GENETIC STUDIES OF THE COMMON EPILEPSIES:
genome-wide association studies in the partial epilepsies

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Declaration

I, Cláudia José Franco Bacanhim Santos Catarino, confirm that the work presented in this thesis is my own.

Dr Dalia Kasperaviciute performed statistical work on the genome-wide association study of partial epilepsies. I performed the statistical work on the genome-wide association study of mesial temporal lobe epilepsy with hippocampal sclerosis and Dr Kasperaviciute subsequently replicated the statistical work. Dr Erin Heinzen and team in Prof. David Goldstein's lab at Duke University did the laboratory work and statistical analyses that led to the description of microdeletions in the cohort of the genome-wide association study of partial epilepsy. Dr Maria Thom reviewed the histopathology of patients with mesial temporal lobe epilepsy and large microdeletions. Dr Joan Liu, Lillian Martinian, Ioannis Liagkouras and Dr Maria Thom performed the neuropathology studies of Dravet syndrome. Vaneesha Gibbons, Robyn Labrum and Rachael Ellis screened *SCN1A* for mutations and deletions in the adults with Dravet syndrome.

Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed:

Date: 5th September 2013

Abstract

This thesis discusses four studies, looking for genetic determinants of common epilepsies:
1) A genome-wide association study (GWAS) of partial epilepsies (PE), which was the first published GWAS in the field of epilepsy (Chapter 4).
2) A GWAS of mesial temporal lobe epilepsy (MTLE) with hippocampal sclerosis (HS) (Chapter 5).
3) A case series of patients with refractory MTLE, operated and found to have large microdeletions at 16p13.11, 15q11.2 and others (Chapter 6).
4) A clinical, genetic and neuropathologic study of a series of patients with Dravet syndrome (DS), diagnosed as adults, including genotype-phenotype correlation analysis (Chapter 7).

The main findings include:
1) The GWAS of PE has not yielded any genome-wide significant association with common genetic variants, possibly because of insufficient power and phenotypical heterogeneity. It is, however, a strong foundation for further studies, illustrating the feasibility of large multicentre GWAS in the epilepsies (Chapter 4).
2) The GWAS of MTLEHS yielded a borderline genome-wide statistically significant association with three common genetic variants close or intronic to the *SCN1A* gene, especially in MTLEHS with antecedents of childhood febrile seizures (Chapter 5).
3) Large microdeletions at 16p13.11 and others were found in patients with MTLEHS and not only in idiopathic non-lesional epilepsies. Good outcome after resective epilepsy surgery is possible in "typical" MTLEHS even with large microdeletions (Chapter 6).
4) The identification of a cohort of adults with DS, not diagnosed as children, allowed the description of long-term evolution through adulthood and recognition of clinical features shared later in the evolution. Over sixty percent had *SCN1A* mutations. Missense mutations were more frequent in patients who survived through adulthood, with truncating mutations and large deletions only found in those who died in early childhood. Medication changes after diagnosis led in some cases to better seizure control, cognition and quality of life. Further evidence for DS as encephalopathy came from post mortem histopathology, with no neuronal loss found in cerebral cortex or hippocampus.

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List of Abbreviations

Abbreviation	Explanation
aCGH	array comparative genomic hybridization
AD	autosomal dominant
ADNFLE	autosomal dominant nocturnal frontal lobe epilepsy
AED	antiepileptic drug
CA	Cornu Ammonis
CB	calbindin
CNS	central nervous system
CNV	copy number variation
CR	calretinin
Cx-43	connexin-43
DNA	deoxyribonucleic acid
DS	Dravet Syndrome
dsDNA	double-stranded DNA
EEG	electroencephalography
EPIGEN	epilepsy genetics consortium, from the UK (London), Ireland (Dublin), Belgium (Brussels) and USA (Duke)
eQTL	expression quantitative trait locus analysis
FHM	familial hemiplegic migraine
fMRI	functional MRI
FS	febrile seizures
GAD	glutamic acid decarboxylase
GEFS+	genetic (generalised) epilepsy with febrile seizures plus
GenEpA	epilepsy genetics consortium, from Finland (Helsinki, Kuopio), Norway (Oslo) and Switzerland (Zurich)
GFAP	glial fibrillary acidic protein
GGE	genetic generalised epilepsies
GWAS	genome-wide association study
HLA	human leucocyte antigen
HS	hippocampal sclerosis

HWE	Hardy-Weinberg equilibrium
ID	intellectual disability
IED	interictal epileptiform discharges
IGE	idiopathic generalised epilepsies
IGSP	Institute for Genome Sciences and Policy, Duke University
ILAE	International League Against Epilepsy
IPI	initial precipitating injury
LD	linkage disequilibrium
MAF	minor allele frequency
MLPA	Multiplex Ligation-dependent Probe Amplification
MPSI	malignant migrating partial seizures of infancy
MRI	magnetic resonance imaging
MTLE	mesial temporal lobe epilepsy
MTLEHS	mesial temporal lobe epilepsy with hippocampal sclerosis
MTS	mesial temporal sclerosis
$Na_v1.1$	voltage-gated sodium channel type 1.1
NGS	next-generation sequencing
NPY	neuropeptide Y
NSE	National Society for Epilepsy (Epilepsy Society, UK)
PET	positron emission tomography
PV	parvalbumin
SIMFE	severe infantile multifocal epilepsy
SMEB	severe myoclonic epilepsy of infancy-borderland
SMEI	severe myoclonic epilepsy of infancy
SNP	single nucleotide polymorphism
SPECT	single-photon emission computed tomography
SUDEP	sudden unexplained death in epilepsy
TLE	temporal lobe epilepsy
UCL	University College London
WHO	World Health Organization
WT	Wellcome Trust
WTCCC	Wellcome Trust Case-Control Consortium

Glossary of commonly used terms

Terms	Definitions
Alleles	Alternative forms of a gene at the same genetic locus.
"Complex" disease	Several genetic variants, interacting with environmental factors, contribute to disease susceptibility.
Complex or prolonged febrile seizures	One or more of the following: focal features; multiple within the same febrile illness or the first 24 hours; duration of 10 minutes or longer. Includes febrile status epilepticus, when duration is longer than 30 minutes (Baulac et al., 2004;Hirtz et al., 1997).
Copy number variation	Genetic variation with departure from the expected diploid representation of the DNA sequence (McCarthy et al., 2008). Includes deletions and duplications.
De novo mutation	Mutation not inherited from one or both parents, but newly occurred in the proband (Baker et al., 2012).
Epileptogenic zone	Theoretical concept, which includes the area that generates the habitual seizures of the patient and also the brain regions still capable of generating seizures once the original seizure onset zone has been resected. The most reliable evidence for its accurate location is seizure freedom after epilepsy surgery (Kellinghaus & Luders 2004).
Genetic heterogeneity	More than one gene contributes to causation or increased susceptibility to one disease or trait.
Genome-wide association study	A dense array of genetic markers is typed in a set of DNA samples informative for the disease (or trait) of interest, with the aim to identify associations between genotype and disease status (McCarthy et al., 2008;National Institutes of Health 2007).
Genomic inflation factor	Defined as the ratio of the median of the empirically observed distribution of the test statistic to the expected median (Devlin & Roeder 1999).
Genotype	There are two copies of each chromosome in each diploid cell, which means two independently inherited DNA sequences per

	locus, the paternal and maternal alleles. The genotype of that individual for that locus are these two alleles (Neale & Purcell 2008).
Haploinsufficiency	An individual heterozygous for a mutation, or hemizygous at a locus due to a deletion, is clinically affected because a single copy of the normal gene does not lead to sufficient protein production to ensure normal function (GeneReviews online).
Haplotype	A series of alleles found at linked loci on a single chromosome (Strachan & Read 1999).
Hardy-Weinberg equilibrium	Theoretical description of the relationship between genotype frequencies and allele frequencies, under the assumptions of a stable population, random mating and absence of selection, new mutations or gene flow (McCarthy et al., 2008).
Heritability	Proportion of the total phenotypic variance that can be accounted for by genetic factors.
Imputation	To infer genotypes at untyped markers.
Incomplete penetrance	A carrier of a pathogenic genetic variant may be asymptomatic.
Linkage disequilibrium	Population correlation between two allelic variants in the same chromosome. Two allelic variants are in LD when they are inherited together more often than expected by chance (McCarthy et al., 2008).
Locus	A specific region on a chromosome.
Locus heterogeneity	Variation at different genetic loci may cause a similar phenotype.
Mendelian disease	Disorder caused almost entirely by a single major gene. Presence or absence of disease depends on the genotype at a single locus (Strachan and Read 1999).
Next generation sequencing	Generic term for high throughput parallel sequencing methods (Baker et al., 2012).
Odds ratio	Measure of association derived from case-control studies. Odds of exposure (to the susceptible genetic variant) in cases compared

	with the odds of exposure in controls (Ioannidis et al., 2009).
Penetrance	Ratio of symptomatic carriers over the total number of carriers of the pathogenic genetic variant.
Phenocopy	Individual who presents the same phenotype as the symptomatic carriers of a pathogenic genetic variant, without being a carrier of the pathogenic genetic variant.
Pleiotropy	Multiple phenotypic effects associated with the same genetic abnormality (Baker et al., 2012;Sivakumaran et al., 2011).
Principal components analysis	Statistical method which transforms a series of correlated variables into a smaller number of uncorrelated factors (Ioannidis et al., 2009).
r^2	Correlation coefficient, measure of linkage disequilibrium. Measure of strength and direction of a linear relationship between genotypes of two variants (Ioannidis et al., 2009).
Symptomatogenic zone	Cortical region giving rise to the symptoms of an epileptic seizure (Lüders & Awad 1992).
Single nucleotide polymorphism	Genetic variant characterized by a change in one DNA base pair, leading to two possible allelic identities. Most common form of genetic variation (Baker et al., 2012).
Variable expressivity	The members of a family with a familial condition and the same pathogenic mutation in one gene may have different phenotypes and phenotypic severity. Between families the phenotypical spectrum may also vary, even when all share the same pathogenic genetic variant.

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Articles in peer-reviewed journals

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1 Chapter Introduction

1.1 Epilepsies – Clinical epidemiology

1.1.1 Incidence and prevalence of epilepsy

Epilepsy is the commonest serious disorder of the brain. It affects all ages and imposes a significant burden around the world. Epilepsy is estimated to affect more than 70 million people worldwide (Ngugi et al., 2010). Its prevalence in Europe is around 8.2 per 1000 people, which corresponds to around 6 million affected (ILAE/IBE/WHO Global Campaign Against Epilepsy 2010). In the UK, there are about 450,000 people with epilepsy (Sander 2003).

Studies in people with new-onset epilepsy estimate that seizure control with antiepileptic drugs (AEDs) is possible in up to 70% of patients (Sillanpaa & Schmidt 2006), but a proportion of people with epilepsy does not receive such treatment: the treatment gap is estimated to be 40% in Europe (ILAE/IBE/WHO Global Campaign Against Epilepsy 2010).

In the other third of people with epilepsy, seizures cannot be controlled by the available medication (French 2007;Kwan et al., 2010). Surgery may be a highly effective treatment for selected patients with pharmacoresistant seizures, but even among those patients who have good odds for a good surgical oucome confirmed by a stringent presurgical evaluation, some are not rendered seizure-free with the appropriate surgery (McIntosh et al., 2001;Spencer & Huh 2008;Thom et al., 2010b). Only a proportion will

experience long-term seizure freedom after surgery: about 66% for patients with temporal lobe resections, 46% with parietal and occipital resections and 27% with frontal lobe resections (de Tisi et al., 2011;Tellez-Zenteno et al., 2005).

1.1.2 Burden of epilepsy and mortality

The implications of having refractory seizures involve many daily life domains, including education, employment (Chin et al., 2007), independent living, mobility and relationships in the family and in society. Refractory epilepsy may encompass important disability and people may be exposed to stigma and prejudice. These problems exacerbate the personal burden of epilepsy and account for much of its societal cost. Recurrent seizures can seriously compromise quality of life and have several associated risks, such as injuries, increased morbidity and premature mortality (Gaitatzis et al., 2004;Tomson et al., 2005).

1.1.3 Definition of epilepsy and epileptic seizure

Epilepsy is considered to be not just one disorder, but a family of disorders of the brain (Berg et al., 2010), characterized by an "abnormally increased predisposition for epileptic seizures and the neurobiologic, cognitive, psychological and social consequences" (Fisher et al., 2005). At least one epileptic seizure is required to make the diagnosis and this can be defined as "a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain" (Fisher et al., 2005).

1.1.4 Classification of the epilepsies and epilepsy syndromes

The epilepsies are a heterogeneous group of conditions, with diverse clinical manifestations, aetiology, treatment options and prognosis.

The concept of classification into generalised and partial seizures dates back to Hughlings Jackson (1931) and became commonly used with the publication by the International League Against Epilepsy (ILAE) of the scheme for classification of epileptic seizures in 1969/70 (Gastaut 1970).

The 1981 ILAE seizure classification defines seizures as generalised when "the first clinical changes (indicate) initial involvement of both hemispheres", while, for partial (focal) seizures, "the first clinical changes (indicate) initial activation of only part of one cerebral hemisphere" (ILAE Commission on Classification and Terminology 1981).

Both the 1981 ILAE classification for seizures (ILAE Commission on Classification and Terminology 1981) and 1989 classification for epilepsy syndromes (ILAE Commission on Classification and Terminology 1989) are still used today, but several proposals have readdressed this classification (Berg et al., 2010;Blume et al., 2001;Engel, Jr. 2001;Luders et al., 1998).

The latest ILAE proposal for classification suggested a revision of terminology and underlying concepts for the classification of seizures, epilepsies and epilepsy syndromes (Berg et al., 2010;Berg & Scheffer 2011).

Partial epilepsies

The partial epilepsies are the most common, accounting for two-thirds of incident cases (Banerjee et al., 2009). They are more likely to be medically refractory than generalised epilepsies, contributing significantly to the total burden of epilepsy.

Partial seizures have their origin "at some point within networks limited to one hemisphere", whereas generalised seizures originate " at some point within rapidly engaging bilaterally distributed networks" (Berg et al., 2010;Berg and Scheffer 2011). A classification of partial seizures according to ictal semiology has been proposed (Blume et al., 2001;Lüders & Noachtar 2001) and older terms such as "complex partial", "simple partial" and "secondarily generalised" have been suggested to be abandoned, because of ambiguity (Berg & Cross 2010).

Mesial temporal lobe epilepsy with hippocampal sclerosis

Mesial temporal lobe epilepsy with hippocampal sclerosis (MTLEHS) can been defined as a common, discrete, frequently refractory "epilepsy syndrome" (Semah et al., 1998). In selected cases it can be "surgically remediable" (Engel, Jr. et al., 2008;Wiebe et al., 2001), constituting the most frequent "aetiology" leading to resective surgery in adults with refractory temporal lobe epilepsy (Falconer et al., 1964).

There is debate as to whether MTLEHS constitutes an "epilepsy syndrome", given its heterogeneity[1] (Berg et al., 2010;Thom et al., 2010b;Wieser 2004) and whether HS can be in fact an "aetiology".

[1] See discussion on the heterogeneity of MTLEHS on Chapter 6, section 6.2.
See also (Thom et al., 2010b).

1.2 Genetic epidemiology of common, "complex" disorders

Genetic epidemiology studies the role of genetic factors and their interaction with environmental factors, as determinants in the occurrence of disease.

I will review the types of available genetic epidemiologic studies, both population-based and family-based and discuss some methodological issues, necessarily briefly, to lay the foundation for the description of the studies described in this thesis and importantly, for the discussion of the findings and of possible next steps forward in this field of research.

Genetic models of disease include several categories:

a. mendelian or monogenic, caused almost entirely by one major gene;

b. oligogenic, involving a few genes;

c. polygenic, with many genetic loci involved, each with a small contribution to the phenotype; and

d. "complex", where multiple genetic variants and environmental factors contribute to disease susceptibility (Strachan and Read 1999).

Important questions when approaching a disease in the context of genetic epidemiology include:

- Does the disease cluster in families (is there familial aggregation)?
- May the observed familial aggregation be explained by environmental factors, or is there evidence that genetic factors play a role?
- Can the mode of inheritance be identified?

- How can the genetic variant(s) responsible for the increased disease risk or susceptibility be mapped and identified?

It is estimated that the human genome codes around 20,000 genes. Around 99.9% of the DNA sequence is the same for everyone; these are the monomorphic loci. For the remaining important proportion of loci, mutations have occurred and were maintained throughout evolution, which changed the DNA base sequence, resulting in different alleles at a given locus, which constitute the polymorphic loci, making us genetically different and, in some cases, also phenotypically different (Neale et al., 2008).

Single-nucleotide polymorphisms (SNPs) arise from a point mutation and have two alleles. Most of the differences in the genomic DNA sequence between any two people are common, with a population frequency above 5%. At an estimated 7 million, common SNPs are the most abundant DNA variation in the human genome (Hinds et al., 2005;Kruglyak & Nickerson 2001) and are the most commonly used form of genetic variation for linkage and association analysis (Neale et al., 2008) - these are the two major analytic methods available for mapping genes involved in susceptibility to disease and traits (Dick 2008).

Mendel's experiments led to the publication of the laws of heredity in 1865 (Dunn 2003). Milestones set over the decades that followed are listed in Table 1.1, which gives a brief chronological overview of gene mapping research.

The discovery of linkage occurred in 1910 (Morgan 1910); only later, the detailed genetic map of *Drosophila* became available; in humans, the first genetic markers available were the blood groups; autosomal linkage in humans was first reported in 1955

(Renwick & Lawler 1955); electrophoretic protein markers became available in the 1950s; the HLA region and restriction fragment length polymorphisms (RFLP) became available for gene mapping in the 1970s. Until the late 1980s, searching for genetic causes of disease required time-consuming linkage analysis, using the existing limited sets of genetic markers in families with Mendelian diseases.

In 1989, microsatellite markers were first described (Litt & Luty 1989;Weber & May 1989). At the end of the 1980s, the Human Genome Project was launched, aiming to map all human genes and sequence all human DNA. This led to substantial technologic developments, tools and strategies to more effectively trace genetic causes of disease (Daiger 2005). In the early 1990s, with the availability of SNPs, there was, for the first time, a set of markers sufficiently numerous and spaced across the entire genome for effective gene mapping (Strachan and Read 1999). By the early 1990s, the technological breakthroughs in SNP genotyping platforms, data storage and statistical methodology, allowed the adaptation of linkage methodology to the whole genome in large collection of families phenotyped for “complex” traits and also led to substantial advances in association analysis studies.

Association analysis became in recent years a method of choice to identify common genetic variants contributing to susceptibility to common diseases (Martin, 2008). The feasibility of genotyping individual samples at hundreds of thousands of SNPs across the genome, has allowed the testing of markers across the whole genome for association and made possible genome-wide methods to control for population stratification, previously a major methodological hurdle.

Since 2005, many successes have been achieved using genome-wide association studies (GWAS) in complex diseases, starting with age-related macular degeneration (Klein et al., 2005), inflammatory bowel disease (Duerr et al., 2006) and type 2 diabetes (Sladek et al., 2007;Wellcome Trust Case Control Consortium 2007). Currently the success stories encompass hundreds of complex diseases (Fig. 1.1), including neuropsychiatric disorders, such as bipolar disease (Ferreira et al., 2008;Wellcome Trust Case Control Consortium 2007), Parkinson's disease (Simon-Sanchez et al., 2009) and multiple sclerosis (Jakkula et al., 2010). The results of published GWAS are regularly compiled in the *NHGRI Catalog of published GWAS*, summarized in graphical form in Fig. 1.1.

High density SNP arrays also allowed the detection of copy number variation (CNV), including deletions and duplications, which are rare genetic variants that have proven relevant to explain part of the genetic variance of many complex traits (Martin 2008).

The more traditional methods of linkage and association are now complemented by new molecular tools, including expression studies, methylation array studies (Martin 2008) and next-generation sequencing methodologies (Do et al., 2012).

Year	What	Human genetic markers (n of loci)	Reference / Author
1865	Laws of heredity	-	Mendel
1822-1911	Biometrics (statistical approach to heredity)	-	Galton
1910	Discovery of linkage	-	(Morgan 1910)
1918	Polygenic inheritance	-	(Fisher 1918)
1921	Detailed genetic map of *Drosophila melanogaster*	-	(Bridges 1921)
1950-1960	Blood groups as human genetic markers	~20	(Lawler & Renwick 1959)
1955	Autosomal linkage first reported in humans	-	(Renwick and Lawler 1955)
1960-1975	Electrophoretic mobility variants of serum proteins	~30	(Lewontin 1991)
1970	HLA tissue types	one haplotype (6p21.3)	(McDevitt & Bodmer 1974)
1979	DNA RFLPs	$>10^5$	(Botstein et al., 1980)
1985	DNA minisatellite VNTRs	$>10^4$	(Nakamura et al., 1987)
1987	Genetic linkage map of the human genome (using RFLPs)	-	(Donis-Keller et al., 1987)
1988	Start of the Human Genome Project	-	(McKusick 1989)
1989	DNA microsatellite markers (di-, tri- and tetranucleotide repeats)	$>10^5$	(Litt and Luty 1989;Weber and May 1989)

1990	DNA SNPs	$>10^6$	-
2000	Completion of the human genome sequence		(International Human Genome Sequencing Consortium 2004)
2002-2005	HapMap project phase I completed: ~1 million SNPs, populations (YRI) Yoruba, Ibadan, Nigeria; (CEU) Utah, USA; (CHB) Han Chinese, Beijing; and (JPT) Japanese, Tokyo.		(International HapMap Consortium 2005)
2000s-	High-density SNP genotyping		-
2005	First success of GWAS in "complex" diseases	-	Age-related macular degeneration (Klein et al., 2005); inflammatory bowel disease (Duerr et al., 2006)
2007	HapMap project phase II completed: ~4.6 milion SNPs		(Frazer et al., 2007)
2007	Copy number variation		(McCarroll & Altshuler 2007)
2010	"1000 genomes" project, pilot data		(1000 Genomes Project Consortium 2010)
2011-	Next-generation sequencing		(Do et al., 2012)
2012	"1000 genomes" project, interim data	$\sim 40x10^6$	(Abecasis et al., 2012)

Table 1.1 Chronology of genetic epidemiology studies and gene mapping of traits and diseases.

