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# Genetic Exploration of Valproate Induced Weight Gain

Joseph M. Chukwu

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# **Genetic Exploration of Valproate Induced Weight Gain**

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**Doctor of Medicine (MD)**

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## Outputs

### Publications

1. Chukwu, J., Delanty, N., Webb, D. & Cavalleri, G. L. 2014. Weight change, genetics and antiepileptic drugs. *Expert Rev Clin Pharmacol*, 7, 43-51(See Appendix 14 for the manuscript)
2. Boyle, M., Chukwu, J., Boyle, M., Connolly, A. & Webb, D. 2014. An audit of first afebrile seizure management in an Irish tertiary pediatric setting. *Eur J Pediatr*, 173, 525-8.

### Conference presentations

1. M O'Dea, D Webb, J Chukwu. A five year review of seizure outcome following VNS insertion for intractable childhood epilepsy- poster presentation at the 4<sup>th</sup> Research and Audit Day Our Lady's Children's Hospital Crumlin on 23<sup>rd</sup> May 2014.
2. J Chukwu, N Delanty, D Webb, GL Cavalleri. Oral Presentation. Pharmacogenomic Risk Factors Associated with Valproate-induced Weight Change in the Paediatric Population. Royal College of Surgeons in Ireland Research Day 2013. Dublin, Ireland, March 2013

3. J Chukwu, S Tirupathi, M King, B Lynch, N Delanty, D Webb, GL Cavalleri. Poster Presentation. Pharmacogenomic Risk Factors Associated with Valproate-induced Weight Change in the Paediatric Population. Molecular Medicine Ireland. Dublin, Ireland, March 2013.
4. J Chukwu, S Tirupathi, M King, B Lynch, N Delanty, D Webb, GL Cavalleri Epilepsy Pharmacogenomics of Valproate induced weight gain Irish Society of Human Genetics Annual Scientific Meeting, 3rd September 2012, Royal College of Surgeons in Ireland
5. J Chukwu, S Tirupathi, M King, B Lynch, N Delanty, D Webb, GL Cavalleri Pharmacogenomic risk factors associated with VPA-induced weight change in the paediatric population – oral presentation at the Epilepsy Genetics (EpiGen) Meeting 5<sup>th</sup> October 2012 at Institute of Neurology Queen’s Square, London
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7. Chukwu J, Gallagher P, McCoy B, Webb D. A study of the value of a sleep EEG record in predicting seizure recurrence in children with a single afebrile seizure – presented at the European Academy of Paediatrics meeting at Istanbul, Turkey between the 5th and 9th of October 2012.
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9. Chukwu J, Gallagher P, McCoy B, Webb D A study of the value of a sleep EEG record in predicting seizure recurrence in children with a single afebrile seizure –presented at the Irish Paediatric Association meeting in Belfast on 19<sup>th</sup> to 20<sup>th</sup> April 2012.
- 10.J Chukwu, S Tirupathi, M King, B Lynch, N Delanty, D Webb, GL Cavalleri Pharmacogenomics of Valproate induced weight gain - 10th European Congress on Epileptology, 30<sup>th</sup> September to 4<sup>th</sup> October 2011, Excel Centre London.

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## List of Abbreviations

A <sub>1</sub>	Test allele
A <sub>2</sub>	Minor allele
ACTH	Adrenocorticotrophic hormone
ADHD	Attention deficit hyperactivity disorder
ADR	Adverse Drug Reactions
AED	Antiepileptic drugs
ANCOVA	Analysis of covariance
BC	Before Christ
BDNF	Brain-derived neurotrophic factor
BMI	Body mass index
BP	The physical position of SNP (base pairs)
°C	Centigrade
CHISQ	Chi square association
CHR	Chromosome code

CMRF	Children's Medical Research Foundation
CNS	Central nervous system
CRF	Case report form
CT	Computed Tomography
CTNBL1	Catenin, beta like 1
CUH	Children's University Hospital Temple Street
df	Degree of freedom
DNA	Deoxyribonucleic acid
DNET	dyplastic neuro-endothelial tumours
DRE	Drug resistant epilepsy
EDTA	Ethylenediaminetetraacetic acid
EEG	Electroencephalography
ETV5	Ets Variant 5 gene
eW	Expected weight
F_A	Case allele frequency

FDA	The Food and Drug Administration
FH	Family history
<i>FLJ35779</i>	Encoding POC5 centriolar protein homolog
FSAI	Food Safety Authority of Ireland
FTO	Fat mass and obesity-associated protein gene
F_U	Control allele frequency
GABA	Gamma-Aminobutyric acid
GAERS	The genetic absence epilepsy-prone rat model
GBP	Great British Pounds
GNPDA2	Glucosamine-6-phosphate deaminase 2
GLUT1	Glucose transporter 1
GPRC5B	G protein-coupled receptor, family C, group 5, member B
GWAS	Genome-wide association study
GWA	Genome-wide association
HGP	Human Genome Project

HIV	Human Immunodeficiency virus
IBM	International Business Machine
ICU	Intensive care unit
IGE	Idiopathic Generalised Epilepsy
ILAE	International League Against Epilepsy
KCTD15	Potassium channel tetramerization domain containing 15 gene
Kg	Kilogram
L	Litre
LD	Linkage disequilibrium or learning disability
LTG	Lamotrigine
M	Molar
m <sup>2</sup>	metres squared
MAF	Minor Allele Frequencies
MAF gene	V-maf musculoaponeurotic fibrosarcoma oncogene homolog

MAP2K5	Mitogen-activated protein kinase 5
MC4R	Melanocortin 4 receptor gene
MES	Maximal electroshock seizure
mg	milligram
MgCl <sub>2</sub>	Magnesium chloride
MRI	Magnetic Resonance Imaging
MTCH2	Mitochondrial carrier 2
n	Number or sample size
NaCl	Sodium chloride
N/A	Not applicable
NCBI	National Centre for Biotechnology Information
NCH	National Children's Hospital Tallaght Dublin
NCRC	National Children's Research Centre, Crumlin
NEGR1	Neuronal growth regulator 1
NG	Nasogastric tube

NHS	National Health Service, United Kingdom
NMISS	Number of non-missing values
NPC1	Niemann-Pick disease type C1
NTC	No template controls
NYU	New York University
OCP	Oral contraceptive pill
OCR	ORACollect DNA collection kit
OG	Oragene DNA collection kit
OLCHC	Our Lady's Children's Hospital Crumlin
OLCH	Our Lady's Children's Hospital
OR	Odds ratio
OW	Observed weight
p	alpha value – level of significance
PCR	Polymerase chain reaction
PEG	Percutaneous endoscopic gastrostomy

PGA	Power for Genetic Association studies
PGB	Pregabalin
PIL	Participant's information leaflet
PLINK	PuTTY Link
PRKD1	Protein kinase D1
Proteinase K	Tritrachium album serine proteinase
PRL	Prolactin
PTER	Phosphotriesterase-Related Protein
PW	predicted weight
QPCTL	glutaminy-peptide cyclotransferase-like RBJ dnaJ homolog subfamily C member 27
RCSI	Royal College of Surgeons in Ireland
REC	Research ethics committees
rs	reference SNP identification number
$r^2$	R-squared (Co-efficient of multiple correlations)
SD	Standard deviation

SDS	Sodium dodecyl sulphate
SE	Standard error
SH2B1	SH2B adaptor protein 1
SLC39A8	solute carrier family 39 (zinc transporter),
SLR	Standard Laboratory Reagent
SNP	Single nucleotide polymorphism
SPSS	Statistical Package for Social Sciences
TE	Tris and Ethylenediaminetetraacetic acid
TMEM18	transmembrane protein 18
TPM	Topiramate
TNNI3K	Serine/Threonine Protein Kinase TNNI3K
TRIS	Tris(hydroxymethyl)aminomethane
μmol	micromole
UK	United Kingdom
USA/ US	United States of America

VGB	Vigabatrin
VPA	Valproic acid, valproate, sodium valproate
WAC	Weight for age centile
WAZ	Weight for age z-score
WC0	Initial weight centile
WC12	Weight centile at 12 months post initiation of VPA therapy
WHO	World Health Organisation
=YEARFRAC	Microsoft Excel function used to calculate the number of whole days between two dates
yrs	Years
ZNS	Zonisamide
z-score	Standard score

## **Student Declaration**

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

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Student Number: \_11112808\_

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The contents of the enclosed manuscript are confidential and should not be disclosed, or disseminated in any way, to any third party other than to staff or students of the Royal College of Surgeons in Ireland or an external examiner appointed for the purpose of reviewing the manuscript.

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## **Summary**

### **Background:**

Childhood obesity has both long- and short-term health consequences and is a growing public health problem in Ireland (O'Neill et al, 2007; Finucane et al, 2008; Riddoch et al, 1991). Medications used in the treatment of chronic medical conditions like epilepsy and psychiatric illness might be contributing to this epidemic (Ben-Menachem, 2007, Pickrell, 2013; Martine). Epilepsy affects about 50 million people worldwide with about 40,000 people living with the condition in Ireland (JEC, 2011, Linehan et al, 2010). Sodium valproate (VPA) is an effective anti-epileptic drug, but up to 71% of the patients exposed to the drug report weight gain as an adverse drug reaction (Biton, 2003). The clinical and genetic risk factors associated with this ADR are unknown.

### **Objective**

To identify clinically relevant predictors of VPA-induced weight change in children with epilepsy within the first year of initiation of therapy.

### **Methods:**

Under a retrospective framework, we recruited and performed clinical phenotyping on paediatric epilepsy patients treated with VPA. We employed maximum percentage weight change, and change in weight-for-age z-score as weight change phenotypes. We extracted DNA from these subjects and

carried out genotyping for 17 SNPs that had previously been robustly associated with changes in BMI and weight in healthy individuals. Meta-analysis of our results was conducted using a fixed-effects model.

### **Results:**

We recruited 251 children, of which 218 underwent genotyping. About 48% of the children experienced a gain in weight of  $\geq 5\%$  from that predicted by weight at initiation of therapy. None of the clinical factors tested significantly influenced weight change. Meta-analysis indicated that the genetic variants rs10938397, rs7498665 and rs1805081 showed nominally significant association with quantitative weight change due to VPA. However none survived correction for multiple testing.

### **Conclusions:**

None of the clinical phenotypes tested in this study was associated with VPA-induced weight change, nor did our results support clinical predictors reported in previous studies. Although three of the SNPs associated with changes in BMI showed nominally significant association with weight change associated with VPA, none of them survived Bonferroni correction. A large multi- centre research is required to provide sufficient power to identify any genetic factors associated with this ADR.

# **Chapter 1: General Introduction/ Background**

## **1. Introduction**

Childhood obesity is a growing and serious public health problem in Ireland (O'Neill et al., 2007, Finucane et al., 2008). Over the last decade the proportion of overweight children in Ireland has trebled (Evans et al., 2011). The health consequences of this increased prevalence of overweight and obesity cannot be overemphasised (Finucane et al., 2008, Riddoch et al., 1991). The increased prevalence of obesity in Irish children has been attributed to various factors including increased consumption of energy-rich food and reduced physical activity (Riddoch et al., 1991, Murrin et al., 2012). Medications used in the treatment of such chronic childhood conditions as diabetes, psychiatric illness and epilepsy may also be contributing to this epidemic (Ben-Menachem, 2007, Pickrell et al., 2013, Martinez-Ortega et al., 2013, Pramyothin and Khaodhiar, 2010, Overbeek et al., 2010, Maayan and Correll, 2011, Dori and Green, 2011, Thon et al., 1992, Davis et al., 2012).

## **1.1 What is epilepsy?**

Epilepsy is defined as a brain disorder which manifests as a tendency to recurrent, unprovoked seizures (Shovron, 2009). About 50 – 80 new cases of epilepsy are diagnosed per 100,000 people per year, while 4 – 10 per 1000 population are living with epilepsy. The lifetime prevalence of seizures is about 10%. The incidence and prevalence of epilepsy are higher in the developing world compared to developed countries. Of the estimated 50 million people with epilepsy worldwide, 40 million (80%) live in the developing world where more than 75% of people living with epilepsy do not receive adequate care (JEC, 2011). In the Republic of Ireland, the prevalence of epilepsy in people over the age of five years is 9 per 1,000 with about 40,000 living with the condition (Linehan et al., 2010). About 1 in 200 Irish children are affected (EpilepsyIreland). Epilepsy is associated with health, social, psychological and economic problems. Epilepsy increases the standardised mortality rate two to three folds (Shovron, 2009). It is also associated with social isolation and problems in personal relationships, education, employment and family life (Shovron, 2009). About 1 in 10 people with epilepsy experience frequent seizures, occurring more than once weekly. Epilepsy demands intense medical resources and associated direct and indirect costs. In the UK for instance, the management of epilepsy takes up about 0.25% of the total health care costs (Shovron, 2009). Using the 2012/2013 NHS health budget of 109 billion GBP, the cost of epilepsy amounts to about 270 million GBP per annum in the UK alone. (NHS, 2013).

## **1.2. How is epilepsy treated?**

Epileptic seizures in children and adults can be managed with anti-epileptic drugs (AEDs), dietary measures and/ or epilepsy surgery procedures. Most children diagnosed with epilepsy are subjected to a trial of one or more AEDs in an attempt to control seizures. Around 24 AEDs have been approved for use for the treatment of epilepsy in the United States (Sirven et al., 2012). The number of available AEDs has increased over the last few years. For example, from 2010 to 2012, five new AEDs have been approved for the US market (Sirven et al., 2012) (see Fig 1 below). This increase in the number of approved AEDs has been associated with slightly improved control of seizures for the majority of the people with epilepsy but this improvement is largely limited to those patients living in developed countries, with access to appropriate health care (Loscher et al., 2013b). Although the new generation AEDs are associated with less severe ADRs their use has not resulted in a significant reduction in the proportion of people with drug-resistant epilepsy (DRE) (Privitera, 2011, Mattson, 1995). Novel approaches to the treatment of DRE are currently being tried (Sorensen and Kokaia, 2013). Gene therapy using cell transplantation and viral vectors is also another promising approach to epilepsy treatment (Riban et al., 2009).

AEDs are functionally and structurally diverse (Howard et al., 2011). Despite the fact that AEDs have been used for many years, the relationship between their clinical effects of seizure control and the mode of action are still poorly understood (Howard et al., 2011). The principal modes of action include membrane stabilization through sodium channel blockage and reduced release of neurotransmitters and enhancement of GABA mediated inhibition (Howard et al., 2011). It is probably due to this wide range of action on different parts of the central and peripheral nervous systems that AEDs are used in many other neurological and psychiatric conditions including neuropathic pain, mania, anxiety, hiccups, hot flushes, pruritus, spasms, trigeminal neuralgia, migraine and restless leg syndrome.

The design and development of AEDs is subject to the established phases of standard clinical trials, but preclinical AED trials are hampered by the limited number of relevant animal models of epilepsy. Experimental models such the electroshock seizure model, maximal electroshock seizure (MES)(Loscher et al., 2013a) test and the pentylenetetrazole seizure models in mice and rats are only suitable for AED discovery targeted at generalized tonic-clonic seizures. While amygdala kindling is used for AED development for partial seizures, the genetic absence epilepsy-prone rat (GAERS) model has been employed for genetic generalised epilepsy (Loscher et al., 2013a).

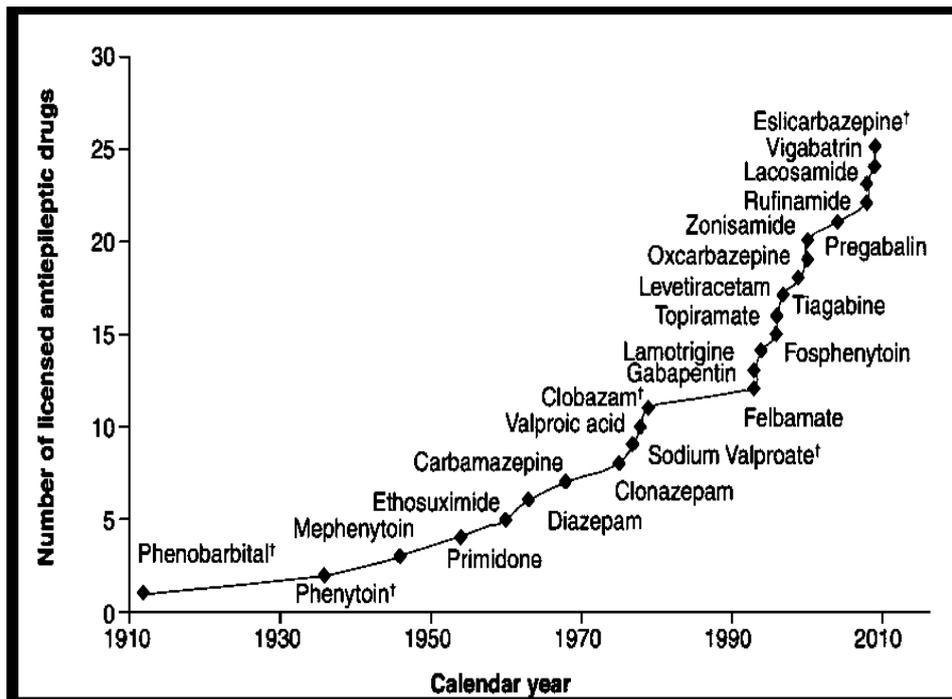


Figure 1. Timeline of antiepileptic drugs approval by the USA Food and Drug Administration in the USA and outside of the USA. +Date of approval outside of the USA

(figure reproduced from (Cavalleri et al., 2011))

### 1.2.1. Drug resistant epilepsy

About a third of people with epilepsy do not respond well to the AEDs that are currently available and have what is frequently referred to as “drug resistant epilepsy” (or “refractory” epilepsy) (Loscher et al., 2013a, Herranz, 2006, Arroyo et al., 2002, Kwan and Brodie, 2000). The International League Against Epilepsy (ILAE) recently defined this as epilepsy “...in which the seizures are not controlled after having correctly taken two appropriate and

well tolerated anti-epileptic drugs.....”(Sanchez-Alvarez et al., 2012). OR “The failure of adequate trials of two tolerated, appropriately chosen and used anti-epileptic schedules (whether as monotherapies or in combination) to achieve sustained seizure freedom.” (Kwan et al., 2010). Study in animal models of resistance indicates that compared to AED resistant animal models, the AED resistant animals had higher frequency of seizures prior to the commencement of AEDs, psychopathology, and hippocampal lesions in addition to the alterations in AED targets and transporters (Loscher et al., 2013a). In patients with DRE the introduction of successive AEDs after the second AED yields diminishing returns in terms of additional proportion of patients that achieve seizure control (Kwan and Brodie, 2000) . While about 50% of patients will be seizure free on the first AED, addition of a second AED will result in an additional 10 – 15% seizure freedom while the introduction of a third and subsequent AED will only result in additional 3 – 5% seizure freedom (see figure 2 below). Even in those patients with severe forms of epilepsy, being treated with multiple AEDs, debilitating ADRs are a great impediment to seizure control as it is difficult to achieve full effective doses, and compliance with therapy is poor as a result (Perucca and Gilliam, 2012, Sanchez-Alvarez et al., 2012). This group of patients therefore bear the combined burden of uncontrollable seizures and severe ADRs resulting from the use of multiple AEDs (Perucca and Gilliam, 2012, Perucca and Meador, 2005).

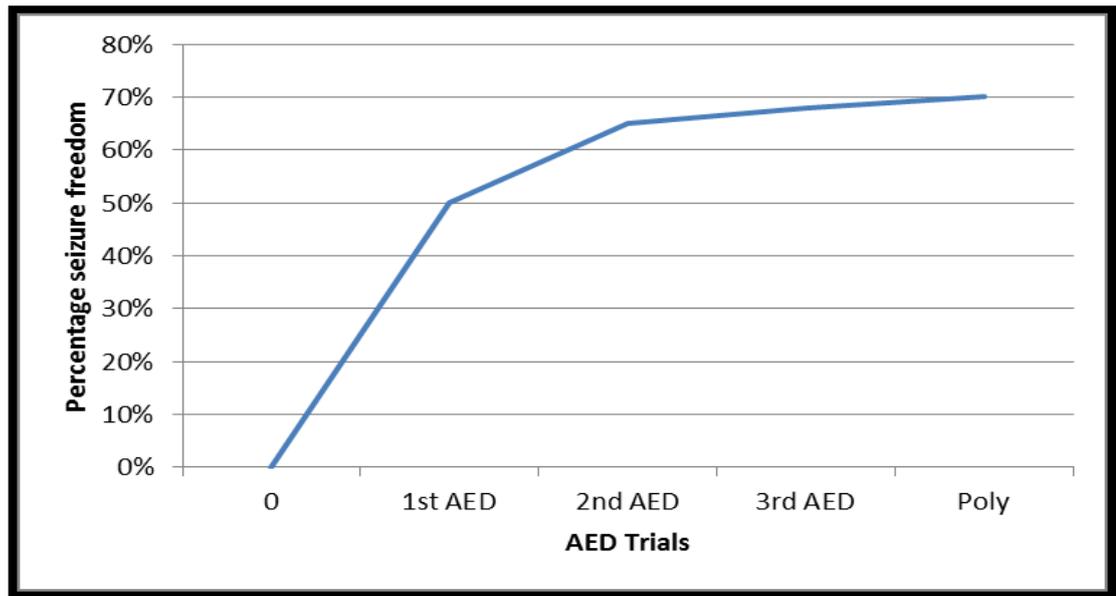


Figure 2: Diminishing returns of seizure freedom from addition of subsequent AEDs

Patients with DRE can be tried on a ketogenic diet. Epilepsy has been treated with fasting and other dietary measures since the year 500 BC (Wheless, 2008). However, the ketogenic has been in use since the 1920s when Wilder and Peterman, two paediatricians at the John Hopkins University, postulated that starvation which results in ketosis resulted in improved seizure control (Wheless, 2008). Its use dropped after AEDs were initially discovered, but there has been resurgence in its use in the last 15 – 20 years (Rogovik and Goldman, 2010). Randomised controlled trials (RCTs) have demonstrated the efficacy of the ketogenic diet in the reduction of intractable childhood epilepsies (Neal et al., 2008, Neal et al., 2009). The classical ketogenic diet is a high-fat (80%), low-carbohydrate (5%) and normal-protein (15%) regime. Other types of ketogenic diets include the medium chain triglyceride (MCT) oil which is low in carbohydrate, modified

ketogenic (Atkins) diet and the use of foods with low glycaemic index (Haberlandt et al., 2013, Klepper and Leiendecker, 2013, Liu and Wang, 2013). The ketogenic is typically used in drug resistant childhood epilepsies, in children whose epilepsy syndrome is not amenable to surgery, in those with Dravet syndrome and myoclonic astatic epilepsy (Rogovik and Goldman, 2010, Wang and Lin, 2013). In those with glucose transporter 1 (GLUT1) and pyruvate dehydrogenase deficiency treatment with ketogenic diet is life-long (De Giorgis and Veggiotti, 2013). Although, this diet is poorly tolerated in adults compared with children but it is suitable for in all ages. This diet should however be avoided in patients with the following metabolic and organ system disorders: porphyria, fatty acid oxidation defect, pyruvate carboxylase deficiency, kidney disease, severe malnutrition, severe gastro-oesophageal reflux and pancreatitis (Wang and Lin, 2013). In addition to performing metabolic screen prior to the initiation of therapy, patients metabolic profile are usually monitored at six-monthly intervals and supplementation with multivitamins calcium, vitamin D and trace elements might be required (Neal et al., 2012).

Surgical procedures are also an option for patients with DRE. The most common paediatric epilepsy surgical procedures are temporal and frontal lobectomies, for children with cortical malformations (Jadhav and Cross, 2012, Harvey et al., 2008). Other procedures include hemispherectomies and multilobar procedures. Children who are unsuitable for resective surgical procedures may benefit from either a callosotomy or the implantation of a device stimulating the vagus nerve (Jadhav and Cross,

2012, Harvey et al., 2008). Seizure freedom rates of up to 80% have been achieved in some selected cohorts of patients who had undergone hemispherectomies or multilobar procedures (Jadhav and Cross, 2012). In addition to seizure freedom, improved seizure control could be achieved in a variable proportion of candidates depending on the causative lesion (Engel, 1996). A randomised controlled trial of anterior temporal lobectomy surgery versus medical treatment in 80 patients with temporal lobe epilepsy clearly demonstrated the superiority of surgical treatment over medical treatment in this condition at the end of the first year of follow up (58% of those that underwent lobectomy were seizure free compared to 8% of medically treated patients) (Wiebe et al., 2001).

The success of epilepsy surgical procedures depends on three main factors: early referral to a specialised epilepsy surgical unit; the nature of the lesion and; the completeness of the resection of the seizure focus (Jadhav and Cross, 2012, Tonini et al., 2004).

Other positive prognostic factors are febrile seizures, mesial temporal sclerosis, tumours and concordance between EEG and MRI (Tonini et al., 2004). The negative predictors of successful epilepsy surgery include the presence of post-operative electroencephalographic (EEG) discharges and the use intracranial monitoring prior to the procedure (Tonini et al., 2004). A dedicated epilepsy surgery team is vital to the success of epilepsy surgery in suitable candidates.

### **1.2.2. Adverse reactions associated with antiepileptic drugs**

An adverse drug reaction (ADR) is defined by the WHO as “ a response to a drug that is noxious and unintended and occurs at doses normally used in man for the prophylaxis, diagnosis or therapy of disease or for modification of physiological function.” (WHO, 1972). ADRs have been a factor in epilepsy treatment since the introduction of bromide (the first AED) more than 160 years ago. However, it is only in recent years that there have been efforts to define, quantify and address the importance of ADRs in epilepsy care (Perucca and Gilliam, 2012).

The consequences of ADRs include early discontinuation of treatment in about 25% of the patients. ADRs impede the attainment of optimal doses and may lead to poor treatment compliance (Cavalleri et al., 2011). ADRs are also associated with increased disability, morbidity and mortality. ADRs not only increase the burden on health care use but also increase health care costs (Perucca and Gilliam, 2012, Perucca and Meador, 2005). In a study of 767 adult and paediatric patients with epilepsy, Buchanan (1992) identified 155 different ADRs (the majority being pharmacodynamic in nature) in 134 of the patients (Buchanan, 1992). The withdrawal and replacement of the offending AEDs was the most common management approach (Buchanan, 1992).

The ideal AED controls seizures without any associated ADR. This does not seem to exist in clinical epileptology as all the AEDs in current use have associated ADRs (Cavalleri et al., 2011). While some of the ADRs are mild and could be ignored, others are severe or even life-threatening.

ADRs associated with AEDs can be classified according to the affected organ or system, severity, associated symptoms, frequency of the ADR or according to the pathophysiological mechanism (Perucca and Gilliam, 2012). Five distinct biological classes have been proposed by Perucca and Gilliam (2005). These are

- (i) sleep, mood and emotion,
- (ii) cognition and coordination,
- (iii) weight and iv) cephalgia and
- (iv) tegument and mucosa.

Other classification systems have also been used including the WHO classification and the classification of ADRs into CNS, Non-CNS manifestations and hypersensitivity (Cavalleri et al., 2011, Perucca and Meador, 2005, Perucca and Gilliam, 2012). Table 1 illustrates the type and frequency of the common CNS, non-CNS and hypersensitivity ADRs of the commonly prescribed AEDs.

Many factors, including genetic variation, are thought to play an important role in the development of AED-induced ADRs (Holmes, 2002 , Cavalleri et al., 2011). The trial-and-error method of AED selection currently being employed by the physicians to determine the most effective AED is contributing to the incidence of treatment failures and AED-induced adverse effects (Yoshida et al., 2011). It is hoped that pharmacogenomic testing would reduce the incidence of these events.

Table 1. CNS and non-CNS side effects of common and non-CNS side effects of common anti-epileptic drugs

Drug	Non-CNS ADRs	CNS ADRs
<b>Carbamazepine</b>	Nausea, vomiting, diarrhoea, hyponatremia, rash, pruritus	Drowsiness, dizziness, blurred or double vision, lethargy, headache
<b>Clobazam</b>	Increased salivation, nausea, vomiting, constipation	Somnolence, aggression, irritability, ataxia, insomnia
<b>Ethosuxamide</b>	Nausea, vomiting	Sleep disturbance, drowsiness, hyperactivity
<b>Ezogabine</b>	Nausea, fatigue, change in colour of urine, dysuria, urinary hesitancy, <b>weight gain</b>	Dizziness, somnolence, confusion, vertigo, blurred or double vision, tremor, abnormal coordination, inattention, memory impairment
<b>Felbamate</b>	Nausea, vomiting, anorexia, <b>weight loss</b>	Insomnia, dizziness, headache, ataxia
<b>Gabapentin</b>	<b>Weight gain</b>	Somnolence, dizziness, ataxia
<b>Lacosamide</b>	Nausea, vomiting, fatigue	Ataxia, dizziness, headache, diplopia
<b>Lamotrigine</b>	Rash, nausea, blood dyscrasias, rash	Dizziness, tremor, diplopia
<b>Levetiracetam</b>	Infection,	Fatigue, somnolence, dizziness, agitation, anxiety, irritability, depression
<b>Oxycarbazepine</b>	Nausea, rash, hyponatraemia	Sedation, headache, dizziness, vertigo, ataxia, diplopia
<b>Perampanel</b>	<b>Weight gain</b> , fatigue	Dizziness, somnolence, irritability, gait disturbance, falls, aggression, mood alteration
<b>Pregabalin</b>	<b>Weight gain</b> , peripheral oedema, dry mouth	Dizziness, somnolence, ataxia, tremor
<b>Primidone,</b> <b>Phenobarbitone</b>	Nausea, rash	Alteration of sleep cycles, sedation, lethargy, behavioural changes, hyperactivity, ataxia, tolerance, dependence,
<b>Rufinamide</b>	Nausea, vomiting, fatigue	Dizziness, somnolence, headache
<b>Tiagabine</b>	Abdominal pain	Dizziness, lack of energy, somnolence, nausea, nervousness, tremor, difficulty concentrating
<b>Topiramate</b>	<b>Weight loss</b> , paraesthesia	Fatigue, nervousness, difficulty concentrating, confusion, depression, anorexia, language problems, anxiety, mood problems, tremor
<b>Valproate</b>	<b>Weight gain</b> , nausea, vomiting, hair loss, easy bruising	Tremor, dizziness
<b>Vigabatrin</b>	Vision loss	Drowsiness, fatigue, dizziness
<b>Zonisamide</b>	Nausea, anorexia	Somnolence, dizziness, ataxia, confusion, difficulty concentrating, Depression

**Legend**

CNS – central nervous system; ADRs – adverse drug reactions

### **1.2.2.1. Weight change is an important adverse drug reaction in epilepsy treatment**

There is an intricate relationship between obesity and epilepsy (Hamed, 2007). While some epilepsy syndromes are associated with obesity, some AEDs are associated with excessive weight gain predisposing the person so treated to obesity, while other AEDs are associated with weight loss (Benson et al., 2010) (see Table 2 below). Indeed, weight change is one of the most common ADRs associated with AED therapy (Pickrell et al., 2013). In some people with epilepsy AED-associated weight change is a treatment-limiting problem in the management of their epileptic seizures (Ben-Menachem, 2007, Biton, 2003). While weight loss is a major issue in growing children and in adults and adolescents with eating disorders, the most concerning weight-related ADR is weight gain which has both short term and long term personal, social, health and psychological consequences (Biton, 2003). Several important AEDs are known to cause some degree of weight change (See Table 2 below). Sodium valproate, carbamazepine, ezogabine, perampanel and pregabalin are associated with weight gain; topiramate, felbamate and zonisamide are associated with weight loss (Ben-Menachem, 2007, Biton, 2003, Zaccara et al., 2013). Lamotrigine, phenytoin and oxycarbazepine are considered weight-neutral AEDs (Biton, 2003).

Table 2: Effects of commonly prescribed AEDs on weight

<u>Weight gain AEDs</u>	<u>Weight neutral AEDs</u>	<u>Weight loss AEDs</u>
Sodium valproate	Lamotrigine	Felbamate
Gabapentin	Levetiracetam	Topiramate
Pregabalin	Phenytoin	Zonisamide
Vigabatrin		
Carbamazepine.		
Ezogabine		
Perampanel		

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**Legend:** AEDs – Anti-epileptic drugs

### **1.2.2.2 Do clinical predictors of antiepileptic drugs-associated weight change exist?**

Since weight-associated ADRs are associated with a high degree of treatment failure, and long-term morbidity, the clinical prediction of these ADRs would be of great value to both physicians and patients with epilepsy. Several clinical predictors of AED-induced weight change have been investigated by several authors in recent years. Although such factors as gender, body mass index at the initiation of therapy, neurocognitive status and primary generalized seizure type have been suggested from the results of different studies of valproate induced weight change, none of these have been consistently replicated (Egger and Brett, 1981a, Novak, 1999, Corman et al., 1997, Egger and Brett, 1981b, El-Khatib, 2007, Wirrell, 2003). Pregabalin induced weight gain appears to be dose related (Arroyo et al., 2004, Stephen et al., 2011). While AED-induced weight loss also appears to be dose-related, in addition, baseline BMI seems to be predictive of this ADR in patients exposed to either topiramate or zonisamide (Ben-Menachem et al., 2003, Gilliam et al., 2003, Arroyo et al., 2005b, Privitera et al., 2003, Reife et al., 2000, Wellmer et al., 2009, Gadde et al., 2012a) (See Table 3 below). A description of some of epidemiology and clinical predictors of weight change associated with a sample of commonly used AEDs will now follow.

#### **1.2.2.2.1. Zonisamide**

Exposure to zonisamide (ZNS) is associated with a mean weight loss of 3.7% of the pre-exposure weight in up to 35% of subjects so exposed (Wellmer et al., 2009, Gadde et al., 2012b, Guerrini et al., 2013, Kothare et al., 2006). This has been demonstrated in a retrospective study involving patients older than 17 years with epilepsy and in a randomized control trial involving obese adults and paediatric patients with epilepsy where ZNS was used for weight reduction. (Wellmer et al., 2009, Gadde et al., 2012b, Guerrini et al., 2013, Brodie et al., 2012, Kothare et al., 2006). The main pre-exposure risk factor for weight loss in ZNS-treated subjects is being overweight pre-treatment (Wellmer et al., 2009). ZNS has been used in weight reduction programs in obese adults (Gadde et al., 2012b).

#### **1.2.2.2.2. Topiramate**

In both randomized double blinded trials and in prospective observational studies, between 16% and 86% of subjects exposed to topiramate (TPM) for a period of 3 to 12 months experienced some weight loss (Gilliam et al., 2003, Arroyo et al., 2005b, Ben-Menachem et al., 2003, Arroyo et al., 2005a, Privitera et al., 2003, Reife et al., 2000). Weight loss on TPM appears to be more common in children than in adults, and occurs within the first 5 months of exposure to TPM (Gilliam et al., 2003, Verrotti et al., 2011, Ben-Menachem et al., 2003, Reife et al., 2000). TPM-induced weight loss may be sustained for two years after the onset of the ADR (Reiter et al., 2004). The degree of weight loss ranges from 3.9% to 7.3% of the pre-exposure weight and has been reported to correlate with dose, pre-treatment weight, reduced caloric intake, higher baseline BMI and duration of treatment (Reife et al., 2000, Sachdeo et al., 1999, Ben-Menachem et al., 2003, Verrotti et al., 2011). However, TPM dose appears to be the only clinical risk factor that has been consistently replicated in these studies (Gilliam et al., 2003, Arroyo et al., 2005b, Reife et al., 2000, Alberici et al., 2009). TPM has been approved as anti-obesity drugs in some countries (Gadde et al., 2012b).

#### **1.2.2.2.3. Pregabalin**

Pregabalin (PGB) is associated with weight gain in about 6 – 16% of those exposed to the medication as has been shown in two prospective

observational studies, two randomized control trials and a meta-analysis of pooled data from 106 studies involving more than 40,000 patients (Stephen et al., 2011, Cabrera et al., 2012, Perez-Vicente et al., 2006, Arroyo et al., 2004, Moon et al., 2010, Ben-Menachem et al., 2003). Although the baseline BMI, PGB dose and duration of therapy have been identified as clinical predictors of this ADR, PGB dose and baseline BMI remain the only predictors that have been consistent replicated (2012, Stephen et al., 2011, Arroyo et al., 2004, Ben-Menachem et al., 2003). See Table 3 below. The enhancement of GABA transmission is the main molecular mechanism thought to be associated with PGB-induced weight gain (Arroyo et al., 2004).

Table 3: Epidemiological studies of the effects of anti-epileptic drugs on weight

AED	Direction of weight effect	% Patients showing effect	Study design & references	Clinical predictor(s)	Observed in adults &/or paediatric subjects?
VPA	Gain	44%	Retrospective (Egger and Brett, 1981b)	Female gender	Paediatric
VPA	Gain	11%	Prospective (Novak, 1999)	Initial weight z-score Initial body mass index	Paediatric
VPA	Gain	71%	Retrospective (Corman et al., 1997))	No history of weight problems BMI at initiation of therapy	Adults
VPA	Gain	44%	Retrospective (Egger and Brett, 1981a)	None tested	Paediatric
VPA	Gain	27% in males 56% in females	Prospective, observational (El-Khatib, 2007)	Gender (female)	51 males; 55 females
VPA	Gain	58%	Retrospective (Wirrell, 2003)	Weight at initiation Neurocognitive status Primary generalized epilepsy	Paediatric
PGB	Gain	11%	Randomised placebo-controlled trial (Siddall, 2006)	None tested	Adults
PGB	Gain	7%-150mg/day 14%-600mg/day	Randomised double-blinded placebo-controlled trial (Arroyo et al., 2004)	Dose	Adults
PGB	Gain	10%	Prospective, observational (Stephen et al., 2011)	Dose	Adults
PGB	Gain	21%-600mg BID 15%-600mg TID	Randomised double-blinded placebo-controlled trial (Beydoun et al., 2005)	None tested	Adults
TPM	Loss	86% at 1 year	Prospective observational (Ben-Menachem et al., 2003)	Baseline BMI	Adults
TPM	Loss	15%-200 or 500mg/day	Randomised double-blinded trial (Gilliam et al., 2003)	Dose	Both
TPM	Loss	69%-400mg/day	Randomised double-blinded trial (Arroyo et al., 2005b)	Dose	Both
TPM	Loss	12%-200mg/day	Randomised double-blinded trial (Privitera et al., 2003)	Dose (suggestive)	Adults
TPM	Loss	13%-600-1000mg/day	Randomised double-blinded placebo-controlled trial (Biton et al., 1999)	Dose (suggestive)	Adults
TPM	Loss	13%-600-100mg/day	Randomised double-blinded placebo-controlled trial (Reife et al., 2000)	Dose	Adults
ZNS	Loss	35%	Retrospective (Wellmer et al., 2009)	Weight pre-treatment	Adults
ZNS	Loss	55%-400mg/day	Randomised double-blinded placebo-controlled trial (Gadde et al., 2012b).	Dose	Adults

**Legend:**  
AED – antiepileptic drug; ZNS – Zonisamide; TPM – Topiramate; PGB – Pregabalin; VPA – Valproic acid; gain – weight gain; loss –weight loss; \* adapted from (Chukwu et al., 2014)

#### **1.2.2.2.4. Sodium valproate**

Among the AEDs associated weight gain, valproate (VPA) appears to be the most problematic in terms of patient management. VPA therapy is associated with weight gain in up to 71% of subjects exposed to the medication (Biton, 2003). The weight gain usually occurs within the first 3 months of initiation of VPA and reaches a peak at 6 months of therapy (Demir, 2000). With VPA treatment, about 10% of the patients experience weight gains  $\geq 10\%$  of the pre-exposure weight.

Several clinical predictors of this ADR have been studied in both adult and paediatric populations. However there has not been any reproducible predictor of this ADR in either of the subject groups.

##### **1.2.2.2.4.1 Adult studies of clinical predictors of VPA-induced weight change**

Corman et al., (1997) in a retrospective record review and interview of 70 adults with epilepsy identified **low or normal initial BMI and a lack of personal history of weight problems** as the two clinical factors predictive of this ADR (Corman et al., 1997). However a previous retrospective study in 63 adults with epilepsy by Dinesen et al., (1984) found no clinical predictors (Dinesen et al., 1984).

El-Khatib et al (2007) identified female gender as a clinical predictor of weight gain in a study of 160 adult patients with epilepsy who have been on valproate therapy for at least six months (El-Khatib, 2007) . This retrospective study was conducted in the neurology outpatient department of a university teaching hospital and was aimed at determining the effects of gender on the degree of VPA-associated weight change and carbohydrate craving among other things. The result indicated that women were much more likely than men to gain weight than men and that the magnitude of weight gain was significantly higher in women than in men (El-Khatib, 2007).

#### **1.2.2.2.4.2. Paediatric studies of clinical predictors of Valproate-induced weight change**

Several candidate clinical predictors have been identified in children. The initial weight z-score and the initial body mass index (BMI) were the two clinical predictors identified by Novak (1999) in a retrospective hospital outpatient-based study of 55 children with epilepsy aged 1.8 to 16.9 years who have been on VPA therapy for 8.6 – 33.8 months. These two parameters showed a positive correlation with weight gain in the subjects studied (Novak, 1999).

