a) GAERS control (n=13 pups from 3 litters), GAERS VPA (n=16 pups from 3 litters), b) NEC control (n=36 pups from 6 litters), NEC VPA (n=23 pups from 4 litters), c) Wistar control (n=24 pups from 3 litters), Wistar VPA (n=24 pups from 4 litters). A Mann Whitney Test was conducted to test for significance. Data are presented as median+range.
Table 1. Number of pups missing one or more vertebral arches in thoracic (T11-T13), lumbar (L1-L6), sacral (S1-S4) or caudal (C1-C2) regions of the spine

<table>
<thead>
<tr>
<th></th>
<th>GAERS</th>
<th>NEC</th>
<th>WISTAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>1/11 (9%)</td>
<td>4/30 (13%)</td>
<td>4/21 (19%)</td>
</tr>
<tr>
<td>VPA</td>
<td>12/12 (100%)</td>
<td>19/20 (95%)</td>
<td>16/20 (80%)</td>
</tr>
</tbody>
</table>

Table 1. Pups that were missing any regions of their spine between T11 and C2 for which full spinal data was available.

Figure 12. Examples of pups with missing vertebral arches a) GAERS VPA exposed pup with missing vertebral arches between T10 and L3 b) NEC VPA exposed pup with no more vertebral arches present after L5) c) WISTAR VPA exposed pup missing vertebral arches at L4 and L5.
4.3.7 Histopathology results

The 6 GAERS control pups showed no lesions of significance. 1/6 of the GAERS VPA had an internal malformation that was not affected by autolysis or tissue degradation. Following the initial analysis by Histopathology at The University of Melbourne, Dr. John W. Finnie a senior veterinary pathologist at the University of Adelaide analysed the pup and summarised the following: “There was a much smaller brain than would be expected at this stage of gestation with a deficiency of some neural elements, constituting a diagnosis of microcephaly. The subarachnoid space was greatly expanded in size which was interpreted as an ex vacua lesion, the space enlarged as a result of the much smaller brain the cranial cavity. Similarly, the ventricles were dilated in response to the cerebrospinal fluid pressure and a deficiency of surrounding brain parenchyma into which the ventricles had expanded.”

![Image](#)

**Figure 13** a) An external image of rat #1406 pup 9 and b) the enlarged subarachnoid space, distorted cortex and ventricular dilation of the brain of #1406 pup 9.

4.3.8 H&E staining results

100% of GAERS, NEC and Wistars in both control and VPA groups had a formed closed spinal cord. 3 pups were excluded these were: 1 GAERS control, 1 NEC VPA and 1 NEC control due to unclear stained sections. Example images for each strain have been presented in Figure 14.
Figure 14. H&E stained images of the spinal cord. a) GAERS control b) GAERS VPA c) NEC control d) NEC VPA e) Wistar control f) Wistar VPA. The position of the neural tube has been indicated as “nt”.

4.4 Discussion

This study has developed a viable animal model that reflects the setting of human women who become pregnant while being treated for epilepsy with VPA. It can be used to assist in identifying factors involved in the pathogenesis of VPA induced teratogenicity. While other animal models do exist, they do not adequately reflect the clinical setting. The presentation of AED induced birth defects is multifaceted and likely to have a complex
pathophysiology meaning it is critical that all potential variables are taken into account. The present model used epileptic strain of rats, doses that were found to be therapeutically effective (from Chapter 3) and a chronic method of administration via the diet. It resulted in clear external morphological differences between exposed and unexposed pups as well as internal vertebral anomalies similar to those observed in humans. The model is currently in the process of being used to assess for mRNA changes in epileptic and non-epileptic rat pup brains exposed chronically to VPA in utero and may be used in the future to assess for any number of potentially involved biological mechanisms.

The rationale for using the GAERS and NEC rats was that the two strains are genetically similar except that GAERs rats have epilepsy and NEC rats do not. GAERS rats are a well-established animal model in epilepsy with all rats exhibiting 1.5 seizures a minute (Marescaux et al., 1992). Although it is known that it is the AEDs which are the dominant factor resulting in the birth defects and not the epilepsy, whether or not epilepsy is a contributing factor has not been well studied. In this study, the GAERS rats exhibited less dramatic differences in weight, length and assessment scores than unexposed GAERS control groups compared with exposed and unexposed group from the other strains (see Figure 10). The reason for this is uncertain but does raise the question of whether or not GAERS rats are actually less susceptible to the effects of VPA. Human rates of birth defects are not as severe as what is observed in most rat and mouse studies with a rate of between 6-17.4% of infants exposed having defects (M. J. Eadie, 2008). Although more studies are required to establish this, this may be another indication that the model is more clinically relevant. The advantage of using the Wistar rats was that they are a genetically varied strain, and it was hypothesized that differences in birth defect rates or types as compared with the other two strains could help to narrow down potential causative genomic factors.

The dose of VPA 20g/kg of food, chosen from the pilot study in chapter 3 works out to be on average 1000mg/kg/rat per day. Prior to administering this dose, a higher dose of 40g/kg was administered to ensure high blood levels, however this made the animals sick. It was therefore decided that the lower dose of 20g/kg would be sufficient. Future studies may assess the effects of a dose between 20g/kg and 40g/kg to mimic doses closer to human levels however this dose is similar to doses used in other previous studies resulting in birth defects in mice and rats (Briner & Lieske, 1995; Ceylan et al., 2001). Previous studies administer the doses as injections, however and although seldom reported, would result in different daily blood levels. The food intake of the rats in all three strains was comparable. On
average control GAERS, NEC and Wistar females ate 12.86, 15.24, 20.89g per day respectively and VPA exposed ate 11.53, 11.25, 12.94g respectively. Differences in food intake can be attributed to the size of the rats with the rats’ weights at the start of the study for GAERS rats: 192.4g for NEC rats: 192.22g and for Wistars: 310.11g. The consequent VPA levels in the mothers’ blood were approaching or above human therapeutic levels. Human therapeutic levels are 300-700um/L (C. U. Johannessen & Johannessen, 2003). The range for GAERS rats was 109.56umol/L-376.53umol/L for NECs was 251.02umol/L-467.37umol/L and for Wistars rats was 216.35umol/L -398.03 umol/l. Interestingly the VPA levels were lower in the GAERS strain as compared with the NEC and Wistars. As a group, the GAERS rats also exhibited less dramatic differences in their VPA group compared with control groups in terms of weight and length. They did, however exhibit the highest number of VPA exposed pups with missing vertebral arches. A correlation test revealed there was no association between blood level and average pup weight for any of the groups. The reason for this could be due to the amount the rats were eating or the time of day the rats were eating. The cardiac blood sample was taken at the time of C-section. C-sections were conducted approximately between 10am and 2pm with later times being optimal due to being closer to the time of birth on day 22. One possibility could be that the GAERS rats ate more at night.

Male rats were exposed to VPA for the duration of mating only. This was to limit any possible affect the father’s exposure could have. The amount of time it took for the females to plug was variable. Few studies have looked at the effect of the father having epilepsy or taking AEDs during conception. Very early studies found contradictory evidence regarding the effect of paternal epilepsy; both that there were intermediate birth defect rates in offspring of fathers with epilepsy (Shapiro et al., 1976) and no association (Annegers, Elveback, Hauser, & Kurland, 1975). Since these early studies, it has been firmly established that it is maternal AED use resulting in the birth defects and not the epilepsy however whether or not the father having epilepsy or taking AEDs contributes to this question has gained little attention in the literature. The most recent study looking at this found that children of fathers with epilepsy that were treated with AEDs had a higher risk of having autistic traits and poor social skills compared with children of untreated fathers with epilepsy at 18 months (Veiby et al., 2013). There has been evidence to suggest VPA can reduce motility of sperm and also result in greater abnormalities in sperm (Isojarvi et al., 2004). Whether or not this could have an affect on the subsequent developing foetus remains a question for future studies to answer.
In the present animal model, there were clear morphological differences between VPA exposed and unexposed pups. The average weights and lengths of the exposed rats was significantly lower than the unexposed rats in all three strains. This is in accord with some human studies which have found that children exposed to AEDs in utero have lower birth weight (Hvas, Henriksen, & Ostergaard, 2000; Kilic, 2014). As a group, infants with reduced birth weight have higher rates of neurodevelopmental problems as well as increased illnesses and reduced growth (Hack, Klein, & Taylor, 1995). Neurodevelopmental problems are another established teratogenic effect of VPA and other AEDs (Meador et al., 2013). Future studies may use the model also assess the long-term behavioural effects of VPA.

It should be noted that low birth weight in AED exposed infants is conflicting in the literature however, with some studies finding untreated women with epilepsy also have offspring with reduced birth weight or that are small for gestational age (SGA) (Hvas et al., 2000; Veiby, Daltveit, Engelsen, & Gilhus, 2009). This raises the question of whether epilepsy is having an effect in another way. This was not the case in this study with the GAERS rats not exhibiting greater reductions in weight than the non-epileptic strains thus confirming that in this study it was the VPA causing the reduction in birth weight. Other earlier reports of fetal valproate syndrome (FVS) indicated that 10% of infants with FVS are SGA (Clayton-Smith & Donnai, 1995). Other studies have found VPA polytherapy to result in reduced birth weight and not monotherapy (Jager-Roman et al., 1986) or that drugs other than VPA such as Topiramate result in more prominent differences and less reduction for other AEDs. (Veiby, Daltveit, Engelsen, & Gilhus, 2014). Reduced birth weight is a common finding in rat and mouse studies (Ehlers et al., 1992b; Mahabady et al., 2011) with some evidence to suggest there may be a dose effect (Vorhees, 1987). Other reports have found an increased risk of SGA for VPA and CBZ (Pennell et al., 2012).

There was no significant difference between the average litter size controls compared with VPA exposed groups for any of the three strains. GAERS was 9.6 and 9, NEC was 9 and 10.1 and Wistar was 13.5 and 11.8 for controls and VPA exposed respectively (Mann Whitney Test: $P=0.2684$, $P=0.2031$, $P=0.3810$). There was also no significant difference in the weight of the placentas of VPA exposed compared with control pups and also no significant difference in the weight of the mother’s heart and liver. All three strains also had significantly reduced external assessment scores in their VPA exposed groups compared with unexposed groups. This scoring system was developed based on the types of pups ob-
erved in the study with all pups falling within one of the categories. Although some pups were born severely underdeveloped, no specific obvious external malformations were identified such as external spina bifida (spina bifida aperta), cleft palate or tail malformations. These are birth defect types commonly observed in studies looking at VPA induced teratogenicity (Menegola et al., 1996; Vorhees, 1987). Although some VPA exposed rats had obvious skin malformations with the skin clearly not developed properly, a clear diagnosis for this presentation has not yet been obtained. It is clear from such studies that different strains of rats, different doses of VPA and different methods of administration result in different birth defect types. To our knowledge, no study has looked at VPA teratogenicity in GAERS or NEC rats however two studies has used Wistar rats. Wistar rats administered VPA at doses of 300mg/kg exhibited cleft palate, spina bifida and exencephaly with an incidence of between 17-20% for each malformation type (Mahabady et al., 2011). Wistar rats administered at doses of 1200mg a day exhibited 100% spina bifida occulta and 3% exencephaly (Ceylan et al., 2001). The different results can be attributed to the method of administration and consequent dose received. Similarly, no study has administered VPA via the diet to assess for birth defects. Most recently a study looking at hippocampus cell structure of VPA exposed pups administered VPA to Wistar rats via the drinking water however they did not report on the morphological effects of the drug (Semmler et al., 2017).

The assessment of internal spina bifida (spina bifida occulta) using the staining methods found no obvious sign of spina bifida when measuring the distances between the vertebral arches. There was no way to adjust for pup size for this particular analysis. The more interesting finding was the missing vertebral arches observed throughout the measuring process. Missing vertebral arches or a “gap”, particularly in the lower lumbar and sacral regions is indicative of spina bifida in humans. In rats, like in humans, there are expected to be a certain number of vertebrae in each segment of the spine; in the cervical region; 7, thoracic region; 13, lumbar region; 6, sacral region; 4 and in the he caudal region; between 26-30 (The University of Massachusetts Amherst, Year Unknown). There was a higher number of pups with missing arches in VPA exposed groups compared with controls for all three strains; GAERS (9.09% controls compared with 100% VPA), NEC (16.66% controls compared with 95% VPA) and Wistar (19.04% controls compared with 80% VPA). Interestingly, out of the controls missing vertebral arches 3/5 NECs and 4/4 Wistars were only missing caudal vertebrae.