Abbreviations: RFLPs, restriction fragment length polymorphisms; SNPs, single nucleotide polymorphisms; VNTRs, variable number of tandem repeats. Sources: (Martin 2008;Strachan and Read 1999).

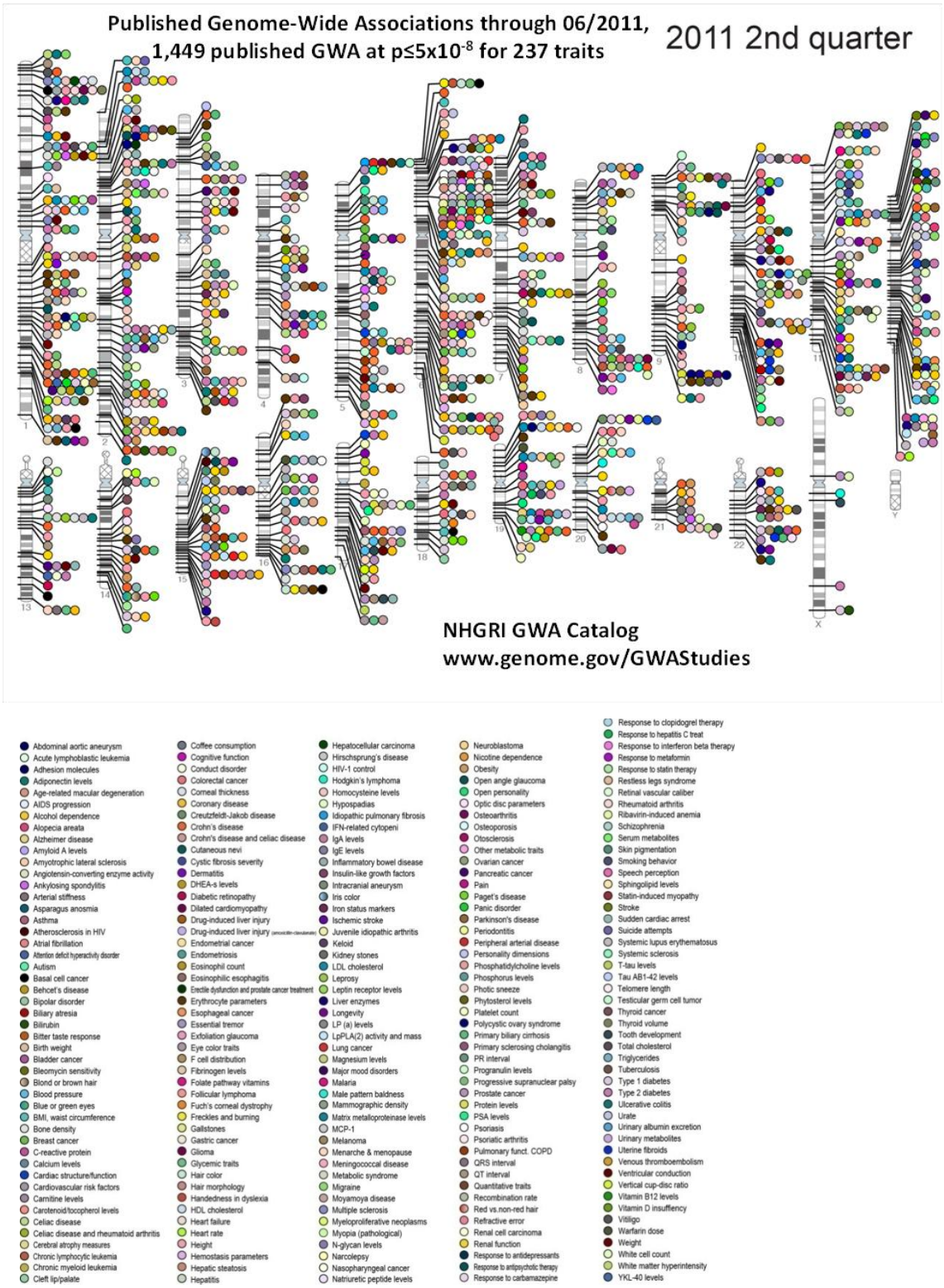

Figure 1.1 Published genome-wide association studies on the online NHGRI GWA Catalog until June 2011. Each colour represents one particular complex disease or trait (colour code explained below).

Source: Online NHGRI GWA Catalog, Hindorff et al., accessed 23/April/2012. Courtesy: National Human Genome Research Institute; http://www.genome.gov/gwastudies.

1.2.1 Family-based genetic studies

1.2.1.1 Segregation analysis

Segregation analysis is performed after familial aggregation of a disease has been established, with the goal of investigating if the patterns of disease observed in families are compatible with a known form of genetic transmission: autosomal dominant, autosomal recessive, X-linked, polygenic, or "complex". Heritability of traits or diseases can be estimated as part of segregation analysis (Strachan and Read 1999).

1.2.1.2 Twin studies

Twin studies can help to demonstrate that genetic factors are important in a disease. Information is collected on sets of monozygotic (MZ) twins and dizygotic (DZ) twins and the concordance rate is compared between the two groups. Higher concordance rates in MZ twins, significantly different from the concordance rates in DZ twins, demonstrate that genetic factors are important for disease susceptibility.

1.2.1.3 Adoption studies

In adoption studies, a comparison is made between adopted children and matched children regarding the phenotype under study, thus looking at the contribution of environmental and genetic factors to susceptibility of disease.

1.2.1.4 Linkage studies

Linkage studies are an important tool to map genetic loci and have been successful in mapping mutations responsible for hundreds of Mendelian disorders and more recently genome-wide linkage studies are used to map susceptibility loci for “complex” disorders (Martin 2008).

Linkage analysis allows the localization of regions of the genome likely to harbour disease loci,with the relative positions of two or more loci inferred by patterns of allele-sharing by relatives (Terwilliger & Ott 1994). After finding a locus linked to the disease or trait, complementary approaches are necessary to identify the causal genetic variants, such as candidate gene studies and fine mapping (Martin 2008).

Parametric linkage analysis tests whether the recombination fraction between two genetic markers, or a genetic marker and a Mendelian disease locus, is different from 0.5, which is the recombination fraction expected between two loci that segregate independently (Terwilliger and Ott 1994). Multipoint analysis is more efficient and preferable to single-point analysis (Strachan and Read 1999).

Linkage analysis methods have been adapted to also analyse common “complex” diseases and traits. As the inheritance model is often unknown, non-parametric linkage is preferred, which makes no assumptions of mode of inheritance, as analysis under the wrong model would lead to loss of power and biased estimates.

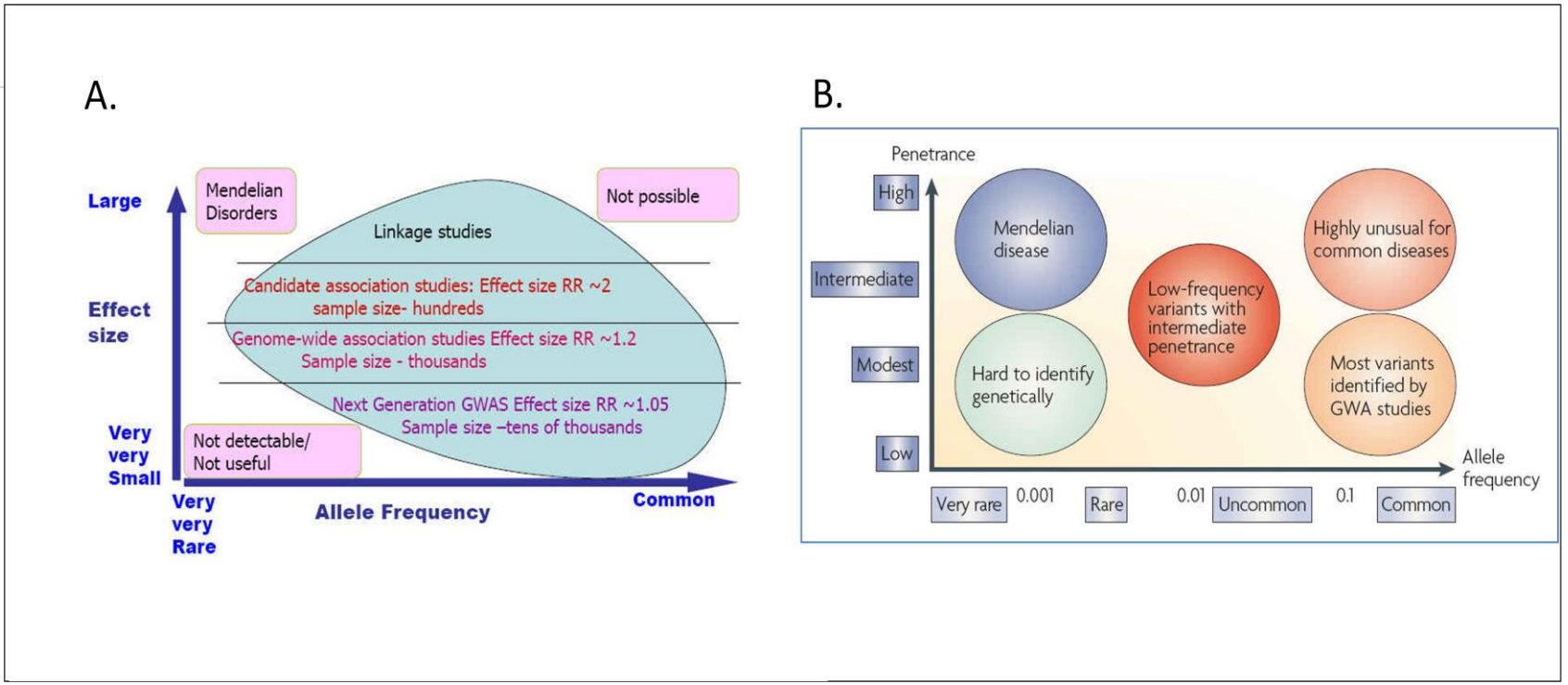

Figure 1.2 Schematic representation of the different types of genetic study designs for studying "Mendelian" and "complex" disorders.
In (A), the blue shaded area represents the currently detectable spectrum of "complex" genetic diseases (Martin, 2009).

(B) Reprinted by permission from Macmillan Publishers Ltd: Nature Review Genetics (McCarthy, M. I. et al., "Genome-wide association studies for complex traits: consensus, uncertainty and challenges", vol. 9, no. 5, pp. 356-369), copyright (2008) (McCarthy et al., 2008).

1.2.2 Population-based genetic association studies

The goal of the genetic association studies is to identify genetic variation contributing to susceptibility to disease or quantitative trait (Martin 2008), by testing for statistical relation between genetic variants and phenotypic variation - disease or trait (Hattersley & McCarthy 2005).

Association studies primarily target common genetic variation, with estimated low conferred risk (odds ratio lower than 1.4), while linkage studies target rarer genetic variation conferring higher risk of disease (Fig. 1.2). Association analysis is different from linkage, correlations are tested independently of ancestral considerations and the statistical methods used are simpler and more powerful (Risch & Merikangas 1996).

The availability of a wide catalogue of DNA sequence variants across the genome that can be used as genetic markers and steady decrease in cost and time of genotyping (Palmer & Cardon 2005), contribute for association methods having become increasingly popular. Careful study design and choice of optimal statistical methods are determinant for the outcome of any association study (Martin 2008).

1.2.2.1 Candidate gene association studies

Population-based genetic association studies were, before the GWAS era, mostly candidate gene studies, where one or a few genetic variants were analysed for association with a disease or trait of interest, based on *a priori* plausibility, as given by biological data, pathophysiological knowledge, animal studies.

There were some successes with the candidate-gene approach, but most of these studies were underpowered, with too small sample sizes. A major challenge presented by

this type of study was to distinguish biologically meaningful relationships from the spurious; this would depend on replication, presence of biological plausibility, among other factors (Tan et al., 2004b). Confounding (e.g. because of population stratification), genotype errors or chance (type I errors arising from multiple testing) could be responsible for a false positive signal of association between a genetic trait or disease and a genetic locus. Other potential problems included misclassification and genetic heterogeneity.

Replication was mandatory to confirm validity of these candidate gene studies and avoid exploring false positives (Ott 2004;Sisodiya et al., 2005). However, there were many results reported that no one could replicate (Martin, 2008), which led to skepticism about this study design.

An interesting recent study investigated whether the thousands of candidate genetic association studies performed in the pre-GWAS era had found any reliable associations for common diseases and phenotypes, by systematically evaluating whether loci proposed as harbouring candidate associations before the advent of GWAS were replicated by GWA studies (Siontis et al., 2010). The authors screened data from GWA studies included in the NHGRI catalog and published before August 2008 (159 articles) and selected variants in candidate loci on the basis of statistical significance (p-value < 0.05), to create a list of independent, non-redundant associations. Only a few of the numerous genetic associations proposed in the candidate gene era have been replicated in GWA studies, but the ones that were conclusively replicated may have large genetic effects (Siontis et al., 2010).

1.2.2.2 Genome-wide association studies

Genome-wide association studies (GWAS; also called whole-genome association studies) comprehensively survey common genetic variants throughout the whole genome, looking for a significant association to common "complex" diseases or traits (Amos 2007;McCarthy et al., 2008;Neale and Purcell 2008;Pearson & Manolio 2008;Teo 2008;Ziegler et al., 2008;Zondervan & Cardon 2007).

GWAS have been very successful in identifying genetic variants associated with a number of "complex" diseases, which in some cases led to insights into genetic architecture and novel pathophysiological pathways (Kraja et al., 2011;Lango Allen et al., 2010;Soranzo et al., 2010). For example, in Crohn's disease, GWAS results highlighted the importance of autophagy and innate immunity as determinants of dysregulated host-bacterial interactions in the pathogenesis of disease (Barrett et al., 2008) and provided novel insights at the genomic level (McCarroll et al., 2008).

One major advantage of the GWA design is that it does not require *a priori* knowledge of pathogenesis, pathways or candidate genes. This important feature distinguishes it from the candidate-gene association studies. Possible limitations include the modest effect sizes of the common genetic susceptibility variants and need for stringent statistical thresholds (Zeggini et al., 2008). Studying a "complex" disorder with GWAS usually requires large numbers of cases and controls (in the order of thousands to hundreds of thousands), necessitating collaboration between groups, combining data across studies and performing multistage analyses (Amos 2007). Strategies to increase power to detect smaller effect loci include international collaborative consortia, thereby

increasing sample size; and also extending SNP coverage, for example through imputation of untyped SNPs.

GWA studies have been possible since 2005, because of several breakthroughs, which occurred in the previous years. Large-scale genomics projects paved the way, by cataloguing and understanding genetic variation (Chanock et al., 2007;Neale and Purcell 2008): the completion of the sequencing of the human genome in 2004 (International Human Genome Sequencing Consortium 2004); the first HapMap map of common genetic variation in man (map of SNP and haplotype data) in 2005 (International HapMap Consortium 2005). Also instrumental was the ever more efficient and affordable technology, with high throughput genotyping chips each containing hundreds of thousands to over one million markers; the development of appropriate statistical strategies and software to circumvent the unique problems of genetic epidemiology; and bioinformatics solutions for storage and flow of the large amount of data generated in the process.

In a GWA study, the genome is interrogated using high-throughput whole-genome genotyping platforms and commercially available chips. Recently, the NCBI database of SNPs, "*db SNP"* build 135, contained 30,443,455 SNPs (www.ncbi.nlm.nih.gov/projects/SNP). It is possible, however, to interrogate only a part of the large total number of SNPs and still capture most of the information on the whole genome, with 500,000 SNPs estimated to provide adequate coverage.

This important fact relates to a property of the genome called linkage disequilibrium (LD), which means that an individual carrying a SNP allele at one locus

often predictably also carries specific alleles at nearby loci. The specific combination of alleles along a chromosome is called a haplotype and the correlation between them is called LD (International HapMap Consortium 2005). In simple terms, this means the genome has a block-like structure and when two markers are in LD (r^2>0.8), one of them captures all the information provided by the two markers. Only the regions of low LD, the so-called hotspots of recombination, require more markers for a less than optimal coverage.

LD exists due to shared ancestry of the contemporary chromosomes. When mutation occurs leading to a causal variant, it is initially "connected" to a unique chromosome surrounded by a unique combination of genetic variants. Crossing-over, recombinations and mutations will act to erode this association over the subsequent generations, at an average rate of 10^{-8} per base pair per generation (International HapMap Consortium 2005).

The two commonly used disequilibrium parameters, which are measures of extent of linkage disequilibrium, are r^2 and |D'|. Let us consider a locus with one rare allele (rare allele C, common allele c) and any other locus (minor allele M, major allele m). Both measures of LD scale the covariance between the loci, $D = p_{CM} - p_C p_M$, but in different ways. $r^2 = D^2 / (p_C p_M (1 - p_C)(1 - p_M))$, so r is the correlation between the two loci, which scales D by the standard deviation of allelic frequency at the two loci. The value of r^2 depends on the allele frequency difference between the two loci (Wray et al., 2011).

Study design of GWA studies

One important factor on which the success of GWA studies depends is study design. This includes the selection of adequate traits to study, careful selection of populations for the study and collaborative approaches (Amos 2007).

GWA studies may have different study designs, presented in Table 1.2. Most frequently used is the case-control design using population controls. This is the study design selected for the GWA studies described in this thesis. Advantages in relation to the cohort or the trio designs include being easier to conduct, less expensive, more efficient.

Other possible GWA study designs are the cohort design and the trio design with family-based controls (Pearson and Manolio 2008). Cohort studies can be underpowered for dichotomous phenotypes, given the limited number of cases for any given disease and therefore meta-analysis is frequently used to overcome the sample size limitations. For a wide range of quantitative traits, longitudinal measures are available, making cohort studies ideal to look for the associated genetic variants, but also into joint effects of genes and environment (McCarthy et al., 2008).

In the family-based trio design of GWA studies, the affected case and both of his/ her parents are included in the study. Only the offspring is classified according to affected status, only affected offspring are included and genotyping is performed in both affected case and parents. The frequency with which an allele is transmitted from heterozygous parents to affected offspring is estimated: under the null hypothesis of no association, the transmission frequency for each allele of a SNP is 50%, while alleles associated with the disease will be transmitted in excess to the affected case (Table 1.2;Pearson and Manolio 2008).

Case-control GWA studies

In a case-control GWA study, the allelic and genotypic frequencies are compared between cases with the disease or trait of interest (presumed to have a high prevalence of susceptibility alleles for that trait) and controls (considered likely to have a lower prevalence of such alleles) (McCarthy et al., 2008).

There are four major parts in a GWA case-control study (Fig. 1.3). First, selection of cases with the trait or disease of interest and suitable controls; second, genotyping with appropriate quality control measures; third, statistical tests for association between the SNPs which passed the quality control steps and the disease or trait; and finally, follow-up of any significant signals, looking for replication of any identified association in an independent population sample and functional studies (Pearson and Manolio 2008).

Assumptions are made that cases and controls are drawn from the same population; that cases are representative of all disease cases; that data (both genotyping and clinical and epidemiologic data) are collected in a similar manner for cases and controls; and that differences in allele frequencies between cases and controls are due to case/control status and not to hidden population substructure or other factors (Amos 2007).

Potential issues are the need to identify and correct for population stratification, which could bias the results; possible overestimation of the relative risk; and case selection may exclude the ends of the spectrum of severity of the disease in question (Amos 2007).

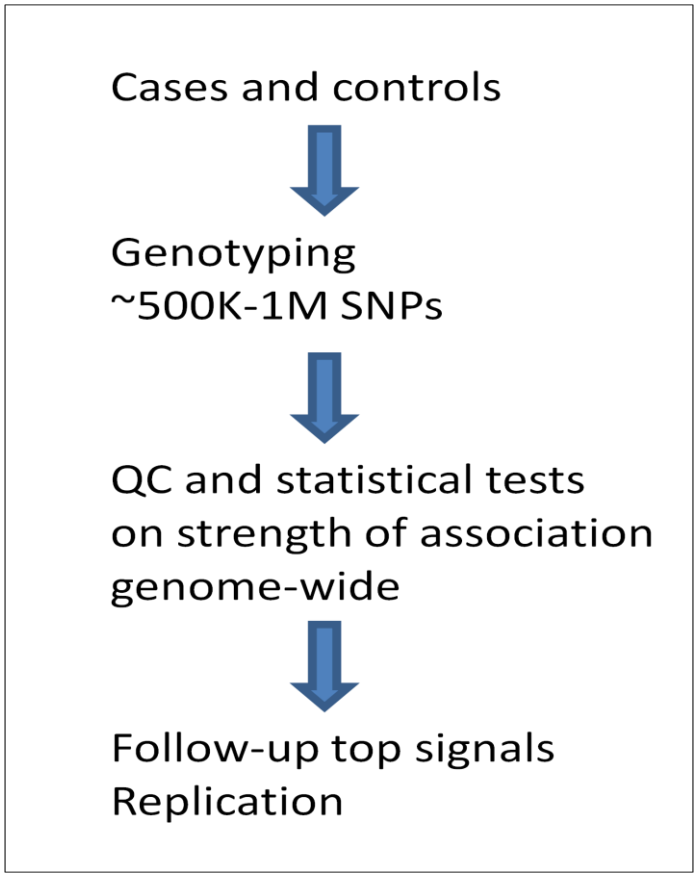

Figure 1.3 Flowchart of the basic principles of case-control genome-wide association studies.