Normal neurocognitive **status and primary generalized epilepsy type** positively correlated with BMI difference in another retrospective study by Wirrell (2003) of 43 children with epilepsy aged 10 – 17 years being treated with VPA for at least 2 months. Although these two factors did not achieve statistical significance in the final analysis both approached statistical significance (Wirrell, 2003).

**Duration of therapy** was a negative predictor of weight change in children with epilepsy being treated with VPA (Sharpe et al., 2009) . In this retrospective chart review conducted in a paediatric neurology office involving 94 children with epilepsy who were aged between 2 – 20 years who have been followed for a period of 4 – 89 months, change in BMI z-score was used as a measure of weight change. There was a negative correlation between change in the BMI z-score and duration of VPA therapy (Sharpe et al., 2009).

Other studies have failed to identify clear clinical predictors for this ADR. For example Demir (2000) in a retrospective study that included 100 children with epilepsy aged 15 months to 18 years on VPA dose of 30 – 50 mg/kg/day over a period of 6 months to 5 years using weight velocity as a measure of weight change found that neither epilepsy syndrome nor seizure control were predictors of this ADR (Demir, 2000, Tanner and Whitehouse, 1976) .

Pre-treatment BMI was not a significant clinical predictor of increased BMI in children who became obese while on treatment with VPA in a prospective study conducted in a paediatric department of a university hospital to determine the incidence and risk factors for the development of metabolic syndrome in VPA-treated children. One hundred and fourteen children with epilepsy on VPA monotherapy, mean age 10.1 ( $\pm$ 4.7) years, were enrolled in the study and they were followed up for at least 24 months (Verrotti et al., 2010).

#### **1.2.2.2.4.3. Studies involving both paediatric and adult patients**

Biton et al (2001) conducted a prospective multicentre double blind controlled trial to evaluate the effect of VPA and lamotrigine on body weight in 141 patients 12 years or older who have been on either medication for less than 90 days. The predictors of weight gain studied included drug dose, plasma concentration and weight at initiation of therapy. None of these predictors correlated with weight change in these patients (Biton, 2001) No clinical predictor of weight gain was identified in a case/control study of 88 female adults and children (age 6 years to 20 years) with epilepsy. The study was conducted in a tertiary endocrine centre in order to determine the effect of VPA on growth and weight gain. The two patient groups studied were 45 girls who were already on VPA treatment and 43 who were yet to start treatment. There was no correlation between the duration of therapy and BMI standard deviation score (de Vries et al., 2007).

In a pooled data analysis of nine open label trials comparing the efficacy and tolerability of the delayed and extended release VPA formulations in 321 adult and paediatric patients aged between 5 years and 85 years being treated with medication for either epilepsy or psychiatric disorders, Smith et al (2004) observed that weight gain was significantly more common with the delayed release, compared to the extended release formulation (Smith et al., 2004).

No common clinical predictor has been consistently observed in adult and paediatric studies. However, this observation is qualified by the fact that it is difficult to compare across studies, due to the variety of study designs applied. For example, groups have used different measures of weight change (BMI z-score, weight velocity, BMI difference, weight for age z-score. Also many of these paediatric studies involved heterogeneous age groups and studied different clinical parameters.

#### **1.2.2.2.4.4 Mechanism underlying VPA-induced weight gain**

The mechanisms underlying VPA-induced weight gain are poorly understood. This might be attributable to the broad spectrum nature of this medication which affects many biochemical pathways (Johannessen, 2000). While this wide range of effects may have some clinical benefit (VPA is also used for bipolar disorder, neuropathic pain, and migraine) it has contributed to the difficulty in understanding both its mechanism of action and the mechanism of its side effects (Johannessen, 2000).

An association between VPA therapy, hyperinsulinaemia/ insulin resistance and weight gain has been observed, although the causal mechanism is not well understood (Verrotti et al., 2009a, Pylvanen et al., 2002) (see Table 4 below). Hyperinsulinaemia is associated with hyperleptinaemia in children with VPA-induced weight gain (Verrotti et al., 2009a, Pylvanen et al., 2002). Direct stimulation of the  $\beta$ -cells of the pancreas and suppression of peripheral insulin uptake are the two main mechanisms VPA cause hyperinsulinism and insulin resistance (Verrotti et al., 2009a). VPA also interferes with hepatic insulin metabolism leading to hyperinsulinaemia without concomitant increase in the serum C-peptide levels (Pylvanen et al., 2006). VPA also Inhibits of GLUT-1 activity in normal astrocytes and fibroblasts resulting in reduced glucose transport (Vorbrodt et al., 2005).

Table 4: Proposed mechanism of valproate-induced weight gain

<b>AED</b>	<b>Molecular mechanism</b>
Sodium Valproate	GABA stimulation of the hypothalamus: Increased (Demir, 2000, Breum et al., 1992, Biton et al., 2003))
Sodium Valproate	Hyperinsulinism and insulin resistance (Demir, 2000, Isojarvi et al., 1998, Verrotti, 2002, Pasquali and Casimirri, 1993);
Sodium Valproate	Hyperleptinemia and leptin resistance (Verrotti et al., 1999a, Verrotti, 2002, Reiterer et al., 1999, Considine, 2005).
Sodium Valproate	Leptin, ghrelin and adiponectin (Diez and Iglesias, 2003, Gil-Campos, 2004, Greco, 2005)
Sodium Valproate	Increased serum level of GLP-1 (Martin et al., 2009)

**Legend:**

AED – antiepileptic drug; GABA – gamma aminobutyric acid

### **1.3. What I set out to achieve:**

This study sets out to test the hypothesis that specific ‘clinical’ factors and genetic variants influence the degree of VPA-induced weight change. By ‘clinical’ factors I refer to patient-specific factors (age, gender, weight centile at initiation) and treatment-specific factors (max VPA dose, serum level, duration of therapy) (see Experimental Chapter 1 section 2.9.3 for full description of the phenotypes).

In terms of genetic variants, I have chosen to focus on a series of 17 variants that have previously been shown to associate robustly with BMI in a healthy population. As a sub-hypothesis, the study sets out to determine whether the effect size (in terms of weight) of these 17 variants changes, when considered in the context of VPA exposure (see Table 20 in the Experimental Chapter 2 for a full description).

These questions were addressed by recruiting and studying 251 paediatric patients on VPA for at least six months, for whom retrospective data was extracted and stored in an electronic database. A comprehensive set of VPA response phenotypes and clinical details were obtained from each participant. This resource was used to explore various ‘clinical’ factors influencing the VPA-induced weight gain. The first 218 children recruited into the study were genotyped against the top 17 SNPs associated with BMI changes in the general population.

While Experimental Chapter 1 will focus on the clinical predictors of VPA-induced weight change, Experimental Chapter 2 will be focused on identifying the genetic predictors.

It was hoped that this work might help further personalise epilepsy care in patients (children in particular) being treated with VPA by minimizing the occurrence of this ADR and maximizing the potential benefits of the treatment for suitable patients. In addition to this, understanding the molecular mechanism of VPA-induced weight gain might enhance the scientific understanding of the contribution of genetic factors to obesity in the general population.

## **Chapter 2: Experimental Design and Methods**

## **2.0. Experimental Design and Methods**

### **2.1. Ethics Approval**

Ethics approval for this study was granted separately by the research ethics committees (REC) of the three institutions involved. Initial ethics approval was granted by Our Lady's Children's Hospital Crumlin (OLCHC) (REC Reference Number: SAC/114/08 and the Children's University Hospital (CUH) Temple Street Ethics Committees (Ethics Protocol Number 08.014) (see Appendix 4). An expedited ethics approval was then granted by the REC of the National Children's Hospital (NCH) Tallaght (REC Reference Number 2011/08/08) on the basis that the project had already being approved by the REC of the other two hospitals (see Appendix 4).

### **2.2. Funding**

Funding for this project was provided by the Children's Medical Research Foundation (CMRF) through the National Children's Research Centre (NCRC) located at OLCH Crumlin, Dublin 12 Ireland (NCRC, 2013, CMRF, 2013). The NCRC provided stipend and college fees for the principal researcher while the RCSI provided laboratory support through the Epilepsy Research Program.

### **2.3. Overall study aim:**

To identify clinically relevant predictors (environmental and/or genetics) of VPA induced weight change.

### **2.4. Study design**

We recruited paediatric patients through outpatient clinics and paediatric wards at OLCHC, CUH and NCH. This was a retrospective, cross-sectional study. There was however an observational, prospective element.

### **2.5. Objectives**

We identified the following specific objectives to achieving our overall study goal:

- i. To identify and recruit in to the study, 250 paediatric patients with epilepsy who been exposed to valproate for at least six months.
- ii. To record and database high quality clinical phenotypes in these 250 patients.
- iii. To execute genotyping of all participants against the 17 SNPs that has been robustly associated with changes in BMI.
- iv. Using the phenotypic and genetic resources, to analyse for environmental and genetic risk factors influencing valproate induced weight change.

The following sections will explain in details the rationale for the study objectives outlined above.

### **2.5.1. Task 1: To identify and recruit in to the study, paediatric patients exposed to valproate**

**Rationale:** The 250 participants we aimed to recruit seemed a reasonable target given the size of the patient population in the three institutions involved in the study. Further, this number was adequately powered to detect the phenotypic and genetic factors that would explain some of the variations in the patterns of weight change in these individuals (see Section 2.10 for sample size and power calculation).

### **2.5.2. Task 2: To prepare high quality treatment phenotypes in participants**

**Rationale:** Collecting and storing clinical information relating to weight change in the study participants is a critical component of this study. Growth in healthy children is natural and predictable. As a result, data on the expected natural growth have been collected in different populations over the years. From these data, epidemiologists have constructed 'growth centile curves' which are routinely used by paediatric health care professionals, to monitor growth and development over time. These centile charts are regularly revised and updated to account for secular changes in growth patterns (Wright et al., 2013, Rao and Simmer, 2012, Grummer-Strawn et al., 2010, Perry et al., 2009).

In Britain and Ireland, Hoey et al (1987) developed a standard centile chart based on data generated from the normal growth patterns in a cross sectional survey of 3,509 healthy Irish children between the ages of five years and nineteen years from varying geographical and socio-economic backgrounds. (Hoey et al., 1987). Prior to this survey study, there was no growth charts for Irish children beyond the newborn period (Hayes et al., 1983). Using the cross-sectional data and information from longitudinal studies, a longitudinal tempo-conditional growth chart for weight and height was constructed for children between 2 years to 18 years old. The method adopted for constructing the growth chart was similar to that adopted by Tanner and Davis (1985) in constructing similar growth standards for North American children ((Tanner and Davies, 1985). The main deficiency of the growth standard developed by Hoey et al (1987) is the lack of growth data on Irish children below the age of two years (Hoey et al., 1987). Hence current growth standard used for Irish children below the age of two years is not based on locally derived data (FSAI, 2011).

Paediatric health care providers measure and plot the weight and height of each child, at every clinic visit, as part of routine paediatric care. Deviations from the predicted growth curve might necessitate further evaluation and possible intervention to return the growth trajectory to the desired path (Panpanich and Garner, 2000). As a result of this long-established 'standard care' procedure, VPA-induced weight gain can theoretically be identified as a deviation over and above the child's natural

growth curve. In this context, we recorded the following parameters on each participant:

- i. All weight and height measurements taken prior to and within the first twelve months after the commencement of VPA
- ii. A range of syndrome and treatment response related phenotypes (see Appendix 6)

### **2.5.3. Task 3: To execute genotyping in all participants**

**Rationale:** The completion of the HGP in 2003 led to great advances in the knowledge of human genetic variations (Sabeti et al., 2007). In addition, the HapMap Project catalogued this information in a manner that allowed the natural redundancy in the human genome to be characterized. The development of Genome Wide Association Studies (GWAS) has provided a framework for the study of the role of common genetic variations in obesity. As of January 2013, over 13 GWAS studies have been published on weight-related phenotypes. The largest of these studies contained data on over 249,000 individuals, leading to the robust association of 32 loci with weight. Loci including *Fat mass and obesity-associated protein gene (FTO)*, *Melanocortin 4 receptor gene (MC4R)*, and *Ets Variant 5 gene (ETV5)* had previously been identified whilst others like *G protein-coupled receptor, family C, group 5, member B (GPRC5B)*, *Serine/Threonine Protein Kinase (TNNT3K)*, *Protein kinase D1 (PRKD1)* were novel (Speliotes et al., 2010). However, despite the size of the study, the 32 loci identified together only

account for 1.45% of inter-individual variations in BMI, the largest locus-specific effect coming from *FTO* (0.34%). In all, about 52 loci for obesity traits have now been discovered through GWAS. These have only modest effect sizes with odd ratios (per allele) of between 1.2 and 1.5 (Farooqi, 2013). However, the genes so far identified may provide insights into the biology of obesity in humans. This study sets out to test the influence of the top 17 SNPs robustly associated with BMI on valproate-induced weight change.

#### **2.5.4. Task 4: To identify the environmental and genetic risk factors influencing valproate induced weight change**

**Rationale:** The analysis of the phenotype data collected in the second milestone will allow for the exploration of the 'environmental' factors influencing valproate-induced weight change. Any relevant 'environmental' factors will then be applied as co-factors in the identification of the genetic factors influencing this ADR. The participants will be genotyped against the 17 top SNPs associated with BMI changes (Skol et al., 2006). Correction for multiple testing in the 17 top SNPs will be done using Bonferroni correction (Hendricks et al., 2013, Rice et al., 2008, Rao and Gu, 2001).

## **2.6. Recruitment centres**

The participants were recruited from the three separate specialized paediatric epilepsy tertiary referral centres in Dublin namely Our Lady's Children's Hospital in Crumlin (OLCHC), the Children's University Hospital Temple Street (CUH) and the National Children's Hospital Tallaght (NCH). In these three hospitals paediatric neurologists (Drs David Webb, Anthony Bouldin, and Blathnaid McCoy at OLCHC; Drs Mary King and Brian Lynch at CUH; Dr David Webb at NCH) run once- (NCH) to twice- (OLCHC and CUH) weekly paediatric neurology clinics where children (from 0 to 18 years) with epilepsy and other neurological conditions are managed (see Appendix 5 for the full contact details of the collaborators). In addition, children presenting with acute neurological conditions including epilepsy are admitted to the hospitals for further management by the neurologist in conjunction with other paediatric specialists. Dublin and adjoining towns are the primary catchment areas for these hospitals, however children with difficult to treat epilepsies are referred from other towns and cities in Ireland. The children in this study were mainly Irish children of male or female gender with epilepsy who were between the ages of 2 years and 18 years at the time of recruitment.

## **2.7. Inclusion criteria**

Potential participants satisfied the following inclusion criteria:

- i. Age: between 2 and 18 years at the time of recruitment
- ii. A diagnosis of epilepsy, defined as recurrent, unprovoked seizures.

- iii. On valproate therapy for a continuous period of at least 6 months at some point in their clinical history.
- iv. Have records of weight at the time of initiation of valproate therapy and at least one other point within 12 months of initiation of valproate therapy;
- v. Must be attending any of the three paediatric hospitals in Dublin participating in this study, within the study period (between June 2008 and June 2013).

## **2.8. Exclusion Criteria**

Those excluded include:

- i. Patients with known chromosomal abnormalities that might affect growth (e.g. Down's syndrome)
- ii. Children on nasogastric (NG) or percutaneous gastro-enterostomy (PEG) feeds.

## **2.9. Recruitment**

### **2.9.1. Ethical considerations**

The ethical issues involved in this research project included: confidentiality, protection of personal clinical and genomic information, reduction of pain and discomfort, informed consent and truthful presentation of research findings. Respect, beneficence and justice which are the basic principles governing research in human subjects as contained in the

Declaration of Helsinki guided this work (Bosnjak, 2001). Confidentiality and anonymity were maintained by ensuring respect for the privacy of personal information. Both clinical and laboratory information were rendered anonymous by developing study codes that de-identified the individual once the clinical and laboratory information left the paediatric hospital. Keys to these codes were only known to the principal researcher and the principal investigator. Prior to enrolment into the study a written informed consent was obtained from all the participants (see Appendix 8). While parents of children who were younger than 16 years signed the consent form on their children's behalf, those children who were older than 16 years signed the consent themselves but in addition required parental permission to proceed with the study. Informed consent was obtained by explaining the research process to the participants; providing them further information which was contained in the participants' information leaflet (PIL) (see Appendices 7 and 8); answering their questions and finally by 'negotiating permission to do' the study (Blaxter et al., 1996). Although children who gave blood samples might have experienced some mild to moderate physical pain as a result, this was alleviated as much as possible by informing them prior to the procedure about what to expect and providing some pain relief in the form of topical anaesthetic cream or spray. As much as possible blood sampling was only undertaken if the child was getting blood tests done for some other clinical indications. No undue suffering was inflicted on any of the participants. Some humane clinical judgement was exercised by the researcher in participants' selection thus seriously ill children in the intensive care unit (ICU) were not

approached. The findings in this study have been presented truthfully (Blaxter et al., 1996).

### **2.9.2. The recruitment process**

The study employed convenience sampling as potential participants were recruited from among the children who attended the neurology clinic or who were admitted to the paediatric ward. Since there was no sampling frame, no form of randomization was employed.

In order to identify children who fulfilled the inclusion criteria, the principal researcher conducted a brief review of the case notes of the potential participants on a clinic day or on the ward. The parents of children meeting the study criteria were approached and informed of the study by either the principal researcher or any of the other members of the neurology team according to guidelines set out in the relevant ethics protocols (See Ethics approval section 2.9.1 above). The purpose and details of the study were explained to the parents and their children. Then the parents and children were provided with the participants' information leaflet (PIL) and written consent statement (see Appendices 7 and 8). They were allowed time to study the PIL and the consent form. Further clarifications regarding the study were provided by the principal researcher or the recruiting staff. Questions regarding the aspects of the study were treated accordingly to the satisfaction of the participants. If the parents and the children were satisfied with the provisions of the study and were freely willing to participate in the

study they would be invited to sign the written informed consent form. The recruiting staff then collected information from the child and the parents and from the case notes using the case report form (CRF – see Appendix 6). A 2 ml saliva or 5 ml blood sample was collected from each participant. The saliva was collected using Oragene DNA collection kits [Oragene DNA OG-500 for saliva collection; Oragene DNA OG-575 or Oracollect OCR-100 for buccal swab collection](DNA Genotek Inc, 2013). If the child is too young to spit saliva either 5ml blood sample in EDTA tube was collected or an oral swab was taken [Oragene DNA OG-575 or Oracollect OCR-100 for buccal swab collection](DNA Genotek Inc, 2013). DNA was extracted from these samples and stored at the Royal College of Surgeons in Ireland (RCSI) Biobanking facility at the Beaumont Hospital Dublin.

### **2.9.3. Phenotype collection and storage**

A secure Microsoft Access database was designed to store the participants' phenotype information in line with the criteria set by the relevant ethics committees (see the Ethical consideration in Section 2.9.1 above). Criteria was set for each phenotype (e.g. definition of each seizure type etc. according the International League Against Epilepsy (ILAE) classification of epilepsy syndromes prior to initiation of recruitment to allow for consistent phenotype assignment (ILAE, 1989). By accessing traditional hard copy patient records and by participants' questionnaire, the following clinical information was recorded for each participant:

The following information was derived from the subjects' medical records:

- i. Basic information – name, date of birth, gender and contact number
- ii. Epilepsy Syndrome covering the 5 axes of classification (semiology, seizure types, syndrome classification, aetiology, co-morbidities)(ILAE, 1989);
- iii. Clinical interpretation of the electroencephalogram (EEG) and neuroimaging data. The recording of the EEG report in the CRF indicated whether the EEG was supportive, unsupportive or unhelpful in the diagnosis of epilepsy. Neuroimaging included magnetic resonance imaging (MRI) or computed tomography (CT) of the brain. The neuroimaging details recorded in the CRF indicated whether the MRI was ‘non-lesional’ in which case the MRI was normal; ‘lesional’ in which case a well-defined abnormality involving a limited portion of the brain was reported ‘global’ in a case where the abnormality in the brain imaging was generalized. In addition, a detailed entry on the abnormalities in the neuroimaging and EEG was made in the free text sections of the CRF (see Appendix 6).
- iv. Information on valproate therapy – start date, finish date, maximum dose, serum levels if available (see Appendix 6).
- v. Data on polytherapy – names and doses of AEDs co-administered during valproate therapy; the commencement and discontinuation dates of the co-administered AEDs (see Appendix 6).
- vi. Data relevant to weight change – all measurements of weight and height and plotting these on centile charts(see Appendix 6);

- vii. Medical history – birth history, neonatal problems (including the presence or absence of perinatal hypoxia and hypoxic ischaemic brain insult, neonatal seizures, hypoglycaemia and other perinatal problems), abnormalities in development, history of febrile seizures, head trauma or CNS infection, and other medical diagnoses.

The following information was derived from parents' and/or the children's reports (investigator administered questionnaire, see Appendix 6):

- i. Family history of obesity – the parents and children were asked whether there was any family member with weight problems which includes overweight and/or obesity. These were dichotomous 'yes' or 'no' options. If the child had a family history of weight problems the degree of relationship to the family member with this problem was enquired into – i.e. whether it was a first degree, second degree or third degree relative (see Appendix 6).
- ii. Activity level – parents were asked about the children's activity level. Activity level was classified in the questionnaire into four categories: zero, low, normal or high. This was a subjective grading of the children's perceived activity level. (See Appendix 6).
- iii. Mobility level- the child's mobility level was graded into three categories which were: normal activity, impaired activity, and wheelchair bound. Information for completing this phenotype was gathered using a combination of the researcher's observation, information volunteered by the parents or information gathered from the medical notes. (See Appendix 6).

- iv. Special diet – parents were asked whether their children were on any special diet. The initial response options were dichotomous ‘yes’ or ‘no’. Parents whose children were on special diets were asked to give details of the type of special diets their children were on. (See Appendix 6).

#### **2.9.4. The Database**

The Microsoft Access database for holding the phenotypic information was designed by a statistician based at the RCSI on the Microsoft Access 2007 platform (see Appendix 3) (Microsoft, 2013). The information on the case report form (see Appendix 6) was made use of in designing the database. It consisted of five sections: the bio data, epilepsy history, VPA specific history, weight and height information and other patient information (see Appendix 6 and the Phenotype Collection and Storage Section 2.9.3 above). The text data collected were linked to database codes to allow for easy transformation of data and integration with data analysis software such as Microsoft Excel and International Business Machine (IBM) Statistical Package for Social Sciences (SPSS)(IBM, 2011)} (see Appendix 2). The database was robust and secured with a password known only to the principal researcher and the principal investigator. The database was installed in a password-protected computer located within the NCRC’s facility at OLGHC in Dublin. Data was entered directly from the CRF to the

database from Monday to Friday. Data was interrogated at monthly intervals to monitor data quality.

## **2.9.5. The growth assessment tools**

### **2.9.5.1. Clinical growth standard for Irish children**

Hoey et al., (1987) developed a standard centile chart based on data generated from the normal growth patterns in a cross sectional survey of 3,509 healthy Irish children between the ages of five years and nineteen years (Hoey et al., 1987). From this cross-sectional data and information from longitudinal studies, a longitudinal growth standard was constructed for children from 2 years to 18 years old. This was supplemented with similar data on British children between the ages of five and 19 years in developing the Hoey and Tanner growth chart (Hoey et al., 1987).

### **2.9.5.2. The WHO multi-centre growth reference for healthy breastfed babies.**

This multi-centre growth study was conducted between 1997 and 2003 and involved a longitudinal follow up of 882 breastfed infants from birth to 24 months in six countries representing all the six regions of Africa, Middle East, Asia, South America and North America. The longitudinal data was combined with data from the cross-sectional survey of 6,669 children aged 18 – 71 months living in ideal environmental conditions to construct the WHO

growth standard for children(de Onis et al., 2009, de Onis et al., 2004) . The aim of the project was to develop a single international reference for the physical growth of children (de Onis et al., 2004). The data has since been expanded to include children over five years old (Turck et al., 2013). This standard has been adopted by about 125 countries by April 2011 (de Onis et al., 2012). Some countries have not implemented this standard because they preferred local growth reference standards (de Onis et al., 2012).

#### **2.9.5.2.1. WHO AnthroPlus for personal computers**

The WHO AnthroPlus for Personal Computers is a software for assessing the growth of the world's children and adolescents, designed and developed by Monika Blössner and colleagues at the WHO's Department of Nutrition for Health and Development, Geneva in Switzerland (WHO, 2009). It incorporates both the WHO Child Growth Standards for 0-5 years and the WHO Reference 2007 for 5-19 years and thus could be used to monitor the growth of children from age 0 – 19 years (Turck et al., 2013). Parameters that could be measured using the WHO AnthroPlus include: weight-for-age, height-for-age and BMI-for-age, head-circumference-for-age, arm-circumference-for-age for children between the ages of 0 and 10 years; the height-for-age and BMI-for age can be determined in children older than 10 years. The software has three modules namely: the Anthropometric Calculator, the Individual Assessment and the Nutritional Survey (Turck et al., 2013).

### **2.9.5.3. The R code for the prediction of weight for age and weight for age centile**

The R code for weight centile prediction was developed by the collaborating statistician, Dr Gabor Borgulya. With this function it was possible to determine the weight for age centile (WAC) at initiation of therapy given the age (in years), gender and weight of the subject at initiation of therapy. This WAC becomes the baseline WAC and was assumed to be constant in children unaffected children by the VPA induced weight change. Using this function it was also possible to determine the expected weight at each visit post-initiation of VPA given the baseline WAC. Hence the degree of deviation of the observed weight from the expected weight could be calculated manually by subtracting the expected from the observed weight.

This function was developed independently for boys and girls. The data used in the development of the code was derived from the original Hoey et al 1987 paper (See section 2.9.5.1 above) (Hoey et al., 1987). While the data in Hoey's paper approximated the boys' centile prediction, it did not approximate those of the girls. Various approaches were undertaken to smoothen the centile curve including removing or modifying some data that were outliers. The smooth interpolation approach of the 3rd, 10th, 25th, 50th, 75th 90th and 97th percentiles for boys and girls, use of loess model in R were some of the approaches used. See Appendix 9 for a full description of the method employed in developing the function.

## **2.9.6. DNA extraction from tissue samples**

The tissue samples used in this study includes whole blood in Ethylenediaminetetraacetic acid (EDTA) tube, saliva and oral/buccal swabs. The following subsections will detail the method of DNA extraction from each of the tissue types.

### **2.9.6.1. DNA extraction from whole blood**

The 5ml whole blood sample collected in EDTA tube was immediately stored in the fridge at 4°C at the Children's Neurosciences Centre, Our Lady's Children's Hospital Crumlin. These samples were then transferred (in batches) for DNA extraction, to the Royal College of Surgeons in Ireland Bio-banking facility at Beaumont Hospital, Dublin.

DNA was extracted from blood using either the manual or the automated methods with the aid of the (Qiacube) machine (Model: Qiagen; Serial no:10632) (QIAGEN N.V, 2013). The Qiacube automated sample preparation uses spin-column kits (see Appendix 12).

#### **2.9.6.1.1 Manual DNA extraction from whole blood**

Manual DNA extraction from blood followed a two-phase protocol.

#### **2.9.6.1.1.1. Phase 1**

The 5ml whole blood sample in EDTA tube was poured into a 50ml universal tube. Standard Laboratory Reagent (SLR) (consisting of 10ml of 2M Tris(hydroxymethyl)aminomethane (TRIS) (pH 7.6), 10ml of 1M Magnesium chloride ( $MgCl_2$ ), 6.6ml M sodium chloride (NaCl) diluted to a volume of 2 litres in distilled water) was then poured into the same tube to fill it up to capacity. The mixture was left to stand in ice for 15 minutes and then centrifuged at 4 degrees centigrade ( $^{\circ}C$ ) at 3500rpm for 15 minutes. The supernatant was then poured away while the pellet was re-suspended in another 50ml SLR by vortexing. The re-suspended pellet was then centrifuged for 10 minutes at 3500rpm at 4 degrees centigrade. The resulting supernatant was again poured away leaving the white pellets. To the white cell pellets, 3ml of Tris-EDTA 10:1/NaCl (0.4M) was then added. This mixture was vortexed until aggregates disappeared. The sample which then contained buffy coat was separated into two aliquots labelled A and B. As a precaution at least one of the aliquots was stored in the freezer for future use (See . Appendix 10).

#### **2.9.6.1.1.2. Phase 2**

The second phase of DNA extraction from whole blood involved the preparation of a Proteinase K buffer solution (1 mg/ml). This was prepared as follows: to a 15ml plastic tube was added 1mL of Proteinase K stock solution, 1mL of 10% sodium dodecyl sulphate (SDS) and 1mL of 0.5M EDTA (pH 8).

This was then made up to 10ml with polymerase chain reaction (PCR)-grade water.

Next 500 microliter ( $\mu$ l) of the Proteinase K buffer solution and 200  $\mu$ l of 10% SDS were added to the white blood cell mixture in a tube. The mixture was then incubated in a water bath at 65°C for 10 minutes while being shaken. The tube was then transferred into an ice/water mix for 10 minutes. One millilitre (1 ml) of 5 molar sodium chloride (5M NaCl) was added to the chilled mixture and it was then vortexed thoroughly. This was centrifuged at 3000 rpm for 15 minutes at 4°C. The resulting supernatant was then transferred to a 50 ml tube containing 5 ml of isopropanol and centrifuged again at 3500 rpm for 15 minutes at 4°C and the supernatant was discarded.

To the pellet, 1ml of 70% ethanol was added and the mixture was transferred to a new DNA storage tube which was centrifuged at 10,000rpm for 15 minutes. The pellet was left to dry for 30 minutes when the supernatant had been discarded. The dried pellet was re-suspended in 1 ml (Tris and EDTA) TE buffer.

#### **2.9.6.1.1.3. Quantifying the extracted DNA**

Nanodrop spectrophotometer (Thermo Scientific, Wilmington USA(Thermo Scientific, 2013) was used to quantify the concentration of DNA extracted. This device measures the DNA concentration in the extract by comparing the wavelength of ultraviolet light absorbed between 260nm and 280nm in 1  $\mu$ l of blank water sample to that of the DNA extract.

#### **2.9.6.1.1.4. Qualifying the extracted DNA**

To determine whether the extracted DNA is free from protein contamination the 260/280 ratio (A<sub>260</sub>/280) was measured. High quality DNA has an A<sub>260</sub>/280 ratio greater than 1.8 as this indicates low protein-contamination. After measuring and recording the DNA concentration and quality, the remaining DNA solution was stored in a freezer at -20°C.

#### **2.9.6.1.2. Automated DNA extraction from blood**

For the automated extraction of DNA from blood, a Qiacube automated sample preparation machine by Qiagen was used. This machine uses the buffy coat obtained from phase 1 of the manual process as input and extracts DNA using spin columns and reagents from the QIAamp DNA Blood Mini QIAcube Kit (Model: Qiagen; Serial no:10632) (Qiagen). The process is considerably more efficient in reducing hands-on work and processing time from whole blood to genomic DNA (See Appendices 10 & 12).

#### **2.9.6.2. DNA extraction from saliva**

An Oragene saliva collection kit (Oragene DNA OG-500) (DNA Genotek Inc, 2013) which contains a nucleic acid stabilization buffer allowing

a sample to be retained for years in a stable form between collection and processing was used for saliva collection. With this kit sufficient quantity of DNA could be obtained for most DNA analysis including PCR and Sanger sequencing. The main disadvantage of using this kit is the risk of contamination of the sample with bacterial DNA from the normal oral flora might make the saliva samples unsuitable for GWAS and next-generation sequencing due to the sensitivity of the enrichment assays.

#### **2.9.6.2.1. Protocol for DNA extraction from saliva**

The protocol for extracting DNA from saliva samples is as follows:

The 2ml saliva sample collected in the Oragene collection kit is mixed with the nucleic acid stabilization buffer immediately after collection by inverting and gently shaking the collection tube for a few seconds.

To ensure adequate DNA release from the sample and permanent inactivation of the nucleases, the sample was incubated at 50°C in a water incubator for a minimum of 1 hour.

The sample volume after incubation was noted before it was then transferred with a pipette to a 15mL centrifuge tube.

A 1/25th volume of Oragene Purifier reagent (DNA Genotek Inc, 2013) was then added to the tube (for example 200µl the purifier was added to a 5ml total sample volume). Mixing was done by vortexing the tube for a few seconds before it was incubated immediately on ice for 10 minutes to remove impurities.

After removal of impurities, the sample was centrifuged at a minimum of 2,500 g at room temperature for 10 minutes. The higher the centrifugal force, the less the amount of turbid material that will be carried over into purified DNA.

The clear supernatant was then carefully transferred with a pipette to a fresh 15 mL centrifuge tube and the pellet was discarded. An equal volume of 95-100% ethanol at room-temperature was added and mixed by gentle inversion 10 times to the clear supernatant. The sample was then allowed to stand at room temperature for 10 minutes to allow the DNA to fully precipitate. The sample was then centrifuged for the second time at room temperature for 10 minutes at a minimum 2,500g. The supernatant was discarded and care was taken to avoid disturbing the DNA pellet. The DNA pellet was dissolved in 1 ml of 10x TE buffer (pH 8.0) for rehydration and transferred to a 1.5 ml micro-centrifuge tube. The rehydrated DNA was centrifuged at room temperature for 15 minutes at 15,000g. The supernatant which contained the extracted DNA was transferred to a fresh 1.5 mL micro-centrifuge tube and labelled. See Appendix 11 for a copy of the protocol.

A Nanodrop spectrophotometer device (Thermo Scientific, 2013) was employed to quantify the DNA extracted. The remaining DNA solution was then stored in a -20°C freezer.

### **2.9.7. KASP™ Genotyping Chemistry by LGC Genomics**

Genotyping of the top 17 top BMI SNPs was carried out at the LGC Genomics (Kbio Sciences) Laboratory based at Hoddesdon in the United Kingdom. The genotyping assay used was the KASP which was based on “...competitive allele-specific scoring of single nucleotide polymorphisms (SNPs) and insertions and deletions (indels) at specific loci” (See Appendix 13). Quality control of the process was carried by the company by testing the assays on the company’s in-house validation DNA. The company was able to determine when the assays were working well when there are distinct clusters and consistently high call rates. Automatic quality control was also carried out on a SNP by SNP basis by using no template controls (NTCs) on each plate to detect contamination. Callable genotypes are those that are > 90%. Chi-squared distribution based on Hardy-Weinberg equation was also applied to determine the quality of the genotyped SNPs (Annon., n.d).

The KASP™ Genotyping involves 35 cycles of PCR initially which was then read on BMG PHERA Star plate reader. A visual inspection of the plates was also undertaken. The plates were then recycled at 3 cycles per recycle, followed by another visual inspection to ensure end point has been reached. Automatic genotype calling was done using the company’s in-house Kraken software. The data quality was checked and cross checked and any errors corrected before being sent to the customer (see Appendix 13).

## 2.10. Sample Size and Power calculations

The power of a study may be described as the ability of that study to give a statistically significant rejection of the null hypothesis (Evans and Purcell, 2012), assuming specific parameters relating to effect size. Overall study power depends on the study design, sample size, magnitude of the effect to be detected and the false positive rate of the test (Evans and Purcell, 2012). Power calculations should be conducted before a study is initiated, in order to determine the design and the sample size (in terms of study participants) required. Power calculations at the conclusion of negative study are useful to determine the detectable effect size given the actual sample size used in the study (Purcell et al., 2003). Power in genetic association studies is dependent not only on sample size but also a range of other parameters which includes (Purcell et al., 2003, Evans and Purcell, 2012):

- i. the frequency of the risk genotype (i.e. how common it is in the population under study);
- ii. the effect size of the risk variant (i.e. the relative risk it confers);
- iii. the genetic model in question (dominant, recessive, multiplicative etc.);
- iv. the prevalence of the trait in the population(s) being studied.
- v. The sample size,  $N$
- vi. The statistical significance level,  $\alpha$

Since the frequency, the genetic model and effect size of the risk variant being looked for in our study is unknown it is difficult to accurately

estimate the power of this study. The power for this study was therefore calculated based on the discrete trait of weight gain due to VPA therapy of >5% of baseline weight. This level of weight gain occurs in about half of the patients exposed to this medication according to data from previous studies (Kanemura et al., 2012, Dinesen et al., 1984, Biton V, 2001, Biton V, 2003). Power in the study was calculated using two main approaches: one for calculating power and sample size in discrete trait association studies (Power for Genetic Association studies (PGA) and the other for calculating power in quantitative trait studies. (GWASPower/QT). The steps involved in calculating power and sample size in this study are described in the following sections.

#### **2.10.1. GWASPower/ QT**

The GWASPower/QT is a statistical power calculator for GWAS and SNP studies developed by Shenghu Wang and Sheng Feng of the Dukes University Durham, USA (Feng et al., 2011). The power calculator helps in estimating the statistical power of detecting SNP associations with quantitative traits in natural populations (Feng et al., 2011). It is useful in exploratory studies such as our study as the phenotype mean of each marker is unknown (Mybiosoftware, 2012). The effect size is inputted as heritability measures. The parameters required for calculating power using this software include:

- i. The heritability or the effect size of the trait (the proportion of the phenotypic variation in the population that could be

ascribed to the genetic variation). This is a compulsory parameter.

- ii. The sample size is a compulsory input parameter
- iii. The type one error rate is also a required parameter
- iv. The linkage disequilibrium between the SNPs and the genetic variant being tested is an optional input,
- v. The number of co-variates and how much of the variance is explained by the co-variates (LD). The covariate input is optional. (See Fig 4 below).

The outputs from the GWASPower/QT calculator include a statistical power and a set of power curves for each of the SNPs being tested. The figure below illustrates the power of study at different effect sizes (Fig 5- below).

Given the number of SNPs tested and the number of individuals being tested what is the power of the study to detect a certain degree of variance/heritability? I.e. how much of the quantifiable trait observed (VPA-induced weight change) is due entirely to genetics as opposed to genetic factors? Genetic and environmental factors could have had varying degrees of influence on this ADR. It could have been 100% genetic and 0%. It is however rare to have a phenotype that is entirely genetic without any environmental contribution. The steps in calculating power using the GWASPower/QT calculator include:

1. The GWASPower/QT software was opened

2. The number of individuals genotyped (214) was entered. Four individuals were excluded from the calculation as their sample did not genotype well.
3. The total number of SNPs (17 SNPs) was entered
4. The LD value was set at value 1 (i.e. the marker correlates 100% with the marker).
5. We assumed there was no covariate so a value of zero was entered.
6. Power was then calculated at different levels of heritability (variance) starting from as low as 0.001(0.1%) to a high of 0.11 (11%).
7. After entering the values the 'RUN' button is pressed and the output displayed is the power.

Eighty percent power, which is considered adequate in GWAS/ SNP studies, was achieved at a heritability of about 5.8%. That is, we had 80% power to detect a variant explaining 5.8% or more of the trait variability.