Vertebral anomalies such as missing vertebral arches have been reported in animal
studies before (Downing et al., 2010; Faiella et al., 2000). The rates at which they occur differ depending on which strain and dose is used. One study assessing B6 and B2 mice observed missing, fused and a-symmetrical vertebral arches and centra (Downing et al., 2010). The prevalence of vertebral anomalies was varied amongst the two strains with a 39% and 94% malformation rate at the 400mg/kg and 800mg/kg doses in B6 mice and 23mg/kg and 53% malformation rate in the D2 mice. A reciprocal cross found vertebral anomalies in both crosses but a higher rate in B6D2 foetuses. The location of the missing arches was not reported. Another study found fusion of vertebral arches rather than the absence of them (Faiella et al., 2000). In both studies, like in the present one, a small percentage of control rats also exhibited spinal anomalies. Other studies have found similarly high rates of spina bifida occulta when administering their highest doses. One study using Wistar observed spina bifida occulta at a rate of 100% in rats treated with 2 x 600mg/kg VPA (Ceylan et al., 2001), and another using found a 95% rate in mice administered 3 x 500mg VPA (Ehlers et al., 1992b). Spina bifida occulta affects the bone and soft tissue and is prevalent in adults. The reported incidence varies and has been reported to be as broad as between 1.2-50% (Eubanks & Cheruvu, 2009). The most common region of spina bifida occulta in humans is the lumbosacral region of the spine and it is most often not regarded as significant, particularly at S3 to S5 where variations are considered as normal (Fidas et al., 1987). This may explain the small percentage of control rats which appeared to have smaller distances in the sacral region (particularly in S2) in our study, as well as in the previous studies discussed. These may have been normal anomalies that coincidentally were higher in controls than treated rats.

The H&E staining analysis found that the VPA did not affect the spinal cord with 100% of the pups having a closed neural tube/spinal cord. This suggests that the form of spina bifida that was occurring was spina bifida occulta which primarily affects bone and soft tissue (Fidas et al., 1987). The reason for this may have been that the dose was too low or that this is the type of malformation the rats were susceptible to.

From the pup histopathological analysis, it was found that one of the VPA exposed GAERS pups assessed had microcephaly. Microcephaly is an abnormally small head circumference which may also be associated with abnormal brain development. It is defined as a head circumference that is either 2 or 3 standard deviations (SD) below the mean. The prevalence of microcephaly in Australia is approximately 5.5 per 10 000 births and 80% of those diagnosed have another concomitant major birth defect (M. Hansen, Armstrong,
Bower, & Baynam, 2017). Microcephaly has been associated with AED induced birth defects in humans, however it has been particularly associated with Topiramate and less so with VPA specifically (Veby et al., 2014). One study found an increased risk of microcephaly for women taking AED polytherapy combinations but not AED monotherapy (Almgren, Kallen, & Lavebratt, 2009). While there was one pup with microcephaly in the small group assessed, it was only a single specimen and so strong conclusions should not be drawn.

As previously mentioned, this model is currently being used to assess mRNA changes in VPA exposed pups compared with VPA non-exposed pups. RNA was extracted from 8 control and 8 VPA exposed GAERS and NEC pups to assess for differences in exposed compared with unexposed brains. The Wistar rats were not included in this part of the analysis as it was expected that the NEC group would be a sufficient control for the early stages of the sequencing and that the Wistars may be used in subsequent analysis. DNA has been the main focus of research until recent times when there has been a shift towards RNA sequencing. RNA sequencing allows us to identify changes in the RNA editing process. RNA editing is a process that occurs post transcriptionally and involves alteration of nucleotides. These changes have the potential to affect the function of important proteins. In this case, we hypothesize that VPA may be causing changes to the RNA editing process that result in differentially expressed proteins and consequently structural defects.

In epilepsy research, RNA sequencing has been used to identify differentially regulated genes in human patients with mesial temporal lobe epilepsy-hippocampal sclerosis (Dixit et al., 2016), and spinal muscular atrophy with progressive myoclonic epilepsy (Kernohan et al., 2017). Mouse models assessing RNA sequencing have assessed status epilepticus (K. F. Hansen, Sakamoto, Pelz, Impey, & Obrietan, 2014) and RNA editing in epileptic compared with non-epileptic mice (Srivastava et al., 2017). The latter was conducted by our collaborators conducting the RNA sequencing for this project. They found differentially regulated genes that correlated with seizure presentation in epileptic mice. These kinds of studies can be used to find genetic mechanisms that are having downstream effects and that may not otherwise be identified.

Through methods other than RNA sequencing, previous rat and mouse studies have assessed potential genetic and epigenetic mechanisms primarily focusing on candidate genes such as those involved in neurodevelopment and folate metabolism. Mouse studies focusing on folate related genes have found variable genetic consequences depending on the strain;
for example, a significant reduction in folate binding proteins FBP-1 in SWV mice exposed to VPA and upregulation of the gene in Lm/BC mice (Finnell et al., 2000). This suggests that strain dependent genetic alterations occur in response to VPA exposure and highlights the significance of using an epileptogenic strain of rats in this study.

A most recent study by Semmler et al. (2017) assessed the hippocampi of rat pups exposed chronically to VPA in utero, where mothers were administered VPA via drinking water at dose of 500 and 825mg/kg. When assessing the pup brains, they found increased neurons and decreased astrocytes in in CA1/2 and CA3 in the hippocampus, an increase in 5-methenyl tetrahydrofolate, a decrease in 5-10 methyltetrahydrofolate as well as decreased homocysteine level. Epigenetic mechanisms such as DNA methylation have also been assessed. Rat pup brains exposed to VPA in utero have DNA de-methylation in WNT1 and WN2 and consequently increased WNT1 and WNT2 mRNA expression (Z. Wang et al., 2010). The WNT/b catenin pathway is involved in brain signalling and neurodevelopment. While previous studies have assessed particular genes and pathways none have looked at global mRNA changes in epileptic rat pups exposed to VPA in utero.

One of the limitations of this study was the difficulty to accurately categorise underdeveloped pups. While the external assessment scoring system was developed, and used, it was not clear exactly what the condition the pups with lower scores had. This may be further assessed in subsequent analysis and may become clearer upon getting results from genetic analysis. Another limitation was the quality of the images used for the internal spina bifida staining analysis. There were a number of ambiguities and unclear spinal segments meaning that not all pups had full spinal information available. A more accurate method, although more expensive, for future analysis may be to use micro CT scans as the primary form of analysis rather than as the validation method. The analysis of the distances was not ideal because we were not able to adjust for pup size and there was no specific measurement that would indicate spina bifida. It was concluded that the measurements obtained couldn’t reveal much, however throughout the process the missing arches were identified. Another limitation was that 1 GAERS VPA and 3 NEC VPA litters were taken out one day early (day 20) and one GAERS VPA litter one day late (day 22) which is not optimal for consistency. Ideally, all factors would be constant and there would also be a constant n number for different data that was presented. Part of the reason for this was that some pups were extracted before some specifics about the analysis were decided upon. It was not logical to entirely exclude these pups as their information was still valuable, however this resulted in a
different number of rats/pups presented in different data sets.

Future analysis will involve refining of the model and using it for genetic analysis. In terms of spinal analysis, factors to assess in the future may include completion of micro CT scans, assessment of the shape of vertebral arches and the number of the ossified parts of the centrum of the spine. Amniotic fluid is another factor to assess in future analysis because as a general observation there appeared to be more fluid in the uterus of VPA exposed pups compared with controls. Producing too much amniotic fluid is called polyhydramnios and is associated with a number of birth defects including open neural tube defects and other congenital defects associated with AED induced birth defects (Rajgire, Borkar, & Gadge, 2017). A future addition to the study may be to assess the effects of concurrent folate administration to the mothers during pregnancy. Administration of vitamin B6 and B12 in conjunction with VPA has been shown to reduce the rate of spina bifida occulta amongst other malformation types (Elmazar, Thiel, & Nau, 1992) but would also be interesting to assess using this model.

4.5 Conclusion

This study has resulted in a viable animal model that could be used in future studies assessing the mechanisms of VPA induced birth defects. The chronic VPA intake through the food resulted in both external and internal morphological differences between VPA exposed pups and control pups in all three strains. It is currently being used to assess for mRNA changes in VPA exposed pup brains and may be used in future analysis to assess for genetic and epigenetic changes and to look for any number of potentially involved mechanisms. Animal models can be very helpful for unveiling potentially involved biological processes and the information gained from such studies can help find a direction in which to analyse human samples.
Chapter 5

Whole genome pharmacogenomic study of susceptibility to birth defects in children born to mothers taking AEDs

5.1 Abstract

**Background:** There is increasing evidence from both human and animal studies that genetic factors modify the risk for the development of congenital physical malformations in children exposed to antiepileptic drugs (AEDs) during the first trimester of pregnancy. Investigating the possibility of genetic influence, previous studies have assessed specific candidate genes but no genomic factor has yet been validated.

**Methods:** To look for potential genomic risk modifiers in humans, the present study uses two approaches: whole exome sequencing (WES) and whole genome DNA methylation scans. The two approaches were used to investigate three hypotheses: 1. There are highly penetrant protein coding variants of large effect that increase the risk of birth defects for pregnant women treated with AEDs, particularly valproate, and 2. Children with AED-induced birth defects, particularly those exposed to valproate will have an increased load of functional *de novo* mutations and/or 3. DNA methylation analysis was used to test the hypothesis that children with AED-induced birth defects, and/or their mothers will show alterations in DNA methylation levels compared with unaffected children and/or mothers.

**Results:** The analysis for Hypothesis 1 is ongoing as samples continue to be collected. Findings for Hypothesis 2 indicate that an increased load of functional *de novo* mutations is unlikely to be a major contributing factor to AED induced teratogenicity. The DNA methylation analysis for Hypothesis 3 is also ongoing and results for this section have not been reported in this thesis.

**Conclusion:** Findings from this small but high-risk cohort indicate that exposure to AEDs during pregnancy does not increase the load of functional *de novo* mutations and is not like-
ly to be a major contributing factor in AED-induced birth defects. Further studies with larger numbers are required to validate these findings. The molecular mechanisms underlying AED teratogenicity remains an important question to be addressed.

5.2 Background

Antiepileptic drugs are established human teratogens however the mechanisms resulting in this teratogenicity are not known. There is ample evidence to suggest that genetic factors play an important role in predisposing a woman to having a child with AED induced birth defects when taking AEDs during the first trimester of pregnancy. The majority of women taking AEDs during pregnancy have healthy children however women who have one child with an AED induced birth defect have an increased risk of having subsequent affected children (Campbell et al., 2013; Vajda et al., 2013). There is also an increased risk of having a child with an AED induced birth defect when a family history of birth defects is present (Tomson et al., 2011). Evidence from animal studies includes different levels of susceptibility in different rodent strains (Faiella et al., 2000; Finnell, Moon, Abbott, Golden, & Chernoff, 1986) and an association between postulated specific malformation types observed and genetic causes. Neural tube defects, for example, have been linked to chromosome 2, 7 and 10 (Rampersaud et al., 2005; Stamm et al., 2008) and also to the grainy head-like transcription factor 3 gene in the curly tail mouse (van Straaten & Copp, 2001) and recently also in humans (Lemay et al., 2017).

Previous studies have focussed on specific candidate genes that may be involved in this teratogenicity. While some associations have been made, no genetic factor has been validated. Candidate genes that have been assessed include genes involved in AED metabolism (CYP2C9, CYP2C19, CYP3A4) transport (ABCB1) and folate metabolism (MTHFR, MTR, MTRR) (Jose et al., 2014). The ABCB1 gene was initially found to be associated with drug resistant epilepsy and therefore is thought to be involved with individual response to AEDs (Siddiqui et al., 2003). In recent studies, it has been implicated along with CYP2C10 in AED teratogenicity (Jose et al., 2014).

Over the last few decades de novo mutations have been associated with numerous early onset diseases such as autism (Iossifov et al., 2014), kabuki syndrome (a disorder characterised by cardiac, skeletal, immunological and developmental defects) (Ng et al., 2010) and Schinzel-Giedion syndrome (a disorder characterised by developmental delay, congenital defects, distinctive facial features and early mortality) (Hoischen et al., 2010).
The findings of studies such as these have determined two main things about *de novo* mutations. 1. There are increased *de novo* mutations in people with genetic disease. 2. There are increased *de novo* mutations as the fathers age is increased (Veltman & Brunner, 2012). These factors along with the nature of AED induced birth defects provide a rationale for assessing *de novo* mutations in children with AED induced birth defects.

In addition to genetic mechanisms, epigenetic mechanisms such as DNA methylation and acetylation may also be involved in the pathogenesis of AED induced birth defects. VPA is a known histone deacetylase (HDAC) inhibitor and is also known to cause DNA demethylation (Gottlicher et al., 2001). Alterations to the methylation process during development can cause aberrant cell expression. Infants exposed to AEDs for longer durations show reduced global methylation in umbilical cord blood and placental tissue (Smith et al., 2012). In rat studies exposure to VPA prenatally, resulted in demethylation of WNT1 and WNT2 in the WNT/beta catenin pathway (Z. Wang et al., 2010). Polymorphisms in gene 5,10 methylenetetrahydrofolate (MTHFR) involved in folate metabolism have been previously shown to result in alterations of DNA methylation. A particular polymorphism 677C>T in this MTHFR gene has been associated with neural tube defects (NTDs) (van der Put, Eskes, & Blom, 1997). People who have the TT genotype have lower folate levels and higher total homocysteine levels compared with other genotypes (Hiraoka & Kagawa, 2017). Kini et al. (2007) investigated whether a combination of genotype (TT) and VPA exposure increased the risk and found that while the rate of malformations was higher, the increase was not the main cause of the teratogenicity.