Abbreviations: QC, quality control.

Design of GWA study	Principles	Advantages	Disadvantages
Case-control	Allele frequencies compared between patients with the disease and disease-free controls.	Shorter timeframe. Easier to conduct. Less expensive. More efficient: large numbers possible. Design of choice; may be only possibility for the study of rare diseases.	More assumptions needed, with danger of biases and spurious associations, if the assumptions are not met; - Usually prevalent cases and selected; may be unrepresentative: e.g. hospital sample may miss the extremes of severity of the disease; - comparability between cases and controls is required; - prone to population stratification. Overestimate RR for common diseases.
Cohort	Large number of individuals observed over time after baseline information collection to assess incidence of disease in subgroups defined by genetic variants.	Incident cases. Fewer biases than case-control studies. Longitudinal health-related measures for a wide range of quantitative traits, lifestyle and exposure data available; may allow evaluation of joint effects of genes and environment (McCarthy et al., 2008).	Typically underpowered for dichotomous phenotypes, given limited cases for any given disease, with GWA data meta-analysis as a solution to overcome sample size restrictions (McCarthy et al., 2008). Expensive; longer follow-up.
Trio	A trio includes affected cases and their parents. Under H_0 of no association, transmission frequency of a SNP is 50%; if there is association between SNP and disease, those alleles will be transmitted in excess of 50% to the affected cases from heterozygous parents.	Not susceptible to population stratification. Does not require phenotyping of parents.	Greater sensitivity to genotyping error: need for more stringent standards of genotyping quality. Logistically more difficult for disorders with older ages of onset.

Table 1.2 Types of genome-wide association studies.

Abbreviations: GWA, genome-wide association; H_0, null hypothesis; RR, relative risk.
In: (Pearson and Manolio 2008).

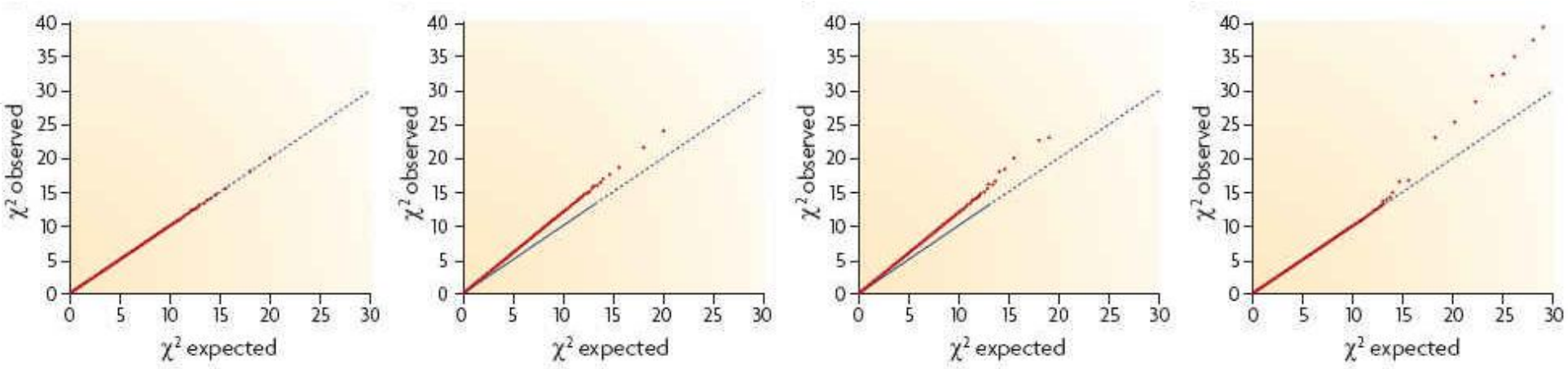

Figure 1.4 Graphs for visualisation of findings in GWA studies: examples of quantile-quantile (Q-Q) plots.

After association analysis, it is critical to test the genome-wide distribution of the test statistic (log-transformed *p* from logistic trend tests) in relation to the expected null distribution. Q-Q plots can mark deviations of the observed distribution from the expected null distribution. True associations show themselves as prominent departures from the null in the extreme tail of the distribution (Barrett et al., 2008;de Bakker et al., 2008).

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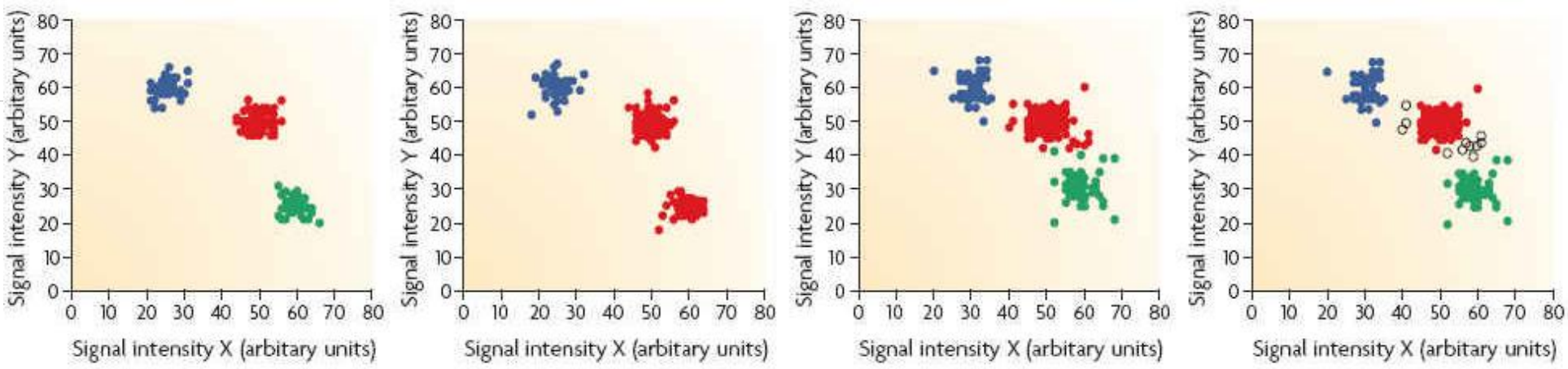

Figure 1.5 Graphs for visualisation of findings in GWA studies: example of signal intensity (cluster) plots. The genotyping raw data are plotted along two axes, one for each allele, defining for each SNP clusters of data corresponding to the three genotype groups, depicted with different colours (blue, AA; red, Aa; green, aa).

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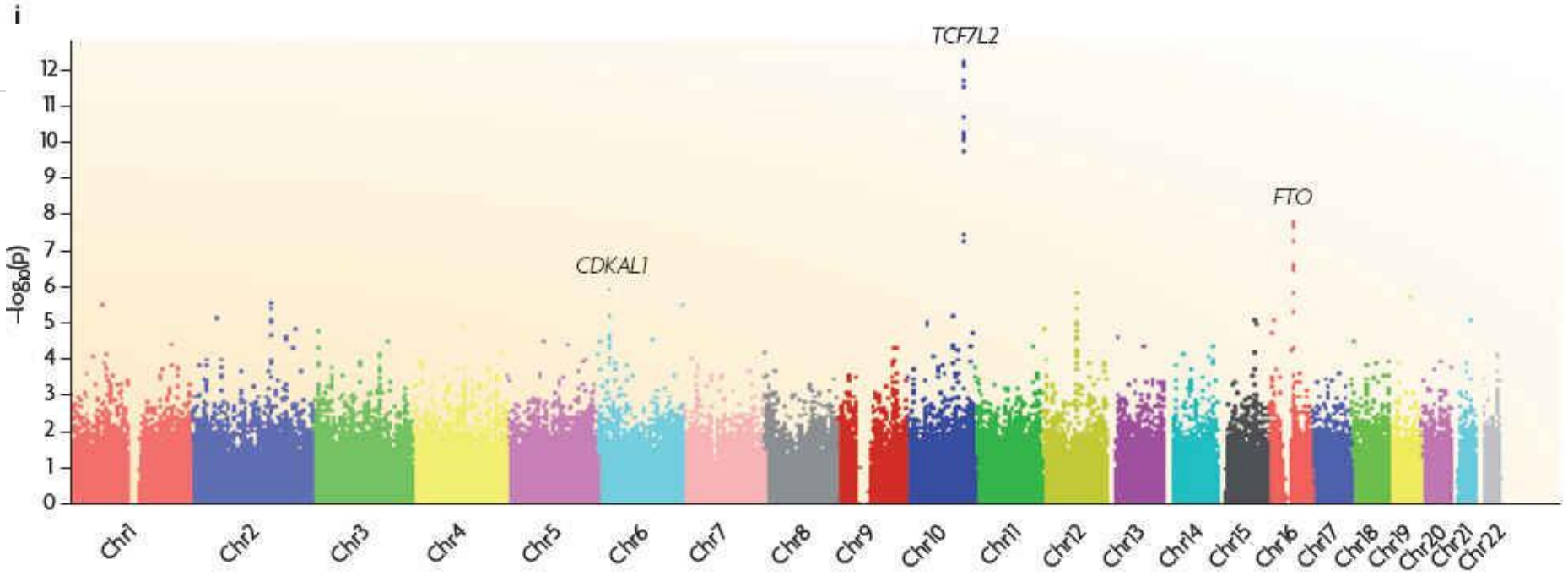

Figure 1.6 Graphs for visualisation of findings in GWA studies: example of a Manhattan plot, which displays the $-\log_{10}$(*p*-value) for every SNP tested in the GWA study, relative to its genomic position in the corresponding chromosome. Each coloured circle represents one SNP, with different colours for each chromosome. This example plots the results of a GWA study of type 2 diabetes (McCarthy et al., 2008).

1.3 Genetic epidemiology of the epilepsies

There are strong arguments making genetics of epilepsy worth researching and indeed one of the priorities in epilepsy research today, as recently recognized in an European statement on research priorities in epilepsy (Baulac & Pitkanen 2008).

Unravelling which genetic variants contribute to increased susceptibility to the epilepsies, both to the common epilepsies and the rare familial epilepsies, is a difficult task, but offers many opportunities. More knowledge on the underlying genetic architecture of the epilepsies is expected to contribute new insights on the pathogenic pathways involved, including mechanistic insights. Better knowledge of the underlying pathophysiology may lead to development of new therapeutic targets and new strategies for pharmaceutical development, to the identification of modifiable non-genetic exposures in the pathogenic pathways, to improvements in predictive models of disease risk (Ioannidis et al., 2009) and improvements in prognosis for many patients.

Genetic epidemiological studies have demonstrated that genetic factors play an important role in the susceptibility to epilepsy. Several genes are already known to contribute to the "familial" epilepsies, but genetic determinants of the common, "sporadic" epilepsies are less well known.

Several lines of evidence suggest that common epilepsies follow "complex" inheritance patterns,which means they are likely the result of multiple genetic factors, with an environmental contribution. Many different genes of relatively small individual effect may contribute to disease susceptibility, or possibly a few genes of large effect, or

both. Population-based genomic studies, using careful phenotyping, are expected to shed some light on the genetic contribution to the common epilepsies.

1.3.1 Evidence for a genetic contribution in epilepsy

From the first half of the twentieth century, family and twin studies of epilepsy indicated the importance of heritable factors in epilepsy.

Familial aggregation studies in epilepsy have provided evidence for the genetic contribution to the risk of epilepsy, by showing that a family history of seizures or febrile seizures is associated with an increased risk of seizures (Ottman et al., 1996a;Ottman et al., 1996b;Ottman 1997a;Ottman et al., 1997b).

A classic study by Lennox (1951), which investigated 4,231 people with epilepsy and 20,000 relatives, was able to show increased familial predisposition for near relatives of people with epilepsy compared to the general population. This was proven not only for the “essential”/ “idiopathic” epilepsies, but also for the “symptomatic” epilepsies, even if higher prevalence of epilepsy was found among close relatives of the “idiopathic” group compared to the “symptomatic” group. Lennox could show that both genetic and environmental factors contribute to epilepsy susceptibility (Lennox 1951;Lennox 1960;Lennox & Jolly 1954).

Fig 1.7 features the Falconer’s polygenic threshold model, which extended Fisher’s polygenic model, to cover dichotomous non-Mendelian traits also. Liability is a theoretical concept, which can be explained as follows: assuming a condition is polygenic and normally distributed, people are affected when the liability is above a certain

threshold value. Siblings of affected people will have a higher average liability than the population mean, with a greater proportion having liability which exceeds the threshold and therefore being affected (Strachan and Read 1999).

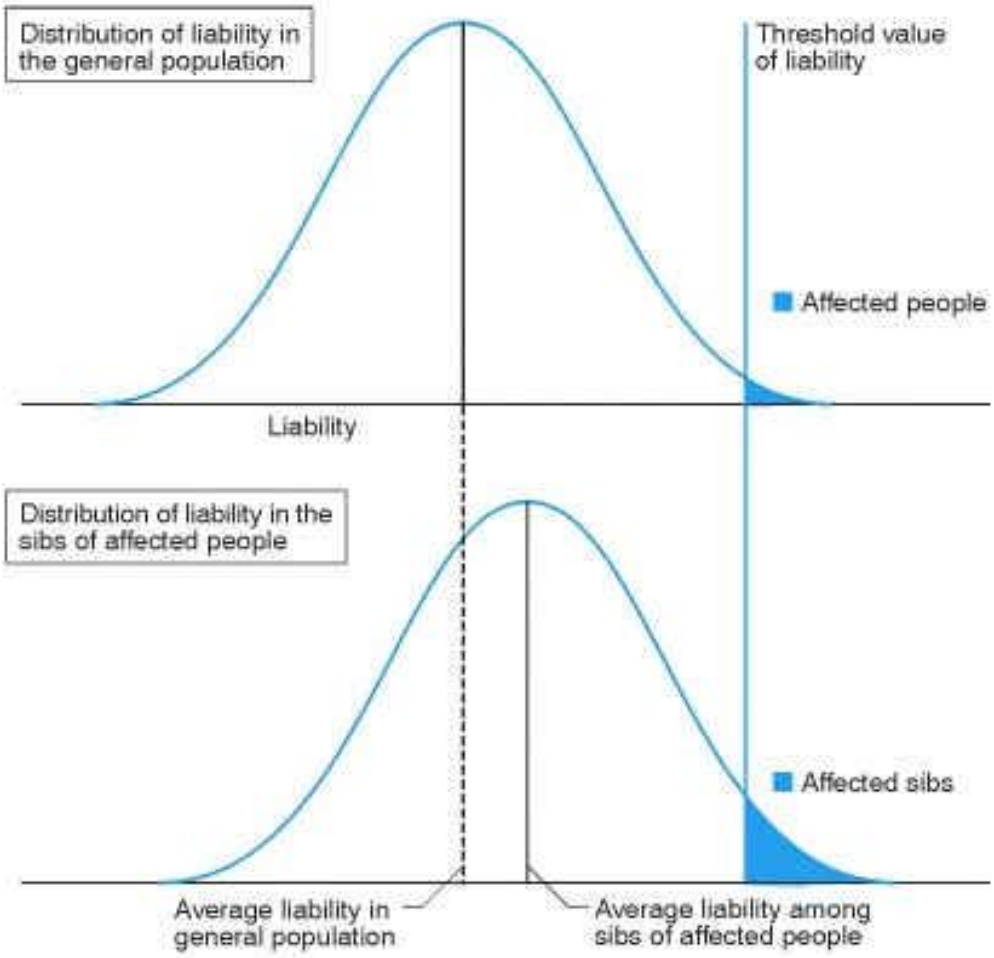

Figure 1.7 Falconer's polygenic threshold model of inheritance for dichotomous non-mendelian traits.

In: Figure 19.3/ page 451, Human molecular genetics, 2nd edn, Strachan, T. & Read, A., Bios Scientific Publishers Ltd, Oxford, Taylor & Francis Group, 1999 (Strachan and Read 1999).

Year	Milestone in epilepsy genetics
400BC	Hippocrates wrote the first book on epilepsy, “On the sacred disease”, stating that it is a disease, which originates in the brain and has a hereditary component.
1951	Lennox, evidence for increased familial predisposition in epilepsy.
1959-61	First twin studies in epilepsy showing contribution of genetic and environmental factors in epilepsy.
1970s onwards	Definition of specific epilepsy syndromes based on clinical, electroencephalographic and imaging findings.
1988	First positive linkage study in epilepsy, suggesting linkage of chromosome 6p region to JME.
1994-1996	Description of pedigrees with familial TLE and description of ADNFLE.
1995	Identification of the first gene for monogenic idiopathic epilepsy: *CHRNA4* in ADNFLE.
1998-2004	Recent twin studies in epilepsy confirm genetic and environmental factors contribute in varying degrees for different epilepsy syndromes.
1998 onwards	More than a dozen genes identified in different familial epilepsy syndromes.
2000	*SCN1A* gene mutations identified in GEFS+.

Table 1.3 Timeline with selected milestones in epilepsy genetics.

Abbreviations: ADNFLE, autosomal dominant nocturnal frontal lobe epilepsy; GEFS+, genetic epilepsy with febrile seizures plus; JME, juvenile myoclonic epilepsy; TLE, temporal lobe epilepsy.

1.3.1.1 Segregation studies

As reviewed by Andermann (2009), the early segregation studies in epilepsy led to the mode of inheritance being hypothesised differently by different authors, from autosomal recessive (Davenport & Weeks 1911), autosomal dominant with modifier genes (Alstrom 1950), polygenic (Conrad 1935a;Conrad 1935b) and multifactorial with contribution of several genes and environmental factors (Andermann 2009;Brain 1926).

These early studies had design flaws, including not taking into account the number of affected relatives or the degree of relationship (Brain 1926;Davenport and Weeks 1911); including other neuropsychiatric disorders in the inclusion criteria for affected relatives; or not considering any control group.

A wide spectrum of variation was found on the estimates of heritability for epilepsy. Reasons may include the fact that epilepsy is a heterogeneous group of disorders, which may have been "lumped" together for these studies, using classifications that evolve in time and investigations that also change with time.

1.3.1.2 Twin studies

Twin studies are a powerful tool to analyse the genetics of the "complex" disorders.

In epilepsy, twin studies suggest that genetic factors are important, with concordance rates in MZ twins consistently higher than in DZ twins (Table 1.4). Diverse methodology has been used in different twin studies, but the more recent studies, including the important work of Berkovic and colleagues (1998), yielded results consistent with the earlier twin studies, concluding that genetic factors influence susceptibility to epilepsy, both for the generalised and partial epilepsies; and not only for

"idiopathic" epilepsies, but also for "symptomatic" epilepsies; and the febrile seizures phenotypes.

Historically, partial epilepsies had been considered as symptomatic or probably symptomatic and perceived as mainly non-genetic. Clinical epidemiological studies and animal studies provided data supporting the importance of genetic factors, even for the "symptomatic" partial epilepsies. Animal model data show there is variation in seizure susceptibility following physical stimuli between different mouse strains, which supports a role for underlying genetic factors (Berkovic et al., 2006b;Frankel et al., 2001), while a recent population-based cohort study of post-traumatic epilepsy showed that patients with family history of epilepsy have a significantly higher long-term risk of epilepsy after mild brain injury (RR 5.75; 95%CI 4.56 to 7.27), or severe brain injury (RR 10.9; 95%CI 4.20 to 24.26) (Christensen et al., 2009). Identifying genetic variants responsible for this differential susceptibility will require further research.

Twin studies have shown different concordance rates in MZ and DZ twins as a function of the type of epilepsy syndrome (Table 1.4). For all epilepsy, concordance rates were 50-60% in MZ twins and 15% in DZ twins; for generalised epilepsies, 65-82% (MZ) and 12-27% (DZ); and for focal epilepsies, 9-36% (MZ) and 5-10% (DZ) (Berkovic et al., 1998). Further, 94% of MZ twins who showed concordance and 71% of concordant DZ twins, had the same type of epilepsy syndrome (Berkovic et al., 1998). The high frequency of concordant MZ twins with the same epilepsy syndrome strongly suggests differential genetic influences over different specific epilepsy syndromes, that is, syndrome-specific genetic determinants (Berkovic et al., 1998;Pandolfo 2011).

Not only have recent twin studies confirmed the evidence for a strong contribution of genetic factors for seizures overall, but also for epilepsy and febrile seizures and also for partial epilepsy and generalised epilepsy (Corey et al., 2011). The authors remark, however, that the proportion of twin pairs concordant for syndrome type decreased as the major syndrome type was increasingly subdivided. The contribution of genetic factors for subdivisions of partial epilepsies – frontal, temporal and occipital epilepsy – was found to be modest, but this study does not take into account specific well-defined syndromes within the partial epilepsies, such as MTLEHS and instead, the authors have possibly "lumped" together several syndromes by using the wider "anatomical" classification of partial epilepsies included in the 1989 ILAE classification (ILAE Commission on Classification and Terminology 1989). The authors acknowledged that the sample was not large enough for subgroup analysis of different types of partial epilepsy (Corey et al., 2011).

Ottman and colleagues looked at concordance for epilepsy phenotype within families. In idiopathic generalized epilepsy (IGE), they found significant clinical heterogeneity within families, with only one third of relatives sharing the same epilepsy syndrome (Kinirons et al., 2008). In previous studies, they had also concluded that genetic factors predispose to epilepsy risk, but suggested that epilepsy syndrome – both for the epilepsy syndrome within the IGEs and the differentiation between generalised and partial epilepsies, - may be determined by other genetic variants, epigenetic factors, environmental factors and interactions between them (Winawer et al., 2003a;Winawer et al., 2003b;Winawer et al., 2005).