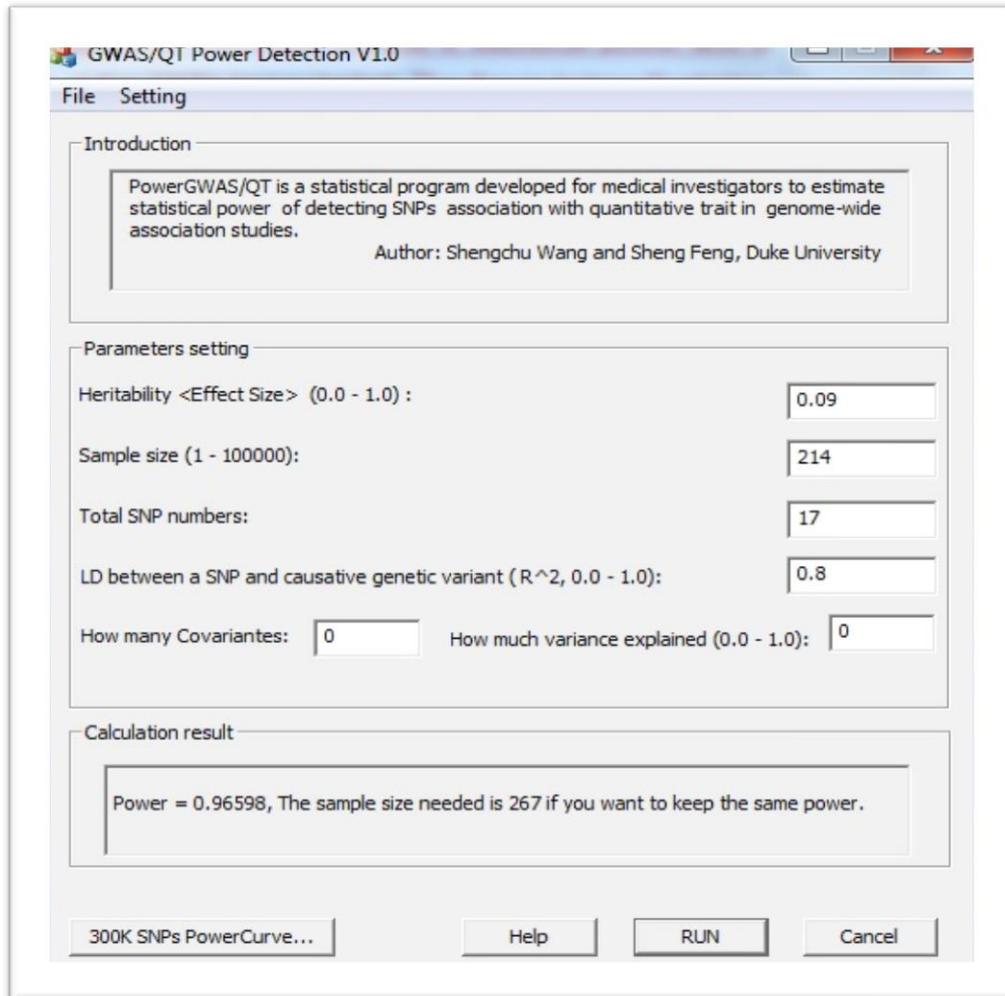


Figure 3: Screen shot of the PowerGwAS/QT calculator

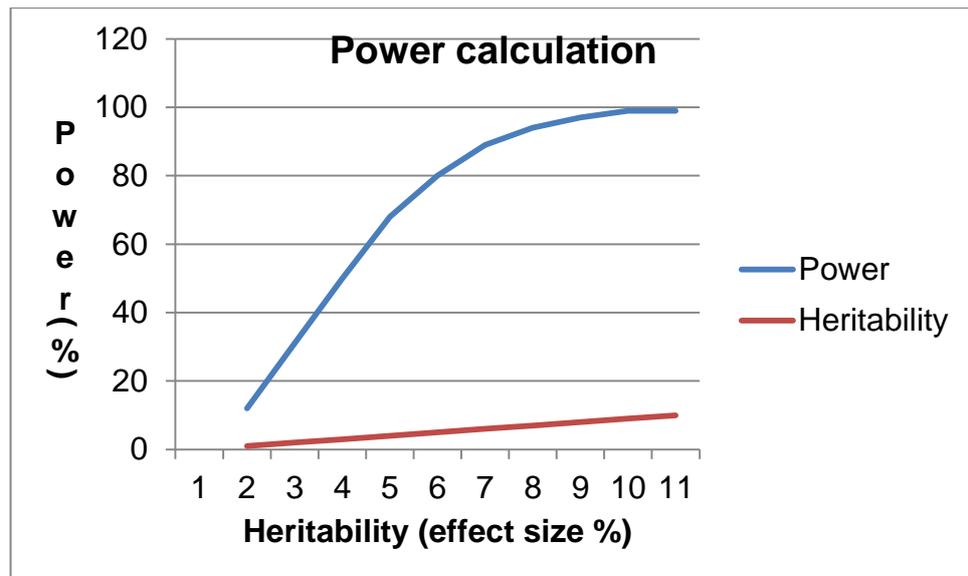


Figure 4: Power calculation using the GWASPower/QT software. The blue curve demonstrates the statistical power at different effect sizes (heritability) of the 17 SNPs genotyped for in the study.

### 2.10.2. Power for Genetic Association Analyses

The Power for Genetic Association Analyses (PGA) software, - a graphical user interface software developed in the Matlab platform for power and sample size calculation in discrete association trait studies, was used to determine power of each of the 17 SNPs to detect varying degrees of effect sizes or heritability (Menashe et al., 2008, NIH, 2013). The PGA has two different types of calculators: **the sample size calculator** and **effect size calculator**. The sample size calculator is based on having a known number of SNPs or sets SNPs and will calculate the number of individuals to test in order to detect an association. The second calculator is able to

calculate the lowest detectable risk with a given sample size. i.e if the number of individuals in the study is known but one needs to determine the lowest level of association that could be detected with the available sample size. The **power** of the study to detect increasing effects sizes (using Odds Ratios) for each SNP based on the known Minor Allele Frequencies (MAF) in the public databases was calculated by assuming a weight gain prevalence of 40% in the sample population exposed to VPA. We assumed that the disease model was co-dominant i.e. a single carrier could show signs of the disease but an individual that inherits two alleles shows stronger signs of the disease. The co-dominant model of inheritance carries the least of assumptions compared to the dominant and recessive models. A co-dominant model with two degrees of freedom assumes that there is a covariate involved while a co-dominant model with one degree of freedom assumes that no covariate is involved. An odd ratio or effect size was assumed- this determines the relative risk of the individual with the disease allele to manifest the disease. The steps taken in calculating power for each of the 17 top BMI SNPs genotyped in this study are as follows:

- i. Based on previous studies it was assumed that the prevalence of weight gain in those exposed to VPA was 40%.
- ii. The MAF of each of the 17 SNPs the subjects were genotyped against was determined from the dbSNP database (NCBI, 2013).
- iii. A co-dominant model with one degree of freedom was assumed
- iv. A relative risk or odd ratio of 2 was assumed
- v. The linkage disequilibrium (LD) value was assumed to be 1 (100%)

- vi. The alpha ( $p$ ) value was set at 0.05
- vii. The control to case ratio was set at 1 (i.e. an equal number of cases and controls)
- viii. The maximum sample size was set at 300
- ix. The 'RUN' button was pressed.
- x. The output for each SNP is illustrated in figure 6 below.

The PGA 1 was used to calculate the sample size while PGA 2 was employed in calculating the effect size. As illustrated in figures 5, at 80% power the study was powered to detect effect sizes as low as about 1.2 and anything higher. As shown in figure 7, a sample size of about 100 was required to achieve 80% power in one of the 17 SNPs (rs10508503) with the lowest MAF of 0.041. For most of the other SNPs sample sizes as low as 50 was required to achieve 80% power (see figure 6 below)

It was decided to recruit one and a half times the required minimum sample size in the study to allow for children with incomplete data. The study therefore set out to recruit 250 children exposed to VPA.

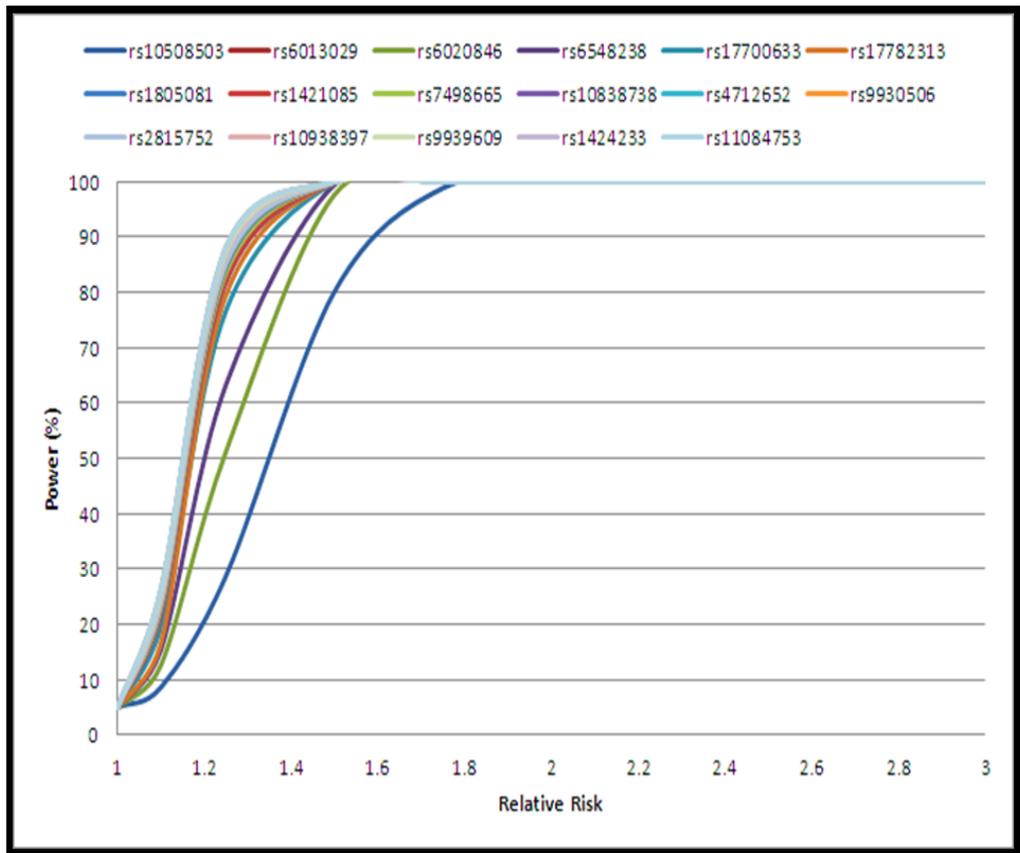


Figure 5: Power of each of the 17 BMI SNPs to detect and effect at varying relative risks. Each curve represents one of the SNPs.

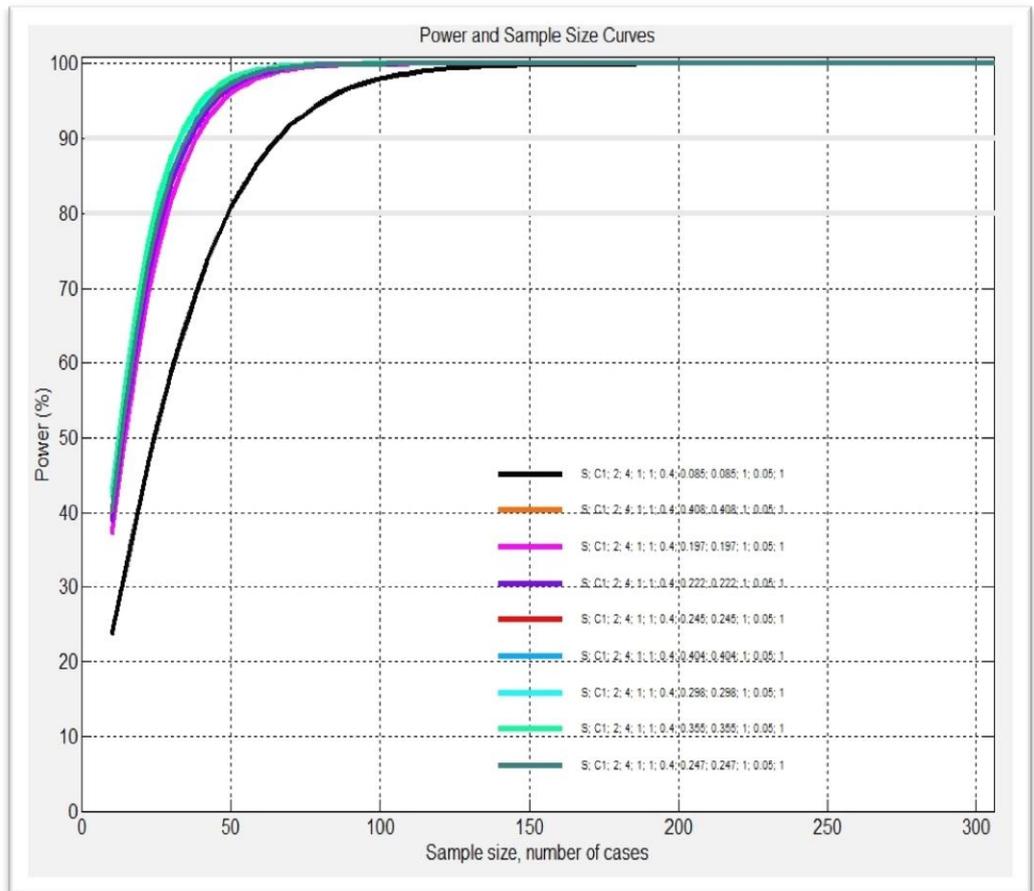


Figure 6: Sample size calculation for some of the 17 BMI SNPs

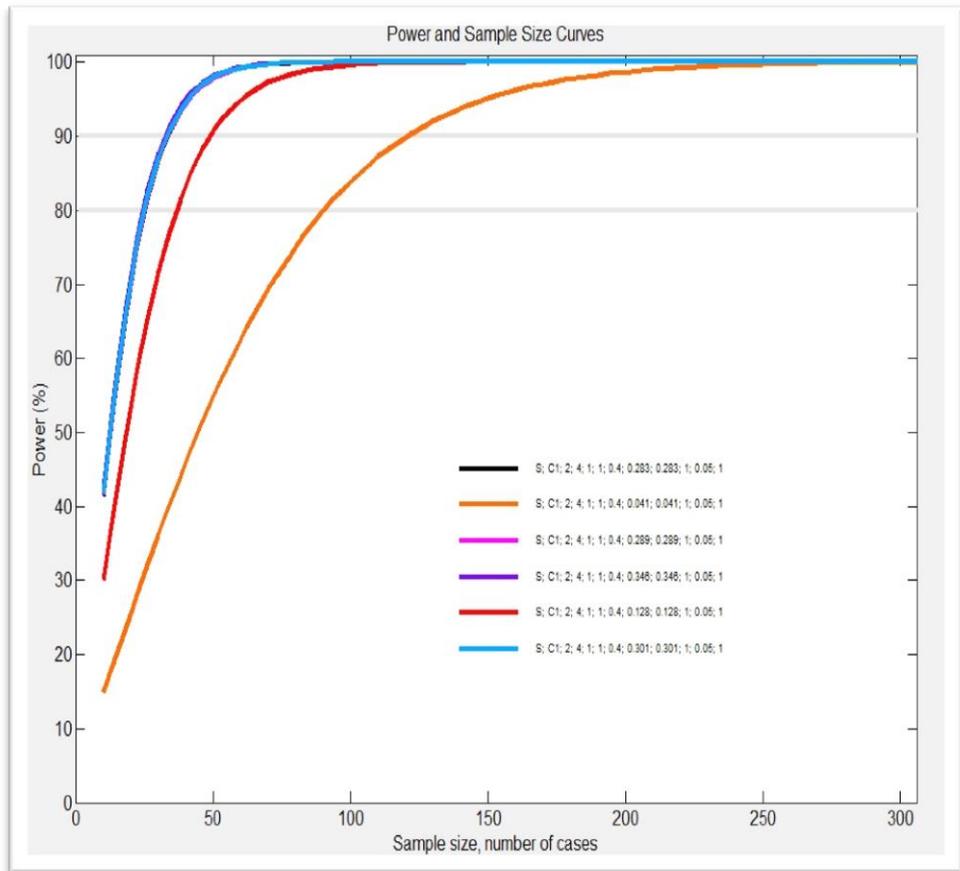


Figure 7: Sample size calculation for the rest of the 17 BMI SNPs

## **Chapter 3: Experimental chapter 1 – clinical predictors**

### **3.0. Experimental chapter 1 – clinical predictors**

#### **3.1. Introduction**

The first stage of analysis focused on identifying environmental factors influencing the valproate induced ADR.

#### **3.2. Valproate**

VPA is a broad spectrum anti-epileptic medication which has been in use since 1973 (See Figure 2 above). Its anti-epileptic effect was discovered by chance in 1963 as it was being used as a solvent for khellin derivatives (Egger and Brett, 1981b, Meunier et al., 1963). It is a branched short chain (8-carbon) fatty acid consisting of 2-propylvaleric acid with the chemical formula  $C_8H_{16}O_2$  (Terbach and Williams, 2009, Drugbank, 2005, Egger and Brett, 1981a). The chemical structure of VPA is illustrated in Figure 9 below.

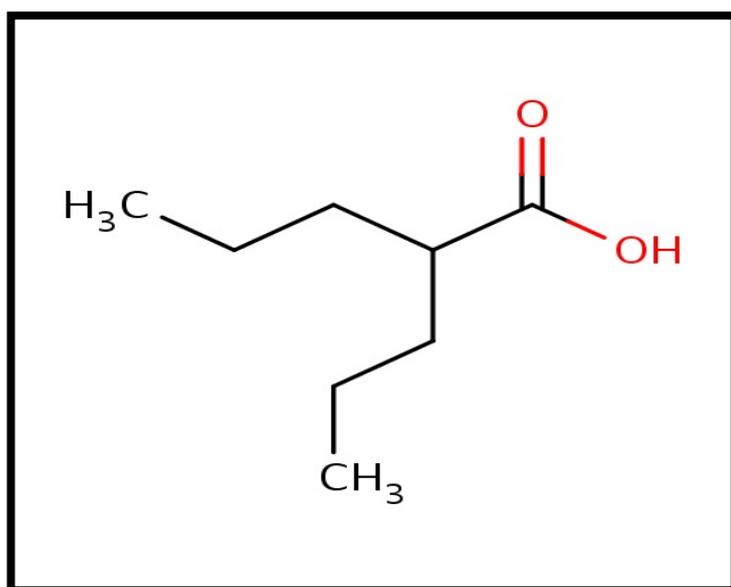


Figure 8: The chemical structure of Valproic Acid

(taken from [www.drugbank.ca](http://www.drugbank.ca) (Drugbank, 2005))

Figure 8 legend: C – carbon; H – hydrogen; O - oxygen

Valproate products include valproate sodium, valproic acid, divalproex sodium and some other generic formulations. VPA is rapidly absorbed from the gastrointestinal tract. It is protein bound and displaces fatty acids from protein binding sites. It penetrates the blood brain barrier hence its use in neurological diseases. The volume of distribution is 11 L/1.73 m<sup>2</sup> for total valproate and 92 L/1.73 m<sup>2</sup> for the unbound valproate. It is completely metabolized by the liver and excreted in the urine mainly as a glucuronide conjugate (Drugbank, 2005).

### **3.2.1. Indications for the use of sodium valproate**

VPA is approved for the treatment of tonic-clonic seizures, absence seizures, complex partial seizures, and seizures in subjects with Lennox-Gestaut syndrome (Egger and Brett, 1981a, Dulac et al., 1982). Other indications include manic, bipolar mood disorders and for migraine headache prophylaxis and treatment. It is also used off label for the treatment of other psychiatric and non-psychiatric conditions such as outbursts of aggression in children with attention deficit hyperactive disorder (ADHD) and in schizophrenia. In combination with highly active anti-retroviral therapy treatment, VPA therapy results in 75% reduction in latent human immunodeficiency virus (HIV) infection (Lehrman et al., 2005, Drugbank, 2005).

### **3.2.2. Mechanisms of action of sodium valproate**

The mechanism of VPA's anti-epilepsy action is not fully understood (Rosenberg, 2007). It has multiple mechanisms of action including the inhibition of gamma amino butyric acid (GABA) transaminase an enzyme that catabolises GABA. Inhibition of GABA transaminase results in increased brain concentration of GABA - an inhibitory neurotransmitter (Drugbank, 2005). In addition to GABA inhibition, VPA inhibits voltage-gated sodium channels and inhibits histone deacetylase (Juengel et al., 2013, Matsushita

et al., 2013, Drugbank, 2005). VPA metabolites have a ketogenic diet-like effect which is thought to contribute to seizure control (Egger and Brett, 1981a)

### **3.2.3. Adverse reactions associated with sodium valproate**

The common ADRs associated with this medication include dizziness, vomiting and abdominal discomfort, tremor and hair loss in about 5-10% of patients; behavioural changes such as irritability in children and depressed moods in adults and weight gain (up to 71%) (NYU, n'd, Biton, 2003, Drugbank, 2005, Egger and Brett, 1981a, Caksen et al., 2002). Other adverse effects associated with VPA include liver toxicity, pancreatitis, hyperammonemia and sedation (Egger and Brett, 1981a).

#### **3.2.3.1. Weight gain as a sodium valproate-induced adverse drug reaction**

Weight gain is one of the most difficult clinical problems encountered with the use of this medication because it has both long- and short-term health consequences. In the short term weight gain is associated with non-compliance with treatment and with discontinuation of therapy in about a third of the patients on this medication (Egger and Brett, 1981a). Given that some of these patients have gained good seizure control on VPA prior to the onset of this ADR and that such seizure control might not be achievable with any other AED, this ADR contributes to failure of medical control of epilepsy. The

affected patients hence are forced to live with the debilitating effect of uncontrolled seizures with associated increased morbidity and mortality. The increased weight associated with this medication might elevate the risk of toxicity from other drugs (especially in children where drugs are calculated in units per kilogram body weight) as the dose of co-administered drugs are adjusted upwards to account for the weight gain (Egger and Brett, 1981a). Other short term complications of weight gain includes psychological morbidities – depression, anxiety, decreased morale and self-confidence, increased risk of bullying especially school-aged children (Egger and Brett, 1981a). Overweight individuals are also at increased risk of respiratory morbidities such as sleep-disordered breathing and obstructive sleep apnoea (Newman et al., 2005)

Long term complications of VPA-induced weight gain include increased risk of the metabolic syndrome characterised by abdominal obesity, dyslipidaemia, hypertension and glucose intolerance (Verrotti et al., 2009c). This syndrome is associated with increased risk of cardiovascular disease and the development of type 2 diabetes (Verrotti et al., 2009c).

VPA therapy is associated with weight gain in up to 71% of the patients exposed to the medication (Corman et al., 1997, Wirrell, 2003). However in most studies the incidence of weight gain is usually about 10% of pre-treatment weight (Biton, 2003). Most patients with this ADR experience it within the first 3 months and it peaks at 6 months after initiation of therapy (Demir, 2000). In addition, there is a wide inter-individual variation in the degree of weight gain on this medication. While a very small proportion of the

subjects lose weight on this medication probably due to the gastrointestinal discomfort caused by the medication, others experience weight gain of up to 71% of their pre-exposure weight ((Egger and Brett, 1981a). Around 10% of patients experience severe weight gain (defined as weight gain  $\geq$  10% of the pre-exposure weight)(Corman et al., 1997). Significant weight gain ( $\geq$ 7%) occurs in even a larger proportion of these patients (Zipursky et al., 2005) .

Previous studies in adults have reported the following two pre-exposure risk factors associated VPA induced weight gain body mass index pre-treatment (Corman et al., 1997);

- i) no personal history of weight problems(Corman et al., 1997) and
- ii) longer duration of therapy (Verrotti et al., Mikkonen et al., 2005a, Prabhakar et al., 2007)

In children the following predisposing factors have been reported:

- i) potentially overweight or overweight at the time of VPA initiation;
- ii) normal neurocognitive status in older children and teens (Wirrell, 2003);
- iii) initial weight Z-score and initial body mass index in children aged 1.8 to 16.9 years (Novak, 1999);
- iv) gender (Egger and Brett, 1981a) and;
- v) pubertal status (Mikkonen et al., 2005b).

It is important to note that the predictors listed above have not been consistently replicated across studies (Egger and Brett, 1981a, El-Khatib, 2007, Corman et al., 1997, Wirrell, 2003) and indeed other studies have not reported any predictors (Dinesen et al., 1984, Pylvanen et al., 2003). As a result, there are no accepted clinical predictors of VPA-induced weight gain. There have been no randomized control trials and some of the studies were underpowered to detect clinically relevant predictors (Biton, 2003).

Since this ADR is very common in both adults and children, and has both short and long term medical and psychosocial implications, this study was undertaken to determine the phenotypic and genetic factors associated with this ADR in children. This analysis is therefore aimed at addressing two basic questions: (1) Does VPA induce a weight change in our patient population? (2) What clinical variables influence this weight change in our patient population?

### **3.3. Methods – Patients cohorts**

For description of patient phenotypes see General Methods Sections 2.6 and 2.7

### **3.4. Methods – regression modelling**

We applied standard multi-linear regression modelling to explore the influence of a variety of clinical variables on weight gain due to VPA. The

clinical variables we tested were: the duration of VPA exposure, maximum dose, serum VPA levels, gender, pre-exposure weight for age centile (WAC), polytherapy, epilepsy syndrome, family history of weight problems, age at initiation of therapy, mobility status and special diet. The main phenotypes we used as a measure of VPA-induced weight gain were:

- i) maximum percentage weight change and
- ii) change in weight for age z-score (WAZ) within the first 12 months of therapy.

Details of the analysis of the influence of clinical factors on maximum percentage weight change are outlined below:

Phenotypic analysis was conducted on the 251 patients recruited into the study (see General Methods Section 2.9.3). We employed two growth monitoring tools to determine the main clinical phenotypes (maximum percentage weight change and average WAZ change) (see Section 2.9.5 in the General Methods Chapter for a full description of the growth assessment tools). The WHO AnthroPlus software was employed to determine the change in weight for age z-score (WAZ) (average WAZ change) in children between two years and ten years at the time of initiation of VPA while the R-code developed from the Hoey et al 1987 growth chart was applied to children  $\geq 2$  years at the time of initiation of VPA (Hoey et al., 1987, WHO, 2013).

### **3.4.1. Determining the maximum percentage weight change using the Hoey et al growth chart and the R code derived from the data in the original paper**

The basic steps in determining weight change (maximum percentage weight change) using the Hoey et al growth charts were (Hoey et al., 1987) (see Section 2.9.5 in the General Methods Chapter):

- i. The weight at initiation of VPA was determined from the clinical records (data stored in Microsoft Access database).
- ii. The subject's age at initiation was calculated in years by subtracting the date of birth from the date of initiation. This was calculated using the Microsoft Excel function (=YEARFRAC).
- iii. The subject's gender was also determined from clinical records (data stored in the Access database).
- iv. The weight for age centile (WAC) at initiation of therapy was determined by entering the gender, age and weight (in kilograms) in the R-function developed from the Hoey et al data. This WAC was taken as the baseline WAC.
- v. It was assumed that, during the first 12 months of VPA exposure, any deviation from 'initiation' centile was attributable to VPA.
- vi. During subsequent visits within the study period, the expected weights (in kilograms) were determined by first determining the child's age at that visit using the Excel function – (=YEARFRAC) then entering the gender, the baseline WAC and the age in the R-function. The R-function outputs the expected weight in kilograms.

- vii. Weight deviation from the expected weight for each visit was calculated by subtracting the observed (measured) weight at that visit from the expected weight (determined using the R-function).
- viii. Percentage weight change was calculated by dividing the weight deviation at each visit by the expected weight at that visit and multiplying by 100.
- ix. The percentage weight change (percentage weight deviation) was calculated for each visit and recorded. The percentage weight change with the highest value within the first twelve months was taken as the maximum percentage weight change and this determines the phenotype for each of the subjects.
- x. Weight deviation at the end of twelve months of therapy was obtained by subtracting the last recorded observed weight from the expected weight at the same period. Observed weight at 12 months (OW12) or any time close to the 12 month period minus the predicted weight at that time period (PW12).

#### **3.4.2. Determining the average weight for age z-score using the World Health Organization Anthro Plus software**

For children between the ages of zero and ten years at the time of initiation of therapy, the WHO-AnthroPlus software was used to calculate the change in weight for age z-score (WAZ) from the time of VPA initiation (WHO, 2013) (see Section 2.9.5.3 for a full description of the WHO

AnthroPlus weight assessment tool). The steps involved in calculating the average WAZ change are outlined below:

- i. The Individual Assessment module of the WHO AnthroPlus was used to calculate the WAZ at initiation of therapy by entering the individual's gender, date of birth, date of first visit and weight at the first visit (see Section 2.9.5.2 in the General Methods chapter).
- ii. For each subsequent visit within the first twelve months, the measured weight and date of visit were entered from which the software calculated the WAZ. This serves as the initiation WAZ.
- iii. At subsequent visits post VPA initiation, the date of the visit, and the observed weight were entered for each individual. The software then calculates the WAZ for each of these visits.
- iv. The difference in WAZ between the initiation WAZ and WAZ at subsequent visits was calculated by subtracting the initiation WAZ from the WAZ at each visit
- v. The mean WAZ difference for each individual was calculated summing all the WAZ difference for all the visits after initiation and dividing this by the number of visits post-initiation of VPA.
- vi. This becomes the main phenotype for determining weight change for those children assessed using the WHO AnthroPlus software (WHO, 2013, WHO, 2009).

### **3.4.3. Derivation of the other clinical phenotypes**

The sources of data for the other clinical phenotypes include the medical notes/ CRF, calculated data and data from the investigator-administered questionnaire (see the Phenotype Collection and Storage section 2.9.3 in the General Methods Chapter): Data obtained from the medical notes and stored in the Access database include the following:

- i. Age at initiation of therapy derived from the subject's date of birth and date of initiation of VPA
- ii. Gender (male or female)
- iii. Epilepsy syndrome – according to ILAE classification 1989 (ILAE, 1989). The epilepsy syndrome classification was converted into a dichotomous data – generalised epilepsy (consisting of idiopathic generalised epilepsy, probable symptomatic generalised epilepsy and symptomatic generalised epilepsy) and focal epilepsy (consisting of simple focal seizures, probable symptomatic focal seizures and symptomatic focal seizures). Only one individual had a syndrome that was unclassifiable.

Co-Administered anti-epileptic drugs (AEDs); although each individual AED was recorded in the CRF this was converted to dichotomous data: VPA monotherapy versus combination therapy. This was for simplicity as there too many categories of AED to make for meaningful analysis.

The following data was calculated based on the information available in the medical notes and stored in the Access database:

- i. Duration of follow up - this was calculated by determining the time interval (in years) between the initiation date and the date of last measured weight within the first 12 month period of initiation of VPA therapy.
- ii. Maximum VPA dose in milligram per kilogram body weight per day (mg/kg/day): The daily dose was determined by dividing the total daily dose of VPA (in milligrams) by the subject's weight at each visit. The maximum VPA dose was assumed to be the maximum dose in mg/kg/day the subject was exposed to during the study period.
- iii. Average serum VPA levels in micromoles per litre, ( $\mu\text{mol/L}$ ) was calculated by summing all the measured serum VPA levels done within the study period and dividing this by the total number of measurements.

The following data was derived from the investigator-administered questionnaire (see Section 2.9.3 Phenotype Collection and Storage in the General Methods Chapter)

- i. Family history of obesity. These were dichotomous 'yes' or 'no' options.
- ii. Activity level – There were four categories of activity level: zero, low, normal or high.
- iii. Mobility level- There were three categories: normal activity, impaired activity, and wheelchair bound.
- iv. Special diet –. The options were dichotomous 'yes' or 'no'.

#### 3.4.4. Data classification

The data gathered were classified into quantitative (Scalar), discrete (Nominal) or ordered categorical (Ordinal) variables for easy analysis with the Statistical Package for Social Sciences (SPSS) version 20 (IBM, 2011) as listed below:

- i. Age at initiation (in years) (quantitative, scalar)
- ii. Gender (Dichotomous, nominal)
- iii. Centile at initiation (quantitative, scalar)
- iv. Dose – maximum dose (mg/kg/day) within 12 months (quantitative, scalar)
- v. Average serum VPA levels ( $\mu\text{mol/L}$ ) (quantitative, scalar)
- vi. Duration of VPA treatment (in years) (i.e. if they have stopped treatment early, before 12 months) (quantitative, scalar).
- vii. Syndromic diagnosis (dichotomous, nominal)
- viii. Co-administered AEDs: VPA only or VPA with one or more other AEDs (dichotomous, nominal)
- ix. Family history of obesity (dichotomous, nominal)
- x. Mobility level - or activity level (normal, impaired, wheelchair bound) (categorical, ordinal)
- xi. Diet -normal diet (yes/no) (dichotomous, nominal)

### **3.4.5. Analyses**

The data was analysed using the SPSS statistical software version 20 (IBM, 2011). A statistician's advice (Dr Patrick Dicker, RCSI) was sought prior to the drafting of the initial analysis plan. The data was entered into the SPSS software and the categorical (nominal) and the ordered categorical (Ordinal) variables were coded to reflect the number of categories while the quantitative (scalar) variables were left intact. The number of categories for some of the categorical variables like number of AED combinations and the epilepsy syndrome was reduced to 2 categories to allow for easier analysis.

The analysis was aimed at answering two basic questions:

- i. Does VPA induce a weight change in our patient population?
- ii. What clinical variables influence this weight change in our patient population?

To answer the first question a one-sample T-Test was performed using Observed Weight at 12 months (OW12) and the Predicted Weight at 12 months (PW12) (see--Section 3.4.1 above) . Also a one-sample t-test was conducted to determine whether VPA treatment induced a significant weight centile change at the level of the treated population as a whole within the first 12 months of treatment? A third one-sample t-test was also conducted to determine whether VPA therapy induced a significant change in the WAZ within the study period (see the section on Determining the average weight for age z-score using the WHO AnthroPlus software above).

To address the second question (What clinical variables influence weight change) we applied Analysis of Covariance (ANCOVA). The following predictors were tested in the ANCOVA model:

- i. Age at initiation (quantitative)
- ii. Gender (discrete, nominal)
- iii. Centile at initiation (quantitative, scalar)
- iv. Dose – maximum dose (mg/kg/day) (quantitative, scalar)
- v. Duration of VPA treatment (quantitative, scalar)
- vi. Syndromic diagnosis (2 categories, nominal)
- vii. Co-administered AEDs – VPA only or VPA + others (dichotomous, nominal)
- viii. Family history of obesity (dichotomous, nominal)
- ix. Mobility level - or activity level (normal, impaired, wheelchair bound) (categorical, ordinal)
- x. Diet - special versus normal (dichotomous, nominal)

### **3.5. Results**

#### **3.5.1. Recruitment and cohort characteristics**

We recruited two hundred and fifty one (251) individuals into the study. The cohort characteristics are presented in Tables 5 to 12 below. More than 61% of the subjects were boys. Approximately half of the children had generalized epilepsy while the remainder had either partial epilepsy or other forms of epilepsy that could not be classified (see Table 9). About two thirds of the subjects were on VPA monotherapy while the rest were on a combination therapy (See Tables 5 and 8). Learning disability (LD) was present in more than half (130/100) of the children. More than a third of those with LD have severe LD. Mild LD was present in 44/130 (36%) while moderate LD was present in 32/ 130 (26%) of those with LD (see Table 5).

Family history of weight problems (obesity or overweight) was reported in 78/225 (35%) of the children. About three quarters of the children had normal or elevated activity levels according to parental reports (see Table 5). VPA was initiated at a mean age of 6 (SD  $\pm$  3.98) years while the subjects were recruited at a mean age of 10.6 (SD  $\pm$  4.3) years. The mean interval between the initiation of therapy and recruitment into the study was 3.2 (SD  $\pm$  3.6) years (see Tables 6 and 7).

Table 5: Descriptive characteristics of the cohort

Characteristics	Characteristics	Frequency (n)	Proportion (%)
<b>Gender</b>			
	Male	153	61.2
	Female	97	38.8
<b>VPA monotherapy</b>			
	Yes	133	64.6
	No	73	35.4
<b>Epilepsy Syndrome</b>			
	Generalized	118	50.0
	Focal	108	46.0
	Other	9	4.0
<b>Learning Disability (LD)</b>			
	Yes	130	56.5
	No	100	43.5
<b>LD severity</b>			
	Mild	44	36.1
	Moderate	32	26.2
	Severe	46	37.7
<b>FH weight problems</b>			
	Yes	78	34.7
	No	147	65.3
<b>Activity Level</b>			
	Zero	5	2.2
	Low	47	20.5
	Normal	139	60.7
	High	38	16.6
<b>Mobility</b>			
	Normal	195	83.3
	Impaired	32	13.7
	Wheelchair	7	3.0
<b>Special diet</b>			
	Yes	12	5.2
	No	219	94.8

**Legend:** n – sample size; % - percentage of sample; LD – learning disability

Table 6: Descriptive statistics at the time of recruitment and at last follow up visit comparing the male with the female participants

<u>Characteristics</u>		<u>Males</u>	<u>Females</u>	<u>p-value (independent sample t-test)</u>
		(N = 151)	(N = 96)	
<b>Start Age (years)</b>	Mean (sd)	6.34 (4.09)	5.45 (3.77)	<b>0.08287</b>
	Median (range)	5.88 (6.4)	4.73 (5.36)	
<b>Last visit Age (years)</b>	Mean (sd)	10.91 (4.34)	10.08 (3.94)	<b>0.12</b>
	Median (range)	11.73(7.15)	9.55 (6.53)	
<b>Length of follow up at last visit (years)</b>	Mean (sd)	4.54 (3.43)	4.56 (3.12)	<b>0.95</b>
	Median (range)	3.49 (4.47)	4.05 (4.08)	
<b>Start weight for age centile</b>	Mean (sd)	0.68 (0.30)	0.57(0.34)	<b>0.03</b>
	Median (range)	0.80 (0.52)	0.66 (0.64)	
<b>WAC at end of 1 year of therapy</b>	Mean (sd)	(0.76) (0.27)	0.61(0.33)	<b>0.0049</b>
	Median (range)	0.87 (0.33)	0.72(0.66)	
<b>WAC last follow up visit</b>	Mean (sd)	0.69 (0.30)	0.62 (0.34)	<b>0.08432</b>
	Median (range)	0.80 (0.42)	0.73 (0.60)	
<b>WAC change start versus last follow up</b>	Mean (sd)	0.03 (0.29)	0.05 (0.24)	<b>0.6614</b>
	Median (range)	0.001(0.226)	0.0226(0.227)	
<b>Maximum VPA_dose_1st Year (mg/kg/day)</b>	Mea (sd)	25 (12)	27 (12)	<b>0.2885</b>
	Median (range)	23 (15)	24 (16)	
<b>Average serum VPA level (µmol/L)</b>	Mean (sd)	442 (232)	435 (183)	
<b>Length of follow up first year (years)</b>	Mean(sd)	0.66 (0.23)	0.68 (0.25)	<b>0.5238</b>
	Median (range)	0.72 (0.36)	0.72 (0.39)	

**Legend**

sd =standard deviation; max = maximum; VPA = valproate; WAC = weight for age centile; WAC – weight for age centile; VPA- valproate;

Table 7: Age at recruitment, VPA start age interval between VPA initiation and recruitment and duration of VPA therapy at the time of recruitment

	<b>Age at recruitment (years)</b>	<b>VPA start age (years)</b>	<b>Interval between VPA initiation and recruitment (years)</b>	<b>Duration of VPA therapy at the time of recruitment</b>
<b>Maximum</b>	18.13	15.95	17.11	11.83
<b>Minimum</b>	2.47	0.12	0.19	0.38
<b>Mean</b>	10.62	5.96	3.22	2.80
<b>Median</b>	10.90	5.23	1.64	2.16
<b>Standard deviation</b>	4.30	3.98	3.64	2.36

**Legend:**

VPA = valproate

Table 8: Anti-epilepsy drugs taken in combination with valproate

<b>Anti-epileptic drug (AED)</b>	<b>Number of subjects</b>	<b>Percentage</b>
Lamotrigine	57	23
Clobazam	28	11
Oxycarbazine	27	11
Carbamazepine	40	16
Ethosuxamide	13	5
Topiramate	14	6
Phenytoin	5	2
Adrenocorticotrophic hormone (ACTH)	10	4
Vigabatrin	7	3
Zonisamide	11	4
Acetazolamide	20	8
Gabapentin	2	1
Phenobarbitone	7	3
Rufinamide	5	2
Felbamate	2	1
<b>Total</b>	<b>248</b>	<b>100</b>

Table 9: Epilepsy syndrome classifications according to ILAE 2004

<b>Epilepsy Syndrome</b>	<b>Frequency</b>	<b>Percent</b>
Idiopathic generalised epilepsy	89	37.9
Symptomatic generalised epilepsy	10	4.3
Probable symptomatic generalised epilepsy syndrome	19	8.1
Benign focal	27	11.5
Symptomatic focal	50	21.3
Probable symptomatic focal	31	13.2
Specific Syndrome	8	3.4
Unclassifiable	1	0.4

**Legend**

ILAE – international league against epilepsy

Clinically significant weight gain (maximum percent weight deviation >5% of expected weight) was observed in 76/157 (48%) of the subjects whose weights were analysed using the Hoey et al growth chart. Forty three out of 157 (27%) of the subjects so analysed had severe weight gain (maximum percent weight deviation  $\geq$  10% of expected weight) while 33/157 (21%) of the subjects experienced moderate weight gain (maximum percent weight deviation between 5% and 9% of expected weight) (see Table 10 below).

Table 10: Weight change categories post initiation of valproate

<b>Weight change category</b>	<b>Number</b>	<b>Percentage</b>	<b>Range of maximum percent weight change</b>
<b>No clinically significant weight gain (maximum percent weight gain &lt; 5%)</b>	81	52%	-13% to 4%
<b>Moderate weight gain (maximum percentage weight gain 5 - 9%)</b>	33	21%	5% to 9%
<b>Severe weight gain (maximum percent weight gain &gt; 10%)</b>	43	27%	10% to 66%
<b>Total</b>	<b>157</b>	<b>100%</b>	

### **3.5.2. Weight for age centile at initiation of valproate therapy**

Analysis revealed that the mean WAC at initiation of VPA therapy was 0.65 (See Table 12 below). Fourteen per cent (14%) of children were overweight. 81% were in the normal weight category while the rest were underweight.

### **3.5.3. Weight for age centile within the first 12 months of valproate therapy**

Within the first twelve months of therapy, the mean WAC of the group had increased from 0.65 to 0.70. The median WAC also increased from 0.76 to 0.82 (see Table 12 below). The proportion of children described as overweight had increased from 14% at initiation of therapy to 34% at the end of the first year of therapy. The proportion of the underweight had decreased

from 5.2% at the initiation of therapy to 2.6% at the within this period. The same pattern though less dramatic change was seen in the normal weight category. There was a slight decrease in the proportion of children in the normal weight category from 81% at the initiation of therapy, to 75% at the end of the first year of therapy See Table 11 below.