Preliminary findings from previous studies provide further evidence that genetic factors are likely contributing factors, however no definitive genetic or epigenetic mechanisms have been validated. We explored this question using different investigative methods in human subjects to identify genomic alterations based on three primary hypotheses:

1. There are highly penetrant protein coding variants of large effect that increase the risk of birth defects for pregnant women treated with AEDs, particularly valproate.
2. Children with AED-induced birth defects, particularly those exposed to valproate will have an increased load of functional *de novo* mutations and/or
3. Children with AED-induced birth defects, and/or their mothers will show alterations in DNA methylation levels compared with unaffected children and/or mothers.
This is the first study to take a whole genome approach to the genomics behind AED teratogenicity.

5.3 Materials and Methods

5.1.1 Study cohort and subject recruitment

The subjects for this study were primarily selected from the Australian Pregnancy Register (APR). The APR was established in 1999 and aimed to follow the outcomes of prospective pregnancies of women with epilepsy taking antiepileptic drugs (AEDs), women with epilepsy not taking AEDs and women taking AEDs for other indications. Potential participants were given information on the study and sent a reply addressed consent form prior to the first interview. Women were interviewed via telephone at 4 time points: at time of signing of consent forms, 7 months gestation, within 6 weeks of delivery and 1 year. Questions regarding the child's development, the child’s and mother’s medical history, treatment history and environmental history were obtained. Three groups of women were recruited for this study:

1. Case subjects: women who took AEDS during the first trimester of their pregnancy and had one or more children with a birth defect (either live births or birth defects detected using ultrasound in a foetus that either died or was terminated)
2. AED exposed control subjects: who took AEDs during the first trimester of their pregnancy and had only healthy children, or children without a birth defect and
3. AED non-exposed control subjects: epileptic women who are not exposed to AEDs

Participants recruited from the APR were recruited by telephone at their one-year interview. After the interview participants were informed of the study and asked to participate. Other sources of recruitment included the practices of consultant neurologists, Prof. Terence O’Brien and Dr. Piero Perucca at The Royal Melbourne Hospital (RMH), Prof. Sam Berkovic at The Austin Hospital and Prof. Torbjorn Tompson at Karolinska Institute in Sweden. Only complete family trio sets were used for the trio analysis and only mothers were used for association analysis however sequencing data of other individuals will be assessed in subsequent analysis. Unaffected siblings of affected children were also included with their parents as control trios. At the time of writing, sample collection for this analysis was ongoing and the final cohort will include additional cases (~17) and controls (~200) from Europe that are provided through our collaboration in the EpiPGX project. The
EpiPGX project is an international collaborative project aiming to identify genomic biomarkers in epilepsy.

After giving verbal consent, participants were sent patients informed consent forms (PICFs) for all family members (mother, father and all children). Ethics approval for this part of the study has been approved from the Melbourne Health Human Resources and Ethical Research Committees (HREC).

5.1.2 Sample Collection

After agreeing to take part in the study, families were sent packs containing consent forms, pathology slips and/or saliva packs. Participants were asked to either get their blood samples (40ml) sent to us from their local pathologist or to provide saliva samples (3ml) in the kits provided. Samples were frozen at -80 degrees upon collection until further processing.

5.1.3 DNA extraction

DNA was extracted from samples from the mother, father and children of all families who sent back DNA using Gentra Puregene blood Kit according to the manufacturers protocol for blood samples. PSP SalivaGene Module 2 extraction kit was used for saliva samples. DNA quality and quantity was measured using the Nano-drop 2000 UV-Vis Spectrophotometer (Thermo Fisher scientific). DNA yield was between: 30ug/ul and 1500ug/ul. DNA quality was required to be between 1.7 and 2. Where DNA quality was not between 1.7 and 2, families were asked to send another sample.

5.1.4 Whole exome sequencing

Whole exome sequencing (WES) was conducted at the Institute for Genomic Medicine, Columbia University Medical Centre, New York, USA. DNA was exome sequenced on the Illumina HiSeq2500 platform with an average 100x coverage of 100-bp paired-end reads. Libraries were constructed using the KAPA Biosystem kit and Nimblegen SeqCap EZ V3.0. Raw sequences data were aligned to the human reference genome (NCBI Build 37) using Burrow-wheel aligner (BWA) alignment tool version 0.5.10 (Li & Durbin, 2009) and variants were called using the GATK best practice protocol. Analysis was restricted to single nucleotide variants and indels within protein coding regions, extending 10 bases into intronic regions. Variants were further filtered based on genotype quality (GQ) >=20, QUAL phred-scaled quality score (indicates confidence of call) >=30 and minimum read depth of
10. Variants were also screened against an in house list of known artefacts and problematic variants. Statistical analysis was conducted using R version 3.4.1 and R studio version 1.0.153.

5.1.5 Testing hypothesis 1

Variants identified will be annotated using Ensembl data source (for genomic context), protein disruption prediction using knowledge based tools including PolyPhen2 and KGGSeq and candidate rare and functional variant identification through filtering on the Genome Aggregation Database (gnomAD). The two primary comparisons will be:

1. All AED associated birth defects vs unaffected controls
2. VPA associated defects vs unaffected controls.

5.1.6 Testing hypothesis 2

Putative de novo mutations were identified using an established trio sequencing protocol (Zhu et al., 2015) applied at the Institute for Genomic Medicine by Dr Petrovski. This protocol identifies variants in children not called in either parent or in an external reference cohorts of 4,503 controls of convenience sequenced at the Institute for Genomic Medicine, 6503 controls of convenience provided by the Exome Sequencing Project (ESP6500SI) (NHLBI GO Exome Sequencing Project; evs.gs.washington.edu) and 60,706 controls of convenience from ExAC (ExAC Broad Institute, Year Unknown).

Hypothesis 2 formally assessed whether there is significant excess of de novo mutations among the case collection compared to existing empirical estimates of ~0.9 protein-coding de novo mutations (mutation rate of ~1.3x10-8) (Besenbacher et al., 2015). Moreover, the rate of de novo mutations was compared between the following groups

1. AED exposed unaffected vs AED exposed affected
2. VPA exposed and affected vs VPA exposed and unaffected

In addition to these groups a third group of unexposed and unaffected was also analysed. Fischer exact tests were used to compared the de novo mutation rate between the groups listed above and a Wilcoxon test was used to determine if there was a significant difference in de novo mutation rate regardless of birth outcome.

5.1.7 Whole Genome DNA methylation Scan

DNA profiling was performed at the Department of Medical Genetics in Utrecht, The Netherlands, using Illumina EPIC chips (850k, 141k type 1and 724k type 2). The chip
contains 850 000 known methylation sites at a single base nucleotide resolution at sites selected on their prior probability of functional consequences e.g. being on or near protein coding regions or on DNAse hypersensitive sites. The DNA methylation protocol was based on bisulphate sequencing. This method involves the administration of bisulphate, which then results in a single base nucleotide reaction that probes for levels of “C” and “T”. Differential DNA methylation will be assessed globally. Association tests will be conducted using various software packages including IMA, LUMI and Minifi.

5.2 Results
5.2.1 Samples genotyped

In total, 153 control and 75 case individual samples, comprising of 61 trios were genotyped (Table 1). While these were the total samples genotyped, different subsets of this group were used for each hypothesis (details in Appendix Tables 2-4). Total samples received throughout the recruitment process have been outlined in the appendix Table 1. The mothers and children were exposed to a range of AEDs (Table 2) and there were a spectrum of birth defect presentations which have been presented as a) children with defects we had samples for (Table 3) and b) mothers of children with defects we have samples for (Table 4) for which there is some overlap. As previously mentioned, recruitment is ongoing and final cohort will include cases (~17) and controls (~200) from Europe that are provided through our collaboration in the EpiPGX project.

### Table 1. Study samples

<table>
<thead>
<tr>
<th></th>
<th>Individuals</th>
<th>Mothers</th>
<th>Fathers</th>
<th>Children</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>153</td>
<td>56</td>
<td>41</td>
<td>56</td>
</tr>
<tr>
<td>Case</td>
<td>75</td>
<td>29</td>
<td>17</td>
<td>29</td>
</tr>
</tbody>
</table>

**Table 1.** The number of samples genotyped for the three types of analysis. Unaffected case siblings have been listed in case families (14 affected children +15 unaffected siblings). For the controls, the number of families is higher than the number of mothers because 4 children and 2 fathers that were genotyped without their mothers. In the cases, the number of families is higher than the number of mothers because there was one child that was genotyped without their mother.
Table 2. Drug exposure of unaffected and affected children genotyped

<table>
<thead>
<tr>
<th></th>
<th>CBZ</th>
<th>LEV</th>
<th>OCBZ</th>
<th>LTG</th>
<th>PHT</th>
<th>TPM</th>
<th>VPA</th>
<th>POLY</th>
<th>NO AEDS</th>
<th>Unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unaffected</td>
<td>9</td>
<td>15</td>
<td>1</td>
<td>7</td>
<td>1</td>
<td>1</td>
<td>7</td>
<td>16</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>Affected</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>6</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviations: CBZ; carbamazepine, LEV: Levetiracetam, OCBZ: Oxcarbazepine, PHT: phenytoin, TPM: Topiramate, VPA: valproate, POLY: polytherapy

Table 2. Drug exposure of all children genotyped in all types of analysis. Unaffected children include the 15 siblings of probands.

Table 3. Birth defect details of children genotyped

<table>
<thead>
<tr>
<th>Birth defect type</th>
<th>#children</th>
<th>Details</th>
</tr>
</thead>
</table>
| Skeletal                        | 3         | 1. Tongue tie, thin lips, dysmorphic facial features, large head, forehead buffing (B,C)  
                                         2. Toe missing on right foot and remaining toes webbed, foot one size smaller than left (B)  
                                         3. Fingers hands fused right hand fingers webbed, left foot toes, right foot one: toe (B) |
| Urogenital                      | 3         | 1. Hypospadias (B)                                                      |
|                                 |           | 2. Undescended testes (C)                                               |
|                                 |           | 3. Hypospadias (B)                                                      |
| Cardiac                         | 1         | 1. 1. Ventricular septal defect (patent foramen ovale small left to right atrial shunt) (C)  
                                         2. Patent foramen ovale (C)                                           |
| Orofacial                       | 1         | 1. Cleft lip (left sided cleft lip and partial right) (C)               |
| Neural tube                     | 1         | 1. Spina bifida (sacral 5x4cm meningocele intact membrane covering) (C)  |
| Skeletal                        | 1         | 1. Clubfoot (B)                                                         |
| Cardiac + neural tube + skeletal | 1         | 1. Spina bifida, ventricular septal defect, atrial septal defect, patent ductus arteriosus, clinodactyly, Arnold Chiari malformation (B,C) |
| Cardiac + skeletal              | 1         | 1. No bone in left thumb, tight fingers in both hands, ventricular septal defect, atrial septal defect (B) |
| Other                           | 1         | 1. Smith-magenis syndrome, chromosome 17 deletion and reverse bilateral clubfeet |

Table 3. The birth defect types of the children genotyped in trio and methylation analysis. Asterisks indicate which type of analysis the child was included in (B=trio analysis, C=methylation analysis).
<table>
<thead>
<tr>
<th>Birth defect</th>
<th>#Children</th>
<th>Details</th>
</tr>
</thead>
</table>
| Neural tube                          | 5         | 1. Anencephaly (A,B,C)  
2. Spina bifida and brain malformation (A,C)  
3. Spina bifida (A,C)  
4. Spina bifida, hydrocephalus (A,C)  
5. Spina bifida (sacral 5x4cm meningocele intact membrane covering) (A,C)  
6. Anencephaly (A)  
7. Spina bifida (A)  
8. Neural tube defects (A)  
9. Spina bifida (A)  
10. Spina bifida and Chiari 2 (A)  
11. Spina bifida (A)                                                                 |
| Skeletal                             | 4         | 1. Tongue tie, thin lips, dysmorphic facial features, large head, forehead buffing (A,B,C)  
2. Clubfoot (A,B)  
3. Toe missing on right foot and remaining toes webbed, foot one size smaller than left (A,B)  
4. I fingers hands fused right hand fingers webbed, left foot toes, right foot one toe (A,B) |
| Urogenital                           | 3         | 1. Hypospadias (A,B)  
2. Undescended testes (A,B)  
3. Hypospadias (A,B)                                                                 |
| Cardiac                              | 2         | 1. Bicuspid aortic valve (A,C)  
2. Ventricular septal defect (patent foramen ovale small left to right atrial shunt) (C)  
3. Ventricular septal defect (A,C)  
4. Patent foramen ovale (C)  
5. Ventricular septal defect (C)  
6. Tetralogy of Fallot (A)                                                                 |
| Orofacial                            | 1         | 1. Cleft lip (left sided cleft lip and partial right) (C)                                                                 |
| Neural tube + cardiac + skeletal     | 1         | 1. Spina bifida, ventricular septal defect, atrial septal defect, patent ductus arteriosus, clinodactyly, Arnold Chiari malformation (A,B,C) |
| Cardiac + skeletal                   | 1         | 1. No bone in left thumb, tight fingers in both hands, ventricular septal defect, atrial septal defect (A,B,C)  
2. Ventricular septal defect, bilateral talipes, Tetralogy of Fallot (C)                                                                 |
| Urogenital + other                   | 1         | Hypospadias, retrognathia & hypertelorism (A,C)                                                                 |
| Multi-organ malformation             | 1         | Chromosomal abnormality (trisomy 21), multi-organ malformations, cardiac malformations, abnormal wall malformations, hydronephrosis (A,C) |
| Other                                | 3         | 1. Left ear nerve missing, right ear malformed cochlear (A)  
2. Smith magenis syndrome, chromosome 17 deletion and reverse bilateral club feet (A)  
3. Dandy Walker Syndrome (C)                                                                 |
defects or a termination of pregnancy. (Variant analysis “A” WES variant analysis, “B” trio analysis C= methylation). Two mothers had more than one child with a defect. One mother had a child with a cardiac defect and one child with a orofacial defect. One mother had one child with hypospadias, retrognathia & hyperterolism and one child with neural tube defects.