First author, year	Type of study	Type of epilepsy	Number of twin pairs	MZ concordance rate (%)	DZ concordance rate (%)
(Lennox 1960)	Clinical series	All types	225	62	15
		"Grand mal"	103	82	15
		"Petit mal"	24	7	0
		"Psychomotor"	42	39	5
		"Focal with lesion"	104	27	13
(Inouye 1960)	Clinical series	Chronic epilepsy	40	54	7
(Harvald & Hauge 1965)	Population-based (Denmark)	Epilepsy	NA	37	10
(Corey et al., 1991)	Population-based (USA-Virginia, Norway)	Epilepsy	280	19	7
(Sillanpaa et al., 1991)	Population-based (Finland)	Epilepsy	188	10	5
(Berkovic et al., 1998)	Clinical series (Australia)	Epilepsy	225	62	18
		Generalised epilepsy	59	82	26
		Partial epilepsy	65	36	5
(Jackson et al., 1998)	Clinical series (Australia)	TLE with HS and prolonged FS in early childhood	3	FS 100	NA
				TLE 0	
				HS 0	
(Miller et al., 1999)	Population-based (US,Virginia)	All types	235	30	13
(Corey et al., 2011)	Population-based (USA, Norway and Denmark)	Epilepsy	598	39	7
		Generalised	153	64	9
		Partial	341	21	4

Table 1.4 Twin studies of the epilepsies.

Abbreviations: FS, febrile seizures; HS, hippocampal sclerosis; NA, not applicable; TLE, temporal lobe epilepsy.

A genetic classification of the epilepsies has been proposed by Andermann (2009), based on the known genetic epilepsy syndromes and corresponding modes of inheritance. The proposed categories are shown below.

a. Monogenic or Mendelian epilepsy syndromes;

b. Single gene disorders associated with epilepsy;

c. Mitochondrial disorders associated with epilepsy;

d. Chromosomal abnormalities associated with epilepsy;

e. "Complex" epilepsy syndromes;

f. Phenocopies.

1.3.2 Monogenic or mendelian epilepsy syndromes

"Monogenic" focal epilepsies

Most of the genetic defects identified thus far in human epilepsies encode for subunits of ion channels, which are critical for normal neuronal excitability. Other genes causing "monogenic" epilepsies have been identified that do not encode ion channels (Mulley et al., 2011b). Table 1.5 lists published "monogenic" partial epilepsy syndromes, including associated genetic loci and causal genes already identified.

The first gene for "monogenic" partial epilepsy was identified in 1995. The *CHRNA4* gene, coding for a neuronal nicotinic cholinergic receptor, was found to be responsible for a proportion of autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) (Steinlein et al., 1995), which had been described on that same year (Scheffer et al., 1995).

Several other familial partial epilepsy syndromes have since been described, including familial mesial temporal lobe epilepsy (FMTLE), autosomal dominant lateral temporal lobe epilepsy (ADLTLE) and familial partial epilepsy with variable foci (FPEVF). Linkage studies and positional cloning have successfully led to localization and subsequent discovery of several causal genes for these familial epilepsy syndromes (Table 1.5).

1.3.2.1 Autosomal dominant nocturnal frontal lobe epilepsy

ADNFLE is characterized by nocturnal seizures, which occur in clusters from sleep and are hypermotor, tonic or have dystonic features (Hayman et al., 1997;Scheffer et al., 1994;Scheffer et al., 1995). Seizure onset is often accompanied by autonomic manifestations (changes in heart rate, breathing, vasomotor tone). Seizures are brief and mostly occur during non-REM sleep. Most individuals retain awareness during seizures. Misdiagnosis was common, particularly with "paroxysmal nocturnal dystonia" (Lugaresi et al., 1986), parasomnias or psychogenic seizures (Lugaresi et al., 1986;Scheffer et al., 1994). Age at seizure onset is around 8–11 years and seizures may persist into adulthood. Neurological examination, brain imaging and interictal EEG are usually normal.

Mutations in *CHRNA4* on chromosome 20q13 can be found in ADNFLE (Steinlein et al., 1995). *CHRNA4* encodes the α4 subunit of the neuronal nicotinic acetylcholine receptor (nAChR), which is a pentameric ligand-gated ion channel distributed throughout the brain. This was the first gene to be linked with familial partial epilepsy.

This was followed by the identification in ADNFLE of *CHRNB2* mutations (De Fusco et al., 2000;Gambardella et al., 2000a). *CHRNB2* also encodes a subunit (β2) of the

neuronal nAChR. More than 100 families have been described, as have "sporadic" cases with de novo mutations.

CHRNA2 was the third neuronal cholinergic receptor gene to be associated with familial sleep-related partial epilepsy, in a clinical and genetic study of a large pedigree of familial epilepsy with nocturnal wandering, ictal fear and movements of the tongue (Aridon et al., 2006). Around 12% of people with ADNFLE are estimated to have mutations in the nAChR subunit genes (Combi et al., 2004).

1.3.2.2 Familial temporal lobe epilepsy

The first description of familial TLE was published by Berkovic and colleagues, almost two decades ago (Berkovic et al., 1994;Berkovic et al., 1996). Large pedigrees with several affected members with TLE have since been described (Cendes et al., 1998;Gambardella et al., 2000b;Santos et al., 2002).

Familial TLE is heterogeneous (Cendes et al., 1998;Santos et al., 2002) and includes distinct syndromes (Table 1.5): familial mesial TLE (FMTLE) (Crompton et al., 2010;Gambardella et al., 2009) and autosomal dominant lateral TLE (ADLTLE) (Winawer et al., 2000;Winawer et al., 2002) have been described. Familial partial epilepsy with variable foci (FPEVF) (Scheffer et al., 1998) may also include patients with TLE.

TLE has also been described in families with genetic epilepsy with febrile seizures plus (GEFS+) (Singh et al., 1999), including patients with *SCN1A* mutations (Abou-Khalil et al., 2001) and patients with *SCN1B* mutations (Scheffer et al., 2007).

Familial mesial temporal lobe epilepsy

Familial mesial TLE (FMTLE) was initially described in 1994. In the first reported families, most cases were "benign" and without MRI changes, with only one out of the 38 patients described having medically refractory TLE (Berkovic et al., 1994).

With the description of more families, phenotypical heterogeneity within and between families became apparent, with family members presenting different seizure types and degrees of severity. Although most were found to have a mild course, some were pharmacoresistant (Berkovic et al., 1996;Cendes et al., 1998;Crompton et al., 2010;Kobayashi et al., 2001).

A comparison between patients with familial MTLE and patients with sporadic MTLE, who underwent epilepsy surgery for refractory epilepsy, showed no significant differences in clinical features and surgical outcome (Andrade-Valenca et al., 2008;Kobayashi et al., 2003a). Surgery was shown to be appropriate for people with familial MTLE, when they are shown to be good surgical candidates (Kobayashi et al., 2003a).

In one study, HS was found on MRI in 34% of asymptomatic first-degree relatives in pedigrees of familial MTLE (Kobayashi et al., 2003b). This could also reflect the advances in MR imaging compared to earlier studies.

Two genetic loci have been linked to FMTLE: one on chromosome 4q (Hedera et al., 2007); and one on chromosome 12q (some also with FS) (Claes et al., 2004) (Table 1.5). So far, no causal gene has been identified for FMTLE. A recent linkage study

identified a locus on 18p11.31 to be associated with hippocampal abnormalities, in one familial MTLE pedigree (Maurer-Morelli et al., 2012).

Autosomal dominant lateral temporal lobe epilepsy

Patients with ADLTLE (or autosomal dominant partial epilepsy with auditory features, ADPEAF), have auditory auras (such as buzzing, humming, voices, music), but may have also "simple partial" seizures with aphasia, vertigo and visual phenomena, or "complex partial" seizures with or without secondary generalization. Age at seizure onset is usually the second or third decade of life (Winawer et al., 2000).

MRI is usually normal in ADLTLE, but one study showed MRI evidence of developmental abnormalities in the lateral cortex of the temporal lobes in a few patients (Kobayashi et al., 2003c).

ADLTLE was mapped to chromosome 10q in 1995 (Ottman et al., 1995) and the causative gene identified in 2002 as *LGI1* (leucine-rich glioma inactivated 1) (Kalachikov et al., 2002). Mutations in *LGI1* are, however, not found in all families with ADLTLE, providing evidence for genetic heterogeneity (Berkovic et al., 2004b) and no clinical feature has been identified that can predict which families with two or more members with TLE with auditory symptoms will have a *LGI1* mutation (Ottman et al., 2004).

Despite the name of the gene, *LGI1* mutations in families with ADLTLE have not been correlated with any significant change in rate of brain tumours or other malignancies (Brodtkorb et al., 2003). The Lgi-1 protein is a subunit of voltage-gated potassium channel K_v1.1-associated protein complexes (Schulte et al., 2006). Evidence for

pathogenicity include data from animal models, with deletion of the $K_v1.1$ potassium channel causing epilepsy in mice (Smart et al., 1998).

A novel *LGI1* mutation was recently described in one pedigree with familial TLE where two or the three affecteds had psychic auras, suggestive of mesial temporal onset (Striano et al., 2011). This mutation was shown not to hamper the protein secretion in vitro, contrary to the previous described *LGI1* mutations and the significance of this finding needs to be clarified.

Deletions may be found in genes where mutations had previously been found for "monogenic" epilepsy syndromes. *LGI1* deletions have also been found in ADLTLE (Fanciulli et al., 2012).

1.3.2.3 Familial partial epilepsy with variable foci

Familial partial epilepsy with variable foci (FPEVF) was first reported in an Australian family (Scheffer et al., 1998). In each affected individual, semiology and EEG seizure characteristics are consistent over time, but can vary between members of each family. Frontal lobe seizures are most common, but some patients have seizures of temporal or occipital onset. Age at onset is variable, peaking at 5 and 25 years. FPEVF was mapped to chromosome 22q (Xiong et al., 1999) and very recently, using whole-exome sequencing, mutations in *DEPDC5* were identified as a common cause of FPEVF (Dibbens et al., 2013).

	MIM	Locus	Gene	Protein function	References
ADNFLE	#600513 (type 1)	20q13.2-q13.3	*CHRNA4*	Nicotinic AChR subunit	(Scheffer et al., 1995;Steinlein et al., 1995)
	#605375 (type 3)	1q21	*CHRNB2*	Nicotinic AChR subunit	(De Fusco et al., 2000;Gambardella et al., 2000a)
	%603204 (type 4)	8p21.2	*CHRNA2*	Nicotinic AChR subunit	(Aridon et al., 2006)
	%603204 (type 2)	15q24	Not known	Not known	(Phillips et al., 1998)
ADLTLE or ADPEAF	#600512	10q24	*LGI1*	K_v1.1-associated protein complexes subunit	(Kalachikov et al., 2002;Morante-Redolat et al., 2002;Scheffer et al., 1998)
					microdeletions (Fanciulli et al., 2012)
FMTLE (Crompton et al., 2010;Striano et al., 2008)	%611630	4q13.2-q21.3	Not known	Not known	(Hedera et al., 2007)
	%608096	12q22-q23.3	Not known	Not known	(Claes et al., 2004)
FPEVF or FFEVF	%604364	22q11-q12	*DEPDC5*	Role in neuronal signal transduction	(Berkovic et al., 2004c;Dibbens et al., 2013;Scheffer et al., 1998;Xiong et al., 1999)
ETL4; familial TLE, 4; occipitotemporal lobe epilepsy and migraine with aura	%611631	9q21-q22	Not known	Not known	(Deprez et al., 2007)
BECTS or BRE; Centralopathic epilepsy; Centrotemporal epilepsy	%117100	11p13	NA *(ELP4?)*	NA	(Strug et al., 2009): genome wide linkage analysis of 38 families, replicated

BFNE or BFNS (Mulley et al., 2011a)	#121200	20q13.33	*KCNQ2*	K^+ channel	(Berkovic et al., 2004a;Biervert et al., 1998;Singh et al., 1998)
	#121201	8q24.22	*KCNQ3*		(Charlier et al., 1998)
BFNIE or BFNIS	#607745	2q24.3	*SCN2A*	Na^+ channel	(Heron et al., 2002)
BFIE or BFIS	#605751	16p12-q12	*PRRT2*	interacts with SNAP25,* regulation of presynaptic Ca^{2+} channels	(Caraballo et al., 2001;de Vries et al., 2012)

Table 1.5 Mendelian or monogenic focal epilepsies: syndromes and associated genes and loci.

Abbreviations: AChR, acetylcholine receptor; ADLTLE, autosomal dominant lateral temporal lobe epilepsy; ADNFLE, autosomal dominant nocturnal frontal lobe epilepsy; ADPEAF, autosomal dominant partial epilepsy with auditory features; BECTS, "benign" epilepsy of childhood with centrotemporal spikes; BFIE, "benign" familial infantile epilepsy; BFIS, "benign" familial infantile seizures; BFNE, "benign" familial neonatal epilepsy; BFNS, "benign" familial neonatal seizures; BFNIE, "benign" familial neonatal-infantile epilepsy; BFNIS, "benign" familial neonatal-infantile seizures; BRE, "benign" rolandic epilepsy; FFEVF, familial focal epilepsy with variable foci; FMTLE, familial mesial temporal lobe epilepsy; FPEVF, familial partial epilepsy with variable foci; $K_v1.1$., voltage-gated potassium channel type 1.1.; MIM, Mendelian Inheritance of Man®; sz, seizure; yr, years.

* SNAP25 is the presynaptic synaptosomal-associated protein 25kDA, involved in fusion of synaptic vesicles to the plasma membrane and calcium-triggered exocytosis (Guerrini & Mink 2012).

1.3.2.4 Genetic or idiopathic generalised epilepsies

Table 1.6 lists genes that have been associated with increased risk of two common genetic/idiopathic generalised epilepsy syndromes: juvenile myoclonic epilepsy (JME) and childhood absence epilepsy (CAE).

Other rare genetic variants, for example in *CACNA1H*, coding the alpha-1H subunit of T-type voltage-dependent calcium channels, can be found in patients with CAE and other GGEs and it has been suggested these genetic variants may contribute to susceptibility to epilepsy, but not be sufficient to cause epilepsy on their own (Heron et al., 2007).

Genome-wide linkage studies of the idiopathic generalised epilepsies have been performed (Chioza et al., 2009;Hempelmann et al., 2006;Sander et al., 2000) and recently, the first GWA study of IGE was published (Steffens et al., 2012).

1.3.2.5 Infantile epileptic encephalopathies

The most relevant infantile epileptic encephalopathies (EE) and mutations in genes that have been associated with infantile EE are summarized in Table 1.7. These include *SCN1A*, *CDKL5*, *STXBP1*, *PCDH19* and *POLG*. More recently, targeted resequencing technology identified mutations in *CHD2*, *SYNGAP1*, *SCN1A*, *SCN2A* and *SCN8A* in a large cohort with epileptic encephalopathies (Carvill et al., 2013). Rare copy number variants have also been described in association with infantile EE (Mefford et al., 2011b).

GGE/ IGE	Gene	Locus	MIM	References
JME	*EFHC1*	6p12.2	#254770	(Suzuki et al., 2004)
	BRD2	6p21.32	#608816	(Pal et al., 2003)
	GABRA1	5q34-q35	#611136	(Cossette et al., 2002)
	CACNB4	2q22-q23	#607682	(Escayg et al., 2000a)
	GABRD	1p36	#613060	(Dibbens et al., 2004)
	CLCN2	3q27.1	#607628	(Saint-Martin et al., 2009)
	KCNQ3	8q24.22	%600669	(Vijai et al., 2003)
	-	15q14	%604827	(Elmslie et al., 1997)
	-	5q12-q14	%611364	(Kapoor et al., 2007)
	-	2q33-q36	%614280	(Ratnapriya et al., 2010)
CAE	*GABRG2*	5q31.1	#607681	(Kananura et al., 2002;Wallace et al., 2001)
	GABRA1	5q34	#611136	(Maljevic et al., 2006)
	GABRB3	15q11-q12	#612269	(Tanaka et al., 2008)
	CACNA1H	16p13.3	#607904	(Chen et al., 2003;Heron et al., 2007)
	-	8q24	%600131	(Fong et al., 1998)

Table 1.6 Two major genetic/ idiopathic generalised epilepsies syndromes and associated genes and loci.

Abbreviations: CAE, childhood absence epilepsy; GGE, genetic generalised epilepsy; IGE, idiopathic generalised epilepsy; JME, juvenile myoclonic epilepsy; sz, seizures; yr, years.Source: OMIM®, www.omim.org [Last accessed 01 June 2012], Copyright® 1966-2014 Johns Hopkins University.

Gene	Infantile EE	Protein	Locus	(Author, year)
SCN1A	Dravet	$Na_v1.1$	2q	(Claes et al., 2001;Harkin et al., 2007)
	SIMFE (Dravet)			
	MPSI			(Carranza Rojo et al., 2011)
CDKL5	Severe EE with early onset, tonic sz and spasms, more in girls	Cyclin-dependent kinase-like protein type 5	Xp22	(Nectoux et al., 2006)
STXBP1	Ohtahara syndrome	Syntaxin binding protein 1	9q34.1	(Deprez et al., 2010)
	Early-onset EE			
PCDH19	Dravet-like, in girls	Protocadherin 19	Xq22.1	(Dibbens et al., 2008)
POLG	Encephalopathy and early-onset epilepsy	Mitochondrial DNA polymerase gamma type 1	15q25	(Horvath et al., 2006)

Table 1.7 Genes associated with infantile epileptic encephalopathies.

Abbreviations: EE, epileptic encephalopathies; MPSI, migrating partial seizures of infancy; SIMFE, severe infantile multifocal epilepsy; sz, seizures; yr, years. Source: OMIM®, www.omim.org [Last accessed 01 June 2012], Copyright® 1966-2014 Johns Hopkins University.

1.3.2.6 Exceptions to Mendelian patterns of transmission in "monogenic" epilepsies

There are several exceptions to the Mendelian patterns of transmission (Table 1.8):

a) Genetic heterogeneity

There are two types of genetic heterogeneity. In allelic heterogeneity, multiple separate alleles at the same locus are responsible for the disease phenotype, with large number of different mutations within one gene being responsible for disease aetiology.

Locus heterogeneity implies different individual genes are responsible for disease aetiology.

b) Pleiotropy

Pleiotropy occurs when multiple phenotypic effects are associated with the same genetic abnormality. Evidence for pleiotropy exists in several human complex diseases and traits and it is already clear that it is a common property of genes and SNPs associated with disease traits, with possible future implications for identification of molecular targets for drug development, genetic risk-profiling and classification of diseases (Sivakumaran et al., 2011).

c) Variable expressivity

Different individuals in one family may have different phenotypes, in terms of epilepsy severity, different epilepsy syndromes, or sometimes haveonly febrile seizures or associated co-morbidities. There is inter-subject phenotypic variability: type, severity and AED response may vary significantly even within members of one family. This may be explained by modifier genetic factors and environmental factors.

An interesting example of variable expressivity is the GLUT-1 deficiency syndrome (De Vivo et al., 1991), caused by heterozygous mutations in the *SLC2A1* gene

(Seidner et al., 1998). Several epilepsy phenotypes have been described (Scheffer 2012). Paroxysmal exercise-induced dyskinesia, dystonia, migraine and hemolytic anaemia are among other possible phenotypes. In two unrelated families, both spanning two generations, 12/15 mutation carriers had epilepsy: eight, GGE with absence seizures with variable age at onset; two, myoclonic-astatic epilepsy; two, partial epilepsy; and two mutation carriers were unaffected. Phenotypic overlap was found between the GGEs observed in this monogenic condition and the common GGEs (Mullen et al., 2010).

d) Phenocopies

A relevant example of phenocopy in the epilepsies is autoimmune limbic encephalitis and subsequent TLE, caused by antibodies to K_v1 potassium channel-complex proteins, including Lgi-1, leucine-rich glioma inactivated 1 protein (Irani et al., 2010). This autoimmune disease "targets" the same protein as familial lateral TLE, where *LGI1* is a known causal gene. This is an example with autoimmune and genetic conditions targeting the same protein (Lerche et al., 2013) and the TLE caused by Lgi-1-associated limbic encephalitis can be considered a phenocopy.

e) Incomplete penetrance

If carriers of a mutation may be asymptomatic, there is incomplete penetrance. For example, estimates in the "monogenic" focal epilepsies range from 54% in ADLTLE (Ottman et al., 2004) to 80% in ADNFLE (Andermann et al., 2005).

Exceptions to "Mendelian" in monogenic epilepsies	Definition
Age-dependent penetrance	Penetrance is the frequency with which a genotype leads to a given phenotype. Age-dependent means this frequency varies with age.
Allelic heterogeneity	Different alleles/mutations within the same gene cause a similar phenotype.
Incomplete penetrance	An individual carrying an allele that normally causes a dominant phenotype may not show that phenotype. Estimated 54% in ADLTLE (Ottman et al., 2004) and 80% in ADNFLE (Andermann et al., 2005).
Locus heterogeneity	Same disease or trait determined by mutations at different loci.
***De novo* mutations**	Gene change present for the first time in one individual as a result of a mutation in a germ cell of one of the parents or in the fertilized egg.
Phenocopies	A phenotype identical to a genetically-determined phenotype, but with environmental causes.
Pleiotropy	A single gene controls several different, often seemingly unrelated, phenotypic effects.
Variable expressivity	Inter-subject phenotypic variability: type, severity, AED response may vary significantly even within members of one family. May be explained by modifier genetic or environmental factors.

Table 1.8 Exceptions to Mendelian patterns of transmission in monogenic epilepsies.

Sources: (Strachan and Read 1999); Genetics Home Reference website [Last access 12/09/2012].