Table 11: Comparison of the proportion of children in the different weight categories at initiation of VPA and at twelve months post initiation

<b>Period</b>	<b>Number</b>	<b>WAC ≤ 0.10</b>	<b>WAC 0.11 - 0.96</b>	<b>WAC ≥ 0.97</b>
<b>Initiation (n)</b>	155	8	125	22
<b>Initiation (%)</b>	100	5.2	80.6	14.2
<b>End of follow up (n)</b>	153	4	115	34
<b>End of follow up (%)</b>	100	2.6	75.2	22.2

**Legend:**  
WAC – weight for age centile

The mean weight for age centile (WAC) for the boys at the time of commencement of VPA was significantly higher than that of the girls (mean WAC of 0.76 (± 0.30) for the boys versus 0.57 (± 0.34) for the girls) (p = 0.005). See Table 6 above. The mean weight deviation from expected weight after 12 months of therapy was 1.56 (± 3.9) kg. More than half of the patients gained 0.64 (range, 29.59) kg. The mean maximum percentage weight deviation from baseline weight was 7.3% (± 11.7 %). The median weight deviation was 4% with a range of 79% as illustrated in Table 12 below.

Table 12: Subjects characteristics pre and post initiation of VPA therapy

	<b>Mean (<math>\pm</math> SD)</b>	<b>Median (<math>\pm</math> Range)</b>
<b>Age at initiation of therapy</b>	6.02 ( $\pm$ 3.99)	5.34 ( $\pm$ 16.00)
<b>Number of visits post-initiation of therapy</b>	2.01 ( $\pm$ 1.05)	1.05 ( $\pm$ 12.00)
<b>Length of follow up within the first year (years)</b>	0.67 ( $\pm$ 0.24)	0.72 ( $\pm$ 1.00)
<b>Maximum VPA dose (mg/kg/day)</b>	25.91 ( $\pm$ 11.71)	23.00 ( $\pm$ 74.00)
<b>Serum VPA levels (<math>\mu</math>mol/L)</b>	439 ( $\pm$ 211)	435 ( $\pm$ 1474)
<b>Weight for age centile at initiation</b>	0.65 ( $\pm$ 0.32)	0.76 ( $\pm$ 1.00)
<b>Weight for age centile at the end of follow up</b>	0.70 ( $\pm$ 0.30)	0.82 ( $\pm$ 1.00)
<b>Weight deviation from expected weight at the end of follow up (kg)</b>	1.56 ( $\pm$ 3.00)	0.64 ( $\pm$ 29.59)
<b>Maximum percentage weight change from expected weight (%)</b>	7.27( $\pm$ 11.70)	4.00 ( $\pm$ 79.00)

**Legend:**

VPA – sodium valproate; SD – standard deviation; kg – kilograms; % - percent

### 3.5.4. Weight for age z-score

Subjects younger than 10 years old with weight data had their mean weight for age z-score (WAZ) determined using the WHO AnthroPlus software (see General Methods Chapter, section 2.9.5.2). The software is not able to determine WAZ for children older than 10 years because weight is not a good measure of growth in those groups of children. There was an overlap in the number of subjects analysed with both the Hoey and WHO WAZ methods for children between the ages of two years and ten years (see Fig 10). All patients between the ages of 2 and 18 years were analysed using

the Hoey chart while all patients between the ages of zero and 10 years inclusive were analysed using the WHO WAZ methods. The mean WAZ change for children between the ages of 0 and 10 years (n= 145) was 0.2172 (SD: 0.70347). (See figure 11 below).

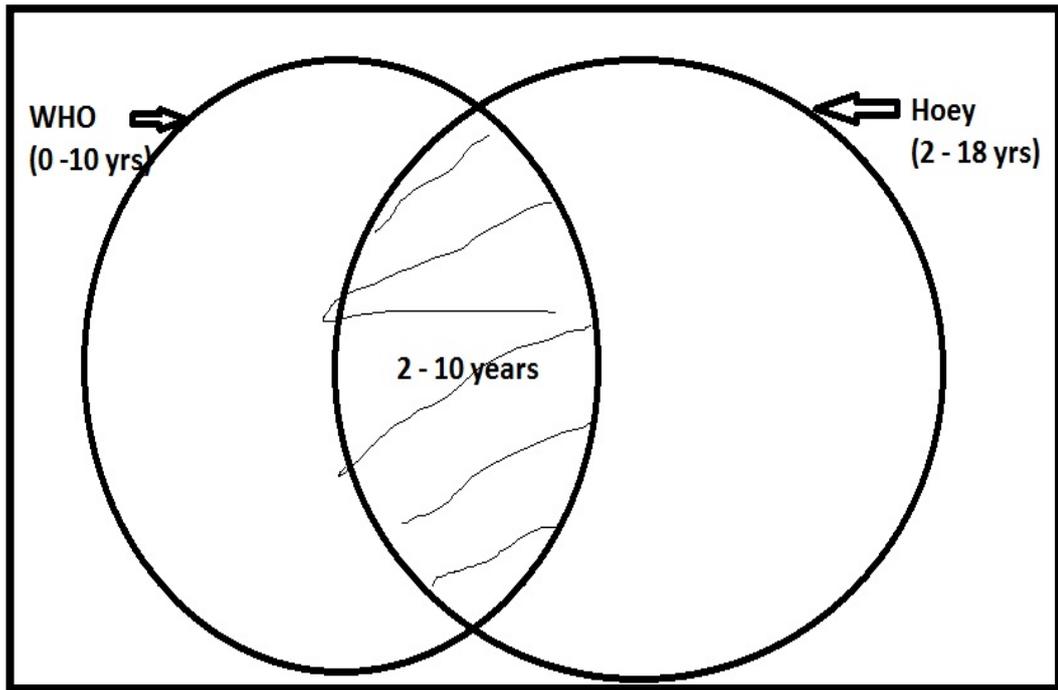


Figure 9: Overlap of the WHO weight for age z-score and Hoey weight for age centile determination

Figure 9 legend: WHO – World Health Organisation; yrs – years;

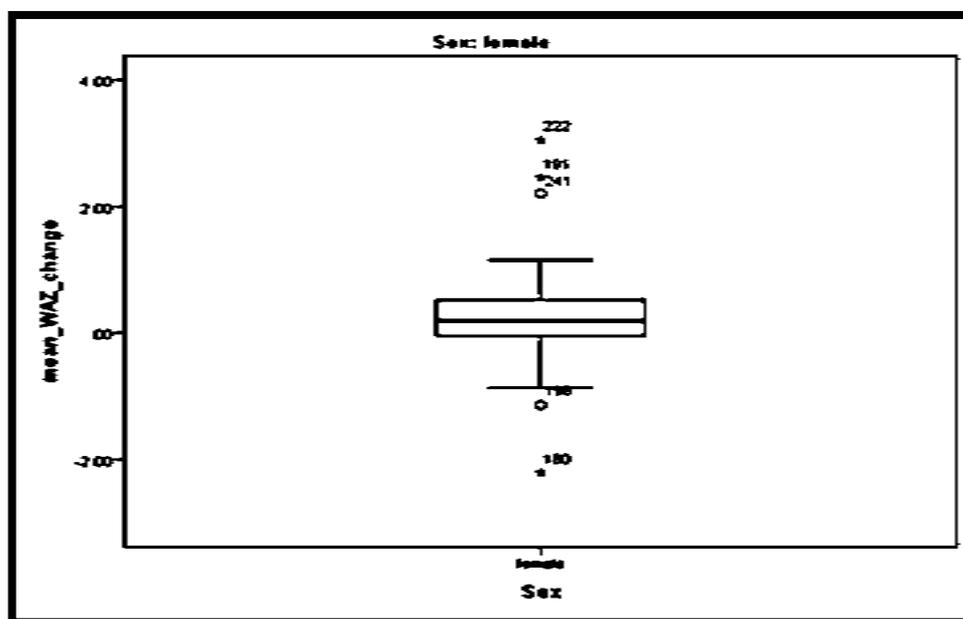
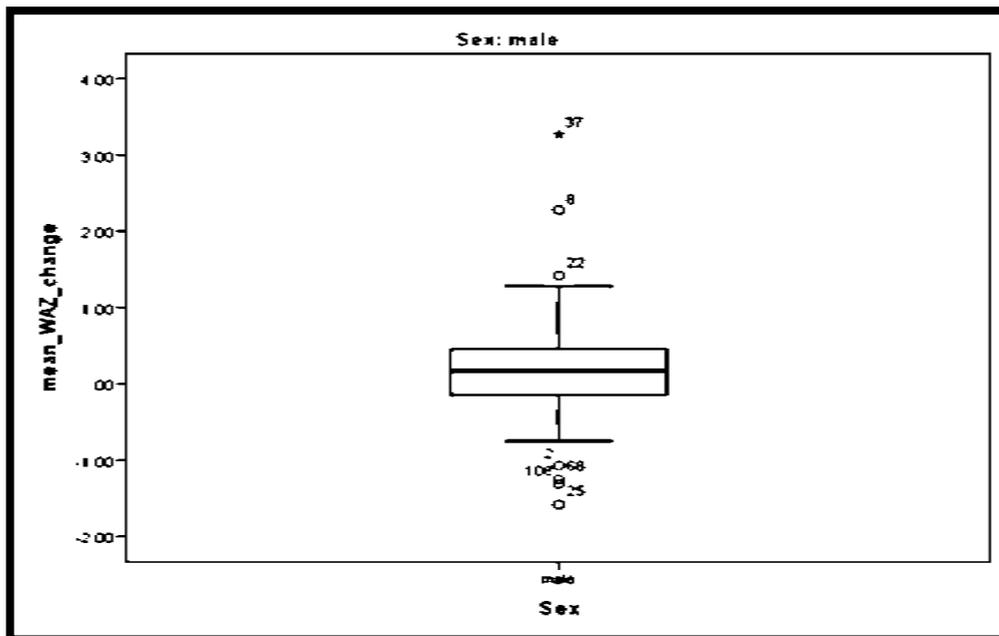


Figure 10: Boxplot of mean weight for age z-score change in boys and girls after 12 months of VPA therapy

### **3.5.5. Valproate dose and serum levels**

The mean maximum valproate dose was 26 mg/kg/day. About half of the subjects were on a maximum dose of 23 mg/kg/day (see Table 12). While the mean maximum doses were slightly higher in girls than boys (27 mg/kg/day versus 25 mg/kg/day), the difference was not significant ( $p= 0.29$ ) (see Table 6). The average serum VPA level was 439 $\mu$ mol/L. There was no significant difference between the boys and the girls in the average serum VPA levels within the treatment period. While the average serum level was slightly higher in boys than in girls (442  $\mu$ mol/L in the boys compared with 435  $\mu$ mol/L in the girls), this difference did not reach statistical significance as illustrated in Tables 6 above.

### **3.5.6. Other antiepileptic drugs**

The AEDs commonly combined with VPA include lamotrigine in about 23% of those on combination therapy, carbamazepine (16%), clobazam (11%) and oxycarbazine (11%). Other AEDs commonly combined with VPA include topiramate (6%), zonisamide (4%), adrenocorticotrophic hormone (ACTH) (4%), phenytoin (2%) and vigabatrin (2%), (See Table 8 above).

### **3.5.7. Length of follow up**

Although the target period of follow up was 12 months, follow up visits occurred at variable time-points during the first year of VPA treatment depending on the severity of the illness, the source of referral and the physicians' practice style. While majority of the patients had complete 12 months follow up data others had less than that. The mean length of follow up was 8 months or 0.67 ( $\pm$  0.24) years. The mean number of visits post-initiation of VPA therapy was 2 visits. Half of the children had only visited once see Table 12 above. Some of the children were not followed up at any of the three tertiary hospitals within the first year of therapy. The follow up data for this group of children was obtained from the district hospitals where they were referred from (if applicable) otherwise their data was excluded from the final analysis.

### **3.5.8. Co-morbidities**

The following co-morbidities were commonly reported in consented subjects – autistic spectrum disorder, attention deficit hyperactivity disorder (ADHD), behavioural difficulties, and developmental co-ordination disorders. Others include: cerebral palsy, hydrocephalus and asthma.

### **3.5.9. Seizure aetiology**

Although majority of the epilepsies were idiopathic in nature there were a number of symptomatic epilepsies and some were probably

symptomatic epilepsies in this group. Common aetiologies for the symptomatic seizures were: tuberose sclerosis, hypoxic ischaemic encephalopathy, viral encephalitis and bacterial meningitis, cortical dysplasia, dysplastic neuro-endothelial tumours (DNET) and Moya-Moya disease.

### **3.5.10. Epilepsy syndrome types**

Epilepsy syndrome was classified according to the ILAE 1989 classification (ILAE, 1989). Epilepsy syndrome could be determined in 235/251 (94%) of participants. About a third of the subjects had idiopathic generalized epilepsy (IGE). The second most common epilepsy classification in the subjects was symptomatic focal seizures reported in about one fifth of the patients. See Table 9 for a breakdown of syndromic classifications in the study cohort.

### **3.5.11. Weight change beyond the first year of therapy**

At the last follow up visit there was no significant difference in the mean WAC between the sexes – 0.69 ( $\pm$  0.30) for the boys versus 0.62 ( $\pm$  0.34) for the girls ( $p = 0.08$ ). There was also no significant difference between the boys and the girls in the mean WAC change at the last follow up visit – 0.03 ( $\pm$  0.29) for the boys versus 0.05 ( $\pm$  0.24) for the girls ( $p = 0.66$ ). See Table 6 above.

### **3.6. Inferential Statistics**

#### **3.6.1. Does sodium valproate treatment induce a significant weight change at the level of the treated population as a whole within the first 12 months of treatment?**

To address this question we conducted a 1-sample T-Test using Observed Weight at 12 months (OW12) and the Predicted Weight at 12 months (PW12) (see Section 3.4.1 for description of the phenotype). There was a significant deviation from the expected weight at the end of the first year of therapy in this population ( $p < 0.001$ ). The result of this test is as illustrated in Table 13.

Table 13: One-Sample Test Maximum Percent Weight Change, maximum weight difference, mean change in weight for age z-score and weight deviation at 12 months (oW12\_eW12): Test Value = 0

	<u>t-statistics</u>	<u>df</u>	<u>Sig.</u> <u>(2-</u> <u>tailed)</u>	<u>Mean</u> <u>Differenc</u> <u>e</u>	<u>95% Confidence</u> <u>Interval of the</u> <u>Difference</u>	
					Lower	Upper
<b>Maximum Percent Weight Change</b>	7.785	156	0.000	7.271	5.43	9.12
<b>Maximum Weight Difference</b>	7.283	156	0.000	2.21554	1.6146	2.81656
<b>Mean WAZ change</b>	3.717	144	0.000	0.21717	0.1017	0.33267
<b>Weight deviation at 12 months of therapy (oW12_eW12)</b>	4.875	148	0.000	1.55812	0.9265	2.18975

**Legend:**

df – degrees of freedom; sig – significance; WAZ – weight for age z-score; oW12 – observed weight at end of twelve months of follow up; eW12 – expected weight at the end of twelve months of follow up

**3.6.2. Does sodium valproate treatment induce a significant weight centile change at the level of the treated population as a whole within the first 12 months of treatment?**

To address this question we conducted a paired sample t-Test using Weight centile initiation (WC0) and weight centile at 12 months (WC12) for each child (See section 3.4.1 for description of the phenotype). We observed a significant difference in the paired t-scores of the WAC between the start WAC and the WAC at the end of the first 12 months of therapy (See Tables 14 and 15).

Table 14: Paired Samples Statistics: paired sample t-test weight for age centile at initiation compared with weight for age centile at the end of the first 12 months of VPA therapy

	<u>Paired Differences</u>					<u>t-statistics</u>	<u>Df</u>	<u>Sig (2-tailed)</u>
	<u>Mean</u>	<u>Std Dev.</u>	<u>Std. error mean</u>	<u>95% C.I of the difference</u>				
				<u>Upper</u>	<u>Lower</u>			
<b>C<sub>0</sub> – C<sub>12</sub></b>	-0.54	0.178	0.014	0.084	0.025	-3.685	43	0.000

**Legend**

C<sub>0</sub> = weight for age centile at initiation of therapy; C<sub>12</sub> = weight for age centile at the end of 12 months of therapy; df – degrees of freedom; std – standard; C.I – confidence interval

Table 15: Paired Samples Statistics: paired sample t-test weight for age centile at initiation compared with weight for age centile at the end of the first 12 months of VPA therapy

	Mean	n	Std. deviation	Std. error mean
<b>WAC at initiation</b>	0.634477	144	0.3147738	0.0262312
<b>WAC at the end of first 12 months</b>	0.689191	144	0.3038613	0.0253218

**Legend**

WAC = weight for age centile; n – sample size; std – standard

**3.6.3. Is sodium valproate therapy associated with a significant maximum percentage weight change in this population?**

To address this question we conducted a one-sample t-test comparing the maximum weight change at time zero with the maximal percentage weight change at the end of 12 months of VPA therapy. The result indicates that there was a significant maximum percentage weight change ( $p < 0.001$ ) as shown in Table 13.

**3.6.4. Clinical predictors of sodium valproate-induced weight gain.**

This analysis focused on the clinical predictors of:

- i) the maximum percentage weight change (during 12 months)
- and

- ii) the weight centile change/ weight deviation at 12 months of therapy.

Analysis of Covariance (ANCOVA) was applied using the 'clinical'/phenotypic factors outlined in the clinical phenotype description section above (Section 3.4.1) In the ANCOVA model the phenotypic factors were tested together and each phenotypic factor was also tested individually. The results are illustrated in Table 17. The reason for adopting both approaches in the ANCOVA is to increase the number of subjects fitting the model. Due to the proportion of subjects with missing data testing the whole cohort together in an ANCOVA will result in only a few of the subjects fitting the model. However, when each clinical profile was tested individually against the maximum percentage weight change, maximum number of subjects fitted into the model. Analysis of co-variates: maximum percent weight change.

This was tested against the following phenotypic factors: age at initiation, length of therapy, epilepsy syndrome, presence or absence of learning difficulty, maximum valproate dose, anti-epileptic combination, family history of obesity, family history of epilepsy, activity level, mobility status, special diet, initiation centile and gender. The fixed factor was gender and the number of subjects that fitted the model was 86 consisting of 53 males and 33 females. The combined model does not have any significant influence on the maximum percentage weight change. As shown in table 16 below even

when the influence of other phenotypic factors was removed from each other  
none of them achieved statistical significance.

Table 16: Results of ANCOVA testing each of the clinical factors together against maximum percentage weight change

<u>Source</u>	<u>Type III Sum of Squares</u>	<u>df</u>	<u>Mean Square</u>	<u>F-test</u>	<u>p-value</u>	<u>Significance</u>
Corrected Model	1331.126 <sup>a</sup>	12	110.927	0.701	0.745	<b>Not significant</b>
Intercept	153.394	1	153.394	0.969	0.328	<b>Not significant</b>
Age at initiation	445.270	1	445.270	2.813	0.098	<b>Not significant</b>
Length of exposure	0.919	1	0.919	0.006	0.939	<b>Not significant</b>
Epilepsy syndrome	62.964	1	62.964	0.398	0.530	<b>Not significant</b>
Learning difficulty	243.079	1	243.079	1.536	0.219	<b>Not significant</b>
Maximum Dose	3.147	1	3.147	0.020	0.888	<b>Not significant</b>
Monotherapy versus polytherapy	24.611	1	24.611	0.155	0.695	<b>Not significant</b>
Obesity FH	0.131	1	0.131	0.001	0.977	<b>Not significant</b>
Activity level	248.119	1	248.119	1.568	0.215	<b>Not significant</b>
Mobility	4.473	1	4.473	0.028	0.867	<b>Not significant</b>
Special Diet	200.605	1	200.605	1.267	0.264	<b>Not significant</b>
Initiation WAC	181.259	1	181.259	0.145	0.288	<b>Not significant</b>
Sex	0.405	1	0.405	0.003	0.960	<b>Not significant</b>
Error	11237.879	71	158.280			
Total	15760.995	84				
Corrected Total	12569.006	83				

**Legend:** a. R Squared = .106 (Adjusted R Squared = -.045); df – degrees of freedom; F - ; FH – family history; WAC – weight for age centile;

#### **3.6.4.1. Testing individual phenotypic factors independently**

ANCOVA was run on individual phenotypic variables to determine their individual influence on the maximum percentage weight change. The result of this analysis indicates that none of the phenotypic variables significantly influenced the maximum percentage weight change. The results of these analyses are outlined in the Table 17 below.

Table 17: Results of ANCOVA testing each of the clinical factors individually against maximum percentage weight change

<u>Dependent variable</u>	<u>Independent variable tested</u>	<u>Number of subjects</u>	<u>r<sup>2</sup></u>	<u>Adjusted r<sup>2</sup></u>	<u>p-value</u>	<u>Significance</u>
Maximum percentage weight change	Maximum dose	128	0.009	-0.007	0.392	Not significant
Maximum percentage weight change	Number of visits	148	0.004	-0.010	0.519	Not significant
Maximum percentage weight change	Start age	148	0.009	-0.004	0.504	Not significant
Maximum percentage weight change	Length of exposure	154	<0.001	-0.013	0.971	Not significant
Maximum percentage weight change	Epilepsy syndrome	148	0.001	-0.013	0.676	Not significant
Maximum percentage weight change	Gender	156	<0.001	-0.006	0.950	Not significant
Maximum percentage weight change	Age at initiation	157	0.009	0.003	0.234	Not significant
Maximum percentage weight change	Length of exposure	155	<0.001	-0.006	0.879	Not significant
Maximum percentage weight change	Learning difficulty	144	0.008	0.001	0.295	Not significant
Maximum percentage weight change	Average serum VPA levels	53	0.015	-0.004	0.384	Not significant
Maximum percentage weight change	AEDs (monotherapy versus polytherapy)	131	0.001	-0.007	0.706	Not significant
Maximum percentage weight change	Mobility	147	0.001	-0.006	0.727	Not significant
Maximum percentage weight change	Special diet	145	0.003	-0.004	0.533	Not significant
Maximum percentage weight change	WAC at initiation	141	0.021	0.014	0.083	Not significant
Maximum percentage weight change	Family history of obesity (degree)	48	0.002	-0.020	0.761	Not significant

**Legend:**  
r<sup>2</sup> - co-efficient of multiple correlation; VPA – sodium valproate; AEDs – antiepileptic drugs; WAC – weight for age centile;

### 3.6.4.2. Deviation from the expected weight at the end of the first 12 months of sodium valproate therapy

Analysis of co-variates was conducted using the weight deviation from the expected weight at the end of twelve months of VPA therapy (oW12\_eW12) as dependent variable and the following phenotypic factors as

independent variables: sex, number of visits, length of exposure to VPA, epilepsy syndrome, maximum VPA dose, learning difficulty, family history of epilepsy, family history of obesity, activity level, mobility and special diet. The number of subjects tested was 83. The age at initiation variable was not tested in this model as it is known that age highly correlates with weight in children therefore the degree of weight deviation from the expected weight at the twelve months of therapy would be highly correlated with the children's age at that point.

The result of this analysis indicates that none of the phenotypic factors significantly influenced the weight deviation. This is displayed in Table 18 below.

Table 18: Analysis of covariates with the dependent variable as oW12\_eW12 with all the clinical phenotypes except age at initiation as co-variates, (N = 83)

Source	Type III Sum of Squares	df	Mean Square	F-test	p-value	Significance
<b>Corrected Model</b>	194.361 <sup>a</sup>	13	14.951	0.824	0.633	<b>N.S</b>
<b>Intercept</b>	148.757	1	148.757	8.203	0.006	<b>N.S</b>
<b>Maximum VPA dose</b>	16.703	1	16.703	0.921	0.341	<b>N.S</b>
<b>Sex</b>	20.019	1	20.019	1.104	0.297	<b>N.S</b>
<b>Number of Visits</b>	4.467	1	4.467	0.246	0.621	<b>N.S</b>
<b>Length of exposure</b>	1.523	1	1.523	0.084	0.773	<b>N.S</b>
<b>Epilepsy syndrome</b>	19.557	1	19.557	1.078	0.303	<b>N.S</b>
<b>Learning difficulty</b>	7.713	1	7.713	0.425	0.516	<b>N.S</b>
<b>Family history of epilepsy</b>	1.350	1	1.350	0.074	0.786	<b>N.S</b>
<b>AED other</b>	3.388	1	3.388	0.187	0.667	<b>N.S</b>
<b>Family history of obesity</b>	36.924	1	36.924	2.036	0.158	<b>N.S</b>
<b>Activity level</b>	1.014	1	1.014	0.056	0.814	<b>N.S</b>
<b>Mobility</b>	54.613	1	54.613	3.012	0.087	<b>N.S</b>
<b>Special diet</b>	57.157	1	57.157	3.152	0.080	<b>N.S</b>
<b>Weight for age centile at initiation</b>	23.855	1	23.855	1.315	0.255	<b>N.S</b>
<b>Error</b>	1251.288	69	18.135			
<b>Total</b>	1563.423	83				
<b>Corrected Total</b>	1445.648	82				

**Legend:** a. R Squared = .134 (Adjusted R Squared = -.029); df – degrees of freedom; VPA – sodium valproate; AED – antiepileptic drug; N.S – not significant

### **3.7. Mean weight for age z-score change**

The last set of regression analysis focused on the mean weight for age z-score as the dependent variable and the phenotypic factors tested in the preceding sections as independent variables. The effects of these factors on the mean WAZ change were tested by including all the factors into the model. The results of the analysis are shown in Tables 19 below.

Table 19: Analysis of covariates with the dependent variable mean WAZ change and the clinical phenotypes as independent variables (n= 29)

Source	Type III Sum of Squares	df	Mean Square	F-test	p-value	Significance
<b>Corrected Model</b>	6.728 <sup>a</sup>	15	0.449	0.479	0.913	<b>Not significant</b>
<b>Intercept</b>	0.758	1	0.758	0.809	0.385	<b>Not significant</b>
<b>Sex</b>	1.279	1	1.279	1.365	0.264	<b>Not significant</b>
<b>Number of Visits</b>	0.202	1	0.202	0.215	0.650	<b>Not significant</b>
<b>Start Age</b>	0.636	1	0.636	0.679	0.425	<b>Not significant</b>
<b>Length of exposure</b>	1.487	1	1.487	1.587	0.230	<b>Not significant</b>
<b>Epilepsy syndrome</b>	0.987	1	0.987	1.053	0.323	<b>Not significant</b>
<b>Learning difficulty</b>	2.174	1	2.174	2.320	0.152	<b>Not significant</b>
<b>Family History of epilepsy</b>	0.637	1	0.637	0.680	0.425	<b>Not significant</b>
<b>Average VPA level</b>	1.184	1	1.184	1.264	0.281	<b>Not significant</b>
<b>Maximum dose</b>	0.105	1	0.105	0.112	0.743	<b>Not significant</b>
<b>AED other</b>	1.435	1	1.435	1.532	0.238	<b>Not significant</b>
<b>Family history of obesity</b>	0.278	1	0.278	0.297	0.595	<b>Not significant</b>
<b>Activity Level</b>	2.610	1	2.610	2.786	0.119	<b>Not significant</b>
<b>Mobility</b>	1.122	1	1.122	1.197	0.294	<b>Not significant</b>
<b>Special diet</b>	1.179	1	1.179	1.258	0.282	<b>Not significant</b>
<b>Centile at initiation</b>	0.212	1	0.212	0.226	0.642	<b>Not significant</b>
<b>Error</b>	12.181	13	0.937			
<b>Total</b>	21.033	29				
<b>Corrected Total</b>	18.908	28				

**Legend** a. R Squared = .356 (Adjusted R Squared = -.387); df – degrees of freedom; VPA – sodium valproate; AED – antiepileptic drugs;

### **3.8. Conclusions**

To the best of our knowledge, this study, investigating the predictors of valproate-induced weight gain in the paediatric population, considers the largest number of subjects studied to date. In this group of children, VPA therapy was associated with a significant weight gain within the first year of therapy. Clinically significant weight gain of  $\geq 5\%$  of expected weight based on pre-treatment WAC was observed in 48% of the subjects. Severe weight gain ( $\geq 10\%$  of expected weight) was observed in 27% of the subjects. The proportion of overweight children increased from a baseline of 14.2% to 22.2% at the end of first year of VPA therapy. The mean maximum percentage weight change within the first year of therapy was 7.3% ( $\pm 12\%$ ). The mean weight for age centile change was 0.04 ( $\pm 0.50$ ), while the mean weight deviation from the expected weight at the end of the first year of therapy was 1.56 kg ( $\pm 3.90$  kg). The mean change in weight for age z-score (WAZ) was 0.22 ( $\pm 0.70$ ). This weight gain was however not sustained on long term averaging four and a half years.

The following 'clinical' factors tested were not significantly associated with the weight gain: sex, number of visits, length of exposure to VPA, epilepsy syndrome, maximum VPA dose, learning difficulty, family history of epilepsy, family history of obesity, activity level, mobility and special diet.

Although some studies have reported that treatment with VPA in children was not associated with a significant weight change the

overwhelming evidence supports the contrary (Bosnak et al., 2003, Caksen et al., 2002). Bosnak et al studied 56 patients aged 6 months to 12 years who were followed for 6 – 40 months (median 15.6 months) and the result indicated no change in mean BMI z-score (Bosnak et al., 2003, Caksen et al., 2002). Casken et al also demonstrated that there was no weight gain in prepubertal children on VPA compared to controls (Caksen et al., 2002). Biton et al, in a randomized double blind trial, compared patients older than 12 years with partial or generalized seizures on valproate with those on lamotrigine. Those on VPA had a mean increase in BMI of  $2.26 \pm 1.59$  kg/m<sup>2</sup> over 32 weeks compared to a BMI increase in BMI of  $0.19 \pm 2.26$  kg/m<sup>2</sup> for those on lamotrigine over the same time period (Biton, 2001). The weight gain associated with VPA therapy occurred within the first ten weeks of therapy (Biton, 2001). VPA therapy is associated with acute weight increase in the first few months of initiation of therapy which stabilizes with continued long term treatment (Sharpe et al., 2009).

However Mikkonen et al (2005) noted that some of the children who became while on treatment with this medication during the pubertal period remained persistently overweight if epilepsy and treatment with this medication was continued into adulthood (Mikkonen et al., 2005a).

Prabhakar et al in a prospective study in adult women treated with VPA followed up for two years, also noted a persistent increase in the body weight and BMI (Prabhakar et al., 2007). The findings in our study do not support the findings in previous studies regarding the persistence of the weight gain after a mean follow up of four and half years.

Regarding the clinical predictors of VPA induced weight gain although clinical predictors have been studied there has not been any reproducible predictor of this ADR. Corman et al., (1997) in a retrospective record review and interview of 70 adults with epilepsy who have been on VPA for a median duration 27 months (range, 3 – 189 months) using weight change > 5% baseline weight as a marker of weight gain investigated the role of the following clinical factors in VPA-induced weight gain: gender, age, BMI, dose, personal or family history of obesity and monotherapy versus polytherapy. The result of the study indicated that **low or normal initial BMI and lack of personal history of weight problems** as the two clinical factors predictive of this ADR (Corman et al., 1997). However a previous retrospective study in 63 adults with epilepsy by Dinesen et al., (1984) found no significant clinical predictors, having examined age, sex, pre-treatment overweight status, duration of treatment, dosage and serum levels of valproate (Dinesen et al., 1984).

Several such studies searching for the clinical predictors of VPA-induced weight change have been conducted in children. Novak (1999) in a retrospective hospital outpatient-based study of 55 children with epilepsy aged 1.8 to 16.9 years who have been on VPA therapy for 8.6 – 33.8 months assessed the following clinical predictors: BMI, weight z-score, gender, age at initiation, monotherapy at start of treatment, duration of follow up, mental retardation, seizure type (generalized versus partial), aetiology (idiopathic or cryptogenic versus remote symptomatic) and dose. Children who were fed by nasogastric tube, those on appetite stimulants (ketogenic diet, oral

contraceptive pills (OCP), corticosteroids) and those with chronic systemic diseases (hepatic, renal failure, IBD, malignant brain tumour, etc.) were excluded. **The initial weight z-score and the initial BMI** were the two clinical predictors reported as significantly associated with weight change in that study.

Wirrell 2003 conducted another retrospective study in 43 children aged 10 – 17 years (mean age 13 (SD 2) years) 77% of whom were on VPA monotherapy at maintenance dose of 18 (SD; 9, range - 4- 40) mg/kg/day. These children were followed up for at least two months. The mean duration of therapy was 17 months (SD 15; range 2 – 54). Those who were followed up for less than two months, or who were on medications that affected weight or who were fed through the nasogastric tube were excluded from the study. The influence of the following clinical factors on weight change was studied: BMI at initiation, BMI category at follow up, BMI difference, age gender, maximum dose, average maintenance dose, average serum level, seizure type, neurocognitive status, duration of treatment, monotherapy versus polytherapy, current VPA usage (discontinued versus continued) and seizure control on VPA. The result of this study indicated that none of the factors tested significantly influenced the BMI difference but two factors: **normal neurocognitive status (p=0.06) and primary generalized epilepsy type** (p=0.07) approached statistical significance(Wirrell, 2003).

Sharpe et al. (2009) reported that the **duration of therapy was a negative predictor** of weight change in children with epilepsy being treated with VPA (Sharpe et al., 2009). In that study a retrospective chart review was

conducted in a paediatric neurology office involving 94 children with epilepsy who were aged between 2 – 20 years on a mean VPA dose of 18.3 mg/kg/day (median 16.1; Range 5 – 39 mg/kg/day) who have been followed for a period of 4 – 89 months (mean 30 months median 26 months). Change in BMI z-score was used as a measure of weight change. The clinical predictors studied included: age at initiation, average valproate dose, average valproate level, duration of treatment, formulation (regular versus sprinkle or extended release), gender, concurrent medication, seizure control and ethnicity. There was a negative correlation between change in the BMI z-score and duration of therapy(Sharpe et al., 2009). This finding is not surprising given that VPA causes an acute weight gain which tends to trend back to normal with continued treatment. In our study although significant weight gain was seen within the first twelve months this was not the case on long term follow up. In our study children followed up for a mean period of 4 years had no significant change in the weight for age centile.

However other studies could not determine any predictors of this weight change. Demir (2000) in a retrospective study that included 100 children with epilepsy aged 15 months to 18 years on VPA dose of 30 – 50 mg/kg/day over a period of 6 months to 5 years using weight velocity as a measure of weight change found that neither the epilepsy syndrome nor the seizure control category was a predictor of this ADR(Demir, 2000, Tanner and Whitehouse, 1976). In our study epilepsy syndrome was assessed there was no correlation between the epilepsy syndrome and the weight change phenotypes used in our study. Although collected information on seizure

control in our cohort we could not analyse the data due to the level of missing data.

Some of the main drawbacks of all VPA-weight change studies to date include the retrospective nature of the studies and the use of different weight change phenotypes which are not very reliable due to the nature of children's growth. Weight for age z-score for example is not a good measure of adiposity as it fails to distinguish shorter fatter children from taller children who are well proportioned (Novak, 1999). BMI has its own drawback as well. Normal BMIs vary across a wide spectrum of patients' characteristics such as race, gender and pubertal status (Novak, 1999, Daniels et al., 1997). One of the other main drawbacks of using weight change or BMI as measure of VPA-induced weight change is the wide within-child variability in these measures over a long period of time (Sharpe et al., 2009, Cole et al., 2005, Mei et al., 2004). These shifts in growth centiles are more common in children younger than six months old but occur less frequently in those older than 60 months (Mei et al., 2004).

The findings in our study are in line with that of previous studies in children and adults many of which have not been able to reliably identify any strong clinical predictors of this ADR. Although some studies have identified candidate clinical predictors these have not been reliably replicated in follow on studies (Sharpe et al., 2009, Wirrell, 2003, Demir, 2000).

Since we have not been able to determine any clinical predictor of this ADR in our study we might assume that this ADR is idiopathic or idiosyncratic. This might point to some genetic contribution to this ADR. The next experimental chapter will therefore explore the genetic contribution to this ADR.

**Chapter 4: Experimental Chapter 2 -  
Genotyping of subjects against the top 19  
single nucleotide polymorphisms associated  
with changes in body mass index**

## **4.0. Experimental Chapter 2 - Genotyping of subjects against the top 19 single nucleotide polymorphisms associated with changes in body mass index**

### **4.1. Introduction**

In Experimental Chapter 1 of this work VPA therapy was shown to be associated with significant weight gain in our paediatric cohort. A weight deviation of 5% or more of the expected weight was observed in 48% of the children treated with this medication. However, our analysis did not identify any significant clinical predictors of this weight gain. Further, we were unable to replicate the clinical predictors proposed in previous studies (in both adult and paediatric populations) to influence this ADR. Given the lack of robust clinical predictors, our findings and the wider literature would suggest this ADR is idiosyncratic and may thus have underlying genetic factors. We therefore set out to characterise genetic factors associated with VPA-induced weight change.

Obesity, defined as body mass index (BMI) greater than 30 kg/m<sup>2</sup>, has a strong genetic component. Heritability studies have shown that genetic variation accounts for between 40-70% of the trait variance in obesity (Maes et al., 1997, Atwood et al., 2002)

Prior to the completion of the Human Genome Project, our understanding of the genetics of obesity came from i) mutations in genes causing monogenic obesity syndromes in humans and ii) animal studies ((Hofker & Wijmenga, 2009). Genes identified through these efforts such as transmembrane protein 18 (*TMEM18*), *FTO*, *MC4R*, *Glucosamine-6-*

*phosphate deaminase 2 (GNPDA2), Brain-derived neurotrophic factor (BDNF), Neuronal growth regulator 1 (NEGR1), SH2B adaptor protein 1 (SH2B1), ETV5, Mitochondrial carrier 2 (MTCH2), Potassium channel tetramerization domain containing 15 gene (KCTD15), dnaJ homolog subfamily C member 27 (RBJ), GPRC5B* are thought to influence the hypothalamic energy regulatory centre (Willer et al., 2009, Speliotes et al., 2010, Loos et al., 2008, Andreasen et al., 2009, Meyre et al., 2009, den Hoed et al., 2013)(Scuteri et al., 2007, Lindgren et al., 2009).

However with the completion of the Human Genome Project there has been an explosion of large population-based GWAS studies focusing on the phenotype of obesity (Willer et al., 2009, Speliotes et al., 2010, Loos et al., 2008, Andreasen et al., 2009, Meyre et al., 2009, den Hoed et al., 2013)(Scuteri et al., 2007, Lindgren et al., 2009).(Speliotes et al., 2010, Thomsen et al., 2012) (den Hoed et al., 2013, Willer et al., 2009, Garver et al., 2013, Czerwensky et al., 2013). However only about 2 – 4% of the variation in weight observed in a population can be accounted for by the SNPs identified through these large GWAS studies.

Extreme obesity might be due to highly penetrant, rare alleles that might be difficult to identify through genome wide association (GWA) studies in the general population; as these studies focus on common variation. Exome or genome sequencing will be required to characterise rare variation in the context of obesity phenotypes.

Variations across the gene encoding the fat mass and obesity-associated protein (FTO) were the first to be associated with predisposition to obesity in a GWA study for type 2 diabetes (Hofker and Wijmenga, 2009, Speliotes et al., 2010). Further studies reported the association of *melanocortin 4 receptor (MC4R)* and *PCSK1* with syndromic obesity in humans and animals (Benzinou, 2008). Since *FTO*, *MC4R* and *proprotein convertase subtilisin/ kexin type 1 (PCSK1)*, other obesity and obesity-related loci have been identified through GWAS including the transmembrane protein (*TMEM18*), glucosamine-6-phosphate deaminase 2 (*GNPDA2*), *brain derived natriuretic factor (BDNF)*, *neuronal growth regulator 1 (NEGR1)*, *scr homology 2 containing putative adaptor protein 1 (SH2B1)* and others (Frayling et al., 2007).

While the initial BMI GWA studies involved large numbers of individuals (25,000 and 32,000) from the general population, the next reported study focused on 1,380 Europeans with extreme obesity. In addition to discovering additional loci, the studies validated two of the three loci (*MC4R* and *FTO*) previously associated with changes in BMI (Hofker & Wijmenga, 2009; Meyre et al, 2009; Thorleifsson, 2009; Willer et al 2009). The largest of the BMI studies involved about 250,000 individuals and revealed 18 new BMI associated loci (Speliotes, 2009) (See Table 20 for a list of some of the BMI loci). Most of these studies have employed meta-analysis of studies from the Caucasian population in order to improve the power to detect further novel loci (Speliotes et al., 2010, Magi and Morris, 2010, Willer et al., 2009)

In all, about 52 loci for obesity traits have now been discovered through GWAS. These have only modest effect sizes with odd ratios (per allele) of between 1.2 and 1.5 (Speliotes et al., 2010, Farooqi, 2013, Maes et al., 2011). Individuals that inherit higher number of these risk alleles are more likely to experience increased BMI compared to the individuals with arraying lower numbers of the risk alleles (Speliotes, 2010).

Among all the BMI loci that have been reported so far, the FTO SNPs account for the greatest proportion of the variance in BMI (about 0.34%) (Speliotes, 2010). *MC4R* is another common obesity locus associated with modest effects on BMI (Willer, 2009). For a given minor allele frequency (MAF) the effect sizes of the more recently discovered loci are smaller than the ones discovered in earlier studies (Speliotes, 2010).

The directional effect of the majority of these alleles seem be similar in both adults and children (Speliotes, 2010, Meyre, 2009). In addition, the alleles associated with increased BMI were closely associated with increased body weight and increased body fat percentage (Speliotes, 2010).