5.2.2 Hypothesis 1. WES variant analysis in mothers

Preliminary analysis was conducted on available samples representing 51 control mothers and 21 case mothers from the Australian data and an additional 5 case mothers from other sources (other sources detailed in Table 6 of the Appendix). Two gene-based association tests were undertaken. These tests compare the burden of variants in cases and controls. The basic burden test assumes all variants to be deleterious when in reality variants may influence the phenotypes differently and may have either a protective or deleterious impact (Wu et al., 2011). To better model this scenario the Sequence-Kernal Association test (SKAT) was applied. Quantile-quantile (q-q) plots were used to visualize the bulk results (Figure 1) and the genomic inflation factor $\lambda$ was calculated as follows. $\lambda = \text{median test stat for observed} / \text{med test stat for the null distribution}$. When the $\lambda$ is greater than one, and the $P$-values mostly fall above the x-y line representing the null hypothesis, as can be seen in Figure 1 a), there is inflation of the false positive rate. This is an indication that the model is not right for the data or there is a factor such as population structure that has not been properly adjusted for or some other quality control issue. The SKAT test shows some under inflation but appears to be the better model for these data. Ideally, the values would mostly follow the red line with a few outliers at the top indicating significant results. It is possible the study is currently underpowered and these tests will be run and evaluated when the additional samples become available.
**Figure 1.** Quantile-quantile plots of observed association model *P*-values (black) against expected *P*-values under the null hypothesis (red) a) Burden Test b) SKAT test. Both graphs show a comparison of the variance between cases and controls at each site.

### 5.2.3 Hypothesis 2. Trio analysis

For hypothesis 2 there were a total of 61 trios that came from 33 parent-child families. Out of this group there were 50 AED exposed and 11 non-exposed pregnancies. There were 9 AED exposed children with birth defects. The WES analysis identified 78 putative *de novo* mutations were identified in 40/61 children in. One of these mutations was present in two children meaning there were 77 unique mutations in total. The variants for each child and their parents were viewed using the Integrated Genomics Viewer (IGV). No trace in a parent was confirmed for 45, and these were considered to be high-confidence variables. We next looked at the variant allele frequencies (VAFs). Under heterozygosity, the allele frequency in the child would not be significantly different from 50%. This was the case for 37 variants as determined by a one sided binomial test. In the remaining 8, the VAF was significantly different to 50% indicating that the mutation occurred post zygotically. These variants will be verified using Sanger sequencing technology.

The 33 variants for which a trace of the variant was observed in a parent exome were further filtered for evidence of mosaic transmission. To eliminate those arising from sequencing artefacts the variants were first filtered based on the following cut offs: less than 2 alternative reads at the locus in the parent exome, a VAF less than 0.10 in the parent exome, or presence of the variant in both parental exomes. We then applied a one-sided binominal test to confirm that the VAF in the parent was significantly different from 50% using a cut-off of *P*>=5 X 10^-6 and that the VAF frequency in the child was not significantly different from 50% (*P*<0.05). These cut-offs have been previously used to determine mosaic transmission (Halvorsen et al., 2016). Three variants met these cut-offs and 4 were borderline variants (Table 6).

There was no difference in *de novo* mutation load in the AED exposed affected (n=9, range=0-2, median=0) and AED exposed unaffected groups (n=41, range=0-2, median=0) (Fischer exact *P*=1). There was no difference between VPA exposed and affected (n=4, range=0-1, median=0) compared with VPA exposed and unaffected (n=9, range=0-2, median=1) (Fischer exact test *P*=0.2657). Similarly, *de novo* mutation load was not significantly different in VPA exposed (n=13, mean=0.769) compared with unexposed to VPA (including
those not exposed to any AED) (n=48, mean=0.729) regardless of birth outcome (Wilcoxon test $P=0.4243$).

CpG islands that are methylated are regions of high mutations (Francioli et al., 2015). Seven of the 45 (15.5%) high-confidence variants fell within CpG islands. To determine if this was high we compared the proportion with that in a publically available de novo data set of variants identified by whole exome sequencing of ~2500 families from the Simpsons Simplex Collection for the study of autism (Iossifov et al., 2014). We found 108 of 1,692 variants identified in this study fall within CpG islands. The proportions were not significantly different ($P=0.073$ Fisher’s exact test).

Of the 3 variants suggestive of mosaic inheritance, one was harboured by a child with hypospadias. This variant lies within the $KAT6B$ gene which is associated with genitopatellar syndrome, a disorder that presents a range of symptoms that includes genital malformations (OMIM: 606170). This was the only variant that was relevant to a child’s phenotype. The second variant met the threshold in one child but not a sibling who also had the variant and similarly the third was part of a cluster of 4 in which the remaining 3 were approaching the cut-off threshold. In the latter, the mother of this child had a sister with congenital heart defect and a brother who died shortly after birth. These findings highlight the difficulty in discerning true mosaic transmission from sequencing artefact using sequencing data alone. An example of a child with mosaic inheritance is shown in Figure 2.

**Table 5.** De novo mutation burden in each group

<table>
<thead>
<tr>
<th></th>
<th>All AED Exposed and affected (n=9)</th>
<th>All AED Exposed and unaffected (n=41)</th>
<th>VPA exposed and affected (n=4)</th>
<th>VPA exposed and unaffected (n=9)</th>
<th>Unexposed and unaffected (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proportion*</td>
<td>4/9 (44%)</td>
<td>19/41 (46%)</td>
<td>1/4 (25%)</td>
<td>6/9 (67%)</td>
<td>9/11 (81%)</td>
</tr>
<tr>
<td>Range</td>
<td>0-2</td>
<td>0-2</td>
<td>0-1</td>
<td>0-2</td>
<td>0-3</td>
</tr>
<tr>
<td>Median</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*Proportion of children with one or more de novo mutations

Table 5. shows the proportion of children that had one or more de novo mutation, the range of the number of mutations each child had and the median number of de novo mutations.
Table 6. Evidence of mosaic inheritance

<table>
<thead>
<tr>
<th>Child ID</th>
<th>Status</th>
<th>Variant (chr-position)</th>
<th>Variant Type</th>
<th>Reference Allele</th>
<th>Alternative Allele</th>
<th>Gene symbol</th>
<th>Child binomial P</th>
<th>Paternal binomial P</th>
</tr>
</thead>
<tbody>
<tr>
<td>con91c1</td>
<td>control</td>
<td>11-2190931</td>
<td>snv</td>
<td>G</td>
<td>T</td>
<td>TH</td>
<td>0.8659</td>
<td>1.19E-11</td>
</tr>
<tr>
<td>con35c1</td>
<td>control</td>
<td>2-207619976</td>
<td>indel</td>
<td>ACAC</td>
<td>A</td>
<td>MDH1B</td>
<td>0.0748</td>
<td>2.13E-06</td>
</tr>
<tr>
<td>con35c1</td>
<td>control</td>
<td>2-207619999</td>
<td>indel</td>
<td>TC</td>
<td>T</td>
<td>MDH1B</td>
<td>0.1725</td>
<td>3.31E-05**</td>
</tr>
<tr>
<td>con35c1</td>
<td>control</td>
<td>2-207620003</td>
<td>indel</td>
<td>GC</td>
<td>G</td>
<td>MDH1B</td>
<td>0.1725</td>
<td>1.12E-05**</td>
</tr>
<tr>
<td>con35c1</td>
<td>control</td>
<td>2-207620005</td>
<td>indel</td>
<td>AC</td>
<td>A</td>
<td>MDH1B</td>
<td>0.2210</td>
<td>1.93E-05**</td>
</tr>
<tr>
<td>f30c1</td>
<td>proband</td>
<td>10-76781833</td>
<td>indel</td>
<td>AGAG</td>
<td>A</td>
<td>KAT6B</td>
<td>0.1055</td>
<td>3.48E-05**</td>
</tr>
<tr>
<td>f1C1</td>
<td>healthy sibling</td>
<td>4-3939007</td>
<td>indel</td>
<td>AAC</td>
<td>A</td>
<td>ALG1L7P</td>
<td>0.0507</td>
<td>8.23E-08</td>
</tr>
<tr>
<td>f1C2</td>
<td>healthy sibling</td>
<td>4-3939007</td>
<td>indel</td>
<td>AAC</td>
<td>A</td>
<td>ALG1L7P</td>
<td>0.0022*</td>
<td>8.23E-08</td>
</tr>
</tbody>
</table>

*below cut-off P > 0.05, **approaching cut-off P < 5.0E-06

Table 6 Results for one-sided binomial tests used to determine mosaic transmission.

**Figure 2.** Example of a *de novo* mutation that may have been transmitted from a mosaic parent from a snapshot from IGV viewer. The grey bars represent reads that map to the location in the genome (Chromosome 11, position 2190931). Red bars indicate the pres-
ence of the variant T allele at this location where the expected reference allele is a G. The variant is observed in 23 of 40 reads that map to this location in the child. In the mother, the T allele is observed in 7 of 66 reads and none are observed in the father.

5.2.4 Hypothesis 3. Whole genome DNA methylation scan

This section of the study is being undertaken by our collaborators in Europe. The DNA methylation scans has been completed and the analysis is underway. In addition to samples from Australia, an additional 30 affected mothers and 3 affected infant samples have been provided through the international collaboration as detailed below.

Affected mothers from other sources:
- 7 from Dr. Chantal Depondt (The University of Brussels)
- 4 from Dr. Graeme Sills (The University of Brussels)
- 2 from Prof. Dr. Holger Lerche (Tübingen University)
- 1 from Susanne Beyer (BonnLAB, Germany)
- 16 from Dr. John Craig (Belfast Health and Social Care Trust)

Affected children form other sources:
- 3 from Dr. John Craig (Belfast Health and Social Care Trust)

5.3 Discussion

To the best of our knowledge, this study is the first to assess pharmacogenomic differences in children with AED induced birth defects using whole exome and DNA methylation approaches. While the WES mothers association analysis and methylation analysis are still in the early stages, this study found from whole exome sequencing trio analysis that increased de novo mutation load is unlikely to be a cause of AED induced teratogenicity. The numbers were small due to the difficulties of recruiting patients for this kind of study given the sensitive nature of the outcome. Despite the small numbers however, women with a broad range of AED exposures and children with a range of AED induced defect types were recruited. The most common AED induced birth defect amongst the case mothers, regardless of whether the child's sample was available were neural tube defects, followed by skeletal, cardiac, urogenital and cardiac defects (Table 4). These defect types are in accord with the most common AED induced defects reported in international pregnancy registers (Morrow et al., 2006; Tomson et al., 2011). The most common drug that unaffected children were exposed to was polytherapy followed by no AEDs followed by Levetiracetam. Out of the cases the most common drug was VPA. This again is in agreement with the literature.
where VPA has been continuously reported to results in the highest number of birth defects (Hernandez-Diaz et al., 2012; Tomson et al., 2011).

In diseases with low genetic heterogeneity it is possible that variants of large effect could be easily identified in a small sample size. This could be because multiple individuals carry the same causal variant or that they carry different causal variants in the same gene. It was hypothesized that variants that may be causal in the mothers are highly penetrant and of a high effect however because the QQ plots were not optimal and it is necessary to further increase the sample size.

In recent years de novo mutations have been associated with a number of severe early onset diseases such as autism (Iossifov et al., 2014), Kabuki syndrome (Ng et al., 2010), epileptic encephalopathies (Epi et al., 2013) and Schinzel-Giedion syndrome (Hoischen et al., 2010). From previous studies such as these, it has become clear that individuals with genetic disease may have a higher rate of de novo mutations than those without and also that increased de novo mutations are associated with increased paternal age (Veltman & Brunner, 2012).

In the present study, the number of de novo mutations in all AED exposed and affected children was not significantly greater than all AED exposed and unaffected children. Similarly, the number of de novo mutations was not increased in VPA exposed and affected compared to VPA exposed and unaffected. This can lead us to conclude than an increase in the rate of functional de novo mutations is unlikely to be a contributing factor to AED induced teratogenicity. The role of specific de novo mutations in in the development of congenital malformations, however, is still an important factor to consider. The results need to be replicated and further investigates in a larger cohort.