1.3.3 Single gene disorders associated with epilepsy

There are also several known monogenic disorders in which a mutation in one gene may lead not just to epilepsy, but also to learning disability or another neurological impairment, or a visible lesion on MRI and therefore fall into the "symptomatic" category, even if monogenic in aetiology. Several malformations of cortical development are examples of single gene disorders associated with epilepsy (Guerrini & Marini 2006), as are some progressive myoclonic epilepsies (Merwick et al., 2012).

The traditional definition of "idiopathic" and "symptomatic", with the implication that genetic disorders are "idiopathic", has been criticised and a proposal put forward for these terms to be reassessed (Berg et al., 2010;Berg and Scheffer 2011).

1.3.4 Mitochondrial disorders associated with epilepsy

Several mitochondrial disorders may have epilepsy as a feature. These may involve either maternaly inherited mutations of mitochondrial DNA, for example myoclonic epilepsy with ragged red fibers (MERRF), or nuclear mutations leading to mitochondrial dysfunction, as seen in epilepsy associated with *POLG* mutations (Horvath et al., 2006) (Table 1.7).

1.3.5 Chromosomal abnormalities associated with epilepsy

1.3.5.1 Chromosomal abnormalities detectable by cytogenetics

More than 400 structural chromosomal abnormalities are associated with epilepsy, including trisomies, partial monosomies, deletions, inversions, translocations and ring chromosomes (Battaglia & Guerrini 2005;Singh et al., 2002). Important examples include

Wolf-Hirschhorn (4p-), Angelman (del 15q11-q13), Miller-Dieker (del 17p13.3) and ring chromosome 20 syndromes (Elens et al., 2012;Singh et al., 2002).

1.3.5.2 Copy number variation

Large copy number variants (CNVs) have been recently identified as an important source of both disease-causing variation and normal genomic variation (Cooper & Mefford 2011).

Large CNVs were found to be associated with neuropsychiatric diseases, such as schizophrenia (Need et al., 2009b;Stefansson et al., 2008;Walsh et al., 2008), autism (Pagnamenta et al., 2009;Weiss et al., 2008) and intellectual disability (Cooper et al., 2011;de Vries et al., 2005;Hannes et al., 2009;Mefford et al., 2007;Mefford et al., 2008;Sharp et al., 2008).

CNVs have also been shown to contribute to susceptibility to epilepsy. Recurrent microdeletions at 16p13.11, 15q11.2 and 15q13.3 were found in generalized epilepsy (de Kovel et al., 2010;Dibbens et al., 2009;Helbig et al., 2009), while in partial epilepsy, recurrent microdeletions were found in 16p13.11, 15q11.2, but not 15q13.3 (Heinzen et al., 2010). The 16p13.11 microdeletion has been shown to be pathogenic and includes candidate genes, the most relevant is *NDE1* (Heinzen et al., 2010;Liu et al., 2012).

A study using array comparative genomic hybridization (array CGH) analysis in 155 post mortem foetal samples had described recurrent 17q12 microdeletions in samples with epilepsy, among other phenotypes, ranging from congenital renal disease to diabetes (Mefford et al., 2007).

In 82 selected patients with medically refractory epilepsy and co-morbidities, ascertained in two tertiary referral centres, array-CGH permitted the identification of CNVs judged of pathogenic significance in 15.6% and its clinical use is now standard in selected cases (Galizia et al., 2012).

A large study used array-CGH technology to look for CNVs in 15,767 children with intellectual disability and congenital defects (1,776 with seizures) and 8,329 unaffected adult controls. Validation was done by customized higher density microarray and fluorescence in situ hybridization. Large CNVs were found to be enriched in children with both neurological and congenital birth defects and with neuropsychiatric diseases. Interestingly, there was an excess of smaller *SCN1A* deletions in the cases with epilepsy (cases were children with a general diagnosis of intellectual disability and/or developmental delay) ($p = 0.019$) (Cooper et al., 2011).

Genetic heterogeneity (Mulley & Dibbens 2009), low penetrance (Dibbens et al., 2009) and variable expressivity (Girirajan et al., 2010;Veltman & Brunner 2010) are features of the epilepsies associated with CNVs. The phenotypic consequences for most CNVs may not yet be well characterized (Cooper et al., 2011). CNVs may encompass many candidate genes. The clinical interpretation of rare non-recurrent CNVs may be, therefore, problematic.

1.3.6 "Complex" epilepsy syndromes

Known "monogenic" epilepsies account for only a small fraction of the epilepsies. Most common epilepsies are thought to have "complex" inheritance, which means many different genetic variants and environmental factors are expected to contribute to susceptibility to the common epilepsies.

Given the technology advances in genetics and imaging, the need to update the classification of seizures, epilepsy syndromes and epilepsies has been recognized and a proposal has been presented (Berg et al., 2010;Berg and Scheffer 2011).

1.3.6.1 Genetics of common epilepsy syndromes

There are several lines of evidence suggesting that "partial" and "generalised" epilepsies have genetic predisposition patterns that may partially overlap.

A study by Ottman and colleagues (1998) analysed the risk of epilepsy among first-degree relatives of 1,498 adults with "idiopathic" or "cryptogenic" epilepsy. In the offspring, the risk for all epilepsies and for partial epilepsy, was greater if the proband's epilepsy was partial rather than generalised. In the parents and siblings, the risk for all epilepsy was greater if the proband's epilepsy was generalised. The authors concluded there may be susceptibility genetic variants increasing the risk for both generalised and partial epilepsies and also different genetic influences acting on susceptibility for either generalised or partial epilepsies (Ottman et al., 1998).

Gene	Epilepsy syndrome(s)	Phenotype MIM No	Author, year
Ion channel genes			
KCNQ2	BFNS	#121200	(Biervert et al., 1998)
KCNQ3	BFNS	#121201	(Charlier et al., 1998)
CHRNA2	ADNFLE	#610353	(Aridon et al., 2006)
CHRNA4	ADNFLE	#600513	(Steinlein et al., 1995)
CHRNB2	ADNFLE	#605375	(De Fusco et al., 2000)
SCN1A	FS	#604403	(Mantegazza et al., 2005)
	GEFS+ (10%)	#604403	(Colosimo et al., 2007)
	TLE in GEFS+		(Escayg et al., 2000b)
	Dravet (70-80%)	#607208	(Claes et al., 2001)
	MPSI	NA	(Carranza Rojo et al., 2011)
SCN2A	Dravet-like EE	#613721	(Ogiwara et al., 2009)
	BFIS	#607745	(Heron et al., 2002)
SCN1B	GEFS+	#604233	(Wallace et al., 1998)
	TLE in GEFS+		(Scheffer et al., 2007)
GABRA1	JME	#611136	(Cossette et al., 2002)
	CAE		(Maljevic et al., 2006)
GABRG2	GEFS+	#611277	(Baulac et al., 2001)
	Dravet	#607208	(Claes et al., 2001;Harkin et al., 2002;Jansen et al., 2006)
CACNA1H	CAE	#611942	(Heron et al., 2007)

Non-ion channel genes			
LGI1	ADPEAF or ADLTLE	#600512	(Kalachikov et al., 2002)
EFHC1	JME	#254770	(Suzuki et al., 2004)
ARX	Early infantile EE (S. Ohtahara)	#308350	(Stromme et al., 2002)
CDKL5	Early infantile EE	#300672	(Weaving et al., 2004)
PCDH19	Dravet-like EE	#300088	(Depienne et al., 2009a;Dibbens et al., 2008)
PRICKLE1	PME	#612437	(Bassuk et al., 2008)
PRRT2	BFIS, FS	#602066, #605751	(de Vries et al., 2012;Gardiner et al., 2012;Scheffer et al., 2012a)
SCARB2	PME with or without renal failure	#602257	(Berkovic et al., 2008)
SLC2A1	GLUT1 deficiency syndrome (several epilepsy phenotypes)	#606777 #612126	(Seidner et al., 1998)
TBC1D24	Familial infantile myoclonic epilepsy	#605021	(Falace et al., 2010)

Table 1.9 Ion channel genes and non-ion channel genes associated with epilepsy.

Abbreviations: ADLTLE, autosomal dominant lateral temporal lobe epilepsy; ADPEAF, autosomal dominant partial epilepsy with auditory features; BFIS, benign familial infantile seizures; BFNS, benign familial neonatal seizures; CAE, childhood absence epilepsy; EE, epileptic encephalopathy; FHM, familial hemiplegic migraine; FS, febrile seizures; GEFS+, genetic epilepsy with febrile seizures plus; JME, juvenile myoclonic epilepsy; MPSI, migrating partial seizures of infancy; NA, not applicable/ not available; PME, progressive myoclonic epilepsy; TLE, temporal lobe epilepsy.

Sources: OMIM®, www.omim.org, Copyright® 1966-2014 Johns Hopkins University; and (Mulley et al., 2011b).

Twin studies have shown different concordance rates in MZ and DZ twins depending on the type of epilepsy syndrome (Berkovic et al., 1998). Interestingly, 94% of the MZ twins who showed concordance and 71% of the concordant DZ twins, had the same type of epilepsy syndrome (Berkovic et al., 1998) suggesting differential genetic influences over different specific epilepsy syndromes (Pandolfo 2011).

The relevant genetic and environmental factors may differ, not only across clinically defined syndromes, but also within syndromes. Different susceptibility genetic variants and environmental factors may influence risk; a single susceptibility locus may influence risk for different syndromes in different patients, which may be due to allelic heterogeneity, pleiotropism, modifier genes and/or modifying environmental factors (Ottman 1997). Genetic variants may contribute to an individual's susceptibility to epilepsy but may not be sufficient to cause epilepsy on their own (Heron et al., 2007).

As an example of genetic variants influencing risk of generalised but not partial epilepsies, the recurrent microdeletions at 15q13.3 were found in genetic/idiopathic generalised epilepsies (de Kovel et al., 2010;Helbig et al., 2009), but not in a large cohort of partial epilepsies (Heinzen et al., 2010). On the other hand, recurrent microdeletions at 16p13.11 and 15q11.2 were found in both GGE/IGE and partial epilepsy (de Kovel et al., 2010;Heinzen et al., 2010), suggesting the existence of genetic variants influencing susceptibility for both generalised and partial epilepsy syndromes (Mefford et al., 2010).

A recent twin study looked for genetic contributions in epilepsy across the 1989 ILAE epilepsy syndromes. A strong contribution of genetic factors was found for several "major" well-characterized syndrome subtypes of IGE, such as CAE, juvenile absence epilepsy, JME, IGE and epilepsy with grand mal seizures on awakening. For the

subdivisions of focal epilepsies – frontal, temporal, occipital, the study concludes that the contribution of genetic factors is modest (Corey et al., 2011), but to "lump" together several different partial epilepsy syndromes into this "anatomical" classification may possibly have diluted any possible existing association signals.

Gene	Polymorphism	Epilepsy syndrome	Phenotype	N of patients	N of controls	*p*-value	Country	Reference
IL-1B	C511T	TLEHS	Susceptibility to epilepsy & role of FS	66	64 TLEHS 89 PEnoTLE	0.0022	Japan	(Kanemoto et al., 2003)
				50	112	0.006	Japan	(Kanemoto et al., 2000)
PRNP	N171S	TLEHS	Susceptibility to epilepsy & response to temporal lobectomy	100	180	< 0.0001	Brazil	(Walz et al., 2003)
	M129V	TLE ("mild")	Susceptibility to epilepsy	289	272	0.006 (OR 1.63; 95%CI, 1.15-2.31)	Italy	(Labate et al., 2007)
		MTLE	Susceptibility to epilepsy	320	558	NS (0.24)	China	(Wang et al., 2008c)
GABBR2	rs967932	MTLE	Susceptibility to epilepsy	318	315	0.018	China	(Wang et al., 2008b)
C3	GF100472	MTLE-FS+	Susceptibility to epilepsy	122	196	0.036	Spain	(Jamali et al., 2010)
GABBR1	G1465A	MTLEHS	Susceptibility to epilepsy	1011	2184	0.004 (OR 5.38; 95%CI, 1.73-16.78)	(meta-analysis of 7 studies)	(Xi et al., 2011)
				102	71	3.78×10^{-8} (OR 10.1; 95%CI, 3.98-25.18)	Argentina	(Kauffman et al., 2008)

PDYN		TLEHS and TLEnoHS	Susceptibility to epilepsy	155	202	NS -- 0.002 (subgroup with FH)	Austria	(Stogmann et al., 2002)
CALHM1	P86L rs2986017	TLE	Susceptibility to epilepsy	560	401	0.015	China	(Lv et al., 2011)
SLC6A4	12-repeat allele	MTLE	AED response	105	81	0.006 (OR 3.88; 95%CI, 1.40-10.7)	Argentina	(Kauffman et al., 2009)

Table 1.10 Population-based candidate gene association studies in temporal lobe epilepsy.

Abbreviations: AED, antiepileptic drug; FH, family history; FS, febrile seizures; HS, hippocampal sclerosis; MTLE, mesial temporal lobe epilepsy; MTLEHS, mesial temporal lobe epilepsy with hippocampal sclerosis; NS, not significant; PEnoTLE, extratemporal partial epilepsy; TLE, temporal lobe epilepsy; TLEHS, temporal lobe epilepsy with hippocampal sclerosis; *SLC6A4*, serotonine transporter gene.

Other genes and loci, not mentioned in the table but "associated with" TLE in at least one published study include *BDKRB1*, *BDKRB2*, *CCL3*, *CCL4*, *5HT-1B*, *SCN3A*, *SCN3B*, *GRIN1*, *PRNP*, 2q, 22q12; and "associated with" FS: 1q, 18qter, 12q, 4q.

Sources: EpiGAD database [Accessed 29 April 2012]; and (Hwang & Hirose 2012;Tan & Berkovic 2010).

1.4 Genetics of temporal lobe epilepsy

Temporal lobe epilepsy (TLE) is the most common form of partial epilepsy in adults (Gastaut et al., 1975;Loiseau et al., 1991;Manford et al., 1992).

The TLEs encompass several different syndromes, both symptomatic and non-lesional and may have neocortical (lateral temporal) or mesial temporal seizure onset zones (ILAE Commission on Classification and Terminology 1989).

Genetic studies have already contributed insights into TLE, but so far the genetic variants identified have explained only a small proportion of the observed cases (Vadlamudi et al., 2003).

The relevance of genetic factors in TLE is well recognized. Epidemiological evidence includes increased familial aggregation (Ottman 1997) and higher concordance rates in monozygotic (MZ) than in dizygotic (DZ) twins (Berkovic et al., 1996;Berkovic et al., 1998) (Table 1.4). Animal models and functional studies have also contributed evidence.

The monogenic epilepsies account for a small percentage of TLE[2] (Table 1.5), while the large majority have sporadic TLE, with "complex" genetic architecture, involving several susceptibility genes and environmental factors.

Population-based candidate gene studies have been performed looking for evidence of genetic susceptibility in "sporadic" TLE. Table 1.10 lists common genetic

[2] See section 1.3.2. - Monogenic epilepsy syndromes (p. 58).

variation shown in at least one study to be associated with TLE. This includes *IL-1β* , found to be associated with TLEHS (Kanemoto et al., 2000); or *PDYN* (Stogmann et al., 2002). A functional polymorphism in the complement C3 gene promoter was found in one case-control study to be associated with susceptibility to TLE and FS (Jamali et al., 2010), but this has not been replicated.

Actually, for most of these associations, the results have not yet been replicated, possibly because of the methodology used, with small sample sizes leading to insufficient power (Labate et al., 2011). Further, many studies did not look at the role of prolonged FS in the association of the candidate gene with MTLEHS or TLEHS and it has been shown in a few studies this may be a determinant in the association (Kanemoto et al., 2000).

One study found an association between age at onset in TLE and genetic variation in *APOE* (Briellmann et al., 2000), but this was a small study, with 43 TLE patients and has not been replicated, so its relevance still needs to be clarified.

An expression study of the entorhinal cortex, with cDNA microarray followed by rtPCR validation, from 11 patients with medically refractory MTLE, yielded 6 candidate genes, *HTR2A*, *NPY1R*, *FHL2*, *C3*, *HLA-DR-γ* and *CD99*, suggesting involvement of neurotransmission and complement systems in TLE pathogenesis (Jamali et al., 2006).

A linkage study in a consanguineous family with FS (1 in 4 affected relatives with FS had TLE with hippocampal atrophy), followed by exon screening of *CPA6* in 138 patients with sporadic TLE, found a novel heterozygous missense mutation in three unrelated patients with TLE (Salzmann et al., 2012).

1.4.1 Mesial temporal lobe epilepsy

MTLEHS is the most frequent type of refractory partial epilepsy seen in the adult epilepsy clinic and represents most of the patients with MTLE. Resective epilepsy surgery has proven to be superior to AEDs in selected patients with refractory MTLEHS (Wiebe et al., 2001), with a long-term seizure freedom rate of less than 60% (de Tisi et al., 2011). HS is the most frequent neuropathologic finding after resective surgery in adults with refractory TLE (Blumcke 2009;Engel, Jr. et al., 2008;Falconer et al., 1964).

For many patients, there is an antecedent history of an "initial precipitating injury" (IPI) (Mathern et al., 1995), such as prolonged FS, trauma, hypoxia, intracranial infection, usually before the age of 5 years. This can be followed by a "latent period" preceding the onset of the habitual seizures, usually at the end of the first decade of life. There may be a period where seizures seem to respond well to AEDs, but the natural history frequently evolves to seizure recurrence in adolescence or adulthood with subsequent refractoriness to medication.

1.4.1.1 Seizure semiology in MTLEHS

A prototypical seizure in MTLEHS consists of an aura, followed by arrest, alteration of consciousness, with amnesia and automatisms. Auras are frequently a rising epigastric sensation, but may include déjà-vu, fear and anxiety, or be non-specific. Limb automatisms, dystonic posturing and oroalimentary automatisms are frequent (Wieser 2004).

Several lateralizing semiological signs have been described, of varying sensitivity and specificity (Loddenkemper & Kotagal 2005). For example, unilateral dystonic

posture is contralateral to the seizure onset zone in 70% of cases (Kotagal et al., 1989), while ipsilateral automatisms are less reliable, correctly lateralizing in 35% of cases (Dupont et al., 1999).

1.4.2 Genetics of sporadic mesial temporal lobe epilepsy

Most cases of MTLE with HS or hippocampal atrophy (HA) are sporadic. Recent segregation studies in MTLE point to a pattern of "complex" inheritance (Crompton et al., 2010), or "multifactorial" aetiology, involving several genes (Secolin et al., 2010).

A study in 66 patients with TLEHS and healthy controls showed an increased family history of seizures, particularly FS, in siblings and parents, with 6% of siblings of TLEHS patients having antecedents of FS (Briellmann et al., 2001b).

Candidate gene association studies of sporadic MTLE have shown association with a few common genetic variants (Table 1.10), but replication is needed to validate the results.

There are data suggesting that genetic variation associated with susceptibility to sporadic MTLEHS may include genetic variation in *SCN1A*. A series of three cases of sporadic MTLE with *SCN1A* mutations was published in abstract form by Scheffer et al. (2011).

1.4.3 Hippocampal sclerosis

HS is the commonest neuropathological finding in patients with TLE, both in surgical series (Blumcke 2009;Bruton 1988;Falconer et al., 1964) and post mortem series (Corsellis 1957;Margerison & Corsellis 1966;Meencke et al., 1996;Thom et al., 2011). Imaging evidence of HS has also been found in patients with "benign" MTLE (Labate et al., 2006).

1.4.3.1 Diagnosis of hippocampal sclerosis

Neuroimaging

MRI findings required for the diagnosis of HS include atrophy, hyperintensity on T2-weighted and fluid-attenuated inversion-recovery (FLAIR) images (Jackson et al., 1990) and loss of the internal architecture of the hippocampus (Fig. 1.8). Use of appropriate MRI protocols and an experienced neuroradiologist are essential to ensure an adequate sensitivity of MRI for detecting HS (Woermann & Vollmar 2009). Volumetry and T2-relaxometry may be useful to confirm HS (Duncan 2010) and several other MRI techniques may increase the diagnostic yield (Duncan 2011).

Neuropathology

Neuropathology is required for a definitive diagnosis of HS (Wieser 2004). Selective neuronal loss and gliosis in the CA1, CA3 and CA4 sectors of the hippocampus are diagnostic features (Blumcke 2008). Also found is reorganisation of the dentate gyrus, with granule cell dispersion and mossy fibre sprouting (Thom et al., 2009b).

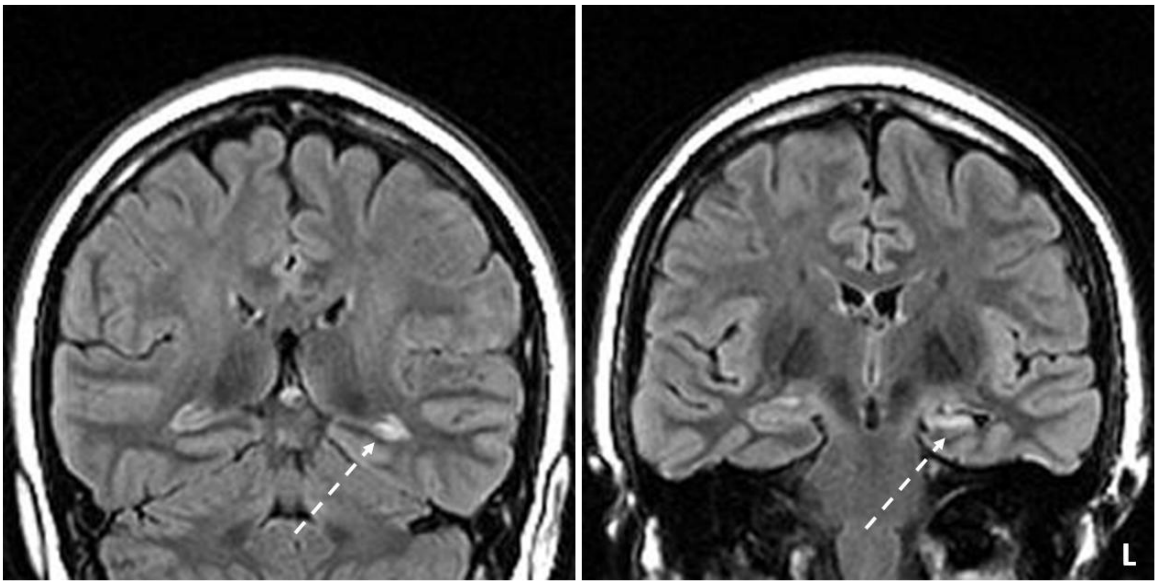

Figure 1.8 Unilateral hippocampal sclerosis. 3-Tesla MRI, coronal images of fluid-attenuated inversion-recovery (FLAIR) sequences, showing radiological features of HS: atrophy, hyperintensity in FLAIR and T2-weighted images and loss of internal architecture of the hippocampus.
Arrows show the location of the hippocampal sclerosis in each image. Abbreviations: L, left.