Table 20: Loci associated with variations in BMI

SNP	Gene	Per allele change in BMI (beta)	Experimental variance (%)	References
R1421085	<i>FTO</i>	0.39	NA	Hofker & Wijmenger, 2009
Rs155902	<i>FTO</i>	0.33	0.34	Willer et al, 2009; Speliotes et al, 2010
Rs17782313	<i>MC4R</i>	0.20	0.10	Willer et al, 2009
Rs571312	<i>MC4R</i>	0.23	0.10	Hofker & Wijmenger, 2009; Speliotes et al, 2010
Rs1805081	<i>NPC1</i>	-0.087	NA	Hofker & Wijmenger, 2009
Rs1424233	<i>MAF</i>	0.091	NA	Hofker & Wijmenger, 2009
Rs10508503	<i>PTER</i>	0.144	NA	Hofker & Wijmenger, 2009
Rs4712662	<i>PRL</i>	0.031	NA	Hofker & Wijmenger, 2009
Rs10938397	<i>GNPDA2</i>	0.19	0.13	Speliotes et al, 2010; Willer et al, 2009
Rs10767664	<i>BDNF</i>	0.19	0.07	Speliotes et al, 2010; Willer et al, 2009
Rs2515762	<i>NEGR1</i>	0.13	0.04	Speliotes et al, 2010; Willer et al, 2009
Rs9816226	<i>ETV5</i>	0.15	0.03	Speliotes et al, 2010; Willer et al, 2009
Rs3817334	<i>MTCH2</i>	0.06	0.01	Speliotes et al, 2010
Rs29941	<i>KCTD15</i>	0.06	0.00	Speliotes et al, 2010
Rs713586	<i>RBJ</i>	0.14	0.06	Speliotes et al, 2010
Rs12444979	<i>GPRC5B</i>	0.17	0.03	Speliotes et al, 2010
Rs2287019	<i>QPCTL</i>	0.15	0.04	Speliotes et al, 2010
Rs1514175	<i>TNNI3K</i>	0.07	0.02	Speliotes et al, 2010
Rs13107326	<i>SLC39A8</i>	0.19	0.03	Speliotes et al, 2010
Rs2112347	<i>FLJ35779</i>	0.10	0.02	Speliotes et al, 2010
Rs2241423	<i>MAP2K5</i>	0.13	0.03	Speliotes et al, 2010
Rs7359397	<i>SH2B1</i>	0.15	0.05	Speliotes et al, 2010, Willer et al, 2009
Rs281575	<i>NEGR1</i>	0.10	0.03	Willer et al, 2009

**Legend:**

*MC4R* –melanocortin-4 receptor; *FTO* -Fat mass and obesity ; *MAF* - *V-maf musculoaponeurotic fibrosarcoma oncogene homolog* ; *PTER* - *Phosphotriesterase-Related Protein*; *PRL* - ; prolactin *BDNF* - *Brain-Derived Neurotrophic Factor* ; *SH2B1* - *Src homology 2 B adapter protein 1* ; *TMEM18* - *Transmembrane protein 18* ; *MTCH2* - *Mitochondrial Carrier 2* ; *GNPDA2* - *glucosamine-6-phosphate deaminase 2*; *NEGR1*- *neuronal growth regulator 1* ; *KCTD15* - *potassium channel tetramerization domain containing 15*; *RBJ* - *dnaJ homolog subfamily C member 27*; *ETV5* - *ets variant 5*; *KCTD15* - *encoding potassium channel tetramerisation domain containing 15*; *GPRC5B* - *G protein-coupled receptor, family C, group 5*; *QPCTL* - *glutaminy-peptide cyclotransferase-like*; *TNNI3K*; *SLC39A8* - *solute carrier family 39 (zinc transporter), member 8*; *TNNI3K*- *fucose-1-phosphate guanylyltransferase and TNNI3 interacting kinase; member 8*; *FLJ35779* - *encoding POC5 centriolar protein homolog*; *MAP2K5* - *mitogen-activated protein kinase 5*; *SH2B1* - *SH2B- adaptor protein 1*; *NPC1* – *Niemann-Pick disease type C1*; *PETR* -

Although genetic susceptibility to VPA-induced weight gain has been proposed and demonstrated in twin studies, no specific gene or SNP has been implicated (Maes et al., 1997, Verrotti et al., 1999b). In this chapter, we

test the hypothesis that valproate induced weight gain correlates with genetic variations that have been shown, in GWA studies, to influence BMI.

## **4.2. Methods**

### **4.2.1. Weight change phenotype**

The primary phenotype used was the maximum percentage weight change from expected weight within the first twelve months of VPA therapy (see section 3.4.1 for the description of phenotype calculation). The second phenotype used was the average change in the weight for age z-score (WAZ). We planned to include any clinical factor shown to be significantly correlated with the weight ADR (see Experimental Chapter 1 above) as a co-factor in the analysis but since the results from the clinical predictor analysis indicated that none of the clinical factors influenced weight gain due to VPA, no co-factor was incorporated in the genetic analysis

### **4.2.2. Cohort**

For a full description of the cohort of subjects in the study see Sections 2.6 and 2,7 in the General Methods Chapter above.

#### **4.2.3. Selection of variants for genotyping**

We selected 17 obesity variants (SNPs) for this study. The SNPs were selected based on the following criteria:

1. The SNPs must have been replicated and validated in previous studies;
2. The SNPs must have been robustly associated with changes in BMI.

These SNPs were sourced from published GWA studies (Klein et al., 2005, Willer et al., 2009, Speliotes et al., 2010, Loos et al., 2008, Andreasen et al., 2009, Meyre et al., 2009, den Hoed et al., 2013, Scuteri et al., 2007). These SNPs have been replicated and validated in several studies and have thus been shown to robustly associate with obesity (Lindgren et al., 2009, Speliotes et al., 2010, Thomsen et al., 2012, den Hoed et al., 2013, Willer et al., 2009, Garver et al., 2013). The selected variants are listed in Table 21 below.

**Table 21: Top 17 SNPs associated with BMI variations in the general population**

CHR	SNP	CHR position	Gene	"weight gain" allele	Effect size (BETA)	Reported by
18	rs17782313 (C/T)	0 57851097	MC4R	C	1.22	(Czerwensky et al., 2013, Cauchi et al., 2009)
18	rs17700633 (A/G)	0 57929432		A	0.21	(Meyre et al., 2009)
16	rs9939609 (A/T)	0 53820527	FTO	A	0.33	(Loos et al., 2008)
20	rs6020846 (A/G)	0 36405667	CTNBL1	G	0.83	(Willer et al., 2009)
20	rs6013029 (G/T)	0 36399580	CTNBL1	T		(Andreasen et al., 2009)
16	rs1424233 (A/G)	0 79682751	MAF	A	1.39	(Andreasen et al., 2009)
18	rs1805081 (A/G)	0 21140432	NPC1	A	0.75	(Meyre et al., 2009)
10	rs10508503 (C/T)	0 16299951	PTER	C	0.64	(Meyre et al., 2009, Garver et al., 2013)
6	rs4712652	0 22078615	PRL	G		(Cotsapas et al., 2009, den Hoed et al., 2013)
16	rs7498665 (A/G)	0 28883241	BDNF and SH2B1	G	0.15	(Cotsapas et al., 2009, Robiou-du-Pont et al., 2013)
2	rs6548238 (C/T)	0 634905	TMEM18	C	0.26	(Willer et al., 2009, Thomsen et al., 2012)
11	rs10838738 (G/A)	0 47663049	MTCH2	G	0.07	(Willer et al., 2009)
4	rs10938397 (G/A)	0 45182527	GNPDA2	G	0.18	(Willer et al., 2009)
16	rs1421085	0 53800954	FTO	T	-0.155	(Speliotes et al., 2010, Lindgren et al., 2009)
1	rs2815752 (A/G)	0 72812440	NEGR1	A	0.13	(Scuteri et al., 2007)
16	rs9930506 (A/G)	0 53830465	FTO	G	-0.118	(Speliotes et al., 2010)
19	rs11084753 (G/A)	0 34322137	KCTD15	G	0.07	(Scuteri et al., 2007)

**Legend:**

MC4R –melanocortin-4 receptor; FTO -Fat mass and obesity ; MAF - V-maf musculoaponeurotic fibrosarcoma oncogene homolog; PTER - Phosphotriesterase-Related Protein; PRL - ; prolactin BDNF- Brain-Derived Neurotrophic Factor ; SH2B1 - Src homology 2 B adapter protein 1 ; TMEM18 - Transmembrane protein 18 ; MTCH2 - Mitochondrial Carrier 2 ; GNPDA2 - glucosamine-6-phosphate deaminase 2; NEGR1- neuronal growth regulator 1 ; KCTD15 - potassium channel tetramerization domain containing 15; CTNBL1 – catenin, beta like 1; A – adenine; G- guanine; T – thymine; C - cytosine

Effect size –the degree of relation between each SNP and BMI

#### **4.2.4. Genotyping**

Genotyping of the 17 SNPs was carried out at LGC Genomics using the KASP Genotyping Chemistry method (See the KASP™ Genotyping Chemistry by LGC Genomics section 2.9.7 in the General Methods Chapter).

#### **4.2.5. Genetic analysis**

The analysis was in two parts. The first part involved linear regression (genotype against the quantitative trait of weight change phenotype) in two subsets of the overall cohort (see section 1.17).

Sub-cohort 1) 123 individuals with the following phenotyping: maximum percentage weight change.

Sub-cohort 2: 73 individuals using the WAZ change in the first year of treatment. There was no overlap in patients between the two cohorts. A fixed effect meta-analysis of the summary statistics resulting from these two sets of linear regression was conducted using the Metal Meta statistical package (Studies) and a total of 196 subjects were included in the meta-analysis.

The second part of the analysis was a case-control association of the discrete trait of ADR-associated weight gain in sub-cohort 1 patients only.

The sub-cohort 1 subjects (whose weight change was analyzed using the Hoey et al growth chart) were divided into three main categories according to their maximum percentage weight change: i) 'controls', ii) 'cases' and iii) 'others' – i.e. those in between the cases and controls. Controls were

considered as those patients whose maximum percentage weight change was less than 5% of the expected weight. Cases were considered as those patients with maximum percentage weight change equal or greater than 10% of the expected weight. Those in the 'others' group (i.e. with maximum weight change greater than 5% and less than 10%) were not considered in the case-control analysis. Case-control analysis was conducted using the Fisher's exact test.

The analysis was conducted using PuTTY-link (PLINK) software (Purcell et al, 2007) a bespoke analysis platform. The steps undertaken in conducting the genetic analysis were as follows:

1. PED and MAP files were generated using the genotyping data. The genotype data was then cleaned up by using the PLINK command tool that includes only the SNPs with more than 90% genotype assay success rate. SNPs with more than 10% missing values (failure rates) were removed. Hence the analysis was carried out with a new PED and MAP files containing the 'cleaned' data.
2. Individuals with incomplete phenotyping (e.g. without complete weight data) were also excluded.
3. The statistical methods employed for the analysis of the association of the quantitative traits of maximum percentage weight gain and average WAZ change using the PLINK software include using the quantitative trait association test module. This tests for association of

quantitative traits with the SNP data using either the asymptotic or empirical significance values (Willer et al., 2009) .

#### **4.2.6. Meta-analysis**

Meta-analysis between the maximum percentage weight change and average WAZ change phenotypes was conducted using the METAL software package (Purcell et al., 2003). METAL is used in meta-analysis of GWA studies by combining either the p-values or the test statistics across the studies. The results outputs are presented in text files (Speliotes et al., 2010). The input file requires the following information: the effect allele, the non-effect allele, the marker SNP, the effect (beta value), the p-value and the weight or sample size (Speliotes et al., 2010).

When carrying out the meta-analysis we ensured there was no overlap in subjects tested across the two phenotypes. The analysis option used was default mode `–SCHEME SAMPLESIZE` command which combines the p-values and the direction of effect from all the studies taking into account the sample sizes of those studies being analyzed (Abecasis et al., 2011). This analysis scheme applies the fixed effect meta-analysis model which assumes that one true effect size underlies all the studies in the analysis and that all the differences in the observed effects are due to sampling error (Borenstein et al., 2009; Abecasis et al, 2011).

#### **4.2.7. Correction for multiple testing**

As the subjects were genotyped for 17 SNPs, any p values generated from significance testing were corrected for the number of independent tests conducted using the Bonferroni method. The p-value derived from each test were therefore be multiplied by the number of tests ( $n = 17$ ) to get the true/corrected p-value.

#### **4.3. Results**

Table 21 above outlines the 17 BMI-associated SNPs that were genotyped in our subjects. It indicates the chromosome number, the position on that chromosome, gene location and reference. The results of our genotyping indicated that none of the SNPs violated Hardy-Weinberg equilibrium after correcting for multiple testing.

Table 22 below outlines the result of the linear regression of the quantitative maximum percentage weight change phenotype against each of the 17 SNPs. This analysis was conducted in 'sub-cohort 1' ( $n=123$ ), on which the maximum percentage weight change phenotype was available. rs10938397 showed a significant association with maximum percentage weight change ( $p$ -value = 0.03, uncorrected). While the 'A' allele of rs10938397 was associated with weight loss, the 'G' allele was associated with weight gain (see figure 11 below). Rs1805081 SNP was borderline significant ( $p$ -value = 0.058, uncorrected). The 'C' allele of the rs1805081 SNP was associated

with weight gain while the 'T' allele was associated with weight loss as shown in figure 12 below.

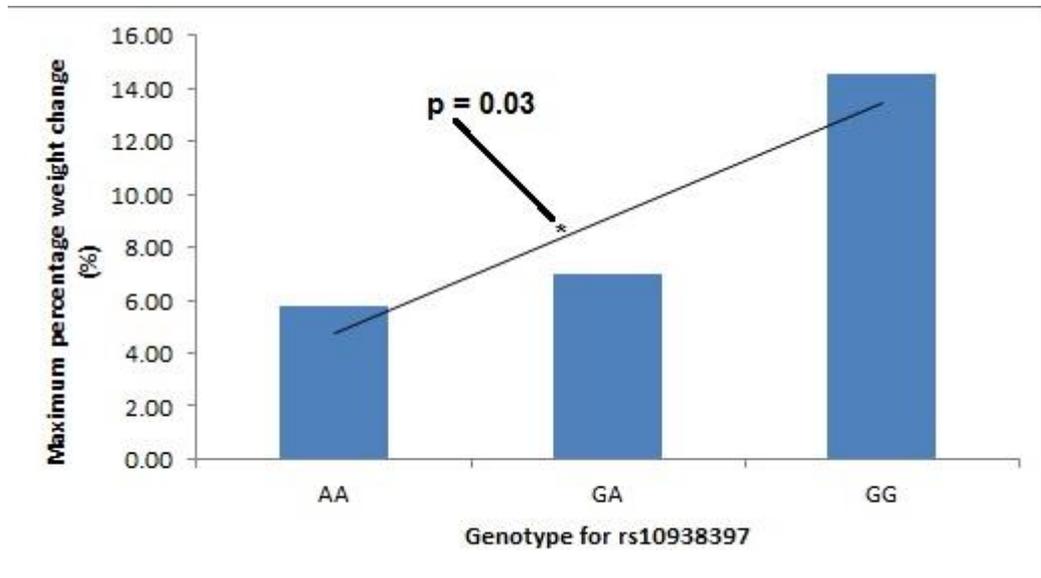


Figure 11: Bar chart showing the average maximum percentage weight gain for the different genotype of rs10938397 for individuals in sub-cohort 1 (weight change data derived from the Hoey et. al growth chart)

Figure 11 legend: AA – adenine/adenine; GA – guanine/adenine; GG – guanine/guanine; p – level of significance

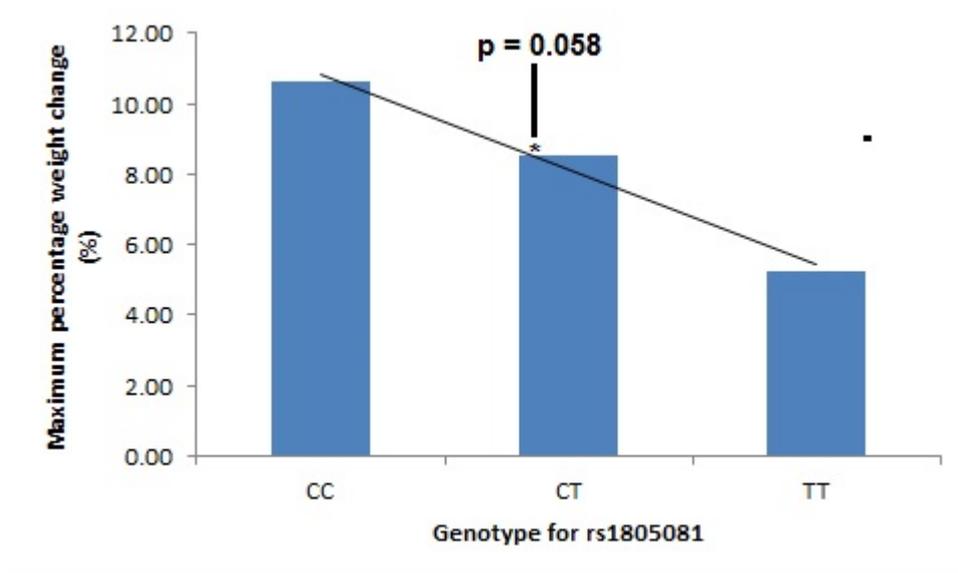


Figure 12: Bar chart showing the average maximum percentage weight gain for the different genotype of rs1805081 for individuals in sub-cohort 1 (weight change data derived from the Hoey et. al growth chart)

Fig 12 legend: CC –cytosine/cytosine; CT – cytosine/thymine; TT – thymine/thymine

p = significance level

Table 22: Association between maximum weight change on Hoey centile and the 17 top BMI SNPs showing the number of non-missing values, the beta standard error R2, T and p-value of the association

CHR	SNP	A1	A2	BP	NMISS	BETA	SE	R2	T	P
1	rs2815752	T	C	72812440	23	.257	1.724	0.000184	1491	0.8817
2	rs6548238	C	T	634905	21	-0.3559	2.003	0.000265	.1777	0.8593
4	rs10938397	A	G	45182527	123	3.529	1.635	0.0371	.159	0.03282*
6	rs4712652	A	G	22078615	121	1.476	1.636	0.006794	.9022	0.3688
10	rs10508503	C	T	16299951	122	1.055	3.115	0.000955	.3386	0.7355
11	rs10838738	A	G	47663049	123	-0.322	1.681	0.000303	0.1916	0.8484
16	rs7498665	A	G	28883241	123	1.511	1.543	0.007863	.9792	0.3294
16	rs1421085	T	C	53800954	120	1.632	1.531	0.009535	.066	0.2887
16	rs9939609	A	T	53820527	123	1.416	1.476	0.00755	.9595	0.3392
16	rs9930506	A	G	53830465	120	0.9769	1.505	0.00356	.6493	0.5174
16	rs1424233	A	G	79682751	123	1.443	1.46	0.008013	.9886	0.3248
18	rs1805081	T	C	21140432	122	3.025	1.578	0.02969	.916	0.05771*
18	rs17782313	T	C	57851097	123	-0.5084	1.912	0.000584	0.2659	0.7908
18	rs17700633	G	A	57929432	122	-0.5882	1.484	0.001308	0.3964	0.6925
19	rs11084753	G	A	34322137	123	1.489	1.745	0.005978	.853	0.3953
20	rs6013029	G	T	36399580	121	6.488	4.111	0.0205	.578	0.1172
20	rs6020846	A	G	36405667	123	5.125	3.347	0.019	.531	0.1284

**Legend:** CHR – Chromosome number; SNP – SNP identifier; BP – the physical position of SNP (base pairs) NMISS – number of non-missing values; BETA = regression coefficient; SE = standard error r2 –regression r-squared ; p – statistical significance. T –Wald test (based on t-distribution); A1 – test allele; A2 – major allele; A – adenine; G- guanine; T – thymine; C - cytosine

Table 23 below outlines the results of the quantitative trait analysis using the WAZ as a dependent variable. This analysis was conducted on subcohort 2 (n=73 individuals), for whom the weight for age Z-score phenotype was available. Five SNPs achieved statistical significance (uncorrected); *rs1805081* (p = 0.03), *rs7498665* (p=0.03), *rs17782313* (p = 0.03),

*rs6020846* ( $p=0.01$ ) and *rs10508503* ( $p = 0.03$ ). (See figures 13 – 17 below).

The only SNP that showed some association across both phenotypes/cohorts was *rs1805081*. However none of the association statistics achieved the Bonferroni corrected threshold of 0.002 (0.05/17). See Tables 24 below.

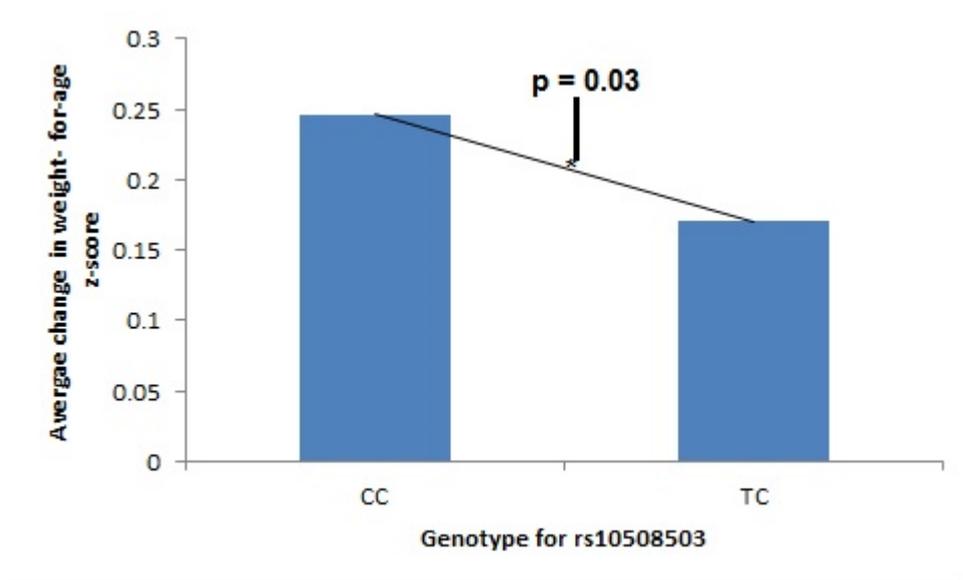


Figure 13: Bar chart showing the average change in weight-for-age z-score for the different genotypes of rs10508503 for individuals in sub-cohort 2 (weight change data derived from the WHO growth chart).

Figure 13 legend: CC – cytosine/cytosine; TC – thymine/cytosine; p – level of significance

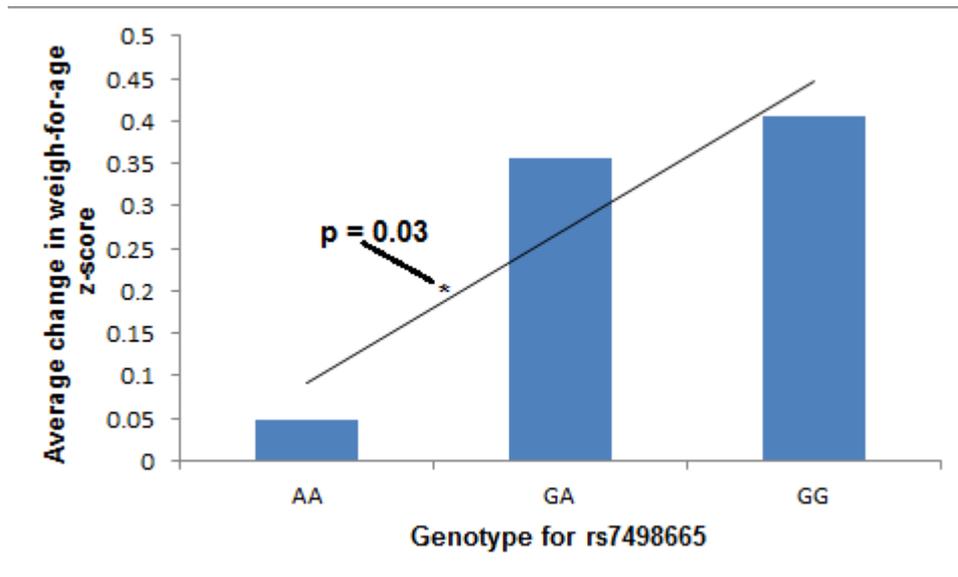


Figure 14: Bar chart showing the average change in weight-for-age z-score for the different genotypes of rs7498665 for individuals in sub-cohort 2 (weight change data derived from the WHO growth chart).

Figure 14 legend: AA – adenine/adenine; GA – guanine/adenine; GG – guanine/guanine; p – level of significance

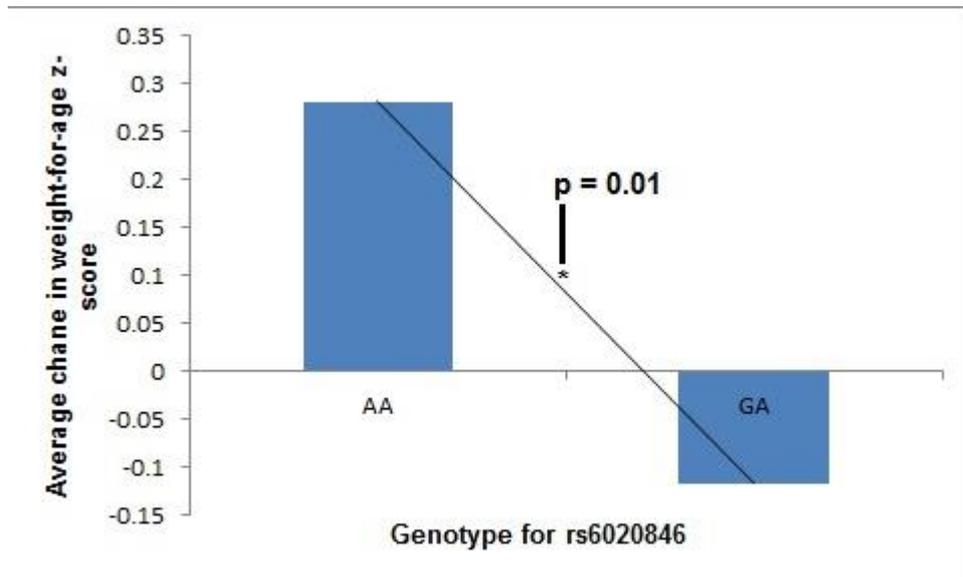


Figure 15: Bar chart showing the average change in weight-for-age z-score for the different genotypes of rs6020846 for individuals in sub-cohort 2 (weight change data derived from the WHO growth chart).

Figure 11 legend: AA – adenine/adenine; GA – guanine/adenine; p – level of significance

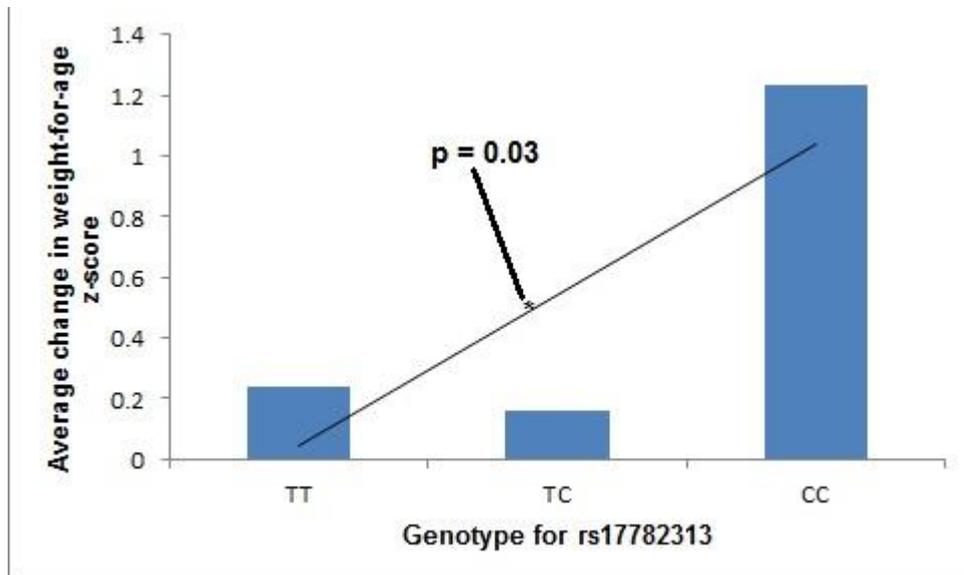


Figure 16: Bar chart showing the average weight-for-age z-score for the different genotypes of rs17782313 for individuals in sub-cohort 2 (weight change data derived from the WHO growth chart).

Figure 16 legend: CC – cytosine/cytosine; TC – thymine/cytosine; TT – thymine/thymine; p – level of significance

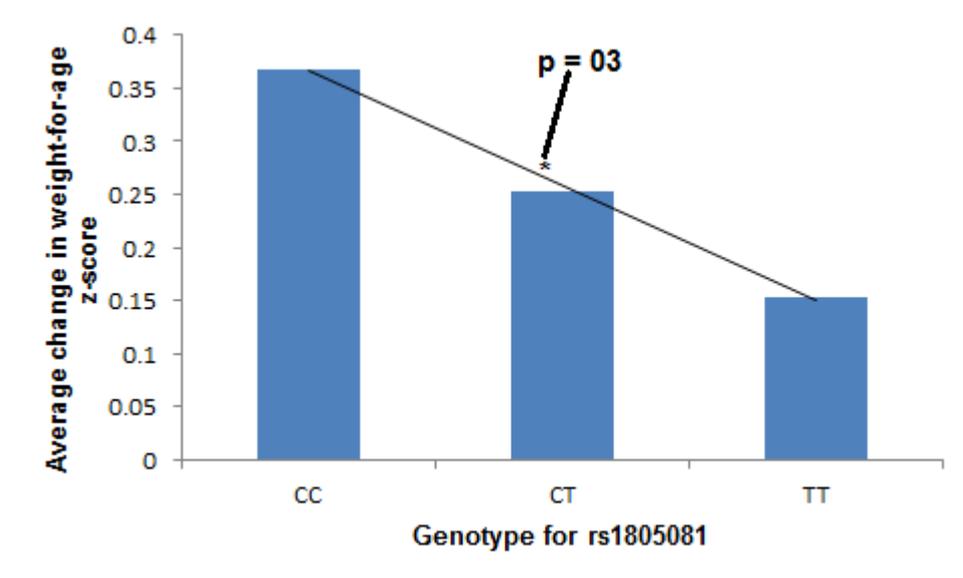


Figure 17: Bar chart showing the average weight-for-age z-score for the different genotype of rs1805081 for individuals in sub-cohort 2 (weight change data derived from the WHO growth chart).

Figure 16 legend: CC – cytosine/cytosine; CT – cytosine/thymine;

TT – thymine/thymine; p – level of significance

Table 23: Linear association between weight for age Z-score change on the WHO weight for age centile chart and the 17 top BMI SNPs in children exposed to VPA for <1 year

CHR	SNP	BP	A1	A2	NMISS	BETA	STAT	P	BONF
1	rs2815752	72812440	C	T	73	9.725	1.084	0.2818	1
2	rs6548238	634905	T	C	73	5.811	0.499	0.6193	1
4	rs10938397	45182527	G	A	73	11.97	1.427	0.1579	1
6	rs4712652	22078615	G	A	73	4.823	0.6465	0.52	1
10	rs10508503	16299951	T	C	73	-32.24	-2.197	0.03128*	0.5318
11	rs10838738	47663049	G	A	73	-5.008	-0.5372	0.5928	1
16	rs7498665	28883241	G	A	71	18.11	2.217	0.02995*	0.5091
16	rs1421085	53800954	C	T	73	3.608	0.4359	0.6642	1
16	rs9939609	53820527	A	T	73	5.353	0.6375	0.5258	1
16	rs9930506	53830465	G	A	72	6.859	0.8441	0.4015	1
16	rs1424233	79682751	G	A	73	-0.7363	-0.08517	0.9324	1
18	rs1805081	21140432	C	T	70	17.43	2.27	0.02637*	0.4482
18	rs17782313	57851097	C	T	72	20.7	2.182	0.03249*	0.5524
18	rs17700633	57929432	A	G	72	-5.018	-0.5127	0.6098	1
19	rs11084753	34322137	A	G	72	-2.329	-0.2633	0.7931	1
20	rs6013029	36399580	T	G	72	-40.8	-1.82	0.07297	1
20	rs6020846	36405667	G	A	73	-47.64	-2.524	0.01384*	0.2353

**Legend:**

CHR – Chromosome code ; SNP name; BP – the physical position of SNP (base pairs) A1 – minor allele; NMISS – number of non-missing values; BETA= beta coefficient, STAT = p – statistical significance. Beta - regression coefficient; BONF – Bonferroni corrected p value; STAT –coefficient t-statistics; A – adenine; G- guanine; T – thymine; C - cytosine

Fixed effects meta-analysis of the unadjusted results of the two sets of quantitative analysis showed that three of the 17 SNPs were significantly associated with quantitative weight centile change rs10938397 ( $p = 0.01$ ; direction of effect = ++), rs7498665 ( $p = 0.04$ ; direction of effect = ++ ) and rs1805081 ( $p = 0.004$ ; direction of effect = ++). See Table 24 below. Note that a positive z-score in Table 24 indicates that the major allele A2 is the weight gain allele while negative z-score in the same table indicates that A1 is the weight gain allele.

Table 24: Meta-analysis of the results of the genetic analysis of the two weight change phenotypes – maximum percentage weight change and change in weight-for-age z-score

SNP	A1 (our result)	A2 (our study)	A1 (original papers)	Weight	Z-score	P-value	Direction
rs9930506	A	G	G	192	1.026	0.3051	++
rs9939609	A	T	A	196	1.144	0.2525	--
rs17782313	T	C	C	195	1.089	0.2763	+-
rs10938397	A	G	G	196	2.553	0.01069	++*
rs7498665	A	G	G	194	2.09	0.03663	++*
rs17700633	A	G	A	194	0.625	0.5323	++
rs6548238	T	C	C	194	-0.165	0.8691	--
rs1421085	T	C	T	193	1.104	0.2698	++
rs1805081	T	C	A	192	2.854	0.004321	+++*
rs4712652	A	G	G	194	1.104	0.2694	++
rs2815752	T	C	A	196	0.775	0.4385	++
rs10838738	A	G	G	196	-0.478	0.6328	--
rs6020846	A	G	G	196	-0.298	0.7659	--
rs10508503	T	C	C	195	1.05	0.2935	+-
rs11084753	A	G	G	195	-0.516	0.606	+-
rs6013029	T	C	T	193	-0.145	0.8845	+-
rs1424233	A	G	A	196	0.728	0.4665	--

**Legend**

A1 – minor allele (test allele); Direction of effect: ++ = both allele 1 and allele 2 in a positive direction; +- = allele 1 in positive direction, allele 2 in negative direction; -+ = allele 1 negative, allele 2 positive; -- = both allele in negative direction; -- = both allele in negative direction. A2 (our study) – other major allele; A – adenine; G – guanine; T – thymine; C – cytosine

**rs10938397:** The ‘G’ allele of rs10938397 was associated with weight gain in the original discovery paper (Abecasis et al., 2011).

. It was reported in that original publication to explain 0.18% of trait variance. In our meta-analysis of the two phenotypes, rs10938397 explains 3.7% of trait variance. The direction of effect in the original was the same as in our study, with the “G” allele as the weight gain allele. See Table 25 and figure 18 below.

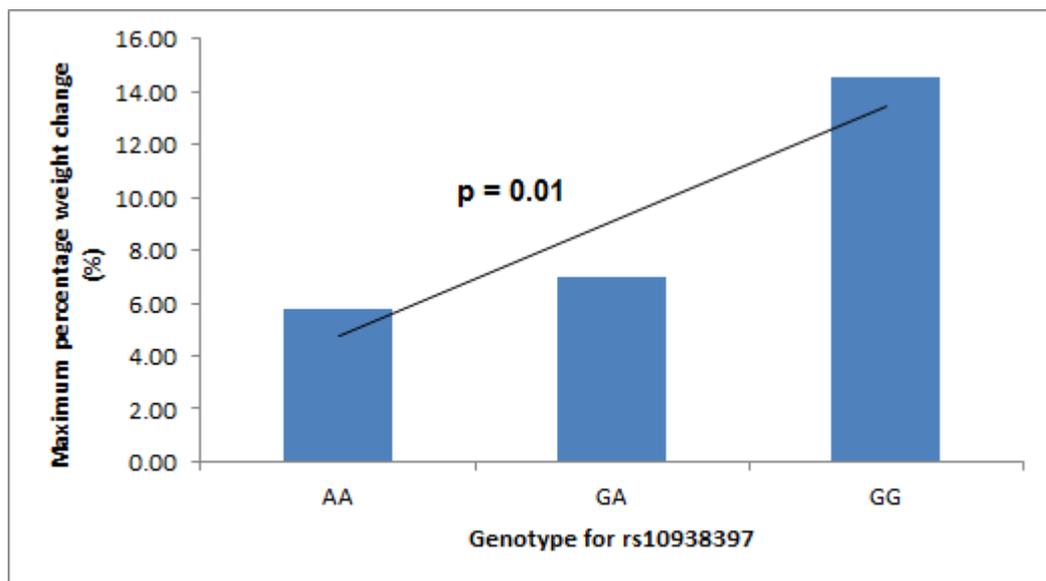


Figure 18: Bar chart showing the maximum percentage weight change for the different genotypes of rs10938397 (weight change data derived from the Hoey et. al growth chart)

Figure 18 legend: AA – adenine/adenine; GA – guanine/adenine;

GG – guanine/guanine; p – level of significance

**rs1805081:** The “T” allele of rs1805081 was associated with weight gain in the original discovery paper (Speliotes et al., 2010). The direction of effect was appears to be the opposite in our study, with the ‘C’ allele being associated with weight gain and the variant explaining 3% of trait variance here., See Table 25 & figure 19

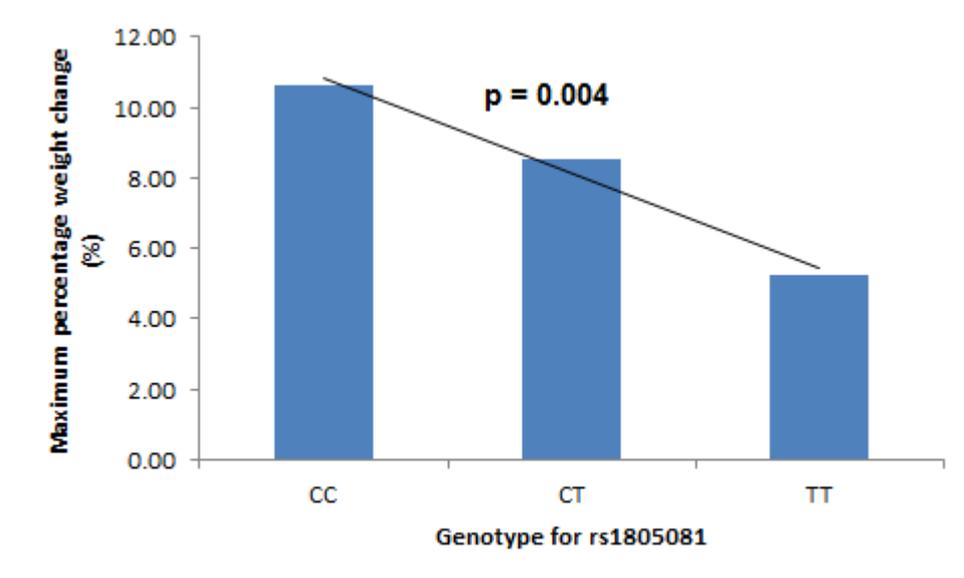


Figure 19: Bar chart showing the maximum percentage weight change for the different genotypes of the SNP rs18505081 (weight change data derived from the Hoey et. al., growth chart)

Figure 19 legend: CC – cytosine/cytosine; CT – cytosine/thymine;

TT – thymine/thymine; p – level of significance

**Rs7498665.** The 'G' allele of this variant was associated with weight gain in the original discovery paper (Meyre et al., 2009). In our analysis the 'G' allele was also associated with weight gain, and explained 0.7% of trait variants. The direction of effect was therefore the same as that of the original publication. See Table 25 and figure 20 below.