One of the most important confounding factors in trio analysis is paternal age. Increasing fathers age is associated with increased de novo mutations (Kong et al., 2012). In the de novo mutation analysis the mean age of the fathers of unexposed unaffected children was 30.9, fathers of exposed unaffected children was 34.6 and the mean age of fathers of exposed and affected children was 33.2. In this study there was no association between paternal age and the number of de novo mutations each child had. Mothers age is also important as de novo mutations do increase with age, however at a slower rate (Goldmann et al., 2016) (Jonsson et al., 2017). The reason for the difference in the rate is that sperm un-
dergoes repeated mitosis (Qin et al., 2007) while ova don’t divide after birth. It has also been found that the type of de novo mutations changes with increasing mothers age (Jonsson et al., 2017) and this will be further assessed in the analysis.

Other notable confounding factors include family history of birth defects with 6/11 (54.5%) unexposed and unaffected, 9/38 (23.68%) exposed and unaffected and 3/9 (33.33%) exposed and affected having a family history of birth defects. This is an interesting observation because the unexposed unaffected group had the highest proportion of children with de novo mutations (81% vs 44% in AED exposed and affected, 46% in AED exposed unaffected, 25% in VPA exposed and affected and 67% in VPA exposed and unaffected) but also had the highest percentage of children with a family history of birth defects. The mother of the unexposed unaffected child with the highest number of de novo mutations (9) had a sister with a congenital heart defect and a brother that died shortly after birth. Out of the affected children; the twins in which one had cardiac and skeletal defects had a cousin with Down syndrome. The mother of a child with skeletal defects had a niece (husband’s sister’s child) with spina bifida. The mother of another child with skeletal defects had a hole in her heart at birth, mitral valve prolapse.

Other potential confounding factors include folic acid exposure where; 2/3 (66.6%) unexposed unaffected, 29/33 (87.9%) exposed unaffected and 6/7 (85.7%) exposed and affected were exposed to folic before conception and all were exposed after conception. Only one exposed and unaffected was exposed to smoking while none of the other groups had alcohol or smoked in the first trimester. Tea/coffee/cola was common amongst all three groups with 3/3 (100%), 26/33 (78.8%) and 3/7 respectively (42.9%) of unexposed unaffected, exposed unaffected and exposed affected respectively. It should be noted that this information was available for women on the register but not those recruited from outside sources. These factors have not been adjusted for in the current analysis but are noted and may be accounted for in downstream analysis.

A limitation of this study is the small sample size, however, a number of previous studies have identified causative de novo mutations in similar or even smaller sample sizes, at least in initial cohorts before being further validated. For instance, 9/10 individuals with kabuki syndrome exhibited a common de novo mutation, 7/7 children with alternating hemiplegia of childhood (Heinzen et al., 2012) and 4/4 children with Schinzel-Giedion syndrome (Hoischen et al., 2010). The small numbers in these previous studies lead us to expect to be
able to observe mutations in genes of large effect in a cohort with a similar sample size. A difference between the studies mentioned and this study, however, may be the broad spectrum of defect types in AED induced birth defects and that is possible and likely that they may not all be caused by one single mutation (Schulpen, Pennings, & Piersma, 2015). Although an increase in load of de novo mutations does not appear to be contributing factor, the role of de novo mutations in specific genes remains worthy of further investigation. As numbers increase, it may become apparent that there are de novo mutations in common genes amongst children with birth defects or in children with specific birth defect types.

The third type of genomic analysis in the process of being conducted is the whole genome DNA methylation scan. The scanning process is currently complete, however as mentioned, the analysis is still in the process of completion. DNA methylation patterns are established during gestation and aberrant methylation has the potential to affect multiple different organ systems. Similarly, AED induced birth defects are non-specific and present in numerous forms affecting various organ systems. Previous studies assessing AED induced methylation changes both from in vitro studies, animal studies and human studies have found AED exposure, particularly VPA exposure, to result in overall hypomethylation. From in vitro studies, VPA has been shown to facilitate DNA de-methylation in neurons (Dong et al., 2007) and result in active DNA de-methylation in embryonic kidney 293 cells (Detich et al., 2003). From animal studies, VPA has been shown to cause DNA de-methylation in WNT1 and WNT2 (Z. Wang et al., 2010) and from human studies, AEDs have been found to result in decreased methylation in umbilical and placental blood of neonates exposed to AEDs in utero (Smith et al., 2012). Hypomethylation is often linked with instability of chromosomes, loss of genomic imprinting and abnormal expression of oncogenes which can all then result in aberrant transcription (Gopalakrishnan, Van Emburgh, & Robertson, 2008; Wilson, Power, & Molloy, 2007). It is hypothesized that there will be hypomethylation globally and in specific genes in affected children compared with unaffected children.

One of the main confounding factors and limitations of this methylation analysis is that methylation patterns change over time and samples from both mothers and children were collected at variable ages and time points after birth. Previous studies have found age-related patterns of both hypo and hyper methylation in specific genes (Johansson, Enroth, & Gyllensten, 2013). A common finding is that global hypomethylation occurs in non-CpG islands and hypermethylation in CpG islands with age (Fraga, Agrelo, & Esteller, 2007).
The control mothers in this study had an average age of 35.5 while the case mothers had an average of 41.8. Similarly control fathers had an average age of 38.9 and case fathers an average age of 45.3. The higher ages in the parents of the affected children could therefore have had an effect not only on the risk of birth defects (Czeizel, 1988) but also on whether or not hyper or hypo methylation occurs. Similarly, the age of parents also has the potential to impact methylation patterns in the child (Adkins, Thomas, Tylavsky, & Krushkal, 2011). The range of the ages of affected children was 6-29 with a mean of 12.9 and the ranges of the ages of the unaffected children was from 2-20 with a mean of 7.9 another factor that could influence the methylation levels in each child.

Other potential confounding factors for the methylation cohort are exposure to folic acid before and after conception, smoking/alcohol and caffeine exposure. This information was available for women on the register but not those recruited from outside sources. ¾ (75%), 16/19 (84.2%) and 6/6 (100%) had folic acid before conception in the unexposed unaffected, exposed unaffected and exposed affected groups respectively. All were exposed to folic acid after conception except one exposed and unaffected pregnancy. None were exposed to smoking/alcohol except one exposed affected. Tea/coffee/cola was common amongst all groups with ¾ (75%), 12/19 (63.2%), and 2/6 (33.3%) having it in the first trimester. The sample size for the methylation analysis is again low, however it was expected that it will be possible to observe differences in methylation levels with such numbers. It was not possible to conduct a power calculation to determine a required sample size for the methylation analysis due to not knowing the effect size before starting the study. The methylation analysis, like the association analysis is ongoing and increases in sample size over time will help combat this problem.

One confounding factor to assess across all mothers and pregnancies was whether or not they had a family history of birth defects. Of the mothers where this information was available, 3/22 (13.6%) case women had a family history of defects and 8/56 (14.3%) controls. There were similar numbers in both groups and although this could be further evaluated.

In addition to sample size, one of the limitations of the three types of analysis is that out of the whole cohort of women, 12/56 (21.42%) control women and 7/25 (28%) case women had one or more spontaneous abortions. The 12 control women had a total of 16 abortions in total and the 8 case women had a total of 12. Spontaneous abortions are com-
mon amongst the population and because no structural defects were identified these women were still categorised as controls. It is difficult to know for sure if these unborn foetuses had any structural defects or if they did not develop due to structural defects. Relatively few studies have evaluated spontaneous abortions and AED exposure. A Danish study conducted in 2014 found that there was an increased risk of spontaneous abortions in women taking AEDs for non-epilepsy indications but not for women taking them for epilepsy (Bech et al., 2014). The EURAP pregnancy register found that the two risk factors for spontaneous abortions are 1. AED polytherapy and 2. having a parent with a major congenital malformation (Tomson et al., 2015a). Recently, The Australian Pregnancy Register assessed the likelihood of malformed foetuses or intrauterine death for multiple pregnancies and found an increased risk of spontaneous abortion if the previous pregnancy was a spontaneous abortion and also an increased risk with AED exposure (Vajda et al., 2017). The same study also found that having spontaneous abortions increased the risk of having a child with a fetal malformation in subsequent pregnancies (Vajda et al., 2017). Out of the cases in the study, 7 women had a child with a birth defect after a spontaneous abortion.

Future analysis for this study include the completion of the WES variant analysis in the mothers and DNA methylation analysis as well as a more extensive whole exome assessment of specific genes in mothers and children. Genes that are identified in the animal experiments may be assessed more closely in the human studies. Other things to consider include assessment of acetylation changes and in the long term, assessment of different tissues such as placental tissue. Ultimately obtaining tissue at the time of birth would be optimal in assessing epigenetic changes such as methylation and this may be assessed in the future.

5.4 Conclusion

This is the first study to use whole genome genetic and epigenetic genomic analysis through an international collaboration. From this study thus far it was found that an increase in de novo mutation load is an unlikely cause of AED induced birth defects. Future directions include WES for genetic variants in the mothers, increasing sample size numbers and potentially assessing specific candidate genes. Progression in animal studies such as those described in Chapter 5 may give insight into potential candidate genes or specific regions to assess more closely.
Outcomes of pregnancies in women taking antiepileptic drugs for non-epilepsy indications

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A R T I C L E   I N F O

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Pregnancy
Teratogenicity
Valproate
Non-epilepsy indications
Bipolar disorder

A B S T R A C T

Purpose: Antiepileptic drugs (AEDs), particularly valproate (VPA), are known to be teratogens when taken by women with epilepsy (WWE), but the risk in women who take these drugs for indications other than epilepsy have been little studied. This study aims to investigate the incidence of birth defects in children born to mothers taking AEDs for non-epilepsy indications.

Methods: The Australian Pregnancy Register (APR), established in 1998, is a prospective observational study operating with ethical approval and informed written consent for participation. Of the 2066 pregnancies enrolled in the Register, 98% are WWE and the remainder received AEDs for other indications. Data from this Register was analysed to study the rates of congenital malformations (CM) in infants exposed to AEDs in utero in WWE compared to those women taking AEDs for other indications.

Results: The malformation rates in pregnancies of WWE taking AEDs (5%), is higher than the rates of infants born to untreated WWE (2%). There were 32 pregnancies enrolled from 29 mothers taking AEDs for indications other than epilepsy (2 women/2 pregnancies were lost to follow up). Out of 30 pregnancies, 9 of which were exposed to VPA, 1 resulted in a child with a malformation (3%) (cleft palate) on 1700 mg/day of valproate.

Conclusions: This is the first attempt to assess the use of AEDs in a prospective study of women who are pregnant but do not have active epilepsy. Although underpowered, this study suggests that women taking AEDs for non-epilepsy indications have a similar risk of having a child with a CM as compared with women taking AEDs for epilepsy. Larger numbers are required to investigate the risk of AED-associated malformations in this important group.

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1. Introduction

Antiepileptic drugs (AEDs) have been used to treat indications other than epilepsy for over 50 years, but this usage has increased over recent years [1]. Non-epileptic conditions include both mood disorders and neurological conditions. The following are the main non-epilepsy indications treated by AEDs and the drugs used to treat them (drugs used to treat are in parenthesis, for abbreviations see below): bipolar disorder (VPA, CBZ, LTG), anxiety (VPA, LTG, TPM, benzodiazepines), migraine (VPA, TPM), neuropathic pain (VPA, CBZ, TPM, PHT), trigeminal neuralgia (VPA, LTG, TPM), schizophrenia (VPA, CBZ, LTG) and multiple sclerosis (CBZ, LTG, GBP). AEDs have been particularly effective in treating bipolar disorder, and some AEDs such as VPA and LTG are now first line treatments [2,3]. Bipolar disorder typically manifests during reproductive years [4], and pregnant women with bipolar disorder who are not medicated have an increased risk of a recurrence of symptoms as well unfavorable pregnancy outcomes, making it difficult for women to safely cease the drug [5]. Similarly, women are 2–3 times more likely to develop multiple sclerosis (MS) than men and over 50% of patients with MS develop their symptoms during their childbearing years [6].

Exposure to AEDs during the first trimester of pregnancy in women with epilepsy is established for most AEDs to increase the risk threefold of having a child with a birth defect. This risk is increased up to 17-fold for VPA [7–9]. However, whether AED teratogenicity is specific to patients with epilepsy, or whether it affects all indications has not yet been well studied. This paper reports the incidence of birth defects for a cohort of women taking...
AEDs for non-epilepsy indications from the Australian Pregnancy Register of Anti-epileptic Drugs.