1.4.3.2 Resective surgery in MTLE

Studies evaluating the impact of the underlying pathology on the disease course of MTLE found that seizure freedom on medical therapy was less frequent in TLE with HS (isolated HS or dual pathology) than in TLE without HS and HS was considered a "major determinant of intractability" (Semah et al., 1998). A group of patients with MTLEHS, compared to patients with MTLE caused by cavernoma, were found to have more frequent pharmacoresistance (Menzler et al., 2011).

Resective epilepsy surgery is superior to medical therapy alone for patients with refractory MTLEHS, who are deemed good surgical candidates (Wiebe et al., 2001). A thorough presurgical assessment is necessary to select the patients with MTLE with good odds of improvement after surgery. "Ideal" candidates have unilateral HS on MRI and concordant clinical, electroencephalography, neuropsychology and imaging data (Duncan 2011). Long-term seizure freedom is seen in 60% of patients with MTLEHS who undergo anterior temporal lobectomy (de Tisi et al., 2011;Tellez-Zenteno & Wiebe 2008).

Several tools help to predict a good outcome after epilepsy surgery, but the literature can be contradictory. Published predictors of favourable outcome of epilepsy surgery include:

- **a.** HS at histopathology of the surgical specimen (Abou-Khalil et al., 1993;Falconer & Serafetinides 1963;Williamson et al., 1993);
- **b.** prolonged FS (Abou-Khalil et al., 1993;Janszky et al., 2003);
- **c.** no history of secondary generalization (Hennessy et al., 2001;Janszky et al., 2005;Jeong et al., 2005);

d. interictal epileptiform discharges (IED) in the ipsilateral temporal lobe only (Hennessy et al., 2001;Radhakrishnan et al., 1998);

e. absolute IED frequency (Krendl et al., 2008);

f. shorter duration of epilepsy (Janszky et al., 2005;Tellez-Zenteno et al., 2005);

g. younger age at surgery (Jeong et al., 2005);

h. extent of resection (Wyler et al., 1995);

i. serial postoperative EEG findings (Rathore et al., 2011).

1.4.3.3 Epilepsy surgery outcome and genetics

Genetic factors may be postulated to contribute to treatment outcome of sporadic MTLE, but no genetic variant has been confirmed to predict outcome after epilepsy surgery.

No difference has been found in outcome after epilepsy surgery between patients with familial MTLE and patients with sporadic MTLE (Kobayashi et al., 2003a).

There are reports in the literature of patients who underwent surgery for medically refractory MTLE and had an identified genetic "cause" for the epilepsy. In a small case series of two GEFS+ patients with MTLE, one with unilateral HS, both with *SCN1B* mutations, both became seizure free after resective epilepsy surgery. This showed that an excellent surgical outcome is possible in patients with refractory MTLE(HS) from GEFS+ families and *SCN1B* mutations, who meet the habitual criteria for resective surgery (Scheffer et al., 2007).

1.4.4 Genetics of hippocampal sclerosis

The frequent association seen between HS and an "initial precipitating insult", especially prolonged febrile seizures (Abou-Khalil et al., 1993;Cendes et al., 1993;Engel, Jr. et al., 2008) led to the theory that these could be causally related (Falconer et al., 1964). Previous hippocampal abnormalities were also suspected to underlie the development of HS after febrile seizures (Fernandez et al., 1998). Although it is still not completely understood the origin and cause of HS, genetic determined mechanisms are expected to play a role (Briellmann et al., 2001b;Cendes 2004). HS is likely to have "complex" inheritance, involving multiple genetic factors and environmental factors.

A frequent family history of seizures, particularly FS, was reported in siblings and parents of patients with TLEHS compared to healthy controls, with 6% of siblings having antecedents of FS (Briellmann et al., 2001b).

In one Australian clinical series, three monozygotic twin pairs, with one twin with TLE, HS and antecedents of FS, were discordant for HS (Jackson et al., 1998) (Table 1.4). As stated by the authors, however, this does not necessarily disprove a genetic component for HS (Briellmann et al., 2001a), with similar reports published for other genetic diseases (Bennett et al., 2008;Caramori et al., 2012;Vogt et al., 2011).

Relatives of probands with familial MTLE may include: patients with HS; patients with hippocampal atrophy (HA) but no signal change on MRI; patients or healthy relatives with normal MRI; and healthy relatives with radiological diagnosis of HS (Kobayashi et al., 2002;Kobayashi et al., 2003b). In one study, one-third of asymptomatic

first-degree relatives of patients with familial MTLE were found to have MRI features of HS (Kobayashi et al., 2002).

Van Poppel et al. (2012) published a series of 20 children with epilepsy with a proven *SCN1A* mutation, including patients with Dravet, GEFS+ and unclassified epilepsy. Ten children (50%) with *SCN1A* mutations had HS: 4 definite unilateral HS, 2 definite bilateral HS and 4 possible HS. Five of six patients with definite HS had antecedents of prolonged FS or febrile status.

A locus on chromosome 18p11.3 has been associated with hippocampal abnormalities in one family with familial MTLE (Maurer-Morelli et al., 2012).

In a recent study, 644 patients with pathologically confirmed Alzheimer's disease, 57 of whom with HS, were genotyped. A significant association was found between HS and a genetic variant of the *GRN* gene, previously shown to be associated with decreased serum levels of progranulin (Dickson et al., 2010).

Further, animal model data have shown that HS may be found in mice with sodium channel defects; for example, this has been shown in transgenic mice with a gain-of-function *Scn2a* mutation (Kearney et al., 2001).

1.5 Febrile seizures

1.5.1 Definition of febrile seizures

Febrile seizures (FS) consist of seizures taking place during fever, without an intracranial infection (Dube et al., 2009;Waruiru & Appleton 2004). The ILAE proposed as definition "a seizure occurring in childhood after one month of age, associated with a febrile illness not caused by a CNS infection, without previous neonatal seizures or a previous unprovoked seizure and not meeting criteria for other acute symptomatic seizures" (ILAE Commission on Epidemiology and Prognosis 1993). The American Paediatric Association, in the 1980 National Institutes of Health consensus statement, defined FS as an "event in infancy or childhood usually occurring between three months and five years of age, associated with fever but without evidence of intracranial infection or defined cause for the seizure" (Freeman 1980).

1.5.2 Epidemiology of febrile seizures

FS are the most common neurological insult in infants and children, with 5% of children under the age of 5 years having at least one FS (Stafstrom 2011). Other epidemiological studies have slightly different estimates of prevalence, also depending on the age groups included: 2 to 5% of children under 6 years of age (Hauser 1994) and 5 to 12% of infants and children up to 6 years of age (Mantegazza et al., 2005).

A difference in prevalence of FS has been reported between western Europe/USA (2-5%), Japan (6-9%) (Nakayama 2009;Tsuboi 1984) and Guam (14%) (Stafstrom 2001),

which may in part reflect the importance of genetic factors in susceptibility to FS, although environmental factors are also postulated to have some influence.

Relatives of children with FS have a higher risk of having "seizure disorders" than the general population, particularly if the proband had FS followed by epilepsy (Hauser et al., 1985).

1.5.3 Classification of febrile seizures

Simple FS, complex or prolonged FS and febrile status epilepticus can be differentiated in terms of several characteristics, including duration: less than 10 minutes, 10 to 29 minutes and longer than 30 minutes, respectively (Hirtz et al., 1997).

Simple FS are brief generalised seizures provoked by fever typically greater than 38.5ºC, in a child aged 6 months to 6 years, with less than 10 minutes' duration, usually not recurring within the same febrile illness (Baulac et al., 2004). Complex or prolonged FS have at least one of the following characteristics: focal jerking or deficit, multiple FS within the same febrile illness period or within the first 24 hours, duration of 10 minutes or more, including febrile status epilepticus (Baulac et al., 2004;Hirtz et al., 1997).

1.5.4 Febrile seizures and subsequent afebrile seizures

The consequences of prolonged FS are not well known, with possible relationship to TLE later in life. Epidemiological retrospective studies have linked a history of prolonged FS with later TLE (Nelson & Ellenberg 1976;Verity & Golding 1991;Waruiru and Appleton

2004), but prospective studies failed to replicate this (Camfield et al., 1994;Nelson and Ellenberg 1976;Tarkka et al., 2003).

About 2 to 10% of children who had at least one febrile seizure will develop subsequent afebrile seizures (Berg & Shinnar 1996). In a recent study, 6% of patients with FS had subsequent afebrile seizures, ten times more than the general population (Neligan et al., 2012). This significant proportion of patients with FS later developing afebrile seizures, suggests the existence of shared genetic determinants between FS and afebrile seizures. Research is therefore needed to look for genetic variants influencing susceptibility to both FS and "common" epilepsies (Mulley et al., 2011c).

The FEBSTAT study is an ongoing prospective study on the consequences of prolonged FS, addressing the important questions of the relationship between FS, MTLE and HS (Hesdorffer et al., 2012;Shinnar et al., 2012).

1.5.5 Genetic studies of febrile seizures

There is evidence for genetic susceptibility to FS (Waruiru and Appleton 2004). Relatives of patients with FS have a higher risk of FS compared to the general population (Briellmann et al., 2001b;Nakayama 2009), with recurrence risk ratios of 3–5 in first degree relatives (Helbig et al., 2008). The heritability of FS has been estimated in two independent studies in Japan, at 75% and 76% (Fukuyama et al., 1979;Tsuboi & Endo 1991).

Table 1.11 lists twin studies of febrile seizures, where the concordance for MZ twins has been consistently shown to be higher than for DZ twins, providing further evidence for the relevance of genetic factors in susceptibility to FS.

Reference	**MZ twin pairs Number (concordance)**	**DZ twin pairs Number (concordance)**
(Berkovic et al., 1998)	38 (41%)	60 (7%)
(Corey et al., 1991)	95 (19%)	157 (6%)
(Tsuboi and Endo 1991)	9 (67%)	7 (14%)
(Schiottz-Christensen 1972)	27 (33%)	37 (14%)
(Lennox-Buchthal 1971)	19 (68%)	46 (13%)

Table 1.11 Twin studies of febrile seizures, showing consistently a higher concordance for monozygotic twins than for dizygotic twins.

Table 1.12 lists genes which have been associated with FS, for both simple FS and genetic epilepsy with febrile seizures plus (GEFS+).

In the rare families with autosomal dominant simple FS, mutations were identified in the *SCN1A* gene (Mantegazza et al., 2005;Scheffer et al., 2009). The *SCN1A* mutation, M145T (loss-of-function) was found to co-segregate in all 12 affected individuals of a large Italian family with simple FS (Mantegazza et al., 2005).

Several other genes have been shown to contribute to generation of phenotypes within the FS spectrum, observation which is consistent with "complex" inheritance for FS (Waruiru and Appleton 2004). Other genetic variation associated with FS include

SCN2A, *SCN9A*, *GABRG2*, *GABRD* and *GPR98* (Table 1.12). Other genetic variants and environmental factors are likely to also contribute to susceptibility to FS.

Published candidate gene association studies of FS include small case-control studies on *SCN1A* and Table 1.13 gives a brief summary of the results of these studies. A candidate gene association study reported an association between SNP rs3812718, related to *SCN1A* and increased risk of FS (Schlachter et al., 2009). Attempts at replication of this result failed, despite identical FS classification and statistical methodology, in Caucasian (Le Gal et al., 2011;Petrovski et al., 2009), Chinese (Zhang et al., 2010) and South Indian cohorts (Balan et al., 2012). The association was tested between the polymorphism and pure FS and also between the polymorphism and partial epilepsy with antecedents of FS and no significant association was found. The small sample sizes of each of these studies meant the studies were possibly underpowered for definitive conclusions.

A meta-analysis pooled the published data on FS in Caucasians, including raw data from studies by Le Gal et al. (2011), Petrovski et al. (2009) and Schlachter et al. (2009). This meta-analysis claimed a significant association ($p = 4.8 \times 10^{-8}$) between rs3812718 in *SCN1A* and FS and between the polymorphism and partial epilepsy with antecedents of FS (Le Gal et al., 2011). Again, the limitations of this study, including the small sample size, do not allow robust conclusions.

Genetic association studies of pure FS and of partial epilepsy with (and without) previous history of FS, merits more research, using larger samples and newer methodology, including GWA studies and next-generation sequencing.

Gene / Protein	Syndrome	Phenotype MIM number	Locus	Author/Year
SCN1A / $Na_v1.1$	GEFS+, type 2 or Familial FS, FEB3A	#604403	2q24.3	(Mantegazza et al., 2005)
SCN2A / $Na_v1.2$	Epileptic encephalopathy, early infantile, 11	#613721	2q24.3	(Ogiwara et al., 2009)
	BFIS 3	#607745		(Berkovic et al., 2004a)
SCN9A / Nav1.7	GEFS+, type 7	#613863	2q24	(Singh et al., 2009)
	Familial FS, FEB3			
GABRG2 / GABRG2	GEFS+, type 3	#611277	5q34	(Baulac et al., 2001)
	Familial FS	#607208		
	Dravet	#611277		(Jansen et al., 2006)
	CAE	#607681		(Kananura et al., 2002)
GABRD / GABRD	GEFS+, type 5	#613060	1p36.33	(Dibbens et al. 2004)
	JME			
	IGE			
GPR98 or *MASS1* / VLGR1	Familial FS, FEB4	#604352	5q14.3	(Nakayama et al. 2002)
IMPA2	Familial FS, FEB6	#609253	18p11.2	(Nakayama et al., 2004)
SEZ6 / SEZ6	FS	NA	17q11.2	(Mulley et al., 2011c;Yu et al., 2007)
CPA6	Familial FS	NA	8q12.1-q13.2	(Salzmann et al., 2012)

Table 1.12 Rare genetic variation contributing to susceptibility to febrile seizures. Abbreviations: AD, autosomal dominant; GEFS+, genetic epilepsy with febrile seizures plus; FS, febrile seizures; MIM, Mendelian Inheritance of Man; mo, months; sz, seizure; yr, years.
Source: OMIM®, www.omim.org [Last accessed 01 June 2012]. Copyright® 1966-2014 Johns Hopkins University.

Reference	**Type of study**	**Ethnicity / Country of origin**	**SNP**	**Number of individuals included in the analysis**	***P* value *allelic tests***	***P* value *genotypic tests***
(Schlachter et al., 2009)	Candidate gene association study	Caucasian	rs3812718, IVS5N+5 G>A (higher freq of A allele in FS)	486 PEnoFS v 701 controls	ns	ns
				90 PE+FS v 701 controls	7.1×10^{-6}	1.5×10^{-5}
				144 pureFS v 701 controls	1.6×10^{-4}	7.1×10^{-4}
				234 (PE+FS and pureFS) v 1187 (controls and PEnoFS)	4.8×10^{-8}	2.5×10^{-7}
(Petrovski et al., 2009)	Candidate gene association study	Australia (generalised epilepsy); UK, Ireland, Belgium, US (partial epilepsy)	rs3812718 or rs922224[a]	124 PEnoFS v 701 controls	ns	ns
				PE+FS	NA	NA
				pureFS	NA	NA
				23 (PE+FS and pureFS) v 825 (controls and PEnoFS)	ns	ns
(Le Gal et al., 2011)	Candidate gene association study	Caucasian	rs3812718 (higher freq of A allele and AA genotype in FS)	113 PEnoFS v 199 controls	ns	ns
				62 PE+FS v 199 controls	ns	ns
				102 pureFS v 199 controls	ns	ns
				164 (PE+FS and pureFS) v 234 (controls and PEnoFS)	ns	ns
(Le Gal et al., 2011)	Meta-analysis	Caucasian	rs3812718	723 PEnoFS v 900 controls	ns	ns
				152 PE+FS v 900 controls	9.1×10^{-6}	8.1×10^{-6}
				246 pureFS v 900 controls	1.4×10^{-3}	3.6×10^{-4}
				421 (PE+FS and pureFS) v 1623 (controls and PEnoFS)	1.5×10^{-7}	4.8×10^{-8}

(Zhang et al., 2010)	Candidate gene association study	Han Chinese	rs3812718 and 7 other SNPs	97 PE+FS v 848 controls	ns	ns
				97 PE+FS v 63 PEnoFS	ns	ns
(Balan et al., 2012)	Candidate gene association study	South India	rs3812718 (higher freq of A allele and AA genotype in MTLEHS)	203 MTLEHS v 282 controls	0.0001	0.0006
				138 MTLEHS+FS v 282 controls	0.001	0.005
				65 MTLEHSnoFS v 282 controls	0.006	0.01
				138 MTLEHS+FS v 65 MTLEHSnoFS	ns	ns

Table 1.13 Candidate gene population-based association studies of the *SCN1A* splice site variant polymorphism rs3812718 and febrile seizures.

Abbreviations: FS, febrile seizures; ns, not significant; PE, partial epilepsy.

a For some patients included in this study, the rs922224 polymorphism, which is a perfect proxy for rs3812718 (r^2=1), was genotyped.

1.5.6 Genetic epilepsy with febrile seizures plus

Genetic epilepsy with febrile seizures plus (GEFS+), previously generalised epilepsy with febrile seizures plus, is a familial epilepsy syndrome, first reported in 1997 (Scheffer & Berkovic 1997). At least two family members must have phenotypes compatible with the GEFS+ spectrum for the diagnosis to be considered.

Typically there is phenotypic heterogeneity within and between families (Scheffer and Berkovic 1997;Singh et al., 1999), although some families may show just one phenotype.

The phenotypic spectrum of GEFS+ has expanded since the initial description. The GEFS+ phenotypes range from mild to severe, from simple FS (mild end of the spectrum), to myoclonic-astatic epilepsy (Singh et al., 1999) and Dravet syndrome (Scheffer et al., 2009;Singh et al., 2001;Veggiotti et al., 2001), on the other end of the spectrum. The clinical picture may include classical FS; FS plus (FS+), where FS persist beyond age 6 years or coexist with afebrile generalised tonic–clonic seizures; or a combination of FS or FS+ with other seizure types, generalised or partial.

The name change from "generalised" to "genetic epilepsy with febrile seizures plus" was proposed after it became clear that not only generalized, but also partial epilepsies, could be recognised in GEFS+ families (Abou-Khalil et al., 2001;Ito et al., 2002;Scheffer & Berkovic 2008;Scheffer et al., 2007). The frequency of "partial epilepsy only" phenotype in GEFS+ families has been estimated at 4% (Scheffer et al., 2012b). This may include "complex partial" seizures, some "with temporal lobe semiology", with or without antecedents of FS, with or without HS.

1.5.6.1 Dravet syndrome is part of the spectrum of GEFS+

Dravet syndrome (DS) is a severe epilepsy syndrome of infantile onset, with multiple seizure types, development delay and poor outcome (Dravet 1978;Dravet et al., 2005), in the severe end of the GEFS+ spectrum (Scheffer et al., 2009;Singh et al., 2001;Veggiotti et al., 2001).

SCN1A mutations, truncating or missense, are found in more than 70% of people with DS (Claes et al., 2001;Claes et al., 2003;Fujiwara et al., 2003;Fujiwara 2006;Harkin et al., 2007;Kanai et al., 2004;Mulley et al., 2005;Ohmori et al., 2002;Sugawara et al., 2002;Sun et al., 2008).This also includes deletions of the *SCN1A* gene (Depienne et al., 2009b;Marini et al., 2009;Mulley et al., 2006).

1.5.6.2 Temporal lobe epilepsy is part of the spectrum of GEFS+

Mutations of the *SCN1A* gene have been found in about 10% of the GEFS+ families (Abou-Khalil et al., 2001;Annesi et al., 2003;Livingston et al., 2009;Nicita et al., 2010), including GEFS+ families with TLE and antecedents of FS (Colosimo et al., 2007).

There is genetic heterogeneity in GEFS+, with mutations in *SCN1B* (encoding the sodium channel β1 subunit) (Scheffer et al., 2007), *GABRG2* ($GABA_A$ receptor γ2 subunit) and other genes, listed in Table 1.10. The phenotypic heterogeneity seen in GEFS+ is likely to be due to modifier genes and environmental factors.

TLE is a possible phenotype within GEFS+ families (Scheffer et al., 2007). In this context, TLE could be hypothesized to be a remote consequence of prolonged FS/ febrile status, or a direct expression of the same underlying genetic variation. Scheffer and

colleagues (2007) screened a large number of individuals for *SCN1B* mutations in GEFS+: several patients with TLE from GEFS+ families, preceded or not by FS, associated or not with HS, were found to have *SCN1B* mutations (Scheffer et al., 2007).