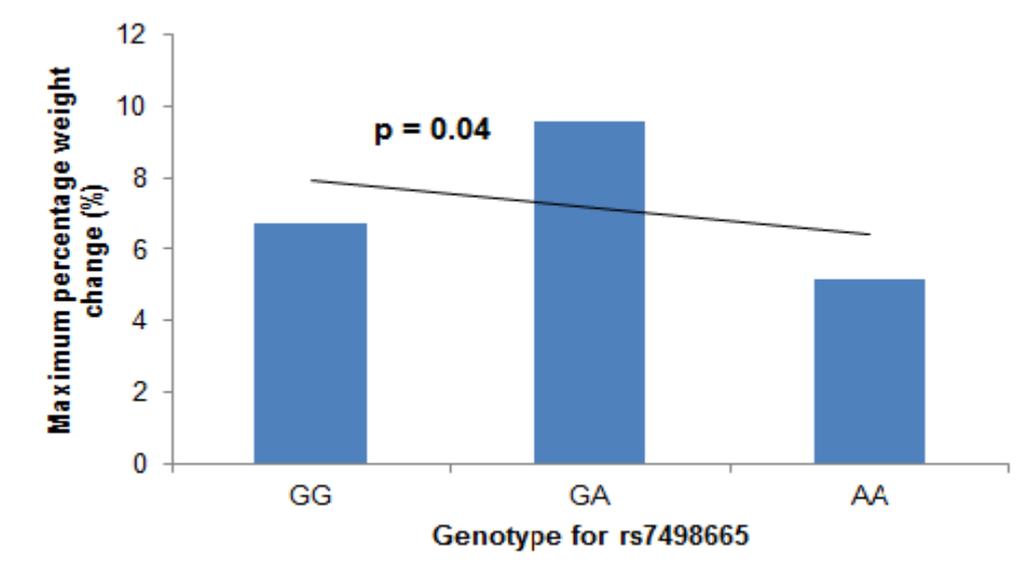


Figure 20: Bar chart showing the maximum percentage weight change for the different genotypes of rs7498665 for individuals in subcohort 1 (weight change data derived from the Hoey et. al growth chart)

Figure 20 legend: AA – adenine/adenine; GA – guanine/adenine;

GG – guanine/guanine; p – level of significance

**rs1421085:** The result of the discrete weight change analysis indicates that only one SNP, rs1421085 ( $p = 0.04$ ; odds ratio= 1.9), was statistically significantly associated with weight change. However Bonferroni correction for multiple testing indicates that none of the SNPs was significantly associated with discrete weight change (see Tables 26 & 27 and figure 21 below).

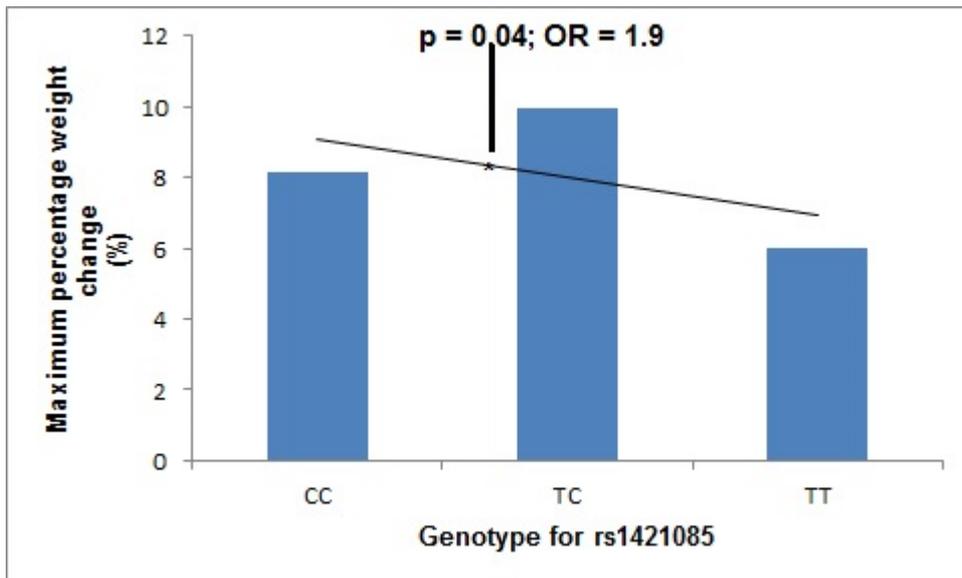


Figure 21: Bar chart showing the maximum percentage weight change for the different genotypes of the SNP rs1421085 for individuals in subcohort 1 (weight change data derived from the Hoey et. al., growth chart)

Figure 21 legend: CC – cytosine/cytosine; TC – thymine/cytosine;

TT – thymine/thymine;  $p$  – level of significance

Table 25: Comparison of the risk allele, effect size and direction of effect between the Speliotos (2010) study by Speliotos et al. and our study

CHR	SNP	Allele associated with weight(original paper)	Allele associated with weight (Our study)	r <sup>2</sup> (our study)	r <sup>2</sup> (Speliotos 2010 study(Willer et al., 2009))	P (Our study)
1	rs2815752	A	T	0.000184	0.04	0.4385
2	rs6548238	C	T	0.000265	NA	0.8691
4	rs10938397	G	G	0.0371	0.08	0.01069
6	rs4712652	G	A	0.006794	NA	0.2694
10	rs10508503	C	T	0.000955	NA	0.2935
11	rs10838738	G	A	0.000303	NA	0.6328
16	rs7498665	G	G	0.007863	NA	0.03663
16	rs1421085	T	T	0.009535	NA	0.2698
16	rs9939609	A	A	0.00755	NA	0.2525
16	rs9930506	A	A	0.00356	NA	0.3051
16	rs1424233	A	A	0.008013	NA	0.4665
18	rs1805081	A	C	0.02969	NA	0.004321
18	rs17782313	C	T	0.000584	NA	0.2763
18	rs17700633	A	A	0.001308	NA	0.5323
19	rs11084753	G	A	0.005978	NA	0.606
20	rs6013029	T	T	0.0205	NA	0.8845
20	rs6020846	G	A	0.019	NA	0.7649

**Legend**

CHR - chromosome code; SNP – single nucleotide polymorphism identifier; r<sup>2</sup> – regression r-squared or the fraction of variance explained by the model; p – statistical significance

Table 26: Discrete factor analysis for maximum weight change (maximum weight change  $\leq$  5% versus maximum weight  $\geq$  10%)

CHR	SNP	BP	A1	F_A	F_U	A2	P	OR
1	rs2815752	72812440	C	0.4028	0.3433	T	0.4485	1.29
2	rs6548238	634905	T	0.1714	0.2045	C	0.7084	0.8046
4	rs10938397	45182527	G	0.4722	0.3433	A	0.07437	1.712
6	rs4712652	22078615	G	0.5	0.4091	A	0.2357	1.444
10	rs10508503	16299951	T	0.05556	0.06061	C	1	0.9118
11	rs10838738	47663049	G	0.3611	0.3433	A	0.8784	1.081
16	rs7498665	28883241	G	0.4444	0.3358	A	0.1335	1.582
16	rs1421085	53800954	C	0.4265	0.2803	T	0.04063**	1.909**
16	rs9939609	53820527	A	0.4444	0.306	T	0.06633	1.815
16	rs9930506	53830465	G	0.4444	0.3125	A	0.06739	1.76
16	rs1424233	79682751	G	0.5	0.4627	A	0.6617	1.161
18	rs1805081	21140432	C	0.3889	0.3561	T	0.6515	1.151
18	rs17782313	57851097	C	0.2639	0.2687	T	1	0.9759
18	rs17700633	57929432	A	0.3429	0.3582	G	0.8781	0.9348
19	rs11084753	34322137	A	0.3333	0.3284	G	1	1.023
20	rs6013029	36399580	T	0.06944	0.02273	G	0.1335	3.209
20	rs6020846	36405667	G	0.09722	0.03731	A	0.1162	2.778

**Legend**

CHR – Chromosome code ; SNP name; BP – the physical position of SNP (base pairs); A1 – minor allele (test allele); A2- major allele; p – statistical significance; OR – odds ratio; SE – standard error; F\_A – Case allele frequency; F\_U - control allele frequency;

Table 27: Discrete factor analysis for maximum weight change (maximum weight change  $\leq 5\%$  versus maximum weight  $\geq 10\%$ ) with correction for multiple testing

CHR	SNP	UNADJ	GC	BONF	HOLM	SIDAK_S S	SIDAK_S D	FDR_B H	FDR_B Y
16	rs1421085	0.04063	0.3066	0.5689	0.5689	0.4405	0.4405	0.2603	0.8464
16	rs9939609	0.06633	0.3592	0.9287	0.8623	0.6175	0.5903	0.2603	0.8464
16	rs9930506	0.06739	0.361	0.9435	0.8623	0.6235	0.5903	0.2603	0.8464
4	rs1093839 7	0.07437	0.3729	1	0.8623	0.6611	0.5903	0.2603	0.8464
20	rs6020846	0.1162	0.4328	1	1	0.8226	0.7093	0.267	0.8683
20	rs6013029	0.1335	0.4537	1	1	0.8654	0.7245	0.267	0.8683
16	rs7498665	0.1335	0.4537	1	1	0.8655	0.7245	0.267	0.8683
6	rs4712652	0.2357	0.5537	1	1	0.9768	0.8477	0.4125	1
1	rs2815752	0.4485	0.7051	1	1	0.9998	0.9719	0.6977	1
18	rs1805081	0.6515	0.8216	1	1	1	0.9949	0.8264	1
16	rs1424233	0.6617	0.827	1	1	1	0.9949	0.8264	1
2	rs6548238	0.7084	0.8518	1	1	1	0.9949	0.8264	1
18	rs1770063 3	0.8781	0.939	1	1	1	0.9949	0.8784	1
11	rs1083873 8	0.8784	0.9391	1	1	1	0.9949	0.8784	1

**Legend**

CHR – Chromosome code ; SNP name; BP – the physical position of SNP (base pairs); UNADJ - Unadjusted p-value ; GC - Genomic-control corrected p-values; BONF - Bonferroni single-step adjusted p-values ; HOLM - Holm (1979) step-down adjusted p-values ; SIDAK\_SS - Sidak single-step adjusted p-values ; SIDAK\_SD - Sidak single-step adjusted p-values; FDR\_BH - Benjamini & Hochberg (1995) step-up FDR control; FDR\_BY - Benjamini & Yekutieli (2001) step-up FDR control

#### 4.4. Discussion

In summary, in the unadjusted linear regression analysis of the quantitative traits the following SNPs achieved nominally significant relationship with maximum percentage weight change and weight for age z-score: rs10938397 (*GNPDA2*;  $p = 0.03$ ), rs1805081 (*NPC1*;  $p = 0.03$ ), rs7498665 (*BDNF* and *SH2B1*;  $p=0.03$ ), rs6020846 (*CTNBL1*;  $p=0.01$ ) and rs10508503 (*PTER*;  $p =0.03$ ). A meta-analysis of the two studies indicates that the following SNPs were nominally associated with weight change; rs10938397 (*GNPDA2*) rs7498665 (*BDNF* and *SH2B1*) and 1805081 (*NPC1*). However, none of these nominally associated SNPs survived Bonferroni correction for multiple testing. It should be noted that Bonferroni correction in this context is highly conservative as there were clear a-priori evidence motivating these tests.

Compared with the original studies, while some SNPs (rs10938397 and rs7498665) that were significantly associated with quantitative weight gain in the general population exert the same directional effect in those affected by the VPA-induced weight change, other SNPs (rs1805081) associated with weight change in the general population have opposite direction of effect in those affected by the VPA-induced weight change. In addition it seems that VPA therapy is associated with markedly increased effect size of some SNPs (rs7498665 and 1805081) on weight change (See Tables 21 and 22 above). Discrete test analysis however indicates that only one SNP (rs1421085 near the *FTO* gene) was significantly associated with discrete weight change in children exposed to VPA therapy. This might be due to the more study power

with the quantitative measure of weight change compared with the discrete weight change analysis.

These results provide some support to the views that some genetic variants associate i) with changes in BMI in the general population are also ii) with VPA-induced weight change and with the degree of weight change associated with exposure to this medication. It appears that individuals who inherit these variants have increased risks of experiencing some weight change when exposed to this medication. The direction of weight/ BMI effect exerted by the exposure of these individuals to VPA seems to be the same as in the general population implying that exposure to VPA enhances the BMI change effects of those SNPs. However in one of the three SNPs that had nominally significant effect on VPA-induced weight change in the meta-analysis result, the direction of weight change in those affected by VPA-induced weight change was in the opposite direction to that of the general population. This indicates that the weight change effect of the SNP can vary between the general population and those affected by this ADR.

## **Chapter 5: Discussion**

## 5.0. Discussion

This study sought to determine the clinical factors influencing valproate induced weight gain in 250 children with epilepsy who have been exposed to VPA for at least six months. It also sought to test the impact of BMI-influencing genetic variations on VPA-induced weight change. While none of the clinical factors tested in our study significantly influenced VPA-induced weight change, it appears that a number of the BMI-influencing genetic variations do influence this ADR.

Regarding the genotyping results, the unadjusted linear regression analysis of the quantitative traits (maximum percentage weight change and change in WAZ) indicates that six of the seventeen SNPs achieved statistically significant relationship and these are rs10938397 ( $p = 0.03$ ), rs1805081 ( $p = 0.03$ ), rs7498665 ( $p=0.03$ ), rs17782313 ( $p = 0.03$ ), rs6020846 ( $p=0.01$ ) and rs10508503 ( $p =0.03$ ). Meta-analysis of the results of independent analysis of WAZ change and the maximum percentage weight change studies indicates that only three of the six SNPs that were significantly associated with quantitative weight change achieved statistical significance and these SNPs are: rs10938397, rs7498665 and 1805081. None of these three signals survived Bonferroni correction for multiple testing, although this correction is very stringent, given that we have clear a-priori motivation for each test. In the discrete weight change analysis however only one SNP (rs1421085: *FTO*) was significantly associated with weight change. Like the other SNPs in the quantitative weight change category, this SNP did not survive Bonferroni correction. Interestingly this

SNP was not among the six SNPs associated with quantitative weight change. Going by the genotyping result the second hypothesis that genetic factors are associated VPA-induced weight change could be accepted as there is a 'suggestive' evidence for this from the results of the genotyping analysis but further testing in a larger cohort of patients exposed to this medication is required in order to confirm or refute this finding. There is definitely more 'suggestive' evidence of 'genetic' predictors of this ADR in this study than there is evidence for 'clinical' predictors.

Previous studies of clinical predictors of VPA-induced weight change in studies involving only children have reported that the following factors as possible positive clinical predictors of the ADR: the initial weight z-score, initial BMI, normal neuro-cognitive status and primary generalized epilepsy type (Novak, 2009; Wirrell, 2003). Duration of therapy was the only negative clinical predictor of this ADR that has been identified (Sharpe et al., 2009). In studies involving both adult and paediatric populations, subjects on delayed release formulations were significantly more likely to gain weight than those on extended release formulations. Although we did not test for all the clinical factors that have been explored in previous studies, our study failed to identify any of the previously identified clinical factors in children such as initial weight for age z-score, neurocognitive status or epilepsy type. We could not test for the influence of BMI on VPA-induced weight change in our study due to incomplete height data. We also utilized a different weight change phenotype (weight for age centile, WAC) than the ones applied in previous studies and we did not investigate the influence of the VPA

formulation on weight gain. Although our study focused on the weight change within the first year of VPA exposure, the subjects had varying durations of exposure to the medication within the first year of therapy. The duration of exposure within the first had no significant effect on the degree weight change. Beyond the first year of therapy, we observed that the weight-for-age centiles (WAC) of the subjects affected by this ADR returned to their pre-exposure status. It should also be noted that several other similar studies in paediatric cohorts have not identified clinical predictors of this ADR (Demir, 2000; Verrotti et al., 2010; Biton, 2001; de Vries et al 2007).

Similar to our effort, most of the previous paediatric studies investigating these clinical predictors have been retrospective in nature (Novak, 1999; Wirrell, 2003; Sharpe et al., 2009; Demir, 2000). However, there has been one prospective open trial in children treated with VPA and one prospective multi-centre double blind controlled trial to evaluate the effect of VPA and LTG on body weight in children over 12 years old (Biton, 2001; Verrotti et al, 2010).

The sample sizes in the previous studies that involved only children had been between 43 and 114 participants (Novak, 1999; Wirrell, 2003, Sharpe et al., 2009; Demir, 2000; Verrotti et al., 2010). To our knowledge our study has the largest sample of children investigated to date for the clinical predictors of VPA-induced weight gain. In this context our findings could be considered as more robust compared to other studies that had smaller sample sizes.

Since we could not replicate some of the clinical predictors in previous studies that involved smaller sample sizes we could assume that this ADR is idiopathic, motivating genetic investigation. Indeed other published studies have also failed to replicate any the previously reported clinical factors. Adult studies seem to have shown similar results indicating that there might be no identifiable clinical predictors of this ADR. However, it should also be noted that the failure to identify the clinical predictors of this ADR might be due to the fact that our study (along with previous efforts) was underpowered to detect such predictors. This might indicate a need to conduct a large multi-centre study with sufficient statistical power to detect the clinical predictors of this ADR.

With the exception of twin studies that have demonstrated the genetic nature of VPA-induced weight change (Verrotti et al., 1999b, Klein et al., 2005), to the best of our knowledge, our work represents the first effort to decipher the genetics of this ADR. In this study we tested the hypothesis that individuals carrying any of a set of 17 variants previously associated with BMI were more likely to be affected by this ADR than those who do not carry these variants. In other words, the presence of any of these variants might enhance the development of the ADR or protect the individuals so affected from developing the ADR. We observed borderline association of some SNPs with VPA-induced weight change all of which had similar directions of effects with studies in the general population indicating that in the presence of any of these SNPs the VPA-induced weight change might be enhanced. However since this VPA-induced weight change occurs over time in children who are

continuously growing, it might be difficult to determine at what point in the development this change occurs. The main scientific question to address, in a case-control study involving children of the same age who are not on the medication, is at what point this change in susceptibility to this ADR occurs. In the short term the findings in our study need to be replicated in other studies.

### **5.1. Strengths of the study**

**It is the first pharmacogenetic study of VPA-induced weight change:** The main strength of the study lies in the fact that it is the first study looking at the pharmacogenetic risk factors associated with VPA-induced weight change in both children. Weight change associated with VPA is a challenging clinical problem in management of epilepsy in both adult and children. Studying the pharmacogenomic risk factors associated with this ADR would enhance the management of people with epilepsy who are being treated with this medication.

**It is the largest study of VPA-induced weight change in paediatrics to date.** This study represents the highest number of children ever recruited into a VPA-induced weight change study to date. In this context it is the best-powered study for clinical predictors of this ADR to date.

**It focused on well-motivated genetic variants:** By focusing on SNPs that had previously been robustly associated with BMI, we enhanced the yield of the genotyping result without increasing the cost. This targeted approach ensured that only the important high yield SNPs, previously associated with changes in weight were genotyped. The cost of conducting GWAs on the whole cohort would have exceeded the budget allocated to this project. However, we have put in place a resource and infrastructure for future GWAS or next-generation sequencing studies.

## **5.2. Limitations of the study**

Despite the strengths of this study, there were several limitations that warrant discussion.

**The retrospective nature of the study,** Missing data is an inherent problem with retrospective studies. Some of the subjects had only one weight measurement after commencing VPA. In some that had more than one weight measurement those measurements were taken at different institutions. This applied in particular to those children who were referred from peripheral hospitals. VPA treatments were sometimes initiated at the referring institution. Sometimes the weight measurements at commencement and/or the drug dosages were not recorded in the referral letters. As no two weighing scales have the same precision of measurements variations in precision could affect the accuracy of determining the VPA effect on the subjects' weight. Another issue that affected data quality was dependence on

paper records, some of which were missing. The recording of clinical phenotypes also varied among physicians. More than twenty physicians were involved in recording of the phenotypes assembled here. Since there was no standard method in place for entering these records each physician involved recorded the phenotypes according to his/her own training and experience levels. Added to this is the fact that these records span over a decade. Since medical knowledge has evolved over this period, there might have been some differences in the categorisation of the clinical phenotypes for example the International League Against Epilepsy (ILAE) epilepsy syndrome classification has changed since the commencement of recording on these children.

The exact date the subject commenced the medication could not always be definitively ascertained for subjects whose treatments were initiated on outpatient basis. Date of issuance of prescription was assumed as commencement date. There might be a discrepancy between the two dates as some subjects might delay the initiation of the therapy by a couple of days. The record used was however the most reliable record available to the researcher as the pharmacy dispensary record is not routinely available to clinicians in this country.

Despite these limitations, we made the best use of resources available. Where possible we contacted the hospitals where the VPA was commenced to gather information on the drug dosages, weight and height information at the commencement of therapy and subsequent weight measurements at follow up visits.

**Our small sample was limited.** Larger sample sizes provide more power to detect true effects. Recruitment was limited by the amount of time available to the researcher to complete the research project (determined by funding), and the logistics associated with the organisation of clinic days in the institutions involved. The researcher had only two years for the recruitment of subjects, sample collection and processing, data collection and analysis and submission of reports. This time frame was inadequate to recruit a larger number of subjects, or to record additional interactions for subjects already recruited (as the subjects attended the neurology clinics approximately once every six months). Indeed, despite these limitations we actually recruited more participants than we had initially targeted.

**The study sites location in tertiary institutions. Given that recruitment was based in tertiary institutions,** the children recruited often had additional medical conditions (beyond their epilepsy) that could impact on weight. This introduces additional confounders to the influence of VPA on weight in these subjects. Ideally the study should be conducted in a district general hospital, restricting recruitment children with epilepsy only. However such an approach would have taken a longer time to complete as it would have been difficult to recruit the required number of subjects in any single district general hospital within the short time frame. The alternative approach would have been to recruit across many district general hospitals at the same time but the logistics of doing this with only one researcher involved would have been extremely challenging.

**The convenience sampling method and the inclusion of children taking combination therapy:** The adoption of the convenience sampling method might have resulted in subjects attending clinics or wards for treatment being more likely to be recruited than those who, for whatever reason, did not present for treatment. Although the profile of those not turning up to the clinic was not determined, it will be difficult to determine how different this group of patients were from the subjects recruited to the study. Ideally a random sampling method with a sampling frame should have been used in order to represent the subset of children that presented to the clinics or wards for treatment. However, limited time and resources were available. We also did include children on combination AED therapy some of which might influence weight change in either direction. AEDs that induce weight gain might have enhanced the weight gain-inducing ADR of VPA while the opposite effect might have applied to the combination therapy that included AEDs associated with weight loss. Ideally subjects on VPA monotherapy should have been studied. Our study findings did not support the assumption that combination therapy has any significant effect on the VPA-induced weight change although we did not analyse the AEDs according to their effects on weight.

**The non-availability of a uniform weight centile tool:** Neither of two growth centile tools available for this study (the Clinical Growth Standards for Irish Children and the WHO Multi-Centre Growth Reference for Healthy Breastfed Babies see Section 2.9.5 in the General Methods Chapter) covered the age spectrum of subjects recruited into the study. This was

mainly due to the data quality in our study. Of the two growth assessment tools used in the study, the weight for age centile (WAC) developed by Hoey et al (1987) covered the age spectrum better than the WHO Anthro tool. This explained the adoption of the maximum percentage weight change derived from the use of the clinical Growth Standards for Irish Children as the main weight change phenotype for this study. However, the Hoey tool has some shortcomings one of which is that it did not have data on the WAC for children less than two years old at the commencement of VPA. The other disadvantage of this tool is that because it is an old tool developed to assess the growth of normal Irish children in the late 1980's and early 1990's, the growth trend depicted by the tool does not reflect the current secular trend in the growth of Irish children. The WHO AnthroPlus tool is more reflective of the current growth trends – but at a global level, not specific to Ireland. Another problem with the WHO AnthroPlus is that it requires height data in order to determine BMI or BMI z-scores. However height data was often not available in clinical notes, especially for children over 10 years of age. Up to the age of ten years weight measurement alone was sufficient to monitor growth trends using the tool but beyond this age BMI centile or BMI z-score is more reliable as the WHO Anthro does not have any weight centile or weight z-score data beyond ten years of age. It would have been ideal to adopt the BMI approach for all ages if the height information was available in most of the patients. Since this was not the case, it was decided to adopt the Hoey et al centile chart as the main weight change measurement tool since it covered most of the subjects. The WHO AnthroPlus tool was used as a

validation tool and also to cover those subjects aged less than two years at the time of VPA commencement. A meta-analysis of the genotyping result from both approaches was carried out to strengthen the result of the test.

Several observers measured the weights using different weight measuring tools: Since this study was multi-centre, the weight measuring was done by several individuals involved in the care of the subjects recruited in the study. There was not standard weighing equipment or protocol for measurement of weight. Inter- and intra-observer error might have occurred during these measurements. These might have contributed to the wrong assignment of weight phenotypes. This problem is magnified by the fact that some children weigh so little that any little discrepancy in the weight due to differences in the weight measuring tool or observer techniques could affect the weight phenotype category. It was difficult to eliminate this problem in our study as in most cases these individuals were recruited years after they had been commenced on the medication.

**The lack of adequate control samples and the use of non-standardized tools to assess level of physical activity:** We did not include any age matched controls who were not taking the medication in our study. It would have been ideal to include controls that have been matched with our cohort for age, gender, epilepsy syndrome and socio-economic status.

**Non-exclusion of children with co-morbid conditions:** In this study we included children with other co-morbid conditions that might have some impact on weight gain. These might have some confounding effects on the

weight gain associated with VPA therapy. Besides some of these children were on other medications other than AEDs that could have some impacts on their weights. Ideally these children should have been excluded from analysis.

**Children on ketogenic diet or other diets that could affect weight were not excluded:** Ketogenic and other 'special' diets have some impacts on weight gain in children. For example, children on high calorie diets for failing to thrive might experience more rapid weight gain than children with the same condition on normal diet. The effect of concomitant administration of high calorie diet and VPA on VPA-induced weight gain has not been studied. However, the number of children in this study on ketogenic or 'special' diets was very small.

**The adoption of maximum percentage weight change as the main weight change phenotype:** We applied the maximum percentage weight change over the first year of study as the weight change phenotype. This might not be the best approach as the maximum weight change might not have represented the individual weight change phenotype accurately. Some of those changes might have been due to measurement errors or might have been related to differences in the observers or in the weighing scales. However this phenotype was chosen to capture many of the individuals who would experience a weight change within the first few weeks of initiation of VPA therapy whose weight revert to normal before the end of the first year. An average deviation from the expected weight over the study period would have been ideal.

**Other issues related to phenotype collection:** A question that was not well structured was that related to activity level. Activity level was classified in the questionnaire into four categories: 'zero', 'low', 'normal' or 'high'. This was a subjective grading of the children's perceived activity level. Several validated and more objective questionnaires are available for the evaluation of the level of physical activity in children (Speliotes et al., 2010, Welk et al., 2000)

A similar issue was noted with the question on mobility level. The response options to this question (mobility) were: 'normal activity', 'impaired activity', and 'wheelchair bound'. Although the researcher gathered the information for completing this question using a combination of the researcher's observation, information volunteered by the parents and/or information gathered from the medical notes, it would have been better to have an objective way of assessing mobility level.

The question on special diet was vague. The parents were asked whether their children were on any special diet. The initial response options were dichotomous 'yes' or 'no'. Parents whose children were on special diets were asked to give details of the type of special diets their children were on. Given that there are a number of special diets children with chronic conditions such as epilepsy could be on, this question was not particularly informative.

### **5.3. Suggestions to improve further studies**

If budget was unlimited, an ideal study design would be a multi-centre prospective study. The study would include children from the ages of 2 years to 16 years on VPA monotherapy. These children should not have any co-morbid medical condition that would impact on weight gain. The study participants should ideally have their weights and heights measured on the day they the VPA was initiated using a uniform measuring scale that is regularly calibrated. Ideally the VPA would be initiated in the institution under direct observation by the medical personnel involved in the study. The definition of the clinical seizure phenotype would follow a standard protocol agreed among the physicians prior to the commencement of the study. Electronic medical record of all the participants would be kept to avoid the problem encountered in this study where some paper records were missing. This database would be backed up regularly in a secure cloud storage system subject to approval by the various ethics committees involved in the study. The sample size should ideally be very large and the study location should ideally be in a secondary care facility. A random sampling method should be employed using a sampling frame which consists of the list of all the children within this age group on VPA monotherapy for epileptic fits.

The weight change phenotype would ideally be change in BMI z-score over the study period using the most recent growth monitoring tool in children. The children would be followed up two monthly for 12 months after the initiation of therapy and weight, height, VPA dose and serum VPA levels would be measured at these time points. A method of checking compliance

with therapy would also be devised and applied. This might involve not only enquiring from the children and their carers but also taking some random serum VPA levels.

A well-designed questionnaire using standardized tools would be used in assessing such measures as activity level, mobility, and diet.

Regarding the genotyping, all subjects recruited to the study would ideally have GWA scans run. Epigenetic studies such as studying the patterns of DNA methylation and histone modifications might also yield some useful information regarding this ADR.

The resources involved in funding this type of study will be enormous given the issues involved and the measures to be taken to ensure high quality phenotyping and genotyping. Conducting such a study in this era when VPA is off-patent would nearly be impracticable as it would be difficult to raise the requisite funds for such a study.

Given the resources available to us in this study, the approach we have taken should therefore be considered in context. It was a pragmatic approach.

#### **5.4. Looking forward**

It is hoped that in the future clinically relevant pharmacogenomic factors associated with this ADR would have been uncovered. This would personalize the care of people with epilepsy treated with VPA as prediction of

this ADR will be easier than what obtains at the moment. As the price of sequencing continually becomes cheaper it is hoped that solution to this this problem will be affordable to all the patients being commenced on this AED in the future.

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# Appendix

## 7.0. Appendix

### 7.1. Appendix 1: DNA collection form

<b>Blood/Saliva Banking – Collection Details</b>
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#### Related Document

LP-HIS-BProcess: Collection, Processing and Storage of Blood for Banking

#### Specimen Identification:

Blood  Saliva  Collection site/ Hospital:

Patient Name:

Date of birth:  Patient Number:

#### Study:

Name of the study:  Study Code:

#### Sample Collection:

Date:  Time:

Number of tubes:

Tube No.	Tube type	Volume (ml)
A		
B		
C		
D		

Time and date in CRC fridge:  Collected By:

Bio-banking technician contacted (date/time):   
(email: *sdonatello@rcsi.ie*; Tel office: 809-3714)

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**Lab use only**

Saliva sample processed at 50C on the ..... for  
.....hour.

Sample processed by technician (date/time):

**Patient Internal Code:**

## 7.2. Appendix 2: Database codes

T CodeValues			
CodeValueId	CodeType	CodeValueDescription	DisplayOrder
1	1	Male	1
2	1	Female	2
1	2	OLH	1
2	2	TSH	2
3	2	NCH	3
3	3	GTCS	1
4	3	Tonic	2
5	3	Clonic	3
6	3	Absences	4
7	3	Drop Attacks	5
8	3	Myoclonic	6
9	3	Simple Partial	7
10	3	Complex Partial	8
11	3	Atypical absences	9
12	3	Secondary generalised	10
13	3	Spasms	11
1	4	Idiopathic Generalised syndrome	1
2	4	Symptomatic GS	2
3	4	Probably symptomatic GS	3
4	4	Benign focal	4
5	4	Symptomatic focal	5
6	4	Probably symptomatic focal	6
7	4	Specific syndrome	7
8	4	Unclassifiable	8
9	4	Dual epilepsy	9
1	5	Yes	1
2	5	No	2

T CodeValues			
CodeValueId	CodeType	CodeValueDescription	DisplayOrder
3	5	Unknown	3
1	6	Yes	1
2	6	No	2
1	7	1 <sup>st</sup>	1
2	7	2 <sup>nd</sup>	2
3	7	3 <sup>rd</sup>	3
4	7	Other	4
1	8	Mild	1
2	8	Moderate	2
3	8	Severe	3
1	9	Supportive	1
2	9	Unsupportive	2
3	9	Unhelpful	3
1	10	Lesional	1
2	10	Nonlesional	2
3	10	Global	3
1	11	Seizure Free	1
2	11	Simple Partial Seizures Only	2
3	11	1-3 Seizures per day (+/- auras)	3
4	11	4 or more seizure days per year	4
5	11	Parital Responder	5
6	11	Possible Surgery/Candidates	6
1	12	ACTH	1
2	12	AZM	2
3	12	CBZ	3
4	12	CLB	4
5	12	CZP	5
6	12	DZP	6
7	12	ETS	7

T CodeValues			
CodeValueId	CodeType	CodeValueDescription	DisplayOrder
8	12	FBM	8
9	12	GBP	9
11	12	LTG	11
12	12	NZP	12
13	12	OXC	13
14	12	PB	14
15	12	PHT	15
16	12	PRM	16
17	12	RNM	17
18	12	TGB	18
19	12	TPM	19
20	12	VGB	20
21	12	ZNS	21
22	12	OTHER	22
10	12	LEV	10
1	14	<3 <sup>rd</sup>	1
2	14	3 <sup>rd</sup>	2
3	14	3-10 <sup>th</sup>	3
4	14	10 <sup>th</sup>	4
5	14	10-25 <sup>th</sup>	5
6	14	25 <sup>th</sup>	6
7	14	25th-50 <sup>th</sup>	7
8	14	50 <sup>th</sup>	8
9	14	50th-75 <sup>th</sup>	9
10	14	75 <sup>th</sup>	10
11	14	75th-90 <sup>th</sup>	11
12	14	90 <sup>th</sup>	12
13	14	90-97 <sup>th</sup>	13
14	14	97 <sup>th</sup>	14

T CodeValues			
CodeValueId	CodeType	CodeValueDescription	DisplayOrder
15	14	>97 <sup>th</sup>	15
1	15	1st Degree	1
2	15	2nd Degree	2
3	15	Other	3
1	16	Zero	1
2	16	Low	2
3	16	Normal	3
4	16	High	4
1	17	Normal	1
2	17	Impaired	2
3	17	wheel chair bound	3

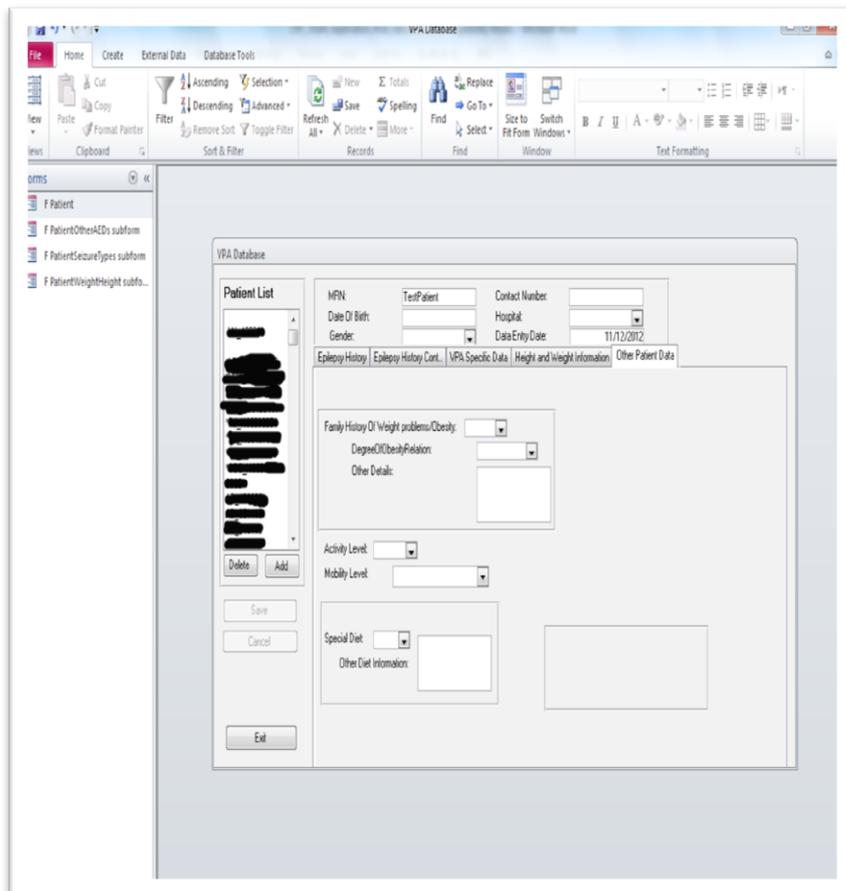
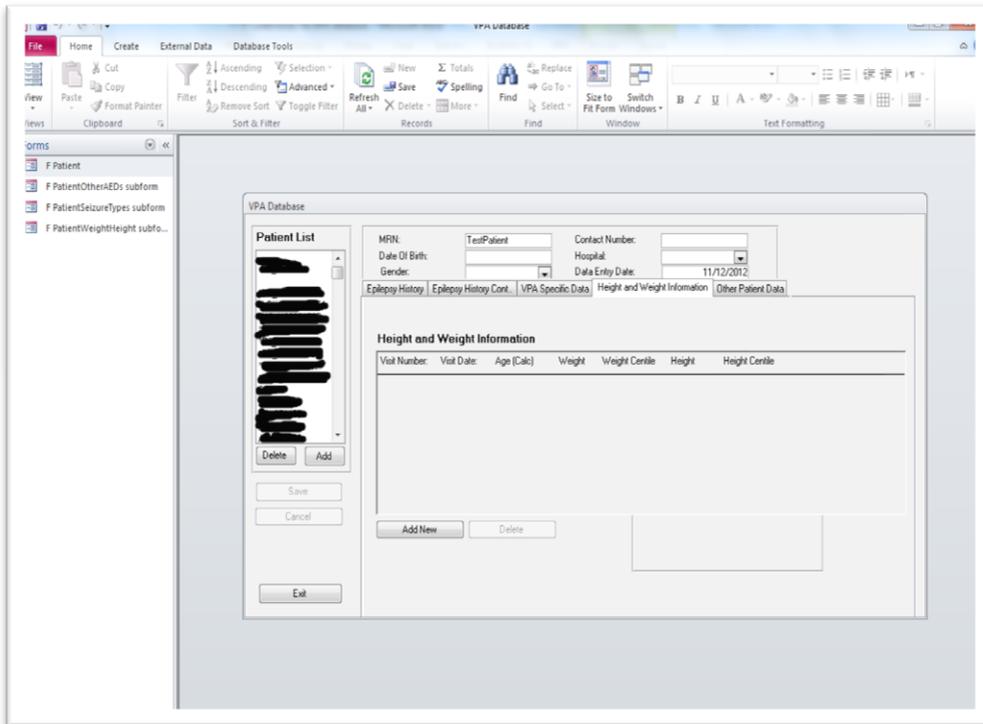
### 7.3. Appendix 3: Screenshots of the Access database

This screenshot shows the 'VPA Database' application window. The 'Patient List' tab is active, displaying a list of patient records on the left. The main form area contains the following fields and sections:

- MRN:** TestPatient
- Date Of Birth:** [Empty]
- Gender:** [Empty]
- Contact Number:** [Empty]
- Hospital:** [Empty]
- Data Entry Date:** 11/12/2012
- Tabs:** Epilepsy History, Epilepsy History Cont., VPA Specific Data, Height and Weight Information, Other Patient Data
- Family History Of Epilepsy:** Degree: [Empty]
- Any History of Febrile seizures:** [Empty]
- Any family history of febrile seizures:** Who: [Empty]
- EEG:** Description: [Empty]
- Any Other Early Risks:** CNS Infections: [Empty], Birth Difficulties: [Empty], Head Injury: [Empty]
- Neuroimaging:** Description: [Empty]
- Number of male siblings:** 0
- Number of female siblings:** 0
- Current Seizure Frequency:** [Empty]

This screenshot shows the 'VPA Database' application window with the 'VPA Specific Data' tab selected. The form contains the following fields and sections:

- VPA Start Date:** [Empty]
- Maximum Dose Tolerated (mg/kg/day):** 0
- Maintenance VPA Dose (mg):** 0
- Termination Date:** [Empty]
- Duration (days):** 0
- VPA Blood Level:** [Empty]
- VPA Seizure Control:** [Empty]
- Other AEDs co-administered:** AED: [Empty]
- Buttons:** Add New, Delete







11<sup>th</sup> July 2008

Dr Bryan Lynch  
EEG Department  
Children's University Hospital  
Temple Street  
Dublin 1

Dear Dr Lynch

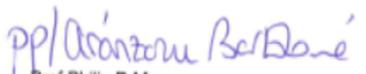
08.014 *Genetic exploration of weight gain associated with valproate therapy;  
proposed by Dr. Bryan Lynch, Consultant Paediatric Neurologist*

We received revised documentation from your Registrar by e-mail on Monday and these were discussed by the Ethics Committee last Tuesday.

The Committee is now satisfied and has approved the project. Could you please forward a brief summary on completion of the project

Thanking you

Yours sincerely

  
Prof Philip D Mayne  
MD, FRCPI, FRCPath  
Medical Secretary to the Ethics Committee

COPY LETTER 19<sup>th</sup> June 2013

Ursula Ryan Ph: 4142342 email Ursula.Ryan@amnch.ie  
Secretariat Fax: 4142371

Dr. David Webb  
Consultant Neurologist  
Our Lady's Children's hospital  
Crumlin  
Dublin 12

August 2nd. 2011

**Re: A Pharmacogenomic exploration of genetic risk factors in valproate-induced weight gain in patients with epilepsy**

*Please quote this reference in any follow up to this letter: 2011/08/08 Chairman's Action*

Dear Dr. Webb,

Thank you for your Submission dated July 19<sup>th</sup>. 2011.

The Vice-Chairman, on behalf of the Research Ethics Committee, has given full ethical approval to this amendment.