- Abbreviations: CBZ: carbamazepine, GBP: gabapentin, LTG: lamotrigine, TPM: topiramate, VPA: valproate

### 2. Participants and methods

#### 2.1. The Australian pregnancy register

The Australian Pregnancy Register (APR) is a national, prospective, observational, telephone interview-based register recruiting three groups of women:

1. WWE taking AEDs during the first trimester of pregnancy
2. WWE not taking AEDs during the first trimester of pregnancy
3. Women without epilepsy taking AEDs during the first trimester of pregnancy

Details of the register have been reported elsewhere [10]. Women were recruited nationwide on a voluntary basis. Eligible women were made aware of the Register through their medical practitioners, health professionals and other relevant sources such as the website and social media. All communication with women was on the telephone. Four interviews were conducted: in the first or second trimester of pregnancy, at 7 months of pregnancy, within the first month of birth and at the end of the first or second year. Details of the pregnancy and birth such as birth defects were recorded into the confidential database and each mother was given an individual identification number.

#### 2.2. Data analysis

All data for this analysis was collected from the APR between mid-1999 and August 2016. Relevant data was found by filtering the database for case subjects without epilepsy and AED exposed controls without epilepsy. Details of each subject’s AEDs and birth outcomes were recorded and assessed for any birth defects. Statistical analysis was not conducted due to the small numbers but comparisons were made between the different groups defined above. Ethical approval was obtained from The Melbourne Health Human Research Ethics Committee (HREC).

### 3. Results

At the time of this analysis there were 2066 pregnancies enrolled in the APR, including 38 twin pregnancies. Of these pregnancies, 32 were from women taking AEDs for a non-epilepsy indication, 2 of which were lost to follow up. 27/30 pregnancies were from unique women, while 3 women had 2 pregnancies each. For 17/30 pregnancies the women were taking folate before conception. All but three pregnancies involved folate supplementation in the first trimester. The indications as listed in Table 1 are: bipolar disorder (n = 16), pain (n = 6), multiple sclerosis (n = 2) anxiety (n = 1), depression (n = 1), hyperekplexia (n = 2), periodic ataxia (n = 1) and sleep disorder (n = 1). Of these 30 pregnancies, 1 resulted in a child with a major congenital malformation (cleft palate) in 2002. The mother of this child was taking VPA 1700 mg for bipolar disorder. She was taking folate before conception and also in the first trimester at a dose of 1 mg. Her pregnancy was registered in the Australian Pregnancy Register before the child with the malformation had been born. She had one induced abortion (maternal choice) earlier in 1993 while not on AEDs, prior to her VPA exposed pregnancy.

Table 1 shows the total number of non-epileptic women in the Australian Pregnancy Register suffering from each non-epileptic indication followed by which AED was taken during pregnancy. There were 32 women taking AEDs for indications other than epilepsy however 2/32 were lost to follow up and are therefore not included. Two of the women with bipolar disorder were on AED polytherapy, both of which included VPA in the combination. The two polytherapy combinations were: 

\[ \text{VPA} + \text{LTG} \] and \[ \text{VPA} + \text{LTG} + \text{GBP} + \text{TPM}. \]

**Hyperekplexia**: a neurological condition in which sufferers experience exaggerated reactions to noise, movement or touch.

Table 2 shows the dose ranges of VPA. All patients suffered a bipolar disorder. The most common doses were above 300 mg and up to 2000 mg which is above the upper limit of permissible doses for pregnant WWE [7,9,11].

Table 3 shows the number of birth defects in all categories of women enrolled in the Australian Pregnancy Register. Those lost to follow up have been excluded (20 pregnancies in total). It should be noted that of the 94 pregnancies resulting in a defect in WWE taking AEDs in the first trimester 5 were twin pregnancies. 2/5 twin pregnancies resulted in both twins having defects while in 3/5 only one of the twins had a defect. There were no twin pregnancies with defects in the other patient groups.

<table>
<thead>
<tr>
<th>VPA doses (mg)</th>
<th>Bipolar Disorder</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–300</td>
<td>1</td>
</tr>
<tr>
<td>301–600</td>
<td>2</td>
</tr>
<tr>
<td>601–1000</td>
<td>2</td>
</tr>
<tr>
<td>1001–1500</td>
<td>2</td>
</tr>
<tr>
<td>1501–2000</td>
<td>2</td>
</tr>
</tbody>
</table>

**Table 2**

VPA doses taken for APR non-epilepsy indications.

**Table 3**

Exposure to AEDs during pregnancy according to non-epilepsy indication.

<table>
<thead>
<tr>
<th>Indication</th>
<th>No of pregnancies</th>
<th>VPA</th>
<th>LTG</th>
<th>GBP</th>
<th>CLZ</th>
<th>PGB</th>
<th>CBZ</th>
<th>AZ</th>
<th>TPM</th>
<th>Polytherapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bipolar Disorder</td>
<td>16</td>
<td>9</td>
<td>9</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Pain</td>
<td>6</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depression</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sleep Disorder</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anxiety</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperekplexia</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Periodic Ataxia</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>9</td>
<td>9</td>
<td>5</td>
<td>6</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

4. Discussion

Fetal exposure to AEDs has been reported to be associated with a multitude of anatomical congenital malformations and cognitive deficits. These malformations were first reported by Holmes et al. (2001) [12] to be caused by exposure of the fetus to AEDs and not due the mother's seizures. Data from previous studies shows conclusively that VPA is the most significant teratogen compared to traditional and second generation AEDs currently available, and that other AEDs pose a less potent teratogenic risk [7,8].

Although this study was underpowered, because of the small number of pregnant women taking AEDs for a non-epilepsy indication, the data suggests that these women have a similar risk of having an infant with a congenital malformation (3%) as WWE taking AEDs (5%). Our study is underpowered partly because of low total denominators but also because of different medication combinations and doses, however this kind of study can potentially deliver relevant results. The incidence of malformations in both the AED-treated groups was slightly higher than that in WWE who were not taking an AED for their entire pregnancy (2%).

The mother of the single child with a congenital malformation who was taking VPA during her pregnancy, was also taking antidepressant sertraline concurrently. In this family consisting of mother, father and one child, the mother has a bipolar disorder and had a previous spontaneous abortion while she was on no medication. Subsequently she had a child with a cleft palate while taking VPA 1700 mg per day. We cannot ignore that the past history of this woman may be a complicating factor as she had epilepsy in childhood which resolved by the age of 14, however this cannot be regarded as active epilepsy. This analysis focused on the indication each woman was taking AEDs for at the time of pregnancy and this case was therefore considered for her bipolar disorder. In order to allay fears of reporting bias it is important to note that the pregnancy was enrolled in the register before the child was born. The mother had been taking folate both before conception and in the first trimester. In this cohort, only 1/30 pregnancies involved folate supplementation before conception and 27/30 in the first trimester. Neurologists are more likely to prescribe folate to women with epilepsy than women taking AEDs for non-epilepsy indications [13], another reason it is critical this patient group is addressed.

Our results, reporting on a single birth defect in a child exposed to high dose VPA during pregnancy for a psychiatric indication, is in accord with the published literature that highlights the risk associated with high dose VPA in WWE. One out of the nine children exposed to VPA during pregnancy one had a defect, which is higher than one would expect by chance. In Australia, the prevalence of oral facial clefts ranges from between 15 and 21 per 1000 births [14], again a smaller percentage than what we observed. The small numbers in this study may be due to underreporting. In Australia, the use of VPA for non-epilepsy indications is increasing. One of our earlier papers shows the increase of AEDs for psychiatric conditions compared with non-epilepsy indications, but it is not numerically defined [15]. A study in the United States found that more women of child bearing age were prescribed VPA for non-epileptic indications than for epilepsy [16]. Although precise numerical data is not available for the Australian population, this highlights the importance and potential underreporting of this patient group. Although there is limited published data regarding outcomes in this group one study found increased spontaneous abortions in women taking AEDs for non-epilepsy indications compared with WWE taking AEDs [17]. This may be another factor to assess in future analysis. Another possible route to take in future analysis would be to follow these children developmentally and assess for developmental disabilities aside from congenital malformations.

5. Conclusion

The question of the teratogenic risk of AEDs prescribed to women for indications other than epilepsy, has not been studied prospectively previously. This is an important issue given the increasing prescription of this class of drugs for non-epileptic indications. The interaction of epilepsy or the genetic background predisposing to epilepsy could influence the rate of malformations in women taking AEDs. Therefore, studies that specifically address this question are urgently required. However, the experience of the APR and other AED pregnancy registers is that this patient group is more difficult to enrol. The question does arise as to whether there is an underreporting of pregnancies in women with indications other than epilepsy. Our study, although underpowered, is the first study to specifically investigate this important question. The data suggest that exposure to a high dose of VPA during the first trimester of pregnancy in women taking AEDs for a non-epilepsy indication may pose a similar risk to that reported in WWE. Further research with larger numbers are required confirm this. This study may be viewed as a preliminary report which needs to be validated by one of the international pregnancy registers. It provides a platform and motivation upon which further research can take place.

Conflicts of interest

None.

Acknowledgments

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References

Chapter 7

General Discussion and Conclusion

7.1 Summary of key findings

The dual aims of this reported PhD thesis were to a) develop animal model that can be used to help unravel causative mechanisms involved in AED induced teratogenicity b) determine genomic markers from whole genome human analysis and to determine if having epilepsy has a role in the onset of these defects. These aims, which have partly been realised have culminated in the development of a viable animal model of AED induced birth defects using a therapeutic dose; we have not been able to identify genomic markers in spite of serious attempts however we found based on a whole genome analysis that increased de novo mutations are unlikely to be a causative mechanism and began to quantify the risk of AED induced birth defects for non-epileptic women. In this thesis, the results of the animal study related chapters were presented in chapters 3 and 4 and human data based studies in chapters 5 and 6. Each chapter included its own Discussion sections where findings were evaluated with reference to the previous literature. The key findings from each chapter as well as limitations and future directions for each section have been summarised in sections 7.1.2-7.1.5.

7.1.2 Determining a therapeutic dose of dietary valproate for epileptic rats (Chapter 3)

One of the main aims was to develop an animal model of VPA induced birth defects that closely models human circumstances and that can be used to assess for contributing causative mechanisms. The first step in the development of this model, explored in Chapter 3, was to find what dose of VPA is therapeutic when administered to Genetic Absence Epilepsy Rats From Strasbourg (GAERS) via the diet. Therapeutic range is the dose or blood concentration at which the drug has its desired therapeutic affect (Cooney et al., 2017). The three doses that were examined in this study were: 5, 10 and 20 g VPA/kg/food. These doses, based on animal weight and food intake, were on average : 308, 651, 1180 mg per day respectively. This study found that while all doses of VPA resulted in a reduction in seizures the highest dose of 20g/kg was the only dose that resulted in significant suppression of seizures in GAERS (29.23% reduction). The VPA blood levels for animals receiving this dose were between 104 and 284umol/L which were approaching human therapeutic levels.
Human therapeutic blood levels of VPA are reported to be approximately 300-700um/L (40-80ug/ml) (C. U. Johannessen & Johannessen, 2003). GAERS is an established model of seizures and previous studies administering acute VPA have observed a reduction in seizures (Dedeurwaerdere et al., 2011; Marescaux et al., 1992; Tringham et al., 2012) however no study has administered VPA via the diet and assessed seizure suppression and blood levels. Comparisons of previous models with our studies may not be strictly appropriate because of the doses, methods of administration and timing but previous studies were reviewed extensively in the relevant chapter. In this study, a dose-dependant relationship was not demonstrated however the highest dose resulted in a significant reduction in seizures and the other doses resulted in a non-significant reduction. While there was significant seizure suppression, and blood levels were approaching human therapeutic levels a dose double the highest dose was administered to rats in order to determine if this would result in higher blood levels, but this was found to make the animals sick. This chapter provided a direction upon which to take the next step, namely the morphological assessment of pups exposed to VPA in utero. Limitations of chapter 3 include the fact that the dose, although supressing seizures and approaching therapeutic blood levels, was still below reported human therapeutic levels. Future directions for further work arising from this section may include attempting a dose that is between 20g/kg and 40g/kg. This may be beneficial to explore if the rates and nature of malformations change amongst the strains.

7.1.3 Development of an animal model of VPA induced birth defects (Chapter 4)

Having determined what dose of VPA is therapeutic in the pilot study in Chapter 3 it was then possible to develop the rest of the animal model (Chapter 4). Previous VPA teratogenicity animal models have used a simplistic approach; using non-epileptic animals, drugs administered via stressful methods such as injection and gavage and administering AEDS at particular time points to achieve a specific outcome (for example neural tube defects on day E9) (Ehlers et al., 1992b; Mahabady et al., 2011). In reality, the cause of AED induced defects is likely to be extremely complex and dependent on multiple factors which should be taken into account when aiming to answer this question. Chapter 4 demonstrated that we have been successful in developing a viable animal model. Three strains were used; inbred epileptic; GAERS, in-bred non-epileptic; non-epileptic controls (NECs) and outbred non-epileptic Wistars. Clear anatomical differences were found between VPA exposed and control pups in all three strains. There was a significant reduction in weight, length and visual assessment scores in VPA exposed compared with control pups. In addition the internal spinal analyses found missing vertebral arches between T11 and C2 predominantly in VPA
exposed pup in GAERS, NEC and Wistars (100%, 95% and 80% respectively) compared with controls (9.09%, 13.3% , 19.04% respectively). VPA is associated with neural tube defects, increasing the risk of spina bifida by 1.5-2.5% (1.1% polytherapy, 2.5% monotherapy) compared to the 0.35% risk of most AEDs (Lindhout & Schmidt, 1986), from human data. From H&E staining analysis it was found that there was no damage to the spinal cord however spina bifida occulta is mainly associated with anomalies of bone and soft tissue. The findings in this section of the thesis show that an association between VPA and neural tube defects exists in the developed animal model. The model is in the process of being used to assess for mRNA changes in VPA exposed compared with control pup brains in the GAERS and NEC strains.