1.6 Relationship between MTLE, HS and FS

MTLE and hippocampal sclerosis are known to be associated with personal antecedents of prolonged FS (Falconer 1971;Kuks et al., 1993) and a family history of FS (Maher & McLachlan 1995;Wallace et al., 1998). Over two-thirds of patients with MTLEHS had FS in childhood (French et al., 1993). The relationship between MTLE and FS is very interesting and complex (Cendes 2004;Harvey et al., 1995). One of the most controversial issues in epilepsy research is whether prolonged FS in fact "cause" HS and TLE (Shinnar 2003;Waruiru and Appleton 2004).

An association between HS and antecedents of FS is documented in clinical reports (Harvey et al., 1995) and surgical series (Thadani et al., 1995). Retrospective studies have shown a significant association between prolonged FS and HS, as diagnosed on MRI or postoperative histopathology (Abou-Khalil et al., 1993;Cendes et al., 1993;Falconer 1971;Falconer 1974). A series of 100 patients who had resective surgery for refractory TLE, showed that a significant proportion of patients with HS had antecedents of prolonged FS in early childhood (30% in the HS group compared with 6% in the group without HS) (Falconer et al., 1964). On the other hand, population-based prospective studies have shown no association between FS and HS (Camfield et al., 1994;Nelson and Ellenberg 1976;Tarkka et al., 2003). The results of large ongoing prospective studies (FEBSTAT study) could clarify these relationships.

In families with FS, TLEHS was shown to develop more frequently in family members who had prolonged FS (Maher and McLachlan 1995).

A hospital-based study looking whether there were clinical differences between patients with MTLEHS and FS and patients with MTLEHS without FS, included 136 MTLE patients from three tertiary referral centres, 79 with MTLEHS (45 histopathologically-confirmed). 52/136 (38%) had antecedents of FS, significantly more frequently in patients with MTLEHS (46.8%) than in MTLE without HS (26.3%, p = 0.0199). 108/136 (79.4%) MTLE patients were drug-resistant. A significantly higher frequency of personal history of FS was found in the drug-resistant group (43.5%) when compared to the drug-responsive group (17.8%; p = 0.008). A significantly higher frequency of HS was also found in the drug-resistant group (64.8%) compared with the drug-responsive group (32.1%; p = 0.0025) (Pittau et al., 2009).

Outcome after epilepsy surgery in MTLE has been reported to be better for patients with MTLEHS and FS than in those without FS, although there are discordant data in the literature. A surgical series of 47 patients with TLE included 19 with FS (17 with prolonged FS). Compared with the group without FS, the group with FS was found to have more frequently an excellent outcome (in 95%), more frequent HS on neuropathology and more frequent family history of FS (Abou-Khalil et al., 1993). In another surgical series, of 133 patients with MTLEHS, 36 had a history of prolonged FS. In the group with prolonged FS, seizure-freedom 2 years after surgery was 91%, compared to 64% in the non-FS group (p = 0.0023) and this difference was significant even after considering other known predictive factors for MTLE (Janszky et al., 2003).

Not concordant with these results is another surgical series, where 118 patients with histopathologically-confirmed HS were followed up over one year. A personal history of status epilepticus was predictive of a negative surgical outcome, but no other variables were found to be significant predictors of outcome, including personal antecedents of FS (Hardy et al., 2003).

Genetics of FS and relationship with MTLE and HS

Several clinical studies have shown a significant association between prolonged FS, MTLE and HS (Baulac et al., 2004;Cendes 2004;Shinnar et al., 2012). How to interpret this relationship is not fully resolved. Complex interactions between genetic factors and environmental factors are likely to be involved in the association of FS with MTLEHS (Cendes 2004), but it is still not clear whether the prolonged FS results from hippocampal damage through genetic predisposition, or prenatal or perinatal insults, or whether it is the prolonged FS that leads to hippocampal damage and later hippocampal sclerosis, in genetically predisposed individuals (Cendes 2004).

There are several possible hypothetical models (not mutually exclusive) for genetic contribution to susceptibility to MTLE, HS and FS.

a) Genetic susceptibility may lower the seizure threshold, so that during a certain age window the stimulus of fever could be enough to reach that threshold, leading to FS;

b) Genetic susceptibility may lead to increased hippocampal susceptibility to damage in face of the challenge posed by prolonged FS;

c) Genetic susceptibility may lead to a cascade of events after FS, leading to epileptogenic mechanisms and chronic epilepsy some time later.

1.6.1 Acute MRI studies in children with prolonged febrile seizures

Imaging studies have shown that prolonged FS/ febrile status epilepticus in early childhood may lead to acute imaging changes in the hippocampus (Farina et al., 2004;Provenzale et al., 2008;VanLandingham et al., 1998) and some of these children may later develop TLE (Cendes 2004).

A case report included repeat MR imaging in one infant, who presented at 15 months with one unprovoked right-sided focal seizure and six months later developed medically refractory TLE with HS seen on MRI and on histopathology after resective surgery. Initial MRI showed diffusion-weighted changes suggestive of edema in the left hippocampus and later MRI showed hyperintensity on T2-weighted sequences of the left hippocampus with atrophy, i.e. unilateral HS (Pinto et al., 2011).

Recently published results of the FEBSTAT prospective study confirm that, in a proportion of children who had prolonged FS, some of the hippocampal changes found on the initial MRI could be biomarkers for later epilepsy (Shinnar et al., 2012), but longer follow-up is needed for definitive conclusions.

1.6.2 Animal models of febrile seizures and epileptogenesis

Animal models can give clues on how prolonged FS can be followed sometimes by chronic epilepsy and may allow a glimpse into epileptogenesis (Bender & Baram 2007). The question of whether specific genetic and acquired predisposing factors are needed for epilepsy to follow prolonged FS may also be addressed with animal models (Dube et al., 2010).

In the 1970s, animal studies with induction of status epilepticus in baboons leading to hippocampal damage (Meldrum et al., 1973;Meldrum & Brierley 1973) added support to the theory that prolonged FS could "cause" HS. More recently, the animal model of FS using induction of hyperthermia with a heated airstream (Baram et al., 1997), significantly contributed to answering these questions. Epilepsy occurs in 35% of rats after these experimentally-induced prolonged FS, which affords an opportunity to look into epileptogenesis and the influence of genetic factors in this process (Bender and Baram 2007;Dube et al., 2010;McClelland et al., 2011).

Animal models also provided some clues that neuronal death is not necessary for acquired epileptogenesis in the immature brain (Dudek et al., 2010). No appreciable neuronal loss nor altered neurogenesis were found in the hippocampus in this animal model of prolonged FS, only mossy fiber plasticity and enhanced hippocampal excitability, postulated to lead to a long-term hyperexcitable hippocampal network and epilepsy later in life (Bender et al., 2003).

Further, interleukin (IL)-1beta levels were found to be chronically elevated only in rats developing spontaneous limbic seizures after febrile status epilepticus, consistent with a role for this inflammatory mediator in epileptogenesis (Dube et al., 2010). This again shows there seem to be objective differences between animals who develop epilepsy after the "initial precipitating insult" and the ones who do not. The relationship between prolonged FS and epilepsy in animal models is a field of intense research (Dube et al., 2010;Stafstrom 2011).

Also in humans, inflammatory mediators (IL-1β, IL-6 and tumor necrosis factor - TNF), have been found to play a key role in rendering the nervous system in proconvulsive, proexcitatory and neurotoxic states (Ismail & Kossoff 2011;Nabbout et al., 2011).

1.7 Role of *SCN1A* genetic variation in Neurogenetics

SCN1A is the major epilepsy gene identified so far. Seizure disorders associated with *SCN1A* mutations range from simple FS to severe epileptic encephalopathies, such as malignant migrating partial seizures of infancy (MPSI) and Dravet syndrome (DS), including severe infantile multifocal epilepsy (SIMFE) (Harkin et al., 2007).

SCN1A has also been shown to contribute to the risk of other neurological disorders, namely familial hemiplegic migraine (Dichgans et al., 2005;Vanmolkot et al., 2007) and elicited repetitive blindness (Vahedi et al., 2009).

Tables 1.14 to 1.16 summarise the epilepsy syndromes and other neurogenetic disorders, for which *SCN1A* genetic variation contributes to increased susceptibility.

SCN1A common variation has been shown to have a role in the pharmacogenetics of some antiepileptic drugs (Tate et al., 2006). Small and possibly underpowered association studies could not replicate this association (Manna et al., 2011;Zimprich et al., 2008). Table 1.17 gives a brief overview of pharmacogenetic studies in epilepsy.

Epilepsy syndrome and/or antecedent history of FS	***SCN1A* mutation**	**Study methodology**	**Pedigree description**	**Author, year**
MTLE and FS: Three (17y, 22y, 38y).	M145T	Linkage analysis on *FEB*1-6 proved linkage to *FEB*3, followed by *SCN1A* seq	One family from Cantabria, Italy/ AD with incomplete penetrance/ 35 individuals over 4 generations, 12 (13 affected, one not tested). All FS were simple FS, before 6yo (5mo-4y, mean 18mo).	(Mantegazza et al., 2005)
TLE (with SPS, very rare CPS and/or SGTCS): Three. TLE and HS on MRI: Two.	M145T (DI-S1)	*SCN1A* seq	Southern Italy, 35 members, 14 affected, 13 alive had had FS <6y.	(Colosimo et al., 2007)
ICE-GTC (CPS since 5y, L HS): one child. Mother, 25y, had FS and later GTCS. Sister had one FS.	F218L (DI-S4)	*SCN1A* seq	One family.	(Livingston et al., 2009)
16/16 affected and tested had *SCN1A* mutation. Right TLE and HS: proband. TLE: 4. PE: 3. Asymptomatic first-degree relative with *SCN1A* mutation: 1.	K1270T (DIII-S2)	*SCN1A* seq	One large GEFS+ family, 27 affected, 18 alive, all 18 had had FS.	(Abou-Khalil et al., 2001)

Table 1.14 Role of *SCN1A* genetic variation in temporal lobe epilepsy, hippocampal sclerosis and febrile seizures.
Abbreviations: aa, amino acid; AD, autosomal dominant; ICE-GTC, intractable childhood epilepsy with generalised tonic-clonic seizures; FS, febrile seizures; HS, hippocampal sclerosis; L, left; PE, partial epilepsy; seq, sequencing; TLE, temporal lobe epilepsy.

1.7.1 *SCN1A* and infantile-onset epileptic encephalopathies

1.7.1.1 *SCN1A* and Malignant migrating partial seizures of infancy

Malignant migrating partial seizures of infancy (MPSI) (Caraballo et al., 2008;Coppola et al., 1995;Marsh et al., 2005) is a severe early infantile onset epileptic encephalopathy (EE), with age-dependent onset, typically from 40 days to 3 months, refractory multifocal bilateral independent seizures, which become rapidly very frequent or continuous and later, infantile spasms, regression, global development delay, cortical visual impairment and microcephaly (Carranza Rojo et al., 2011).

A recent study in patients with MPSI screened genes known to be associated with infantile EEs, including *SCN1A*, for mutations and CNVs. Two of fifteen patients had pathogenic genetic variation: one *de novo SCN1A* missense mutation and one *de novo* 11.06 Mb deletion of chromosome 2q24-2q31.1, which included *SCN1A* (Carranza Rojo et al., 2011).

1.7.1.2 *SCN1A* and Dravet syndrome

SCN1A mutations are detected in 70-80% of patients with Dravet syndrome (DS) (Claes et al., 2001;Harkin et al., 2007;Sun et al., 2008). Most are *de novo* (Harkin et al., 2007) and may be truncating (40%), missense (40%), splice site, or small intragenic mutations (Harkin et al., 2007). Microdeletions and duplications including the *SCN1A* gene are found in 10% of *SCN1A*-negative cases (Marini et al., 2009;Mulley et al., 2006). Next-generation sequencing may allow the identification of *SCN1A* genetic variation in *SCN1A*-negative DS cases.

About 5% of cases carry familial mutations (Nabbout et al., 2003). The relatives of patients with familial mutations, who also carry the *SCN1A* mutation, may have GEFS+ phenotypes of varying severity, but usually mild (Kimura et al., 2005). This variable expressivity suggests other genes and possibly also environmental factors, may be contributing to the seizure phenotype.

Both somatic and germline mosaicism have been recorded and, as a consequence, an unaffected parent, or one with a mild phenotype (for example, simple FS) may have one or several children with DS (Depienne et al., 2006;Gennaro et al., 2006;Marini et al., 2006;Morimoto et al., 2006).

DS includes SMEI and severe myoclonic epilepsy of infancy-borderland (SMEB), where one or two cardinal features of SMEI may be missing (Harkin et al., 2007). *SCN1A* mutations may also be found in SMEB (Fujiwara et al., 2003;Fukuma et al., 2004;Harkin et al., 2007), both missense and truncating mutations. Interestingly, the same mutation may lead to SMEI or SMEB in different individuals (Harkin et al., 2007;Mulley et al., 2005).

MRI brain scan in patients with Dravet syndrome and *SCN1A* mutations may show abnormalities. HS has been reported in a few patients with DS and *SCN1A* mutations, including one child out of 58 who had MRI after the age of 4 years (Striano et al., 2007b). One adult DS patient with an *SCN1A* mutation, from a series of 22 adults with Dravet syndrome (12 screened for *SCN1A* mutations), had unilateral HS on MRI performed at the age of 22 years (Catarino et al., 2011b). These data do not seem to support an association between prolonged FS and HS in Dravet syndrome (Guerrini et al.,

2011). In a retrospective review of MRI brain scans of 20 children with *SCN1A* mutation (Van Poppel et al., 2012), 10 had definite or possible hippocampal sclerosis; five had DS. Prospective studies are needed to further address this question.

1.7.1.3 *SCN1A* and vaccine encephalopathy

A retrospective clinical and genetic study of 14 cases with alleged vaccine encephalopathy found that 12/14 had diagnostic criteria for DS. *SCN1A* mutations were found in 11/12 DS cases, 5 truncating and 6 missense (Berkovic et al., 2006a).

More recently, a retrospective study replicated this finding. Five children with a diagnosis of alleged vaccine encephalopathy by pertussis vaccination in infancy, had clinical histories compatible with DS and all five had *SCN1A* mutations (Reyes et al., 2011).

In the study of DS presented in Chapter 7, eleven of the 22 adult DS patients had a close temporal relation of seizure onset and vaccination, 9/11 had a previous diagnosis of alleged vaccine encephalopathy and 4/11 had *SCN1A* mutations, one truncating, 2 missense and one splice site mutation (Catarino et al., 2011b).

A retrospective study of 40 DS patients, all with *SCN1A* mutations (12 missense, 18 truncation and 10 other), looked for clinical differences between patients with seizure onset within 2 days after vaccination and patients with seizure onset 2 days or more after or before vaccination. Mean age at onset was 28.4 weeks (SD 5.9) in the first group and 26.2 (SD 8.1) in the second, with both groups with a similar long-term outcome. Vaccination might, therefore, trigger earlier onset of DS in children who would develop

the disease anyway, but no evidence was found for influence in the long-term outcome (McIntosh et al., 2010).

1.7.1.4 *SCN1A* and GEFS+ syndrome

GEFS+ was initially identified in large pedigrees with autosomal dominant inheritance and incomplete penetrance (Scheffer and Berkovic 1997). Pathogenic rare genetic variation has been identified in large autosomal families, but GEFS+ also occurs in small families, many unrecognized and can be considered a "complex" disorder, involving several genes and an environmental contribution (Scheffer et al., 2009).

Only a minority (10%) of families with GEFS+ have mutations in *SCN1A* (Abou-Khalil et al., 2001;Annesi et al., 2003;Livingston et al., 2009;Nicita et al., 2010) and rarely in *SCN1B* and *GABRG2* (Baulac et al., 2001). In the majority of GEFS+ families, no molecular basis has been so far identified (Scheffer et al., 2009).

A study of twelve GEFS+ families with patients with an *SCN1A* mutation, showed an earlier median age at onset of FS than the general population (Sijben et al., 2009). This may be the first genetic factor described to modulate age at onset of FS, but the findings need to be replicated.

Epilepsy syndrome(s)	***SCN1A* mutations**	**Patients included**	**Author, year**
Partial epilepsy (with antecedents of febrile status epilepticus)	Heterozygous, de novo, missense	1 patient (4y)	(Okumura et al., 2007)
Migrating partial seizures in infancy	R862G 11.06Mb deletion of 2q24-2q31.1 (deletion includes *SCN1A*)	2 children (out of 15 screened)	(Carranza Rojo et al., 2011)
Panayiotopoulos syndrome	F218L	1 family, 2 siblings and asymptomatic father	(Livingston et al., 2009)
Hemiconvulsion-hemiplegia syndrome in DS	R1892X	1 patient (2y)	(Sakakibara et al., 2009)
Rasmussen syndrome	R1575C	1 patient	(Ohmori et al., 2008a)

Table 1.15 Role of the *SCN1A* gene in other epilepsy syndromes.

Abbreviations: DS, Dravet syndrome; EE, epileptic encephalopathies; FS, febrile seizures; GEFS+, genetic epilepsy with febrile seizures plus; TLE, temporal lobe epilepsy.

1.7.1.5 *SCN1A* and partial epilepsy, including MTLEHS

SCN1A mutations may contribute to susceptibility to partial epilepsy, depending on other genetic or non-genetic factors.

A 12 year-old boy with an *SCN1A* mutation had a history compatible with intractable childhood epilepsy with generalised tonic-clonic seizures (ICE-GTC), with prolonged generalised clonic febrile seizures at 10 months, afebrile prolonged generalised clonic nocturnal seizures from 3 to 5 years of age and complex partial seizures from the age of 5. He had moderate global learning disability, behavioural problems and autistic features. MRI at the age of 10 years showed left HS. He had a left temporal lobectomy. His mother had febrile seizures and generalised tonic-clonic seizures and his sister had one febrile seizure, the family history is compatible with GEFS+ (Livingston et al., 2009).

A 3.5 year-old child had one prolonged hemiclonic seizure with secondary generalisation in context of fever aged 5 months, followed by frequent generalised febrile seizures and onset of partial seizures from the age of 2.5 years, was found to have a de novo missense *SCN1A* mutation (Okumura et al., 2007).

SCN1A mutations have also been found in GEFS+ families with partial epilepsy (Abou-Khalil et al., 2001;Ito et al., 2002). TLE with or without HS is a possible phenotype in GEFS+ families carrying *SCN1A* mutations (Abou-Khalil et al., 2001;Ito et al., 2002;Scheffer et al., 2007) (Table 1.14).

A large family has been described with several affected members with TLE with or without HS and antecedents of simple FS, with a *SCN1A* mutation segregating with the epilepsy and FS (Colosimo et al., 2007).

1.7.2 *SCN1A* and other neurological diseases

Other neurologic diseases where *SCN1A* mutations proved to contribute to susceptibility include some acute encephalopathies, familial hemiplegic migraine and elicited repetitive blindness, as shown in Table 1.16.

1.7.3 *SCN1A* mutations in asymptomatic individuals

SCN1A mutations have rarely been found in asymptomatic individuals. These include unaffected relatives in GEFS+ families (Abou-Khalil et al., 2001) and healthy adult controls (Klassen et al., 2011).

The missense mutations of the *SCN1A* gene, M1841T (Annesi et al., 2003) and R1916G (Combi et al., 2007), each identified in one GEFS+ family, lead to almost complete loss of function of $Na_v1.1$, which could be attributable to folding defects. These folding defects of the $Na_v1.1$-mutants could be "rescued" through molecular interactions with accessory/ modulatory proteins (such as the beta1 subunit of the sodium channel), or pharmacological chaperones, such as sodium channel blockers (Rusconi et al., 2007;Rusconi et al., 2009).

Neurological disease	***SCN1A* mutations/deletions**	**Study methodology**	**Author, year**
Acute encephalopathy with seizures	V982L (PE and AE) M1977L (GEFS+ and AE) R1575C (AERRPS)	3/87 Japanese children with AE and 0/100 healthy controls had *SCN1A* missense mutations; 1 affected sister had also AE but no *SCN1A* mutation.	(Saitoh et al., 2012)
Acute encephalopathy		1/15 Japanese patients with AE had *SCN1A* mutation.	(Kobayashi et al., 2010)
Familial hemiplegic migraine (FHM3)	Q1498K	Genome-wide linkage analysis of 2 FMH families (without mutations in *CACNA1A* or *ATP1A2)*	(Dichgans et al., 2005)
	T4946A	10 FMH families (without mutations in *CACNA1A* or *ATP1A2)* screened: 1 North American kindred with *SCN1A* mutation	(Vanmolkot et al., 2007)
Elicited repetitive daily blindness, associated with hemiplegic migraine.	mutations	2 families with FMH and ERDB, sequencing of *CACNA1A, ATP1A2, SCN1A*	(Vahedi et al., 2009)

Table 1.16 Role of the *SCN1A* gene in other neurologic diseases.

Abbreviations: AE, acute encephalopathy; AERRPS, acute encephalitis with refractory repetitive partial seizures; CPS, complex partial seizures; ERDB, elicited repetitive daily blindness; FS, febrile seizures; GEFS+, genetic epilepsy with febrile seizures plus; PE, partial epilepsy; TLE, temporal lobe epilepsy.

Recently, Noebels's group performed exome sequencing of 237 ion channel genes, in groups of unaffected individuals and patients with sporadic idiopathic epilepsy. Interestingly, they found rare missense variation in known "Mendelian" disease genes in both groups, thereby showing that pathogenic ion channel mutations may confer a certain risk or increase susceptibility, but the final phenotype will depend on each individual's full complex allelic architecture (Klassen et al., 2011).

Previous work from this group had shown that animals with specific mutations in two ion channel genes, *Kcna1* and *Cacna1a*, experienced fewer seizures and had a higher survival rate than single-mutant animals; this protective interaction between pathogenic ion channel variants could alter the clinical expression of epilepsy (Glasscock et al., 2007).