Yours sincerely

---

Ms. Ursula Ryan  
Secretary,  
SJH/AMNCH Research Ethics Committee

## 7.5. Appendix 5: Names and addresses of collaborators

1. Prof Norman Delanty FRCPI  
Consultant Neurologist  
Director of Epilepsy services  
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RCSI, Education & Research Centre  
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Crumlin, Dublin 12.  
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## 7.6. Appendix 6: Case Report Form

Database to include all of the information from the patient

### I. Demographics:

1. Identity: name
2. Identity MRN
  - a. Variable
3. DOB
4. Sex (Male/Female)
  - a. discrete
5. Hospital (OLH/ TSH/NCH)
  - a. Discrete
6. Contact number
  - a. Variable/number

### II. Epilepsy History:

1. Semiology
  - a. free text box
2. Seizure types
  - a. One or a combination of (drop down menu with new row for additional?):
    - i. GTCS
    - ii. Tonic
    - iii. Clonic
    - iv. Absences
    - v. Drop attacks
    - vi. Myoclonic
    - vii. Simple partial
    - viii. Complex partial
    - ix. Atypical absences
    - x. Secondary generalised
    - xi. Spasms
3. Syndrome classification
  - a. Discrete

- i. Idiopathic Generalised syndrome/
- ii. symptomatic GS
- iii. probably symptomatic GS/
- iv. benign focal/
- v. symptomatic focal
- vi. probably symptomatic focal
- vii. Specific syndrome
- viii. Unclassifiable
- ix. Dual epilepsy

Free text box at bottom (i.e. if you think either 7, 8 or 9 above you will fill in free text)

4. Aetiology

- a. free text box

5. Co-morbidities

- a. free text box

6. Family history of epilepsy

- a. Discrete (yes/no)
- b. Degree (discrete – option of 1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup>, other)

7. Learning disability

- a. Discrete(yes/no)
- b. Severity: (option of Mild, Moderate or Severe)

8. EEG

- a. Discrete – option of
  - i. Supportive
  - ii. Unsupportive
  - iii. Unhelpful

Add free text box

9. Neuroimaging

- a. Discrete - option of
  - i. Lesional
  - ii. Non-lesional
  - iii. Global

Add free text box

10. Current (at time of data collection) seizure frequency

- a. Seizure free
- b. Simple partial seizures only
- c. 1-3 seizures days per year (+/- auras)
- d. 4 or more sz days/year
- e. Partial responder
- f. Possible/surgery candidates

11. Early risks:

- HI
- CNS Infection
- Birth problems

12. Siblings

- Boys (no)
- Girls (no)

13. Febrile seizures

- Patient (Y / N)
- Family (Y / N)

**III. VPA specific variables:**

1. Start date
2. Maximum dose tolerated (mg/kg)
  - a. Variable/number
3. Maintenance dose in (mg/ kg)
  - a. Variable/number
4. Termination date ('blank' = ongoing. If that calculator box is easy i.e. calculate numbers of months exposed to drugs.. but don't worry if messy)
  - a. Variable/figure
5. Other AEDs co-administered. (one or a combination of.. a drop down is fine even if it looks messy.. extra column for each additional drug? i.e. patient can be exposed to several)
  - a. ACTH
  - b. AZM

- c. CBZ
- d. CLB
- e. CZP
- f. DZP
- g. ETS
- h. FBM
- i. GBP
- j. LEV
- k. LTG
- l. NZP
- m. OXC
- n. PB
- o. PHT
- p. PRM
- q. RNM
- r. TGB
- s. TPM
- t. VGB
- u. ZNS
- v. Other

with free text box

6. VPA blood levels:

- a. free text box

7. Seizure control on VPA:

- a. Seizure free
- b. Simple partial seizures only
- c. 1-3 seizures days per year (+/- auras)
- d. 4 or more sz days/year
- e. Partial response
- f. Possible/surgery candidates
- g. Unsure

#### **IV. Weight related data:**

This is the type of 'table' we're looking to populate. I.e. patient starts drug on visit 0, we record date, weight, height etc. On visit 2 we do same and on so...

Parameter	Visit 0 (start)	Visit 1	Visit 2	Visit 3	Visit 4
Date					
Weight (kg)					
Height (cm)					
Weight centile					
Height centile					

We somehow need to design 'form' questions that will populate this table. We thought something along lines of below but feel free to adjust as you see fit.

1. Date of visit
2. Weight at visit (kg)
  - a. Figure
3. Weight centile at visit (discrete, one of..)
  - a. <3<sup>rd</sup>
  - b. 3<sup>rd</sup>
  - c. 3-10<sup>th</sup>
  - d. 10<sup>th</sup>
  - e. 10-25<sup>th</sup>
  - f. 25<sup>th</sup>
  - g. 25<sup>th</sup>-50<sup>th</sup>
  - h. 50<sup>th</sup>
  - i. 50<sup>th</sup>-75<sup>th</sup>
  - j. 75<sup>th</sup>
  - k. 75<sup>th</sup>-90<sup>th</sup>
  - l. 90<sup>th</sup>

- m. 90-97<sup>th</sup>
- n. 97<sup>th</sup>
- o. >97<sup>th</sup>

4. Height at visit (centimetres)

5. Height centile at visit (discrete, one of..)

- a. <3<sup>rd</sup>
- b. 3<sup>rd</sup>
- c. 3-10<sup>th</sup>
- d. 10<sup>th</sup>
- e. 10-25<sup>th</sup>
- f. 25<sup>th</sup>
- g. 25<sup>th</sup>-50<sup>th</sup>
- h. 50<sup>th</sup>
- i. 50<sup>th</sup>-75<sup>th</sup>
- j. 75<sup>th</sup>
- k. 75<sup>th</sup>-90<sup>th</sup>
- l. 90<sup>th</sup>
- m. 90-97<sup>th</sup>
- n. 97<sup>th</sup>
- o. >97<sup>th</sup>

## **V. Patient related variables**

1. Family history of weight problems/obesity

- a. Discrete (yes or no)
- b. Degree of relation
  - i. 1<sup>st</sup> degree
  - ii. 2<sup>nd</sup> degree
  - iii. other
- c. AND FREE TEXT BOX

2. Activity level (discrete, one of:)

- a. Zero
- b. Low
- c. Normal
- d. High

3. mobility (discrete, one of:)

- a. normal
- b. impaired
- c. wheel chair bound

4. special diets:

- a. discrete - yes or no

(+ free text box)

## 7.7. Appendix 7: Participant's Information Leaflet

### INFORMATION LEAFLET

#### INFORMATION FOR PATIENTS/PARENTS/GUARDIANS REGARDING THE STUDY

**1. Study Title**

A genetic exploration of weight gain associated with valproate therapy.

**2. What is the purpose of the study?**

Epilepsy affects about 30,000 people in Ireland. The treatment of epilepsy is mainly by anti-epileptic medications (AEDs). Side effects to AEDs are commonplace. Weight change is an adverse effect of several important AEDs. It can be a treatment-limiting problem in the management of patients with epilepsy. The most common AEDs associated with weight gain are sodium valproate (Epilim) and vigabatrin (sabril), and with weight loss are topiramate (topamax) and zonisamide (zonegran).

Why these side effects are seen in only some people are not known. It is likely that there may be some genetic or "in-built" factors have a role to play.

To identify these "in-built" factors, we are carrying out a genetic study on patients with epilepsy who are on or who have been on valproate (Epilim) therapy for at least 6 months. To do so, it is necessary to collect a blood sample from as many patients as possible. This information will improve our understanding on why some patients develop this problem of weight gain and may have also some implication for the broader understanding of obesity in general population.

**3. Why have I been chosen?**

You/your child have been chosen because you have been on Epilim therapy for at least 6 months. Your record of previous medications and responses to various medications along with the weight measurements are available in this hospital. This should provide information about your type of epilepsy and the way you respond to medications. We are trying to study at least 300-400 patients

**4. Who is organizing the study?**

This is an investigator- initiated study.

Principal Investigator: Dr David Webb Consultant Paediatric Neurologist, Crumlin

Other Investigators: Dr Blathnaid McCoy Consultant Paediatric Neurologist, Crumlin

Dr Norman Delanty Consultant Neurologist, Beaumont

Dr Mary King Consultant Paediatric Neurologist, Temple Street

Dr Bryan Lynch Consultant Paediatric Neurologist, Temple Street

Dr Tony Bouldin, Consultant Paediatric Neurologist, Temple Street, Dublin 1

Dr Gianpiero Cavalleri Research lecturer in Epilepsy Genetics, and Pharmacogenetics, RCSI, Dublin 2

Prof David Goldstein, Institute of Genome Sciences and Policy, Duke University, North Carolina, USA

Dr Joseph Chukwu, Clinical Research Fellow, National Children's Research Centre Crumlin

The Research fellow is funded by the National Children's Research Centre Crumlin and the Irish Epilepsy Association- "Brainwave"

Duration of the study: approximately 2 years

**5. What will happen to me if I take part?**

If you/your child agree to participate, you/your child will have to meet with the researcher once off for this study. This can be arranged to coincide with a clinic visit and you don't have to come specifically for this. The researcher will take a detailed history from you/ your child and also go through the records and collect all the information and store it in a safe place. A blood or spit sample will be collected from you/your child. The blood sample will be collected by a needle prick. This may cause a bit of bruising and discomfort but it will pass without side effects. This blood or spit sample will be analyzed and will be looked at in the light of all the information we have gathered from you and your child's records.

**6. Are there any disadvantages in taking part in this study?**

There are no disadvantages in taking part in this study. Your care with your doctor will proceed in the same way as before.

**7. What are the possible risks of taking part?**

The only risk involved is physical distress that you/your child may have from blood sampling. There may be a small bruise and you may be uncomfortable for a little after. We will try to avoid these complications as much as possible.

**8. What are the possible benefits of taking part?**

You may not receive any direct benefit from taking part in the study. However, information obtained during the course of the study may help us to understand better your condition or illness. The information we get from this study may help us to treat future patients with epilepsy better by predicting the side effect in advance and take measures to counteract that.

**9. Is my doctor being paid for including me in the study?**

No.

**10. Confidentiality – who will know I am taking part in the study?**

All information, which is collected about you during the course of the research, will be kept strictly confidential. Any information about you, which leaves the hospital, will contain no information as to your identity so that you cannot be recognised from it.

**11. GP Notification**

Your GP will not normally be informed that you are taking part in this study as it does not alter your/your child's management plans

**12. Hospital Ethics Committee Approval**

Our Lady's Hospital for children, Crumlin has approved the study. Ethics Committee Mater Misericordiae Hospital has also approved the study and we will be recruiting children from Temple Street hospital.

**13. What will happen to the results of the study?**

Normally the results of the research will not be made known to you or your child. But if you would like to know the progress you can contact your research doctor or your specialist doctor.

**14. Voluntary participation**

It is up to you/your child to decide whether to take part or not. If you decide to take part you will be given an information leaflet and consent form. Even if you decide to take part, you/your child are free to withdraw at any time and without giving a reason. This will not affect the standard of care you will receive. Your doctor will not be upset if you decide not to take part.

If you need any further information please contact one of the following doctors:

Dr Bryan Lynch : 01-8094200

Dr Mary King: 01-809 4232?

Dr Tony Bouldin 01....

## 7.8 Appendix 8: Consent Form

*(Form to be on headed paper)*

### CONSENT FORM

**Reference Number:**

1. I confirm that I have read and understand the information leaflet dated 15/07/11 version 2 for the above study and received an explanation of the nature, purpose, duration, and foreseeable effects and risks of the study and what my/my child's involvement will be.
2. I have had time to consider whether to take part in this study. My questions have been answered satisfactorily and I have received a copy of the Patient Information Leaflet.
3. I understand that my/my child's participation is voluntary and that I am/we are free to withdraw at any time without my/my child's medical care or legal rights being affected.
4. I am willing to allow access to my/my child's medical records by representatives of the sponsor, Ethics Committee or local or foreign regulatory authorities but understand that strict confidentiality will be maintained. The purpose of this is to check that the study is being carried out correctly.
5. I agree to allow my child to take part in the above study.
6. I understand that my name will not be used in any report that comes from the blood samples. In turn, I understand that I cannot restrict the use of the information that comes from studying the blood samples. I accept that the information may be given to medical regulatory authorities in Europe or elsewhere. I am aware that the study resource will be owned and used by both academic and commercial researchers. These include, but are not limited to, the Royal College of Surgeons in Ireland.

7. I have been made aware of my rights concerning the collection, processing and use of the data for this procedure. I agree to the collection, processing and use of personal data as described in this document.

8. I would consent for my data (both clinical and DNA) to be used for further studies.  
Tick: Yes/ No

\_\_\_\_\_  
\_\_\_\_\_  
**Name of Research Participant/Parent/Guardian**      **Date**      **Signature**  
**(in block letters)**

\_\_\_\_\_  
\_\_\_\_\_  
**Name of Person taking consent**      **Date**      **Signature**  
**(if different from doctor/researcher)**

\_\_\_\_\_  
\_\_\_\_\_  
**Doctor/Researcher**      **Date**      **Signature**

1 copy for research participant, 1 copy for researcher, 1 copy to be inserted into the hospital notes

## 7.9. Appendix 9: R-Function for growth percentiles

-

MATURE DRAFT

DR GÁBOR BORGULYA

### DEVELOPING CHILD GROWTH WEIGHT PERCENTILE FUNCTIONS

#### Contents

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#### 1. The objective of this project

The aim of this project was to develop an approximate function that take the age, sex and weight of children as inputs and output their weight percentile as output in the weight distribution of children of the same sex and age; and the inverse function that outputs weight. Ideally the function would have been fitted on data of Irish children born in the 1990ies to match the population of the study where the function is planned to be applied.

#### 2. Data sources

In the current study less perfect data were Saud Alhusaini provided me[1] with the paper Standards from birth to maturity for height, weight, height velocity, and weight velocity: British

Date: 15/JUN/2012.

children, 1965. I. J. M. Tanner, R. H. Whitehouse and M. Takaishi Arch Dis Child 1966 41: 454-471 doi: 10.1136/adc.41.219.454 to use as a possible data source. We found less old data for a more distant population, the USA. Saud recognised a paper referencing the above as a better data source candidate Hoey HM, Tanner JM, Cox LA (1987). Clinical growth standards for Irish children. Acta Paediatr Scand Suppl (338): 1-31. Gábor found this article as a paper print in the SGUL library, then the paper was scanned at RSCI (1987\_Hoey.pdf).

### 3. Modelling cross sectional percentiles vs. individual growth curves

An alternative approach to the original question would have been modelling individual growth curves rather than percentiles of the population, but the paper gave only limited information on this. A curve of the typical individual is shown on page 467 of the Tanner et al. paper. The population distribution can be viewed as the superposition of a large number of individual growth curves, this explains the widening of the distribution during adolescence: although children approximately follow percentile curves as they develop the age at the onset of the intensive growth associated with puberty is also variable.

## 4. The parametric approach

4.1. Results for boys. The first approach to fit the required function was completed on the 14/May/2010[2] and the following R code was developed:

```
## Weight and weight centile prediction for boys

weight=function(age,centile,m0=-5.538068e+01,m1=-3.401687e+01,m2=1.714676e
+00,m3=-2.914505e-02,a=4.252609e-08,b=3.180275e-05,c=8.994375e-03,hi=
4.382704e-05,pa=1.434529e+01,right=1.983228e-05,left=-5.357727e-01,la
=-2.733154e-08,lk=3.430904e+00,shift=1.006158e+00,a2=4.560486e+02,b10
=2.416257e+03,me15=1.557696e+01,sd2=1.301421e+00,c62=1.183027e+04,ra=1,rk
=1){
w=pnorm(age,me15,sd2)
medianWeight=(1-w)*(a2*age+b10)+w*c62
lambda=m0+m1*age+m2*age^2+m3*age^3
mean=a*lambda^2+b*lambda+c
sd=hi+((age>=pa)*(hi-right)+(age<pa)*(hi-left))*(1/(1+Mod(
complex(real=age-pa)^((age>=pa)*rk+(age<pa)*lk))*((age>=pa)*
ra+(age<pa)*la))^(-1)
medianWeight*(Mod(complex(real=(qnorm(centile)*sd+mean)*lambda+1)^(1/
lambda))^(-shift)
}
f=function(ce,a,w)(weight(a,ce)-w)
centile=function(age,weight)uniroot(f,c(0.0001,0.9999),a=age,w=weight)
$root

## Example usage:

#1. On which percentile is the boy who is 12 years old and 40 kg of weight? (68%)
centile(12,40)

#2. What is his expected weight one year later? (44.5 kg)
weight(12+1,.68)
#or more precisely,
weight(12+1,centile(12,40))
```

The above R function corresponds to the Table 9 of the Hoey paper. It approximates the percentiles of boys - the percentiles of the girls are different and imprecisely approximated by this function.

4.2. Attempt for girls. Unfortunately fitting the same formula for girls was unsuccessful: while for boys the result was a strictly monotone function, the best fit after optimisation for girls was non-monotone. As non-monotony could have lead to unexpected contradictions during the applications of the function this result was discarded.

While it may have been possible to modify the function used in this parametric approach to give an acceptable fit a new approach was used where notable non-monotony can not occur.

## 5. The smooth interpolation approach

5.1. Smooth interpolation for the 50th percentile of boys. Each percentile curve is interpolated using a smooth function. Here I describe the details of this function using the example of the 50th percentile of boys.

5.1.1. Error in the source data. The table 9 of the Hoey paper contains the following data:

age	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	...
pc50	12.7	13.5	14.6	15.3	16.4	16.3	18.3	19.3	20.3	21.3	22.4	...

^^^

The weight of 16.3 kg at the age of 4.5 is an outlier, it breaks the monotonously increasing series corresponding to growth of the boys, it must be error. The differences between consecutive weights suggest that the true weight should be around 17.4. So in the following calculations I assume that the digit 7 was mistyped and I use 17.3 instead:

age	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	...
pc50	12.7	13.5	14.6	15.3	16.4	17.3	18.3	19.3	20.3	21.3	22.4	...

^^^

5.1.2. Fitting the interpolation curve. In the smooth regression approach the published percentile curve was approximated with local polynomial regression fitting[4] using the loess[3] function of R[5]. This ensures that the approximating curve precisely goes through each given age-weight coordinate pair.

```
boy_centile50_model = loess (pc50 ~ age , data=boy_pc50 , control=loess.control(
  surface="interpolate" ), span=5/nrow(boy_pc50))
```

The fitted model can be used to construct a function that predicts the 50th percentile weight corresponding to any age (also ages between the tabulated ones). The 50th percentile weights of boys are shown in figure 1 on page 4, black circles correspond to the Hoey data, the green curve corresponds to the interpolation curve.

A parameter of the loess model is the span - the higher the value the smoother the curve is, at the cost of not exactly going through each point. Various settings are plotted in figure 2 on page 5;  $span = 5/nrow$  corresponds to the smoothest curve (brown) that still crosses each point.<sup>1</sup>

<sup>1</sup>This was later changed to 7, see section 5.7 on page 9.

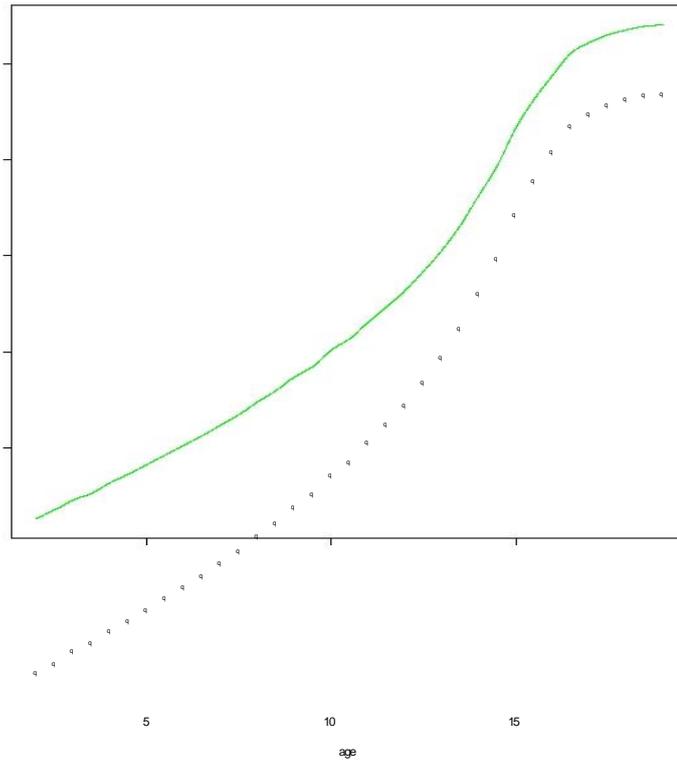


Figure 1. 50th percentile weights of boys and loess interpolation curve

5.2. Weight-centile interpolation of 15-year-old boys. Each weight corresponding to a given percentile (at a given age) is interpolated using the 7 weights corresponding to the 7 percentiles tabulated by Hoey et al (3rd, 10th, 25th, 50th, 75th, 90th and 97th). To get a good approximation of the weight at any percentile a second smooth function is fitted on these 7 weight values. Here, however, we have to use an other kind of function, one that ensures that the fitted weight-centile curve (for the given age) has the appropriate quantiles at each of the 7 values. This property was achieved using multiple normal distributions fit on quantiles. A normal distribution is usually parametrised using its mean and variance, i.e. two parameters. Thus two points of a probability density curve identify a normal distribution. For a given percentile the closet lower and closest higher percentile are used (e.g. the weight corresponding to the 31th percentile is interpolated from a Gaussian distribution for on the weights of the 25th and 50th percentiles). For very low or very high percentiles outside the tabulated range the two lowest (3rd and 10th) or two highest percentiles (90th and 97th) are used, respectively.

```
boy_15y_weights = c ( 38.0 ,      42.0 , 47.2 , 53.3 , 58.9 , 65.1 , 72.2 )
```

```
z                = c ( .03 ,      .10 ,  .25 ,  .50 ,  .75 ,  .90 ,  .97 )
```

```
boy_15y = function ( ct ) {
  if ( ct == 0 ) return ( 0 )
  if ( ct == 1 ) return ( Inf )
  HighIndex = which . max ( c ( z , 1 ) >= ct )
  if ( HighIndex == 1 ) { HighIndex = 2 } else { if ( HighIndex == 8 ) HighIndex
    = 7 }
  LowIndex = HighIndex - 1
}
```

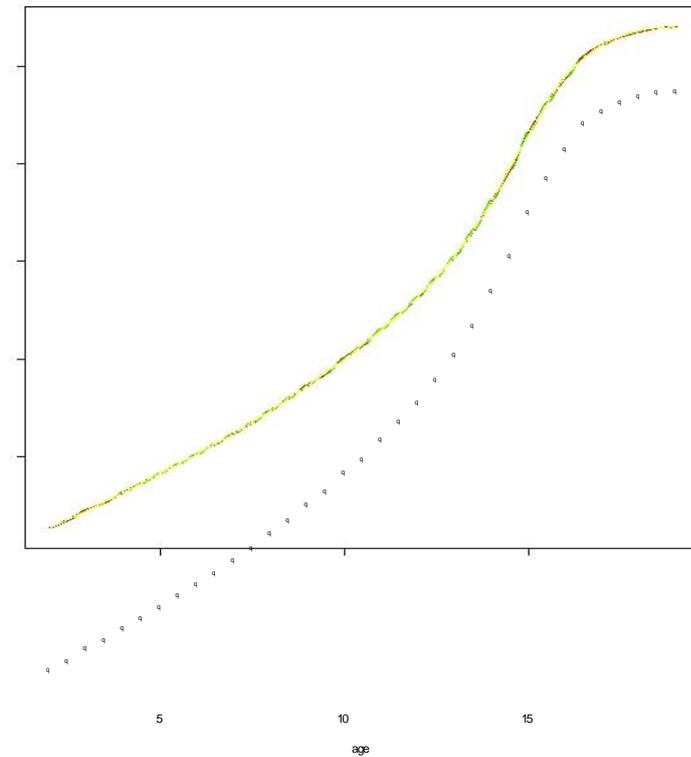


Figure 2. Various span settings in the loess interpolation

```
LowCentile = z[LowIndex]           ; LowWeight = boy_15y_weights[LowIndex]
HighCentile = z[HighIndex]         ; HighWeight = boy_15y_weights[HighIndex]
x = c(LowCentile, HighCentile); y = c(LowWeight, HighWeight)
```

```
approx_mean = boy_15y_weights[4]           # median
approx_sd    = boy_15y_weights[4] - boy_15y_weights[3] # pc50 - pc25
```

```
f1 = function(par) {
  fitted_mean = par[1]
  fitted_sd   = par[2]
  yhat = qnorm(x, mean = fitted_mean, sd = fitted_sd)
  SqErr = sum((y - yhat)^2)
}
```

```
par = c(fitted_mean = approx_mean, fitted_sd = approx_sd)
o = optim(par, f1, method = "BFGS")
par = o$par
fitted_mean = par[1]
fitted_sd   = par[2]
```

```
qnorm(ct, mean = fitted_mean, sd = fitted_sd)
}
```

As it can be seen in figure 3 on page 6 this method has achieved both first order smoothness of the interpolating curve and it matches the tabulated weight-percentile coordinate pairs. The

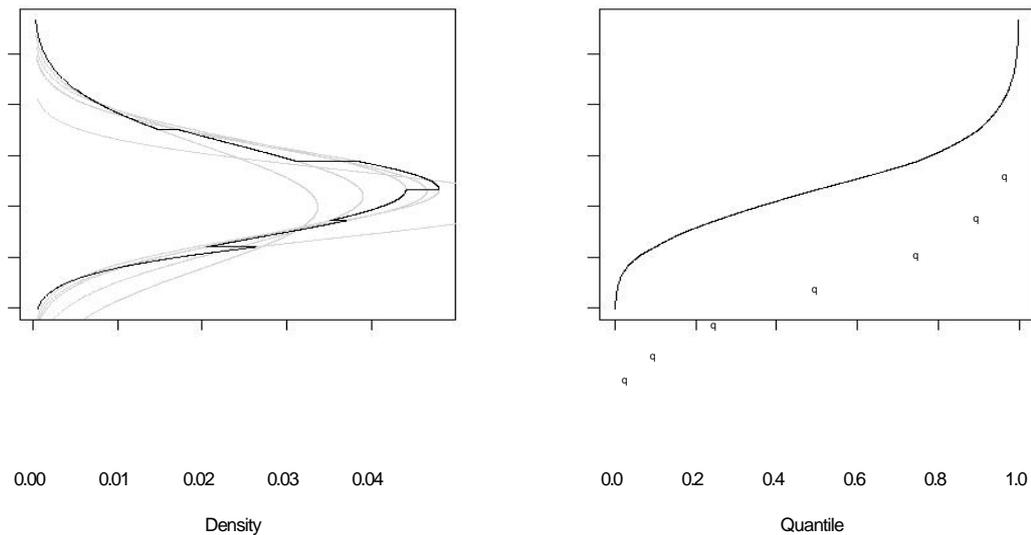


Figure 3. Density curve and quantile curve of the weight of 15-year-old boys fitted on the 7 tabulated data points

curve is not smooth, however, in the second order, which is unnatural. Better second order smoothness could be achieved using transformation of the weight values, but in practice this would mean negligible gain of accuracy.

5.3. Smooth interpolation of all the 7 percentile curves of boys. Exactly the same method was used as above:

```

boy_centile03_model=loess (pc03 ~ age , data=boy7 , control=loess.control(surface
    "interpolate"),span=5/nrow(boy7))
boy_centile03=function (age) predict (boy_centile03_model , data.frame (age=age))
boy_centile10_model=loess (pc10 ~ age , data=boy7 , control=loess.control(surface
    "interpolate"),span=5/nrow(boy7))
boy_centile10=function (age) predict (boy_centile10_model , data.frame (age=age))
boy_centile25_model=loess (pc25 ~ age , data=boy7 , control=loess.control(surface
    "interpolate"),span=5/nrow(boy7))
boy_centile25=function (age) predict (boy_centile25_model , data.frame (age=age))
boy_centile50_model=loess (pc50 ~ age , data=boy7 , control=loess.control(surface
    "interpolate"),span=5/nrow(boy7))
boy_centile50=function (age) predict (boy_centile50_model , data.frame (age=age))
boy_centile75_model=loess (pc75 ~ age , data=boy7 , control=loess.control(surface
    "interpolate"),span=5/nrow(boy7))
boy_centile75=function (age) predict (boy_centile75_model , data.frame (age=age))
boy_centile90_model=loess (pc90 ~ age , data=boy7 , control=loess.control(surface
    "interpolate"),span=5/nrow(boy7))
boy_centile90=function (age) predict (boy_centile90_model , data.frame (age=age))
boy_centile97_model=loess (pc97 ~ age , data=boy7 , control=loess.control(surface
    "interpolate"),span=5/nrow(boy7))
boy_centile97=function (age) predict (boy_centile97_model , data.frame (age=age))

```

5.3.1. Error in the source data. Plotting the data revealed another error in the Hoey table: weight 15 kg at the age of 4.5 years, 25th percentile must be error, I substituted it with 16, that is realistic.

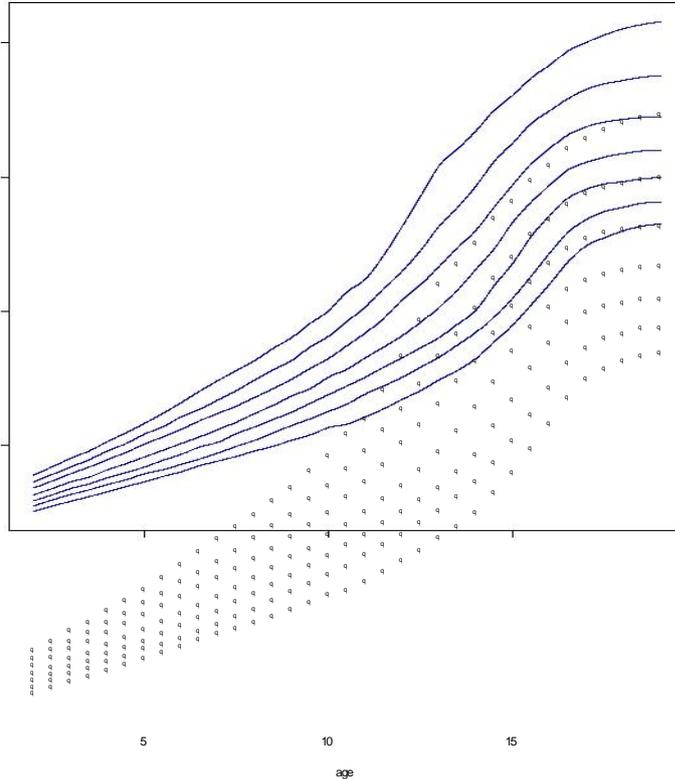


Figure 4. Weight percentile curves of 15-year-old boys: circles, raw data; lines, local polynomial regression fitting curves

5.3.2. Results. The results can be seen in figure 4 on page 7.

The original series of points look somewhat irregular as some points, this is maybe due to sampling error in the Hoey paper.

5.4. Weight(age, centile) function. To get the weight(age, centile) function we first need to interpolate the 7 centiles for the given age using the boy\_centileXX functions, then interpolate between the centile, combining the above tabulated age with any percentile and tabulated percentile with any age approaches. Let's thus modify the boy\_15y\_weights function:

```
boy_weight_functions = c ( boy_centile03 ,
    boy_centile10 ,
    boy_centile25 ,
    boy_centile50 ,
    boy_centile75 ,
    boy_centile90 ,
    boy_centile97 )
...
boy_weight = function ( age , ct ) {
  boy_weights = sapply ( boy_weight_functions , function ( x ) x ( age ) )
  ...
}
```

```

LowCentile   = z [ LowIndex ]           ; LowWeight = boy_weights [ LowIndex ]
HighCentile  = z [ HighIndex ]          ; HighWeight = boy_weights [ HighIndex ]
...
approx_mean  = boy_weights [ 4 ]         # median
approx_sd    = boy_weights [ 4 ] - boy_weights [ 3 ] # pc50 - pc25

```

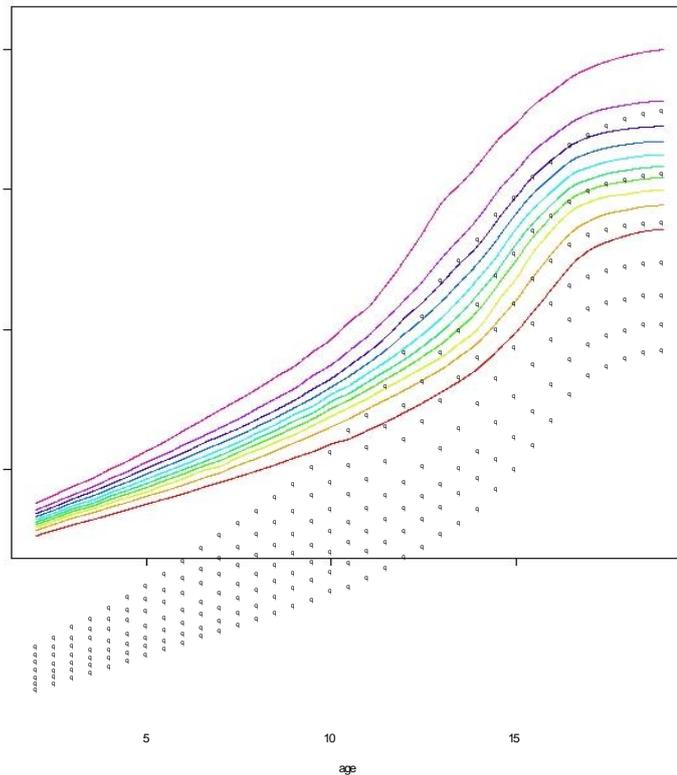


Figure 5. Fitted weight percentile curves (5th, 15th, 25th, . . . , 95th) and the original data points

The result can be seen in figure 5 on page 8

5.5. Centile(age, weight) function. The centile(age, weight) function is the inverse function of the weight(age, centile) function. While an analytic solution could be developed let's use a numerical inverse as that is easier and quicker to program and its main limitation the slower execution does not make a difference in the planned application of the function.

```

boy_centile0 = function(centile, age, weight) (boy_weight(age, centile) - weight)
boy_centile = function(age, weight) uniroot(boy_centile0, c(0.0001, 0.9999),
  age=age, weight=weight)$root

```

These results could be best visualized with a color coded or topographic chart. As this is not important for the understanding of the method I have not prepared this graph.

5.6. Application to the data of girls. There were no methodical changes when the data of girls was modelled.

## 5.6.1. Steps.

- (1) Data entry: typing in the Hoey paper table 10 data
- (2) Fitting the 7 local polynomials
- (3) Combining the 7 curves with interpolations between them
- (4) Coding the inverse function.

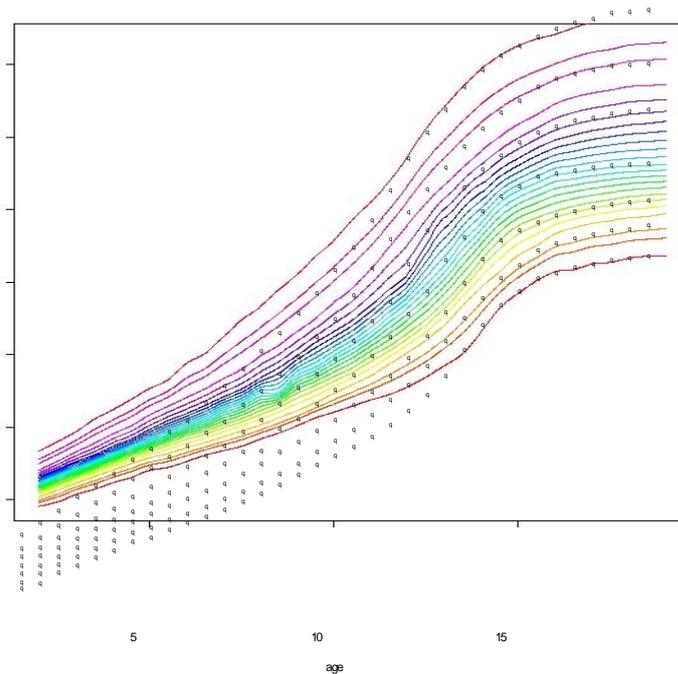


Figure 6. The original fitted weight percentile curves (1st, 3th; 5th, 15th, 25th, . . . , 95th; 97th, 99th) and the original data points of girls

5.6.2. Testing. The original results can be seen in figure 6 on page 9.

The weight 24.8 kg (at 8.5 years, 50th percentile) is obviously error as is less than the 24.7 half a year earlier and girls do not shrink. As I could not explain the outlier with mistyping I omitted this original data point.

After these 3 errors identified in the Hoey tables I convinced myself that the tabulated data were inaccurate. Hence I gave up the condition that the fitted percentile curves should go through each data point, and favouring smoother curves (less declines in growth velocities) I increased the smoothing factor to  $span = 7/nrow$ .

5.7. Increase the span parameter for boys. To keep the methodology uniform across the analysis I introduced the  $span = 7/nrow$  increase in smoothing into the modelling of the weights of boys as well.

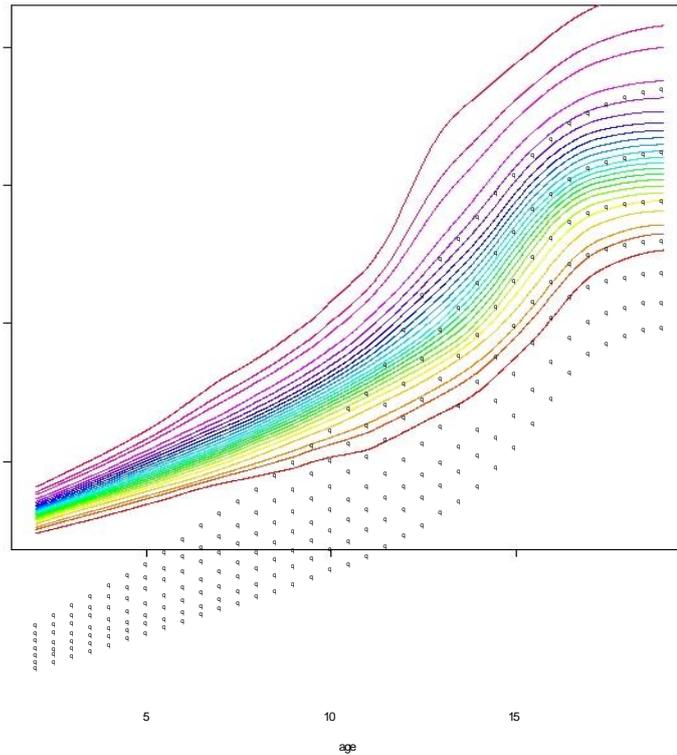


Figure 7. Fitted weight percentile curves (1st, 3th; 5th, 15th, 25th, . . . , 95th; 97th, 99th) and the original data points of boys

## 6. Results

6.1. The results as figures. The results for boys in figure 7 on page 10, the results for girls can be seen in figure 8 on page 11.

6.2. How to use the developed interpolation functions?

- (1) Open and run the "20120702182159GM child weight percentiles.R" program in R.
- (2) Follow the example below:

```
> boy_weight ( 11 , . 6 0 )
[ 1 ] 34.45404
> boy_centile ( 11 , 34.45 )
[ 1 ] 0.5997207

> girl_weight ( 11 , .60 )
[ 1 ] 34.52648
> girl_centile ( 11 , 34.5 )
[ 1 ] 0.59811
```

Here we calculated the weight of 11 year-old 60th percentile boys, then the percentile of 11 year-old boys with the weight of 34.45. The second part did similar calculations for girls.

- (3) These 4 functions can be built into analyses in R or other applications.

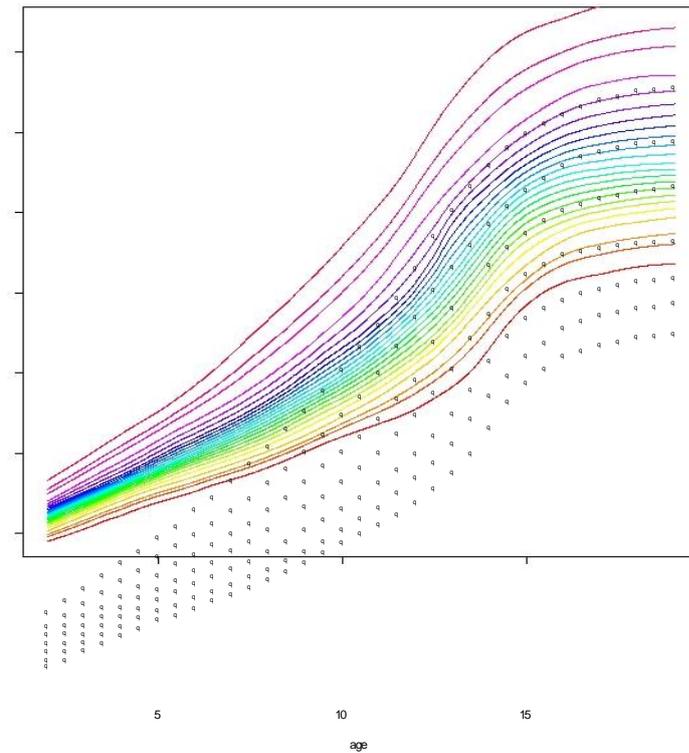


Figure 8. Fitted weight percentile curves (1st, 3th; 5th, 15th, 25th, . . . , 95th; 97th, 99th) and the original data points of girls

## 7. Summary

During the analysis of the Hoey data some errors were identified and substituted with similar, but realistic values assuming mistyping (see sections 5.1.1 (page 3) and 5.3.1 (page 6)). As no substitution was straightforward one data point was omitted, see section 5.6.2 (page 9).

Due to the errors in the published data smoothing was also introduced.