One of the limitations of chapter 4 was that it was difficult to quantify types of birth defect. The pups were given visual assessment scores rather than being labelled as a “yes” or “no” about having a defect. It was difficult to know what the precise nature of the birth defects were, however this may be better understood upon completion of genetic analysis. Another limitation was the quality of the spinal analysis images. There were difficulties in deducing precise locations of vertebrae and whether or not the staining technique was adequate. For this reason, micro CT scans are being conducted to validate the findings. Finally, there were different n numbers for different data sets which was not ideal. Part of the reason for this was that some pups were extracted before some specifics about the analysis were decided upon. The study is ongoing with micro CT scans of the pups and mRNA analysis currently in the process of completion. Further studies using the animal model may include: assessment of methylation levels in the pups and/or mother’s livers, assessment of DNA changes in pup brains (also available), and evaluation of embryonic fluid.

7.1.4 Whole genome analysis of affected children and their families (Chapter 5)

In order to assess for genetic and epigenetic biomarkers Chapter 5 took a whole genome approach to AED induced teratogenicity using human samples. The primary finding from this chapter was that an increased number of de novo mutations are an unlikely cause of AED induced birth defects. A precise risk of certain de novo mutations however may still be explored. No association, however, between a birth defect type and a de novo mutation were found in this study possibly due to small numbers. This finding means one causative mechanism can be ruled out and allows for more time and resources to assess other potential contributing mechanisms. The results for hypothesis 1 and 3 were not able yet complete and
so not able to be included in this thesis, however will likely clarify many new findings in the coming months.

Although chapter 5 was limited by sample size, conclusions could be drawn about the trio analysis. Hypothesis 2 was not able to yet be analysed due to the study being underpowered as indicated in the QQ-plots. Other limitations include the fact that different tissues were compared (blood vs saliva) and that samples for all individuals were taken at variable time points after birth. This is a limitation for the analysis of methylation data, as methylation patterns change over time (Johnson et al., 2012). The findings could be related to the age of the mothers, fathers or children. The completion of the DNA methylation analysis, mother variant analysis and genome wide association studies are currently ongoing. Future studies may include assessment of specific candidate genes or genes that were found to be of interest in the animal model mRNA analysis.

7.1.5 Quantifying the risk of AED induced birth defects for non-epileptic women (Chapter 6)

In recent years, the administration of AEDs for epilepsy has decreased and the prescriptions for non-epilepsy indications has increased (Vajda, Horgan, et al., 2012). This may be due to the fact that the teratogenic risk of AEDs for women with epilepsy has been well documented, while this risk for women taking AEDs for non-epilepsy indications has been largely underreported in the literature. This chapter focused on this important patient group. This study found a birth defect rate of 3.3% birth defects in children women taking AEDs for non-epilepsy indications compared with a rate of 5% in children of women with epilepsy taking AEDs. This indicates that women taking AEDs for non-epilepsy indications have a similar risk of having a child with a defect than women with epilepsy taking AEDs. In our study, out of the nine women taking VPA, one had a child with a defect. This is a much higher number than one would expect and again highlights the potential similar risk between epileptic and non-epileptic women. Comparing malformation rates between different indications is extremely valuable in determining if epilepsy is a contributing factor via genetic factors (discussed in chapter 6). Differences in dosing for these non-epilepsy indications, however are important to note. In some cases, lower doses are prescribed for non-epilepsy indications and may thus result in lower rates of malformations. Although not observed in this study, this was found in a recent study regarding Topiramate (Sonia et al., 2018).
It is essential that the degree of risk is defined for this particular group not only to control the prescribing of AEDs in these women but also to help to better understand the pathogenesis of AED induced teratogenicity in epilepsy as well. This study, accepted for publication in Seizure: European Journal of Epilepsy provides motivation for other international registers to do the same thing, ultimately increasing numbers and helping to be able to make definitive conclusions about this important question.

Chapter 6 was also limited by the small sample size meaning no firm conclusions could be drawn. Another limiting factor was the fact that the woman with the child with a birth defect had previously had childhood epilepsy which was not active. The AEDs were prescribed for non-epilepsy indications (bipolar). The genomic implications of this highlight again the importance of increasing numbers to confirm the finding. Future directions will include a greater effort to recruit non-epileptic women taking AEDs as a part of the register. This may include promoting the Australian Pregnancy Register at psychiatric clinics. In addition, the involvement of other international pregnancy registers will be critical to arrive at conclusions about this topic. It is expected that other projects will conduct similar studies upon the publication of our results.

7.2 Research implications

A major outcome of the work for this thesis study is the development of an animal model that closely replicates a human clinical setting and that can be used not only to assess numerous factors with the data produced from the current model (mRNA, DNA and organ analysis) but that can also be replicated in the future to better understand other factors involved in the pathogenesis of AED induced birth defects. The concurrent human whole genome assessment has so far ruled out one potential mechanism; that de novo mutations are a cause of AED induced birth defects- making us one step closer to identifying potential causative mechanisms. The human studies involved the recruitment of many families, many of which the DNA has been extracted for and sequenced and still able to be analysed in a number of different ways. The assessment of the risk of women with non-epilepsy indications suggested that having epilepsy is unlikely to be a contributing factor to the AED induced birth defects and the results from the animal model are in agreement with this. Larger human studies are required however to confirm this and the publication of our Chapter 6 study is expected to result in a collaborative effort from other international registers to conduct similar studies. The work in this PhD has paved the way for significant array of potential studies
that could continue to expand on what has already been found and can be used for numerous future analyses.

**7.4 Final Conclusion**

The question of AEDs and teratogenicity is a complex one and continued research in this area will undoubtedly reach conclusive answers. This thesis has contributed to one segment of a very complex research question and developed a methodology and platform upon which future research can build upon. It is likely that the genetic architecture of AED induced defects are not caused by one single causative mechanism but rather a combination of factors. There are numerous factors that need to still be assessed both using human samples and the established animal model. Ultimately increasing the numbers in the human study, using the developed animal model to assess for genomic factors and combining the information derived from the two processes, over time will help us to find an answer to this critical question that affects millions of women globally.
Table 1. Family recruitment contact table

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Families Identified</td>
<td>165</td>
<td>47</td>
</tr>
<tr>
<td>No of families genotyped (1 or more of family members)</td>
<td>71</td>
<td>29</td>
</tr>
<tr>
<td>No of families with samples not yet sequenced</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>Declined to participate</td>
<td>85</td>
<td>16</td>
</tr>
</tbody>
</table>

Table 1. The families that were contacted and accepted to take part in the study, how many of those families went on to send one or more samples and how many were genotyped. This is an estimate as not all families contacted at the start of the study were recorded.

Table 2. Total samples received throughout study

<table>
<thead>
<tr>
<th></th>
<th>Individuals</th>
<th>Families</th>
<th>Mother</th>
<th>Father</th>
<th>Children</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>295</td>
<td>87</td>
<td>83</td>
<td>64</td>
<td>148</td>
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<tr>
<td>Cases</td>
<td>104</td>
<td>32</td>
<td>33</td>
<td>18</td>
<td>53</td>
</tr>
</tbody>
</table>

Table 2. The total number of samples received throughout this study from Australian sources (recruitment through both the APR and private practice). Not all of these samples were sent for genomic analysis due to a) samples arriving after samples were sent for sequencing b) problems with DNA purity c) samples missing in the freezer.
Table 1. Control families in association, trio and methylation analysis

Antiepileptic drug, birth outcome and analysis type for every pregnancy for every control woman included in the analysis. (A)=WES mother variant analysis (B)=Trio analysis (C)=Methylation analysis *=father sample used for trio analysis **=father sample used for methylation ***=fathers sample used for both. VPA doses have been included where data was available.