The parametric approach yielded a compact black box solution for boys, but it failed for girls. A more complex double smoothed interpolation approach was successful for both boys and girls, also giving full methodical transparency.

The result is an embeddable R module providing 4 functions for the user:

- (1)  $boy\_weight(age/year, centile) \rightarrow weight/kg$  for boys
- (2)  $boy\_centile(age/year, weight/kg) \rightarrow centile$  for boys
- (3)  $girl\_weight(age/year, centile) \rightarrow weight/kg$  for girls
- (4)  $girl\_centile(age/year, weight/kg) \rightarrow centile$  for girls

## 8. Further possibilities

### References

- [1] Saud Alhusaini. "Growth Curve Paper" E-mail to Gábor Borgulya. 2010-02-24 23:42:23.
- [2] Gábor Borgulya. "Re: Growth Curve Paper" E-mail to Saud Alhusaini. 2010-05-14 21:33:07.
- [3] B. D. Ripley, based on the cloess package of Cleveland, Grosse and Shyu (currently available as dloess at <http://www.netlib.org/a/>: the R implementation is based on an 1998 version)

- [4] W. S. Cleveland, E. Grosse and W. M. Shyu (1992) Local regression models. Chapter 8 of Statistical Models in S eds J.M. Chambers and T.J. Hastie, Wadsworth & Brooks/Cole
- [5] R Development Core Team (2012). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL <http://www.R-project.org/>  
E-mail address: [gabor@borgulya.hu](mailto:gabor@borgulya.hu)

## 7.10. Appendix 10: Protocol for Isolation of White Blood Cells

### ISOLATION OF WHITE BLOOD CELLS

1. Label 50 mL tubes with the study progressive number and patient initials.
2. Pour the blood into the 50ml tube and rinse the blood tube with SLR, previously autoclaved.
3. Fill the tube to 50 mL point with SLR.
4. Leave tubes fully covered on ice for 15 minutes.
5. Centrifuge at 3500 rpm for 15 minute at 4 degrees (no brake).
6. Carefully pour off the supernatant into a urine bottle containing some powder Virkon.
7. Resuspend the pellet in 10 mL SLR.
8. Vortex until pellet is fully resuspended.
9. Fill the tubes with SLR to 50 mL.
10. Centrifuge for 10 min at 3500 rpm at 4 degrees C (no brake).
11. Carefully pour off supernatant in a urine bottle with Virkon (leave the last mL).
12. You are left with white blood cell pellet. You can proceed to DNA extraction step or stored it at -20 degrees C.

### SLR BUFFER RECIPE (FOR LYSIS OF RED BLOOD CELLS)

2M Tris HCl, pH 7.6 (10mM)      10 ml

2M MgCl<sub>2</sub> (5mM)                      5 ml

5M NaCl (10mM)                      4 ml

Make up to 2L with de-ionised water. Autoclave for 15 minutes at 121C.  
Store at RT.

## 7.11. Appendix 11: Protocol for extraction of DNA from saliva samples

### DNA EXTRACTION FROM SALIVA SAMPLES

#### **SAFETY PROCEDURES**

Labcoat and nitrile gloves must be worn at all times, since sample may be contaminated with infectious agents.

Used labware may contain hazardous infectious agents from the purification process. The waste has to be collected and disposed in hazardous bags in the yellow bins.

#### **DNA EXTRACTION**

Notes:

- Once collected, Oragene saliva samples are stable at room temperature for years without processing.
  - DNA solvent used: TE buffer (10mM Tris-HCl, 1 mM EDTA, pH 8.0).
1. Mix the Oragene/saliva sample in the Oragene vial by inversion and gentle shaking for a few seconds.
  2. Incubate the sample at 50°C in an air incubator for at least 3 hours (the sample may be incubated also overnight if more convenient).
  3. Transfer the content of the entire sample to a 15mL centrifuge tube. Note the volume of the sample.
  4. Add 1/25<sup>th</sup> volume Oragen Purifier (e.g. 160µl for 4mL of sample) to each tube and mix vortexing for a few seconds.
  5. Incubate on ice for 10 minutes.
  6. Centrifuge at room temperature for 10 minutes at as high speed as is practical. Minimum 2500xg
  7. Carefully transfer the majority of the clear supernatant with a pipette to a fresh 15 mL centrifuge tube. Discard the pellet.
  8. Add an equal volume of RT 95-100% ethanol to the clear supernatant. Mix gently by inversion 10 times.
  9. Allow the sample to stand at RT for 10 minutes to allow the DNA to fully precipitate.
  10. Centrifuge at RT for 10 minutes at as high speed as is practical. Minimum 2500xg.
  11. Carefully remove the supernatant with a glass or plastic pipette and discard it. Take care to avoid disturbing the DNA pellet.

12. Dissolve the DNA pellet in 1 ml of DNA solvent and transfer to a 1.5 ml microcentrifuge tube.
13. Ensure complete rehydration of the DNA prior to any subsequent step. To improve rehydration may use:
  - a. Vigorous pipetting and vortexing
  - b. Incubation at 50°C for 1 hour with occasional vortexing
  - c. Incubation at RT for 1 or 2 days (suggested)
14. Centrifuge the rehydrated DNA at room temperature for 15 minutes at 15000xg (13000rpm).
15. Transfer supernatant to a fresh 1.5 ml microcentrifuge tube without disrupting the pellet.

### **Quantification of DNA by Absorbance**

Dilute the DNA 1:10 in TE buffer. Use TE as blank.

OD 260 should be between 0.1 and 1.5 and OD260/280 should be higher than 1.6.

## 7.12. Appendix 12: Protocol for Using the Qiacube DNA isolation machine

### SOPs FOR USING QIACUBE

**Equipment name:** Qiacube

**Make/model**                      Qiagen

**Serial no:**                              10632

#### SAFETY PROCEDURES USING QIACUBE

- Sample used with this instrument may contain infectious agents. Labcoat, safety glasses and nitrile gloves must be worn at all times when using Qiacube.
- All users have to be suitably trained before using QIACUBE.
- Used labware may contain hazardous chemicals or infectious agents from the purification process. These wastes have to be collected and disposed in hazardous bags in the yellow bins.
- Always have the front door closed when the machine is operating.

#### WHAT TO DO BEFORE USE

##### *Sample preparation*

- Read the handbook supplied with the Qiagen kit and the relevant protocol sheet available at [www.qiagen.com/MyQIAcube](http://www.qiagen.com/MyQIAcube) (protocol sheet for DNA QIAamp mini blood extraction is provided here).
- Make sure all samples and all discarded material have been removed from previous runs and surfaces have been cleaned before starting a new run.
- Use the rotor adaptor rack when placing the collection tube and the spin column in the rotor adaptor in the position suggested by the relevant protocol sheet.

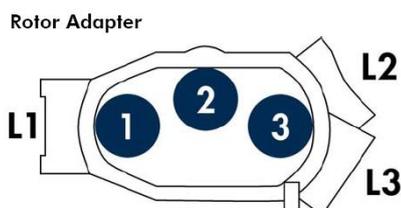
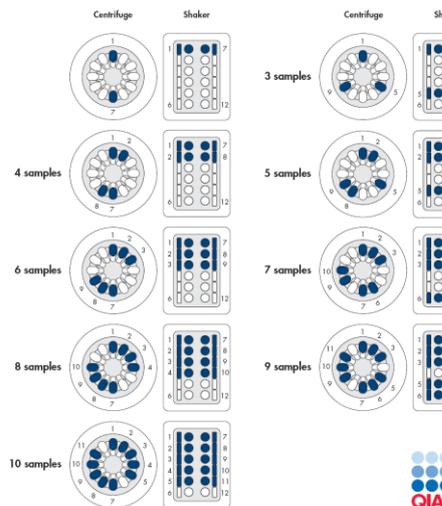
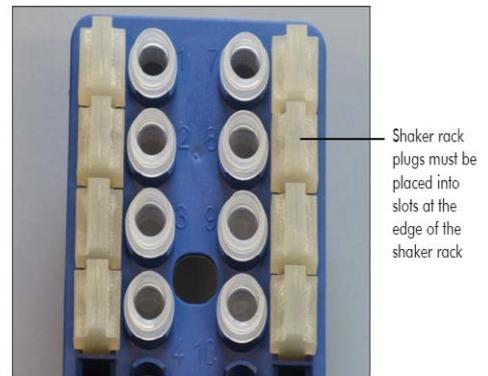


Figure 1

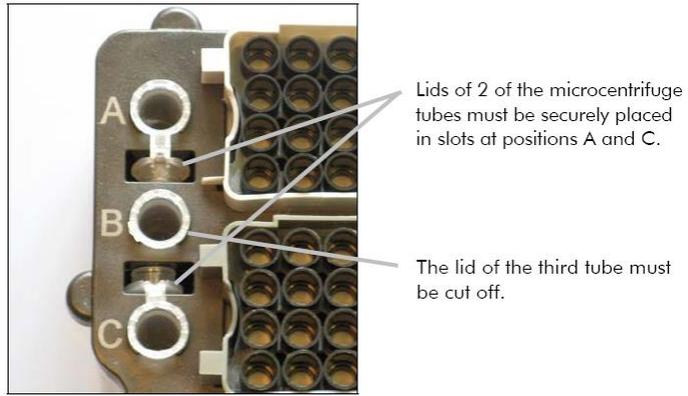
- Place the sample to be processed in the specific tubes: conical tubes with screw cap provided by Qiagen, when using blood, 2 ml tubes with cap when working with other material.
- Load the sample tubes in the shaker and the rotor adaptors in the centrifuge making sure to match the numbers and according to loading chart (Figure 2A, available in the QIAcube accessory drawer or at [www.qiagen.com/MyQIAcube](http://www.qiagen.com/MyQIAcube)). Remember to use as many white rubbers in the specific slots to indicate the number and the position of the samples used (see Fig 2B).
- Make sure the set of reagents are those needed for that specific protocol. Uncap the reagent bottles and place the caps in the Qiagen accessory drawer. Check that their amount as well as the amount of tips is enough for the run.
- Prepare the Protease tube with the volume required for the number of samples used (check protocol sheet). Place the Protease tube in position A making sure the lid is placed in the slot (see Fig 2C).



Loading chart



Loading the shaker rack with samples tubes that have screw caps.



**Correct loading of accessory buffers.**

Figure 2

### SWITCHING ON

- Switch on the Qiacube at the power switch. Select the appropriate application from the main menu. Relevant protocols have to be uploaded to the machine before hand (see Installing New Protocols section)
- Select the correct kit name by pressing up and down arrows.
- To start a protocol run, press “Start” and follow the check steps.
- Close the lid and press “Start” again.
- The machine will perform a loading check. Do not leave until the protocol starts!

### SWITCHING OFF

- If the run was successfully completed a message with “Run completed” will appear on the screen. In case some error has occurred, take note of the error number and contact Qiagen Technical service if necessary.
- Take the adaptors out of the centrifuge and load them accordingly on the rotor adaptor rack. Take the spin column out of the tube, close the lid of the sample tube and discard of the rotor adapter and spin column in a biohazard bag.
- Discard the used sample tubes and tips (placed in the front right drawer) in a biohazard bag.
- Dispose of the biohazard bags in the yellow bins.
  
- Re-Cap the reagent bottles and place the protease tube back in the fridge.

### MAINTENANCE:

Cleaning reagents allowed: Mild detergents and 70% Ethanol

#### *Regular maintenance*

- Remove used disposable labware and unwanted samples or reagents from the worktable.

- Empty the waste drawer and if necessary clean the liner with a mild detergent (micosol) or 70% ethanol and then rinse with distilled water.
- If necessary wipe the worktable surface with distilled water and then 70% ethanol. DO NOT USE 70% ETOH ON THE FRONT DOOR!

#### *Periodic maintenance*

- Refer to Qiacube user manual (Qiacube accessory drawer, [www.qiagen.com/MyQIACube](http://www.qiagen.com/MyQIACube), or User manual file cabinet)

#### INSTALLING NEW PROTOCOLS

- New protocols for the QIACube can be downloaded at [www.qiagen.com/MyQIACube](http://www.qiagen.com/MyQIACube)
- To transfer new protocols to the QIACube, first save them to the qiacube memory stick (kept in the QIACube accessory drawer). Create a folder named “New\_Protocols” on the memory stick.
- Select the protocol to be downloaded and save it to the “New\_Protocols” folder.
- Connect the USB key to the QIACube (USB port is on the front).
- From the main menu, press “Tools”, “Data Exchange”, scroll up and down to find the desired protocol, and then press “Select”. Select “Protocols” and either “Load individually from USB” or “Load all from USB”.

#### SERVICE ENGINEER:

- Qiacube technical service 1800 555 049

## KASP

### How KASP works

The purpose of this document is to provide an explanation of how KASP™ genotyping chemistry works and also to provide information on how data is collected and analysed in our service laboratory.

#### KASP overview

KASP genotyping assays are based on competitive allele-specific PCR and enable bi-allelic scoring of single nucleotide polymorphisms (SNPs) and insertions and deletions (Indels) at specific loci.

The assay-specific KASP Primer mix and the universal KASP master mix are added to DNA samples, a thermal cycling reaction is then performed, followed by an end-point fluorescent read. Bi-allelic

discrimination is achieved through the competitive binding of two allele-specific forward primers, each (transfer) cassettes; one labelled with FAM™ dye and the other with HEX™ dye.

with a unique tail sequence that corresponds with two universal FRET (fluorescence resonant energy

#### Detailed explanation of KASP genotyping chemistry

KASP™ genotyping assays are based on competitive allele-specific PCR and enable bi-allelic scoring

The assay-specific KASP Primer mix and the universal KASP Master mix are added to DNA samples, of single nucleotide polymorphisms (SNPs) and insertions and deletions (Indels) at specific loci.

a thermal cycling reaction is then performed, followed by an end-point fluorescent read. The KASP Primer mix contains three assay-specific non-labelled oligos: two allele-specific forward primers and one common reverse primer. The allele-specific primers each harbour a unique tail sequence that corresponds with a universal FRET (fluorescence resonant energy transfer) cassette; one labelled with

FAM™ dye and the other with HEX™ dye. The KASP Master mix contains the universal FRET

cassettes, ROX™ passive reference dye, taq polymerase, free nucleotides and MgCl<sub>2</sub> in an optimised

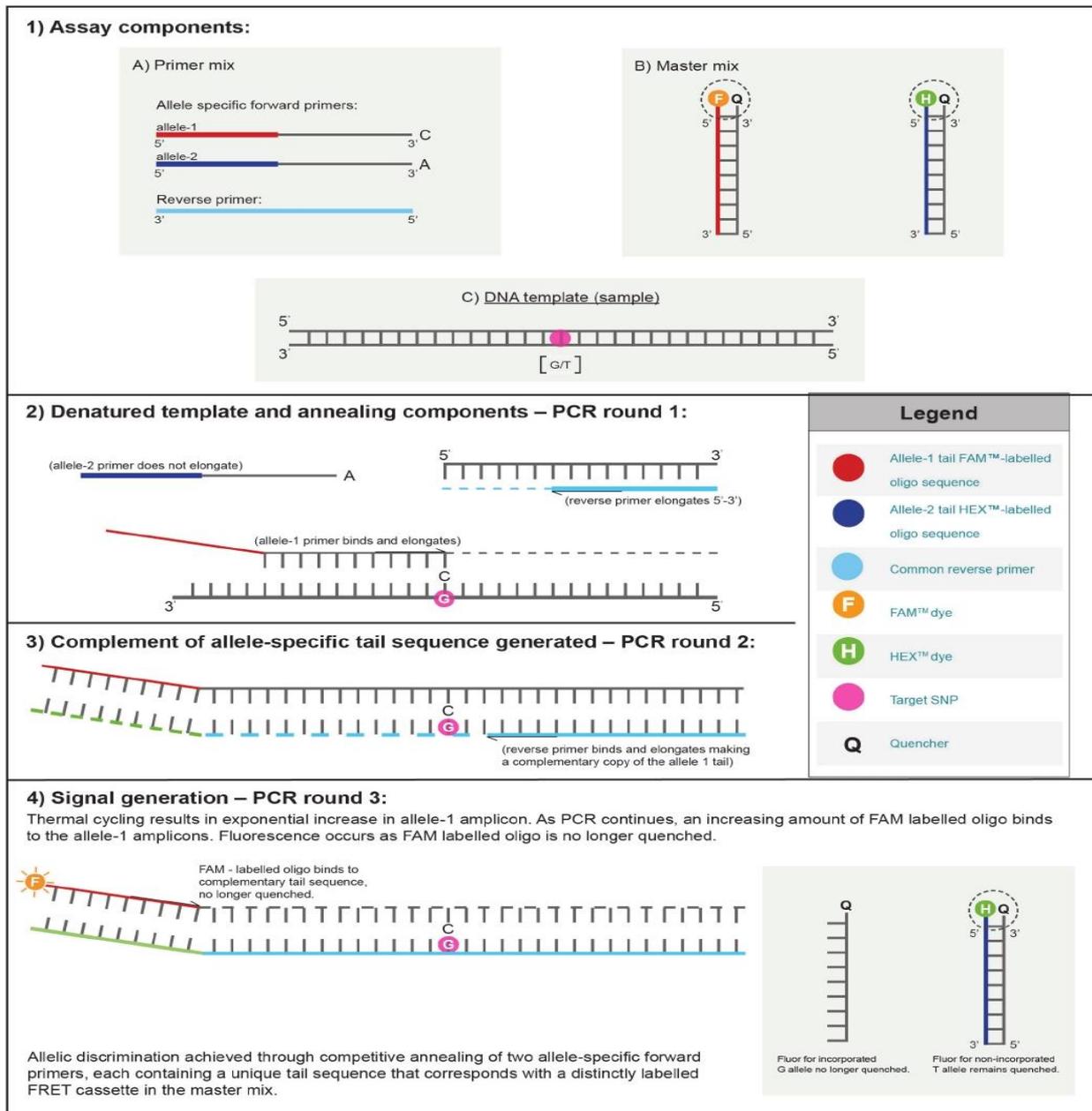
buffer solution. During thermal cycling, the relevant allele-specific primer binds to the template and elongates, thus attaching the tail sequence to the newly synthesised strand. The complement of the allele-specific tail sequence is then generated during subsequent rounds of PCR, enabling the FRET

cassette to bind to the DNA. The FRET cassette is no longer quenched and emits fluorescence.

Bi-allelic discrimination is achieved through the competitive binding of the two allele-specific forward

primers. If the genotype at a given SNP is homozygous, only one of the two possible fluorescent

signals will be detected. If the genotype is heterozygous, a mixed fluorescent signal will be detected.



#### 7.14. Appendix 14: Manuscript of the review paper published

### **Weight Change, Genetics, and Anti-Epileptic Drugs**

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<sup>2</sup>The Division of Neurology, Beaumont Hospital, Dublin, Ireland,

<sup>3</sup>Molecular and Cellular Therapeutics, the Royal College of Surgeons in Ireland, Dublin, Ireland.

\* Author for Correspondence. [gcavalleri@rcsi.ie](mailto:gcavalleri@rcsi.ie)

### **Summary**

Weight gain caused by antiepileptic drugs (AEDs) constitutes a serious problem in the management of people with epilepsy. AEDs associated with weight gain include sodium valproate, pregabalin, and vigabatrin. Excessive weight gain can lead to non-compliance with treatment and to an exacerbation of obesity-related conditions. The mechanisms by which AEDs cause weight gain is not fully understood. It is likely that weight change induced by some AEDs has a genetic underpinning, and recent developments in DNA sequencing technology should speed the understanding, prediction,

and thus prevention of serious weight change associated with AEDs. This review focuses on the biology of obesity in the context of AEDs. Future directions in the investigations of the mechanism of weight change associated with these drugs and the use of such knowledge in tailoring the treatment of specific patient groups are explored.

## **The Biology of Weight**

Many biological factors have been implicated in the pathogenesis of obesity. An imbalance between energy intake and energy expenditure presents an over-simplistic view of the underlying mechanism of obesity. A complex interaction between environmental factors, central nervous system (CNS) neurotransmitters (e.g. serotonin, glutamate, GABA etc), neurotransmitter receptors, peripheral endocrine systems and some Circadian rhythms influence food energy intake and expenditure [1,2]. In the vast majority of cases, the role that an individual's genetic makeup plays in the pathogenesis of this imbalance is polygenic. Food intake and thermogenesis are controlled by a complex interaction between the CNS and the peripheral nervous system (PNS). The peripheral and central control pathways of energy balance are linked at multiple points. The anabolic and catabolic neuropeptides are the two classes of neuropeptides involved in energy balance.

## **The Genetics of Weight Regulation**

Obesity is a multifactorial disorder with a significant genetic component. Indeed family and twin studies indicate that BMI has a heritability of between 0.7 - 0.8 [3,4]. Monogenic obesity is rare in humans but understanding the genes underlying these rare cases can provide a window on the biology of obesity more generally. Around 5% of early onset, severe obesity are explained by pathogenic mutations in the gene coding for the G-protein coupled melanocortin 4 receptor (MC4R) (Yeo et al., 1998<sup>[5]</sup>). The central melanocortin system regulates food intake, energy expenditure, body weight and glucose homeostasis through MC4Rs located in the hypothalamic and extra-hypothalamic nuclei [6]. Individuals carrying the *MC4R* mutations are typically of tall stature and experience severe early-onset obesity and hyper-insulinaemia [7,8]. Mutations in the *MC4R* gene are the most common cause of familial obesity. A recent genome-wide association study (GWAS) study indicates that common variation across *MC4R* in the general population also contributes to BMI variations and to increased risk of sporadic obesity [9]. A much smaller number of familial obesity cases can be explained by mutations in the leptin and pro-opiomelanocortin (*POMC*) genes. Mutations in both the leptin gene and its associated receptor can lead to severe, early-onset obesity with associated hyperphagia. Leptin is a peptide hormone produced primarily in the white adipose tissue. It functions, through receptors on the hypothalamus, to inhibit appetite [10]. Mutations in *POMC* leads to obesity of later onset than those cases due to loss of MC4R signalling [11].

Gene variants with minor allele frequency of < 5% have been mapped for many familial syndromes that have obesity as a central component of the clinical phenotype. Examples include Albright hereditary osteodystrophy (mutation in *GNAS1*), Bardet-Biedl syndrome (mutations in various *BBS* genes), Fragile X Syndrome (mutations in *FMR1*), lipodystrophy syndromes (mutations in *LMNA*), and Prader-Willi syndrome (deletions involving 15q11-q13). These variants are highly penetrant in patients and families with severe obesity as the predominant presenting feature and display classical Mendelian patterns of inheritance [11].

Despite the above examples, the vast majority of the genetic influence on obesity is polygenic – a combination of genetic variation across a number of genes, each with individually small effect sizes. The development of GWAS has provided a framework for the study of the role of common genetic variation in obesity. As of January 2013, over 13 GWAS studies have been published on weight-related phenotypes. The largest of these studies contained data on over 249,000 individuals, leading to the robust association of 32 loci with weight. Loci including *FTO*, *MC4R*, *ETV5* had previously been identified whilst others like *GPRC5B*, *TNNI3K*, *PRKD1* were novel [12]. However, despite the size of the study, the 32 loci identified together only account for 1.45% of inter-

individual variation in BMI, the largest locus-specific effect coming from *FTO* (0.34%). In all, about 52 loci for obesity traits have now been discovered through GWAS. These have only modest effect sizes with odd ratios (per allele) of between 1.2 and 1.5 [11]. However, genes involved may provide insight to the biology of obesity. For example several are located in regions containing genes encoding for neuronal regulators of appetite, energy balance and neuroendocrine function [12].

### **Weight Change in Epilepsy**

Weight change of itself is not a central feature of epilepsy. However, particularly in refractory epilepsy, a sedentary life-style due to regular seizures increases the risk of obesity [13]. In addition, depression is common in people with epilepsy, which might contribute to the increased prevalence of obesity [14]. Exercise-induced seizures, AED side-effects and conflicting medical advice regarding the safety of exercise contribute to the problem. Very few studies have explored the issue of weight change in individuals with epilepsy. In a Norwegian study the exercise pattern of patients with epilepsy closely matched those of age- and sex-matched individuals in the general population [15]. A survey of exercise patterns in 412 adults with epilepsy indicated that most undertook light exercise three or less times per week and that there was no difference in frequency and intensity between

those who reported that seizures were a barrier to exercise than those who did not [16]. The only study of epilepsy-related weight change in children and adolescents concluded that juveniles with epilepsy exercised less and were therefore more likely to be overweight than their siblings without epilepsy [17].

Most of the weight changes in patients with epilepsy are related to treatment. AEDs may be associated with weight gain, weight loss or they may be weight neutral (see Table 1). The principal AEDs associated with weight gain are sodium valproate, gabapentin, pregabalin, vigabatrin, and carbamazepine [18]. Those AEDs associated with weight loss are topiramate, zonisamide, and felbamate [19]. Unsurprisingly, AEDs associated with weight gain are of greatest concern for patients and their clinicians since weight gain not only affects body image and self-confidence but can also contribute to chronic illness related to obesity such as hypertension, type 2 diabetes mellitus, dyslipidaemia, and atherosclerosis [14,20-22]. These adverse effects may also affect seizure control as they could impair adherence to the treatment regimen. Since AEDs are often taken for many years, physicians and their patients should be aware of the adverse metabolic effects of some AEDs [23]. The adverse health effects of weight gain can be exacerbated by the multiple medications taken by some individuals with epilepsy [21]. Best practice in epilepsy care indicates that in selecting AEDs, the patient's profile should

carefully considered – this includes weight prior to the initiation of therapy and other medical or psychiatric morbidities [13]. Ideally an AED should be a weight neutral unless the patient's profile dictates otherwise [22]. Regular weight checks after the initiation of treatment are important to monitor adverse reaction.

## **AED-associated Weight Change**

**Sodium valproate (VPA):** VPA, a broad-spectrum AED used in the treatment of both generalized and partial seizures has been reported to cause weight gain in up to 71% of exposed patients, although typically the rate is around 10% [22]. Weight gain usually occurs within the first 3 months and peaks at 6 months after initiation of therapy ([24]). The degree of weight gain is variable, with about 10% of the patients experiencing severe weight gain. Factors proposed to influence VPA-induced weight gain include body mass index pre-treatment ([25] [26-28]), gender [29], post-pubertal status [30], age at which treatment was first initiated [31], neuro-cognitive status [32], and a diagnosis of primary generalized epilepsy[32]. It has also been reported that post-pubertal girls are more likely to gain weight than post-pubertal boys or prepubertal children of any gender [26,29,33-35] and that a longer duration of therapy correlates with weight gain [26,28,36]. Other studies have not reported predictors [37,38]. It should be noted that none of the proposed predictors listed above have been consistently replicated [25,29,32,39]. As a result, there are no accepted clinical predictors of VPA-induced weight gain. There have been no randomized control trials and some of the studies were underpowered to detect these predictors [22].

The mechanisms underscoring VPA-associated weight gain are unclear. VPA therapy is associated with hyperinsulinaemia [40,41], although the causal mechanism is poorly understood [42]. Insulin, a peptide hormone, plays a central role in energy metabolism in the well-fed state. Increased blood glucose in the well-fed state stimulates insulin secretion. Insulin then promotes glycogen storage in the liver and muscle and converts the excess glucose to fatty acids and triglycerides in the adipose tissue. Interference with hepatic insulin metabolism leads to hyperinsulinaemia without concomitant increase in the C-peptide levels [43]. Interestingly, VPA inhibits GLUT-1 activity in normal astrocytes and fibroblasts resulting in reduced glucose transport [44].

VPA therapy is also associated with hyperleptinaemia and leptin resistance [42,45-48]. A high soluble leptin receptor/ leptin ratio has been observed in children with epilepsy [49]. Serum leptin levels strongly correlate with BMI in children on VPA therapy [50]. The relationship between increased leptin levels and increased BMI appears more marked in girls than in boys [46,51]. VPA therapy has also been associated with increased ghrelin levels at 6 months along with increased BMI in pre-pubertal children taking VPA compared to controls [52]. Ghrelin is a peptide hormone secreted by the stomach and proximal small intestine. It is the only hormone known to stimulate appetite and promote food intake, resulting in a positive energy balance and weight gain.

Indirect evidence from animal studies indicates that VPA therapy exerts a time- and dose- dependent inhibitory effect on adiponectin gene expression in mature mouse adipocytes [53]. The mechanism of this effect is probably through the inhibition of histone deacetylase activity. Adiponectin is an adipokine produced by the adipose tissues and acts in the brain and peripheral organs to regulate glucose and in fatty acid metabolism [54]. Adiponectin decreases gluconeogenesis while increasing glucose uptake. It also promotes fat catabolism by promoting  $\beta$ -oxidation and clearance of triglycerides. Greco et al [55] noted that adiponectin levels were lower in obese epileptic patients when compared to those who did not gain weight [55]. Adiponectin deficiency can predispose to the metabolic syndrome [54].

Other studies have suggested a role for a variety of other neurohumoral factors in VPA-induced weight gain, such as glucagon-like peptide-1 [56], neuropeptide Y [45,48,57], galanin [48] GABA [58-60]).

**Pregabalin (PGB):** In controlled trials of pregabalin (PGB), a weight gain of 7% or more was noted in about 8% of subjects treated with PGB, compared to 2% of those treated with placebo. A meta-analysis of pooled data from 106 studies involving 43,525 patients treated with 150 - 600 mg of PGB indicates that the majority of patients maintained their weight within 7% of baseline weight while about 16% gained  $\geq 7\%$  of baseline weight [2]. In contrast to VPA, dose [61] and duration of exposure are appear as

robust predictors of PGB-induced weight gain [62]. This ADR is not associated with baseline BMI, gender, or age [63]. Diabetic patients treated with PGB have been shown to gain an average of 1.6 kg (range: -16 to 16 kg), compared to patients treated with placebo who gained an average of 0.3 kg (range: -10 to 9 kg) [63]. The dose-dependent effect of PGB-induced weight gain has been demonstrated in a double blind placebo-controlled trial in epilepsy which showed a 12 to 14% weight gain for those patients treated with 600mg of PGB compared to 10% gain in those treated with 150mg and 6% in the placebo group [13,64,65]. Although the exact mechanism of PGB-induced weight gain is unknown, enhancement of GABA transmission may play a role [64].

**Topiramate (TPM):** The reported incidence of weight loss in adults and children treated with TPM for 3 months to up to 1 year ranges from 16% up to 86% [66-70]. In a randomized double-blind trial of TPM in adults and children weight loss was noted in 6 - 15% of adults on 25 - 500mg/day, but not in any of the children on similar doses [67]. Most weight loss occurs within 3 – 5 months of exposure [66,70,71] and may be sustained for up to two years [72]. The degree of weight loss also varies widely and ranges between 3.9% and 7.3% [66]. The degree of weight loss correlates with dose, pre-treatment weight, reduced caloric intake, higher baseline BMI and duration of treatment [66,70,71,73]

The mechanism of weight loss is not understood [71,74]. TPM depresses appetite in children that lost weight while on the

medication [71,75]. Other mechanisms may include significant reduction in the serum galanin (a neuropeptide involved in the regulation of feeding behaviour ) levels [76], stimulation of lipoprotein lipase in brown fat [77,78] and [79] uncoupling of proteins 1 and 2 in mitochondria [80,81] increase in serum level of adiponectin.

Interestingly, TPM therapy is associated with increased insulin sensitivity and has demonstrated efficacy in type-2 diabetes [82-84]. Short term therapy with low dose TPM does not affect insulin sensitivity in obese non-diabetics [85]. TPM can preserve pancreatic  $\beta$ -cell activity in type-1 diabetics with epilepsy being treated with TPM [84]. In animal models with type 2 diabetes, TPM acts both as an insulin secretagogue and as an insulin sensitizer [86].

**Zonisamide (ZNS):** Up to 35% of patients exposed to zonisamide (ZNS) experience some weight loss. The mean weight loss is usually around 3.7% +/- 9.1% [87]. ZNS used in the treatment of psychiatric conditions also results in weight loss [88]. There is inter-individual variability in the weight change ranging from -36% to +32% of pre-initiation weight [87]. In a randomised control trial in obese subjects treatment with 400mg of ZNS, a third of patients reported >10% weight loss [89]. BMI at initiation of therapy appears to correlated with degree of weight loss although this observation requires replication [87].

Inhibition of lipogenesis through its effect on the mitochondrial carbonic anhydrase isoforms CA VA and CA VB may be the main molecular mechanism for ZNS induced-weight loss. Weight loss in ZNS therapy is associated with low serum leptin levels in obese females [90]. Indeed, ZNS and TPM have been approved as anti-obesity drugs in some regions [91,92].

**Others:** Additional AEDs including carbamazepine (CBZ) and gabapentin (GBP) have been reported to lead to weight gain. Whilst felbamate (FBM) has been associated with weight loss. However, the degree of weight change associated with these AEDs is less than for VPA, GBP, TMP and ZNS.

CBZ is a sodium channel blocker and one of the most commonly prescribed AEDs. Weight gain has been reported in around 5% of individuals on CBZ treatment, an effect that appears idiosyncratic [93]. Work in murine and human cell lines has provided strong evidence that CBZ influences weight by targeting adipocytes to alter adipose tissue metabolism and differentiation [94].

GBP is a gamma-aminobutyric acid (GABA) analogue that also acts on presynaptic calcium channels. Several observational studies have reported quantitative data on weight gain associated with GBP use. A study involving 610 epilepsy patients on GBP as an add-on therapy reported weight gain in 9% of patients, an effect that appeared dose dependent [95]. An earlier study in 44 epilepsy patients on high-dose GBP therapy reported weight gain in 23% of patients [96].

FBM acts as a modulator of GABA receptors and blocker of NMDA receptors. The use of FBM is restricted to very severe forms of epilepsy such as Lennox-Gastaut syndrome, due to associated toxicity (which can lead to liver failure). A study of 65 children and adolescents on FBM therapy reported weight loss in 75% of patients, with the degree of weight loss correlating with age [97]. Significant weight loss (>5%) has been reported in long-term use of FBM in children [98].

**Clinical summary:** It is thus clear that a significant number of commonly used AEDs can impact on body weight in many individuals with epilepsy. The mechanism of weight change effected by these AEDs is poorly understood, and may in large part have an underlying pharmacogenomic basis. Weight loss and weight gain induced by AEDs may contribute to the important co-morbidities of epilepsy, such as endocrine disturbance, risk and effects of seizure-related injuries, and lipid homeostasis. The treating physician should consider the potential effects of AEDs on

weight when initiating treatment in an individual patient. Thus, for example, valproate should generally be avoided when initiating therapy in someone with epilepsy and co-morbid obesity, whereas zonisamide would be best avoided in an individual with low body mass, or a history of anorexia. Similarly, early consideration should be given to switching to an alternative therapy if a recently initiated AED appears to be negatively impacting weight and well-being in a patient with epilepsy. Finally, predictive pharmacogenomic analysis would be an important advance in individualized pharmacotherapy in the epilepsies.

### **Five-Year View**

Progress in genetic mapping has accelerated our understanding of genetics in the causation of human disease, response to treatment, drug resistance and adverse drug reactions [99,100]. In epilepsy there has been a significant increase in pharmacogenomic research over recent years [101,102]. GWAS and next-generation sequencing now allows a comprehensive screening of genetic variations across different patient populations. These techniques offer the potential not only to enhance the understanding of the molecular mechanisms underlying some epilepsy syndromes, but also to provide insight into the underlying mechanism of some previously unrecognised obesity syndromes. Improved knowledge of the molecular mechanism of epilepsy and obesity will not only enhance the understanding of the relationship between the two diseases but will also help in understanding the role of AEDs in

weight change. Well-powered GWAS- and next-generation sequencing-based mapping efforts have yet to be applied to weight-change related ADRs in epilepsy. Such an effort for AED-associated weight change may further allow us to understand the complex biology of obesity. Large consortia-based efforts including but not limited to EPIPGX and EPI4K provide a framework for discovery in this context [103,104].

### **Key Issues**

1. Obesity is a common public health problem with long-term medical and psychological implications
2. Weight change is a common problem in patients with epilepsy, often due to treatment with AEDs such as sodium valproate.
3. Anti-epileptics and anti-psychotic treatments contribute to the rising prevalence of obesity. While most of these drugs cause weight gain, others cause weight loss while others are weight neutral.

4. The biology of obesity is very complex and scientific knowledge in this area is rapidly evolving.
  
5. Anti-epileptic and anti-psychotic medications interfere with the key biological mechanisms regulating energy balance. This might explain some of the weight-related adverse effects attributed to these medications.
  
6. The development of gene sequencing technology has enhanced the understanding of the biology and genetics of obesity, and may also help us to understand AED-induced weight change.

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Table 1: A summary of epidemiological studies exploring AED-induced weight change.

AED	Dir. of effect	% Patients showing effect	Study design & ref	Clinical predictor(s)	Observed in adults &/or paed. subjects?
VPA	Gain	44%	Retrospective [105]	<ul style="list-style-type: none"> <li>Female gender</li> </ul>	Paediatric
VPA	Gain	11%	Prospective [106]	<ul style="list-style-type: none"> <li>Initial weight Z-score</li> <li>Initial body mass index</li> </ul>	Paediatric
VPA	Gain	71%	Retrospective [25]	<ul style="list-style-type: none"> <li>No history of weight problems</li> <li>BMI at initiation of therapy</li> </ul>	Adults
VPA	Gain	44%	Retrospective [29]	<ul style="list-style-type: none"> <li>None tested</li> </ul>	Paediatric

AED	Dir. of effect	% Patients showing effect	Study design & ref	Clinical predictor(s)	Observed in adults &/or paed. subjects?
VPA	Gain	27% in males 56% in females	Prospective, observational [39]	<ul style="list-style-type: none"> <li>Gender (female)</li> </ul>	51 males; 55 females
VPA	Gain	58%	Retrospective [32]	<ul style="list-style-type: none"> <li>Weight at initiation</li> <li>Neurocognitive status</li> <li>Primary generalized epilepsy</li> </ul>	Paediatric
PGB	Gain	11%	Randomised placebo-controlled trial [107]	<ul style="list-style-type: none"> <li>None tested</li> </ul>	Adults

AED	Dir. of effect	% Patients showing effect	Study design & ref	Clinical predictor(s)	Observed in adults &/or paed. subjects?
PGB	Gain	7%-150mg/day  14%-600mg/day	Randomised double-blinded placebo-controlled trial [108]	<ul style="list-style-type: none"> <li>Dose</li> </ul>	Adults
PGB	Gain	10%	Prospective, observational [61]	<ul style="list-style-type: none"> <li>Dose</li> </ul>	Adults
PGB	Gain	21%-600mg BID  15%-600mg TID	Randomised double-blinded placebo-controlled trial [65]	<ul style="list-style-type: none"> <li>None tested</li> </ul>	Adults
TPM	Loss	86% at 1 year	Prospective observational [66]	<ul style="list-style-type: none"> <li>Baseline BMI</li> </ul>	Adults

AED	Dir. of effect	% Patients showing effect	Study design & ref	Clinical predictor(s)	Observed in adults &/or paed. subjects?
TPM	Loss	15%-200 or 500mg/day	Randomised double-blinded trial [67]	<ul style="list-style-type: none"> <li>Dose</li> </ul>	Both
TPM	Loss	69%-400mg/day	Randomised double-blinded trial [68]	<ul style="list-style-type: none"> <li>Dose</li> </ul>	Both
TPM	Loss	12%-200mg/day	Randomised double-blinded trial [69]	<ul style="list-style-type: none"> <li>Dose (suggestive)</li> </ul>	Adults
TPM	Loss	13%-600-1000mg/day	Randomised double-blinded placebo-controlled trial [109]	<ul style="list-style-type: none"> <li>Dose (suggestive)</li> </ul>	Adults

AED	Dir. of effect	% Patients showing effect	Study design & ref	Clinical predictor(s)	Observed in adults &/or paed. subjects?
TPM	Loss	13%600-100mg/day	Randomised double-blinded placebo-controlled trial [70]	<ul style="list-style-type: none"> <li>Dose</li> </ul>	Adults
ZNS	Loss	35%	Retrospective [87]	<ul style="list-style-type: none"> <li>Weight pre-treatment</li> </ul>	Adults
ZNS	Loss	55%-400mg/day	Randomised double-blinded placebo-controlled trial [110].	Dose	Adults

**Table 2: Reported possible molecular mechanisms of AED-induced weight change**

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<b>AED</b>	<b>Molecular mechanism</b>
Sodium Valproate	GABA stimulation of the hypothalamus: Increased [24,111,112])
Sodium Valproate	Hyperinsulinism and insulin resistance [24,113-115];
Sodium Valproate	Hyperleptinemia and leptin resistance [114,116-118].
Sodium Valproate	Leptin, ghrelin and adiponectin [55,119,120]
Sodium Valproate	Increased serum level of GLP-1[121]
Pregabalin	Enhancement of GABA transmission [108]
Topiramate	Increased expression of uncoupling proteins 1 & 2[66,80,81,122-124]
Topiramate	Reduced serum galanin levels [74]
Topiramate	Inhibition of mitochondrial carbonic anhydrase isoforms VA and VB [125,126];

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Topiramate	Stimulation of lipoprotein lipase in brown fat [77-79]
Zonisamide	Inhibition of mitochondrial carbonic anhydrase isoforms (VA and VB)[22,125-127]

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