<table>
<thead>
<tr>
<th>#</th>
<th>Mother I.D</th>
<th>Pregnancy 1</th>
<th>Pregnancy 2</th>
<th>Pregnancy 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CON1PM (A,C)</td>
<td>CON1C1 LTG/LEV LIVE BIRTH WITHOUT DEFECTS (C)</td>
<td>CON1C2 LTG/LEV LIVE BIRTH WITHOUT DEFECTS (C)</td>
<td>CON1C3 LTG/LEV LIVE BIRTH WITHOUT DEFECTS</td>
</tr>
<tr>
<td>2</td>
<td>CON3PM (A) **</td>
<td>CON3C1 CBZ/LEV LIVE BIRTH WITHOUT DEFECTS</td>
<td>SPONTANEOUS ABORTION</td>
<td>CON3C2 CBZ/LEV LIVE BIRTH WITHOUT DEFECTS</td>
</tr>
<tr>
<td>3</td>
<td>CON4PM (B) *</td>
<td>CON4C1 NO MEDS LIVE BIRTH WITHOUT DEFECTS (B)</td>
<td>CON4C2 NO MEDS (CBZ AT 17 WEEKS) LIVE BIRTH WITHOUT DEFECTS (B)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>CON7PM (A,B,C) ***</td>
<td>CON7C1 VPA 1000mg LIVE BIRTH WITHOUT DEFECTS (B,C)</td>
<td>CON7C2 VPA 1000mg LIVE BIRTH WITHOUT DEFECTS (B,C)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>CON10PM (A,C) **</td>
<td>CON10C1 CBZ LIVE BIRTH WITHOUT DEFECTS (C)</td>
<td>CON10C2 CBZ LIVE BIRTH WITHOUT DEFECTS (C)</td>
<td>CON10C3 CBZ LIVE BIRTH WITHOUT DEFECTS</td>
</tr>
<tr>
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<td>CON13PM (A,C)</td>
<td>CON13C1 LTG LIVE BIRTH WITHOUT DEFECTS</td>
<td>LTG SPONTANEOUS ABORTION</td>
<td>CON13C2 LTG* LIVE BIRTH WITHOUT DEFECTS (4th interview not yet done)</td>
</tr>
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<td>7</td>
<td>CON17PM (A,B,C) ***</td>
<td>CON17C1 LTG/TPM 3rd int. LEV* LIVE BIRTH WITHOUT DEFECTS (B,C)</td>
<td>CON17C2 LTG/TPM/LEV LIVE BIRTH WITHOUT DEFECTS (B,C)</td>
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</tr>
<tr>
<td>8</td>
<td>CON19PM (A)</td>
<td>CON19C1 CBZ LIVE BIRTH WITHOUT DEFECTS</td>
<td>CON19C2 CBZ LIVE BIRTH WITHOUT DEFECTS</td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>Mother I.D</td>
<td>Pregnancy 1</td>
<td>Pregnancy 2</td>
<td>Pregnancy 3</td>
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</tr>
<tr>
<td>9</td>
<td>CON21PM (A)</td>
<td>CON21C1 VPA 1000mg LIVE BIRTH WITHOUT DEFECTS</td>
<td>CON21C2 VPA 1000mg LIVE BIRTH WITHOUT DEFECTS</td>
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<tr>
<td>10</td>
<td>CON28PM (A) **</td>
<td>SPONTNEOUS ABORTION VPA</td>
<td>CON28C1 VPA 400mg LIVE BIRTH WITHOUT DEFECTS</td>
<td>CON28C2 VPA 400mg LIVE BIRTH WITHOUT DEFECTS</td>
</tr>
<tr>
<td>11</td>
<td>CON33PM (A,C )</td>
<td>CON33C1 LTG LIVE BIRTH WITHOUT DEFECTS</td>
<td>CON33C2 LTG LIVE BIRTH WITHOUT DEFECTS</td>
<td>CON33C3 LTG LIVE BIRTH WITHOUT DEFECTS</td>
</tr>
<tr>
<td>12</td>
<td>CON34PM (A,C) **</td>
<td>NO MEDS INDUCED ABORTION MATERNAL CHOICE</td>
<td>CON34C1 VPA 400mg LIVE BIRTH WITHOUT DEFECTS</td>
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<tr>
<td>13</td>
<td>CON35PM (B,C) ***</td>
<td>CON35C1 NO MEDS (CEASED VPA BEFORE PREG) LIVE BIRTH WITHOUT DEFECTS (B,C)</td>
<td>NO MEDS INDUCED ABORTION MOLAR PREGNANCY</td>
<td>CON35C2 NO MEDS LIVE BIRTH WITHOUT DEFECTS (B,C)</td>
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<td>14</td>
<td>CON36PM (A,C)</td>
<td>CON36C1 VPA 200mg LIVE BIRTH WITHOUT DEFECTS (*n meth)</td>
<td>CON36C2 VPA 200mg LIVE BIRTH WITHOUT DEFECTS (C)</td>
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<tr>
<td>15</td>
<td>CON38PM (A,C)</td>
<td>CON38C1 CBZ (CEASED TPM AND VPA BEFORE PREG) LIVE BIRTH WITHOUT DEFECTS</td>
<td>CON38C2 CBZ LIVE BIRTH WITHOUT DEFECTS</td>
<td></td>
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<tr>
<td>16</td>
<td>CON39PM (A)</td>
<td>CON39C1 LTG/TIAGABINE LIVE BIRTH WITHOUT DEFECTS</td>
<td>CON39C2 LTG/TIAGABINE LIVE BIRTH WITHOUT DEFECTS</td>
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<tr>
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<td>CON40PM (A)</td>
<td>CON40C1 LEV/LTG LIVE BIRTH WITHOUT DEFECTS</td>
<td>CON40C2 LEV/LTG LIVE BIRTH WITHOUT DEFECTS</td>
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<tr>
<td>18</td>
<td>CON42PM P2 TWINS (C)</td>
<td>CON42C1 VPA1500mg/LEV/ETX LIVE BIRTH WITHOUT DEFECTS</td>
<td>CON42C2, CON42C3 LEV/ETX/CLZ LIVE BIRTH WITHOUT DEFECTS (C) LIVE BIRTH WITHOUT DEFECTS (C)</td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>Mother I.D</td>
<td>Pregnancy 1</td>
<td>Pregnancy 2</td>
<td>Pregnancy 3</td>
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<td>19</td>
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<td>LTG</td>
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<td>LIVE BIRTH WITHOUT DEFECTS</td>
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<td></td>
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<td></td>
<td></td>
<td>(C)</td>
<td></td>
<td>(C)</td>
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<td>VPA 1000mg</td>
<td>VPA1000mg/CLZ</td>
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<td></td>
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<td>LIVE BIRTH WITHOUT DEFECTS</td>
<td>LIVE BIRTH WITHOUT DEFECTS</td>
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<tr>
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<td></td>
<td>(B,C)</td>
<td>(B,C)</td>
<td></td>
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<tr>
<td></td>
<td>(A,C)</td>
<td>LEV/LTG</td>
<td>LEV/LTG*</td>
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</tr>
<tr>
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<td>LIVE BIRTH WITHOUT DEFECTS</td>
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<tr>
<td></td>
<td></td>
<td>(C)</td>
<td>(C)</td>
<td></td>
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<tr>
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<td>CON61PM</td>
<td>CON61C1</td>
<td>CON61C2</td>
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<td>(C)</td>
<td>CBZ</td>
<td>CBZ</td>
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<tr>
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<td>LIVE BIRTH WITHOUT DEFECTS</td>
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<td>(C)</td>
<td>(C)</td>
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<td>(A,B)</td>
<td>LEV/LTG</td>
<td></td>
<td></td>
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<tr>
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<td>*</td>
<td>LIVE BIRTH WITHOUT DEFECTS</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>(B)</td>
<td></td>
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<tr>
<td>25</td>
<td>CON66PM PREG 4: TWINS</td>
<td>SPONTANEOUS ABORTION CBZ/TPM</td>
<td>CON66C1</td>
<td>SPONTANEOUS ABORTION CBZ/TPM</td>
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<tr>
<td></td>
<td>(A,B)</td>
<td></td>
<td>CBZ/TPM/LACOSAMIDE</td>
<td>CBZ/TPM/LACOSAMIDE</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td></td>
<td>LIVE BIRTH WITHOUT DEFECTS</td>
<td>LIVE BIRTH WITHOUT DEFECTS</td>
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<td>CON71C1</td>
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<td>(A)</td>
<td>LEV/OXCARB AZEPINE</td>
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<td>CON75PM</td>
<td>CON75C1</td>
<td>CON75C2</td>
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<td>(A)</td>
<td>LTG</td>
<td>LTG</td>
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<td></td>
<td></td>
<td>LIVE BIRTH WITHOUT DEFECTS</td>
<td>LIVE BIRTH WITHOUT DEFECTS</td>
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</tr>
<tr>
<td>#</td>
<td>Mother I.D</td>
<td>Pregnancy 1</td>
<td>Pregnancy 2</td>
<td>Pregnancy 3</td>
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<tr>
<td>----</td>
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<tr>
<td>28</td>
<td>CON78PM</td>
<td>CON78C1 NO AEDS LIVE BIRTH WITHOUT</td>
<td>CON78C2 CBZ LIVE BIRTH WITHOUT</td>
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<td></td>
<td>DEFECTS</td>
<td>DEFECTS</td>
<td></td>
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<tr>
<td>29</td>
<td>CON82PM</td>
<td>CON82C1 NO AEDS TILL 19 WEEKS(LTG)</td>
<td>CON82C2 LTG LIVE BIRTH WITHOUT</td>
<td></td>
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<td>(A)</td>
<td></td>
<td>LIVE BIRTH WITHOUT DEFECTS</td>
<td>DEFECTS</td>
<td></td>
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<tr>
<td>30</td>
<td>CON88PM</td>
<td>CON88C1 LEV/CLOBAZAM LIVE BIRTH</td>
<td>CON88C2 LEV LIVE BIRTH WITHOUT</td>
<td>CON88C3 LEV</td>
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<tr>
<td>(A,B)</td>
<td></td>
<td>WITHOUT DEFECTS</td>
<td>DEFECTS</td>
<td>LIVE BIRTH WITHOUT</td>
</tr>
<tr>
<td>*</td>
<td></td>
<td>(B)</td>
<td>(B)</td>
<td>DEFECTS</td>
</tr>
<tr>
<td>31</td>
<td>CON91PM</td>
<td>CON91C1 LEV LIVE BIRTH WITHOUT</td>
<td>CON91C2 LEV LIVE BIRTH WITHOUT</td>
<td></td>
</tr>
<tr>
<td>(A,B)</td>
<td></td>
<td>DEFECTS</td>
<td>DEFECTS</td>
<td></td>
</tr>
<tr>
<td>*</td>
<td></td>
<td>(B)</td>
<td>(B)</td>
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</tr>
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<td>CON95C1 CBZ LIVE BIRTH WITHOUT</td>
<td>CBZ SPONTANEOUS</td>
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<td>DEFECTS</td>
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<td>Pregnancy 1</td>
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<td>38</td>
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<td>CON140PM</td>
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<td>SPONTANEOUS ABORTION LEV/TPM</td>
<td>CON140C2 LEV/TPM LIVE BIRTH WITHOUT DEFECTS (B)</td>
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<td>CON143C2 TPM LIVE BIRTH WITHOUT DEFECTS</td>
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<td>DEFECTS</td>
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<td>47</td>
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<td>SPONTANEOUS ABORTION OXCARBAZEPINE</td>
<td>CON157C1 OXCARBAZEPINE LIVE BIRTH WITHOUT DEFECTS (B)</td>
<td>CON157C2 LEV LIVE BIRTH WITHOUT DEFECTS (B)</td>
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<td>49</td>
<td>CON161PM</td>
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<td>CON161C3, CON161C4 LTG LIVE BIRTH WITHOUT DEFECTS</td>
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<tr>
<td>50</td>
<td>CON162PM</td>
<td>CON162C1 NO MEDS LIVE BIRTH WITHOUT DEFECTS (B)</td>
<td>CON162C2 NO MEDS</td>
<td>CON162C3 LEV/CBZ LIVE BIRTH WITHOUT DEFECTS</td>
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<tr>
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<td>(A,B) *</td>
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<td>LIVE BIRTH WITHOUT</td>
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<tr>
<td>51</td>
<td>CON163PM</td>
<td>CON163C1 LTG LIVE BIRTH WITHOUT DEFECTS (B)</td>
<td>CON163C2 LTG</td>
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<tr>
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<td>(A,B) *</td>
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<td>DEFECTS</td>
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<tr>
<td>52</td>
<td>CON166PM</td>
<td>CON166C1 VPA 700mg (dose increased to 800mg daily at 24 weeks) LIVE BIRTH WITHOUT DEFECTS (B)</td>
<td>CON163C2 LTG</td>
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<tr>
<td></td>
<td>(A,B) *</td>
<td></td>
<td>LIVE BIRTH WITHOUT</td>
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<tr>
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<td>DEFECTS</td>
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Table 1. Continued

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<th>Pregnancy 4</th>
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<td>SPONTANEOUS ABORTION NO AEDS</td>
<td>CON168C1 NO AEDS</td>
<td>CON168C2 LEV</td>
<td>CON168C2 LEV</td>
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<td>OUT DEFECTS</td>
<td>OUT DEFECTS</td>
<td>OUT DEFECTS</td>
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<td>CON171C2 VPA 800mg</td>
<td>CON171C2 VPA 800mg</td>
<td>CON171C2 VPA 800mg</td>
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<td>OUT DEFECTS</td>
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<tr>
<td>55</td>
<td>CON173PM (A)</td>
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<td>CON173C2 LTG</td>
<td>CON173C2 LTG</td>
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<td>OUT DEFECTS</td>
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Table 2. Control families with more than 4 pregnancies

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<th>Pregnancy 4</th>
<th>Pregnancy 5</th>
<th>Pregnancy 6</th>
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<tr>
<td>56</td>
<td>CON49PM (B,C) ***</td>
<td>NO AEDS (CBZ WEANED PRIOR TO PREG) INDUCED ABORTION MATERNAL CHOICE</td>
<td>CON49C1 NO MEDS (CEASED CBZ FOR PREG) LIVE BIRTH WITHOUT DEFECTS (B,C)</td>
<td>CON49C2 NO MEDS LIVE BIRTH WITHOUT DEFECTS</td>
<td>CON49C3 NO MEDS SPONTANEOUS ABORTION</td>
<td>NO MEDS SPONTANEOUS ABORTION</td>
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Table 3. Children samples were sequenced for without their mother

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<th>AED and outcome</th>
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<tr>
<td>1</td>
<td>CON16C1 (C)</td>
<td>CBZ LIVE BIRTH WITHOUT DEFECTS</td>
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<tr>
<td>2</td>
<td>CON29C2 (C)</td>
<td>No AED (started LTG at 16 weeks) LIVE BIRTH WITHOUT DEFECTS</td>
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<td>3</td>
<td>CON52C1 (C)</td>
<td>LTG LIVE BIRTH WITHOUT DEFECTS</td>
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<tr>
<td>4</td>
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Table 4. Fathers genotyped without mothers

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<td>2</td>
<td>CON16PF (C) ***</td>
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<tr>
<td>#</td>
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<tr>
<td>5</td>
<td>F6PM</td>
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<tr>
<td>6</td>
<td>F7PM</td>
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<tr>
<td></td>
<td>(C)</td>
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<tr>
<td>7</td>
<td>F9PM</td>
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<tr>
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<td>(A,B,C)</td>
</tr>
<tr>
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<td>***</td>
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<tr>
<td>8</td>
<td>F10PM</td>
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<tr>
<td>#</td>
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<tr>
<td>9</td>
<td>F12PM</td>
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<td>(A,B,C) ***</td>
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<tr>
<td>10</td>
<td>F13PM</td>
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<tr>
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<td>(C) **</td>
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<tr>
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<td>F17PM</td>
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<td>#</td>
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<td>------------</td>
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<tr>
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<td>F18PM (A,C)</td>
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<tr>
<td>13</td>
<td>F20PM (A)</td>
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<td>14</td>
<td>F21PM (A)</td>
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<td>F31PM (A)</td>
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<td>F32PM (A,B)</td>
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<td>18</td>
<td>F33PM (A,B)</td>
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<tr>
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<td>F36PM (A,B) *</td>
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<td>F38PM (A,B) *</td>
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<td>F44PM (A,B) *</td>
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<td>23</td>
<td>CON24PM (changed to case) (C)</td>
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<td>24</td>
<td>CON25PM (changed to case) (A,C)</td>
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Table 5. Case children sampled without mother or father

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<tbody>
<tr>
<td>1</td>
<td>F11C4 (C)</td>
<td>VPA 700mg</td>
<td>Live birth with defects: Patent foramen ovale</td>
</tr>
<tr>
<td>2</td>
<td>F14C1 (C)</td>
<td>VPA 500mg BD</td>
<td>Live birth with defects: Dysmorphic features</td>
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Table 6. Samples received from outside sources

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<th>Source</th>
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<th>AED exposed to</th>
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<td>Prof.Torbjorn Tompson</td>
<td>TOP due to spina bifida</td>
<td>VPA</td>
</tr>
<tr>
<td>27</td>
<td>002PM (A)</td>
<td>Prof.Torbjorn Tompson</td>
<td>TOP due to neural tube defects</td>
<td>VPA</td>
</tr>
<tr>
<td>28</td>
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<td>TOP due to spina bifida</td>
<td>VPA</td>
</tr>
<tr>
<td>29</td>
<td>004PM (A)</td>
<td>Prof.Torbjorn Tompson</td>
<td>TOP due to spina bifida and Chiari 2</td>
<td>VPA</td>
</tr>
<tr>
<td>30</td>
<td>32609PM (A)</td>
<td>Prof. Sam Berkovic</td>
<td>Live birth with spina bifida</td>
<td>VPA/CBZ</td>
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</table>

Abbreviation TOP= Termination of pregnancy
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