Mechanism	AEDs	Gene	Author, year
Drug targets	CBZ, PHT	*SCN1A* / AED resistance	(Tate et al., 2005) (Manna et al., 2011;Zimprich et al., 2008)*
Drug transport	multidrug	*ABCB1* / AED resistance	(Soranzo et al., 2004) (Tan et al., 2004a;Zimprich et al., 2004)*
Drug metabolism	PHT	*CYP2C9*	(Chaudhry et al., 2010;Mamiya et al., 1998)
	PHT, DZP	*CYP2C19*	
HLA	CBZ	HLA-A*3101 / CBZ-induced hypersensitivity in Europeans	(McCormack et al., 2011)
	CBZ	HLA-B*1502 / CBZ-induced hypersensitivity in Chinese	(Chung et al., 2004)

Table 1.17 Association studies and pharmacogenetics of epilepsy.

Abbreviations: AED, antiepileptic drug; CBZ, carbamazepine; DZP, diazepam; PHT, phenytoin.

* Negative replication studies.

2 Chapter Methods

2.1 Introduction

This chapter outlines the general procedures and statistical methodology used for the genome-wide association studies. A case-control approach was used to look for genetic variants associated with susceptibility to partial epilepsy and to mesial temporal lobe epilepsy with hippocampal sclerosis. The chapters dedicated to each of these two genome-wide association studies (Chapter 4, Genome-wide association study of partial epilepsies; Chapter 5, Genome-wide association study of mesial temporal lobe epilepsy with hippocampal sclerosis) refer to sections in the present chapter for description of methodology used and will expand in more detail on the specifics of each study.

2.2 Ethics approval and patient consent

This project was approved by the relevant local Ethics Committees of the participating institutions. In London, approval was obtained from the Joint Research Ethics Committee of the National Hospital for Neurology and Neurosurgery and the UCL Institute of Neurology. All patients provided written informed consent. Informed assent from relatives or legal guardians was requested in the case of adults with intellectual disability.

2.3 Patient recruitment, inclusion criteria and study cohorts

2.3.1 Patient recruitment strategy

The EPIlepsy GENetics (EPIGEN) Consortium and the GenEpA Consortium are multicentre collaborative efforts, looking for genetic variants contributing to epilepsy.

In London, patient recruitment has been ongoing since 2001, at the specialized adult epilepsy clinics of the National Hospital for Neurology and Neurosurgery, at both the Queen Square and the Chalfont Centre sites. I participated in the recruitment of patients in clinic from 2007 to 2010.

Adults with epilepsy were recruited from the epilepsy clinics in the participating centres (Table 2.1). The recruitment was carried out in normal clinic attendances; no additional procedures, visits or patient contact were made.

The study information sheet[3] was distributed to patients by the nurses prior to the clinic and each patient was given the opportunity to ask any questions regarding the study while in clinic. Patients with a definite diagnosis of epilepsy were asked to participate, after reading the information sheet and asking any question arising. All who agreed then signed the consent form.

2.3.2 Inclusion criteria

The inclusion criteria for the population-based genetic study of epilepsies are: adults with a definite diagnosis of epilepsy and able to provide written informed consent.

[3] The study information sheets and participant consent form are available in Appendix 2.

For the genome-wide association study (GWAS) of partial epilepsies, patients were included only if they matched the International League Against Epilepsy (ILAE) 1989 criteria defining partial (focal) epilepsy, "seizure semiology or findings at investigation disclose a localized origin of the seizures" (ILAE Commission on Classification and Terminology 1989). The epilepsy diagnosis and the epilepsy syndrome classification were reviewed by a senior Consultant Epileptologist and myself.

Patients were included for the GWAS of mesial temporal lobe epilepsy with hippocampal sclerosis (MTLEHS) if they had a compatible electro-clinical syndrome (Wieser 2004), including imaging and/or histopathological confirmation.

2.3.3 Exclusion criteria

Patients were excluded from the study if the diagnosis of epilepsy became questionable or was ruled out after further clinical investigation. If written consent was not retrievable from the study box-files or individual clinical notes, or if patients later withdrew consent, patients were also excluded from the study.

2.3.4 Study cohorts

The study was conducted at the National Hospital for Neurology and Neurosurgery, at both the Queen Square and the Chalfont Centre sites. This project was a collaborative effort, involving several other international centres: for the EPIGEN Consortium (www.epilepsygenetics.eu), UCL Institute of Neurology, National Hospital for Neurology and Neurosurgery Queen Square and Chalfont Centre sites, London, UK; Center for

Human Genome Variation, Institute for Genome Sciences and Policy (IGSP), Duke University, Durham, North Carolina, USA; Beaumont Hospital, Dublin, Ireland; Erasme Hospital, Université Libre de Bruxelles, Brussels, Belgium; for the GenEpA consortium, University Hospital Zurich, Switzerland; University of Eastern Finland, Kuopio University Hospital and University of Helsinki, Helsinki University Central Hospital, Finland; and University of Oslo, Norway. On the GWA study of MTLEHS (stage one/ discovery phase), there was one more collaborating centre, from Vienna University, Austria. Numbers of cases and controls included in the GWA studies for each participating centre are listed in Table 2.1.

2.3.5 Control cohorts

Controls for the GWA study were derived from several cohorts, listed in Table 2.2, across the populations from which the cases had been extracted, with only two exceptions: the cohorts from Belgium and Norway had no specific controls available for inclusion in this GWA study.

Use of shared controls has become frequent in GWA studies across disciplines since the success of the WTCCC study in 2007 (Wellcome Trust Case Control Consortium 2007) and a number of shared control sets are available online.

	UK	Ireland	Belgium	USA	Finland	Switzerland	Norway	Austria	Total
				GWA study of PE					
Patients	1422	670	580	780	428	235	212	NA	**4327**
Controls	5667	211	0	1165	757	285	0	NA	**8085**
				GWA study of MTLEHS					
Patients	331	148	77	97	116	182	70	166	**1187**
Controls	5667	211	0	1165	757	285	0	338	**8423**

Table 2.1 Patients and controls included in the GWA studies: numbers genotyped for each sub-cohort.

Abbreviations: GWA, genome-wide association; MTLEHS, mesial temporal lobe epilepsy with hippocampal sclerosis; NA, not applicable; PE, partial epilepsies.

Centre	Control cohorts[b]	N	Description	References
UK	1958 British birth Cohort	2045	UK 1958 British Birth Cohort (children born to parents resident in Great Britain during the week of 3-9 March 1958).	(Bartley et al., 1994)
UK	WTCCC UK Blood service sample	3622	UK national repository of anonymised DNA samples from 3,622 consenting blood donors. Collection established for the WTCCC studies.	(Wellcome Trust Case Control Consortium 2007)
USA	Duke memory study	1165	Cognitively normal controls, who had taken part in a genetics of memory study at Duke. 84% of participants filled in a questionnaire about history of neurological conditions and subjects who reported a history of seizures were excluded.	(Cirulli et al., 2010;Need et al., 2009a)
Ireland	Irish controls	211	Irish neurologically-normal controls from the Study of Irish Amyotrophic Lateral Sclerosis.	(Cronin et al., 2008)
Finland	Vantaa85+	469	Vantaa-85+ study: people aged 85 or over living in the city of Vantaa (Southern Finland), on April 1, 1991.	(Myllykangas et al., 2005;Peuralinna et al., 2008)
Finland	Finn-GSK	288	Controls without neurological conditions, recruited and genotyped for this study	*Present study*
Switzerland	Swiss controls	285	Controls without neurological conditions, recruited and genotyped for this study	*Present study*
Austria[a]	Austrian controls	338	Controls without neurological conditions, recruited and genotyped for this study	*Present study*

Table 2.2 Control cohorts used in the genome-wide association studies of partial epilepsy and of MTLEHS.

Abbreviations: N, number of individuals in each cohort; WTCCC, Wellcome Trust Case Control Consortium.

a The Austrian centre only participated in the GWAS of MTLEHS; b No control samples were available from the Belgium or Norwegian centres.

2.4 Phenotyping and creation of London-EPIGEN database

The demographic and clinical details of patients included in the study were stored for each centre in a database. This study necessitated the collection and storage of large amounts of detailed clinical data, obtained mainly from case notes and hospital electronic clinical databases.

The database for the London cohort was created and stored at the UCL Institute of Neurology, Queen Square, in Microsoft® Office Excel format, for the GWA studies presented here and as a large dataset for further studies.

Subjects in each participating cohort have had detailed phenotyping, often over extensive periods of follow-up. The majority of participants come from tertiary referral centres for epilepsy, with comprehensive assessment, investigations, classification and follow-up and all have been evaluated by experienced consultant epileptologists. In the great majority of cases, the syndromic epilepsy diagnosis was reached through clinical appraisal, EEG and brain imaging. Most patients will have long histories of epilepsy and therefore probably more diagnostic clarity regarding their epilepsy syndrome.

2.4.1 Definition and prioritisation of the phenotypes to study

The definition and prioritisation of clinically relevant questions to be addressed with the GWA study of partial epilepsy were discussed and appropriate phenotypes were agreed across the collaborating sites, so that consortium-wide clear definitions, well-characterised phenotypes and homogeneous phenotyping were available across the different cohorts.

2.4.2 Phenotyping

Approximately 2,950 patients with epilepsy from the London cohort were identified and entered in the electronic database created for the GWA studies until March 2011, with 2,910 patients classified by me for epilepsy syndrome and the other main phenotypical variables. Patients classified as partial epilepsy and mesial temporal lobe epilepsy with hippocampal sclerosis were included in the GWA studies described in this thesis.

The clinical records of the patients included in the GWA studies were reviewed by me, both in case notes and hospital electronic databases and the available data reviewed, collated and entered by me in the database. The epilepsy syndrome used was the one stated in the most recent clinic letter. If this was not available or there were discrepant data, the available electro-clinical data were reassessed by me to arrive at the most probable syndromic diagnosis. Any change of epilepsy syndrome diagnosis over time was noted and the first diagnosis, current diagnosis and reason for the change were noted by me in the database.

An audit of the phenotypical information included by me in the database was performed just before the first batch of DNA samples was genotyped. Of the 1,812 patients with epilepsy initially genotyped as part of the first stage of the GWA study of partial epilepsy, 1,606 (89%) were audited. The clinical information for the epilepsy syndromic classification was checked by a senior epileptologist. There were 66 discrepancies in the classification (4%), which led to reclassification in 12 cases (0.8%). Furthermore, the diagnosis of MTLEHS was audited by re-checking all case notes, blinded to the first assessment. Any discrepancy was noted and entered into the database.

Phenotypes	Categories	Definition
Epilepsy syndrome	1 = partial	The diagnosis of partial epilepsy was made according to the International League Against Epilepsy classification (ILAE Commission on Classification and Terminology 1989) - "seizure semiology or findings at investigation disclose a localized origin of the seizures" and reviewed by a consultant epileptologist.
	2 = generalised	
	3 = unclear whether 1 or 2	
	4 = both 1 and 2	
TLE	Yes/No/Unclear	The diagnosis of TLE was made according to the ILAE classification (ILAE Commission on Classification and Terminology 1989).
MTLE	Yes/No/Unclear	MTLE of any aetiology (cryptogenic; tumoral, infectious, etc). May include bilateral HS, or dual pathology, when the electroclinical syndrome is MTLE.
MTLEHS	Yes/No/Unclear	The diagnosis of MTLEHS was made by a compatible electro-clinical syndrome (Wieser 2004); all cases had imaging and/or histopathological confirmation. Individuals with bilateral HS and/or dual pathology were excluded.
HS	Yes/No If yes, unilateral/bilateral.	Hippocampal sclerosis visible on the MRI brain scan. Unilateral or bilateral. Radiological diagnosis, independent of epilepsy syndrome.
Aetiology	Unknown/known Description	Probable / possible aetiology of the epilepsy.
MRI brain scan findings	Description	Description of the MRI findings.
Febrile seizures	Yes/No/Unclear	Personal antecedents of childhood FS, according to ILAE definition (ILAE Commission on Epidemiology and Prognosis 1993). Indication of degree of probability and source of information.
Any SG	None - No; ≥1 – Yes	One or more partial seizures with secondary generalization.
Habitual SG	None, or rare, or only at epilepsy onset before starting medication - No; otherwise – Yes.	Habitual partial seizures with secondary generalization.
Age at onset of habitual seizures	Age in years	Age at onset of habitual seizures.

Self-reported ethnicity	Description	Self-reported ethnicity as recorded.
Seizure-related family history	Yes/No If yes, description	Description of family history of epilepsy or febrile seizures.

Table 2.3 Phenotypic definitions used in the discovery phase of the GWA studies.

Abbreviations: HS, hippocampal sclerosis; MTLE, mesial temporal lobe epilepsy; SG, secondary generalization; TLE, temporal lobe epilepsy.
Note: The "unclear" category includes cases where it was not possible to arrive at a robust epilepsy syndromic diagnosis with the available data.

2.4.3 Creation of the London-EPIGEN database

The primary justification for creating the London-EPIGEN database was the expected value of the infrastructure for the present studies and also for future studies. The database was built using Microsoft® Office Excel for Windows, version 2003.

Basic demographic data (name, surname, date of birth, hospital number) were initially entered for each patient for whom a hard-copy consent form was archived in the box-files kept for the study.[4] A systematic effort was also made to look and retrieve consent forms from case notes, for any patients with a DNA number assigned for the EPIGEN study.

[4] This was done for organizational purposes, to identify all existing duplicates. Identifiers were then deleted from the database, and the data anonymised.

All DNA numbers included in previous studies were entered into the database. A search for all other DNA numbers corresponding to the individuals in the London-EPIGEN database was conducted from the UCL Institute of Neurology Neurogenetics Laboratory database. DNA numbers from the Neurogenetics Lab database were retrospectively searched for all patients with consent forms filed for the EPIGEN study. For those patients prospectively entering the study after the database was set-up, DNA numbers were made available by the Neurogenetics Lab and entered in the database.

For some individuals, data available from previous studies were retrieved. Where possible, case notes were reviewed to confirm any existing relevant data.

Data on the database were regularly audited for quality. Any duplicates were removed, after the information pertaining to that one case was concatenated. Any discrepancies between data from different sources were noted. All sources of information were noted into the database.

2.4.4 Phenotyping data from collaborating centres

The phenotype data were collected for each centre according to the definitions agreed across the consortium for these GWA studies, listed in Table 2.3, and transferred from all centres to London, where it was entered into phenotype files for data analysis**.**

All centres involved were tertiary epilepsy referral centres, phenotyping definitions and criteria were previously agreed across the Epigen consortium and with the Genepa consortium, and the criteria used (ILAE definitions) previously discussed and agreed on.

Problems identified	Solutions implemented
No unique patient identifiers after anonymising data.	Give each patient an alphanumeric unique identifier.
Patients may change surname, e.g. through marriage. Alternative first names (abbreviations, occasional use of middle name in addition to first name),	Include columns for name, surname, alternative name, alternative surname. Check each patient's name, surname, hospital number and date of birth in the hospital electronic records and clinical notes.
More than one patient may have the same name and surname.	Record on the database when two patients share the same name and surname; ensure that these are checked and they are two different people.
Alternative hospital numbers, Some patients have more than one hospital number.	Record all hospital numbers and check each patient's electronic hospital record for any more hospital numbers.
Alternative DNA numbers, Patients may have up to five DNA numbers.	Record all DNA numbers. Review Neurogenetics laboratory database for all samples marked "EPIGEN", "population-based genetic study", or any other alternative name of the study.
Some patients may share the same date of birth.	Recheck information in hospital electronic records.
Discrepancies in the source databases.	Record on the database any discrepancies found between data sources and any reasons for them and date the record. Keep activity log in the database.
Patient names may be misspelled.	Check in hospital electronic records the correct name and note in the database any previous misspellings and where the error stemmed from.
Patient names may be written with the first and surnames the wrong way around.	Check in hospital electronic records and record in database.
Patient hospital numbers, DOB may occasionally be incorrect.	Check in hospital electronic records and record in database.
Labels of the EPIGEN study were very heterogeneous in the first years.	Check all possible labels for the study in the Neurogenetics laboratory database.
Difficulties in automatic comparison of databases, due to misspellings, or missing information.	Check each patient record one by one. Cross-check all available data sources. Record which source of data had inaccuracies.

Table 2.4 Issues during the creation of the clinical database and solutions found.

2.5 Laboratory methods

2.5.1 DNA extraction

Blood samples were obtained for all patients included in the study. DNA was extracted from blood samples using standard protocols. For some patients with MTLEHS (n=63), who had resective epilepsy surgery, DNA was extracted from resected brain tissue (Heinzen et al., 2010).

2.5.2 DNA quantification and standardization

2.5.2.1 Spectrophotometry

DNA was quantified using a spectrophotometer: the determination of nucleic acid concentration was done by measuring absorbance at 260 nm (Sambrook et al., 1989). After the concentration of DNA was determined, the samples were diluted manually. All aliquoting was performed by two people, to allow for double-checking of the procedure.

2.5.2.2 Automated DNA standardization

The Picogreen® method was used for rechecking dsDNA concentration at Duke University IGSP Laboratory. This method consists of fluorometric quantitation of dsDNA (Ahn et al., 1996).

2.5.3 DNA storage and transport

All DNA samples collected since 2001 were reviewed, catalogued and stored in a dedicated freezer.

For this project, transfer of DNA to Duke University, Durham, NC, was approved by the relevant ethics committees. DNA samples for all included patients from the London cohort and the majority of the samples from collaborating centres, were sent to Duke University. All samples were sent in an anonymised state, in dry ice, by express mail. The identification numbers for all samples were independently checked by me and another researcher, the samples were packed and the information on which samples were sent was entered by me into the London-EPIGEN database.

2.5.4 Genotyping

Genotyping was performed at the genotyping facility of the Institute for Genome Sciences and Policy (IGSP) of Duke University, for the majority of samples included in these GWA studies. For the Wellcome Trust Case-Control Consortium controls and the controls for the Irish, Finnish-Vantaa85+ and Swiss cohorts and both cases and controls of the Austrian cohort, genotyping was performed elsewhere, as shown in Table 2.5.

The majority of samples for the GWA studies was genotyped on the Illumina Human610-Quad chip. A subset of cases was run on other Illumina Infinium™ Beadchips genotyping arrays, which are listed in Table 2.6.

Control cohorts[a]	Source of genotyping data
UK controls (WTCCC 1958 Birth Cohort controls)	Wellcome Trust Case Control Consortium phase 2, September 2009 data release
UK controls (WTCCC UK Blood service sample controls)	
Duke controls	Duke University IGSP
Ireland controls	Genotype data downloaded from the dbGaP database (http://www.ncbi.nlm.nih.gov/gap), with dbGaP accession number phs000127.v1.p1.
Finland controls (Finn-GSK controls)	Duke University IGSP
Finland controls (Vantaa85+ controls)	(Genotype data received as Beadstudio files)
Switzerland controls	Duke University IGSP
Austrian controls[b,c]	(Genotype data received as Beadstudio files)

Table 2.5 Source of genotyping data for the control cohorts.
Abbreviations: IGSP, Institute of Genome Sciences and Policy (Duke University, NC, USA); WTCCC, Wellcome Trust Case-Control Consortium.
a There were no control samples from the Belgium and Norway centres.
b The Austrian cohort participated in the GWA study of MTLEHS, but not in the GWA study of partial epilepsy.
c Also applies to the genotyping data from Austrian cases.

2.5.4.1 Description of the genotyping process and technology used

The basic workflow of the genotyping process through to genotype calling, for the Illumina Infinium™ assay, is schematically presented in Fig. 2.1.

This Illumina system uses a complex bead array and relies upon a fluorescent reporter mechanism, with a locus specification step at the beginning of the process. This step creates a specifically addressed oligonucleotide chain, which is then amplified by a process in a very similar way to whole-genome amplification (Neale and Purcell 2008).

After whole-genome amplification of the DNA sample, the amplified product is fragmented, then precipitated and resuspended. The Beadchips are prepared for hybridization in the capillary flow-through chamber. The samples are applied to it and incubated. After hybridization of the unlabeled DNA fragment to the 50mer oligonucleotide probe[5] on the array and enzymatic extension, products are then fluorescently stained and the intensities of the beads' fluorescence is detected and finally analyzed using Illumina Beadstudio v3 software, for automated genotype calling.

Detailed genotyping quality procedures were in place, with optimisation of logistics, e.g. regarding plate lay-out and inclusion of duplicate samples (Fellay et al., 2007).

[5] The expression 'mer' denotes the length of the oligonucleotide: '50 mer' means the number of nucleotide units is 50 (Oxford Dictionary of Biochemistry and Molecular Biology, Second edition, Ed Cammack R et al. 2006).

Illumina genotyping chip	Number of probes in chip	Used in the GWA studies of PE and MTLEHS Patients	Used in the GWA studies of PE and MTLEHS Controls
HumanHap300v1	317,503	Ireland, Austria	Austria
HumanCNV370v1	370,404	0	Finland
HumanCNV370-Quadv3	373,397	0	Finland
HumanHap550v1	555,352	0	USA
HumanHap550v3	561,466	UK	Ireland, USA
Human610-Quadv1	620,901	UK, Ireland, Belgium, Finland, USA, Norway, Switzerland	Finland, USA, Switzerland
Human1Mv1	1,072,820	0	USA
Human1M-Duov3	1,070,000	0	Finland
Human1.2M-Duo Custom	1,199,187	0	UK (WTCCC)

Table 2.6 Types of Illumina whole-genome genotyping Infinium™ Beadchips used in the GWA studies of partial epilepsy and of MTLEHS.

Abbreviations: MTLEHS, mesial temporal lobe epilepsy with hippocampal sclerosis; PE, partial epilepsy; WTCCC, Wellcome Trust Case-Control Consortium.
Source: www.illumina.com , "Infinium HD DNA analysis Beadchips", Illumina technical note, 2008. Infinium is a registered trademark of Illumina.

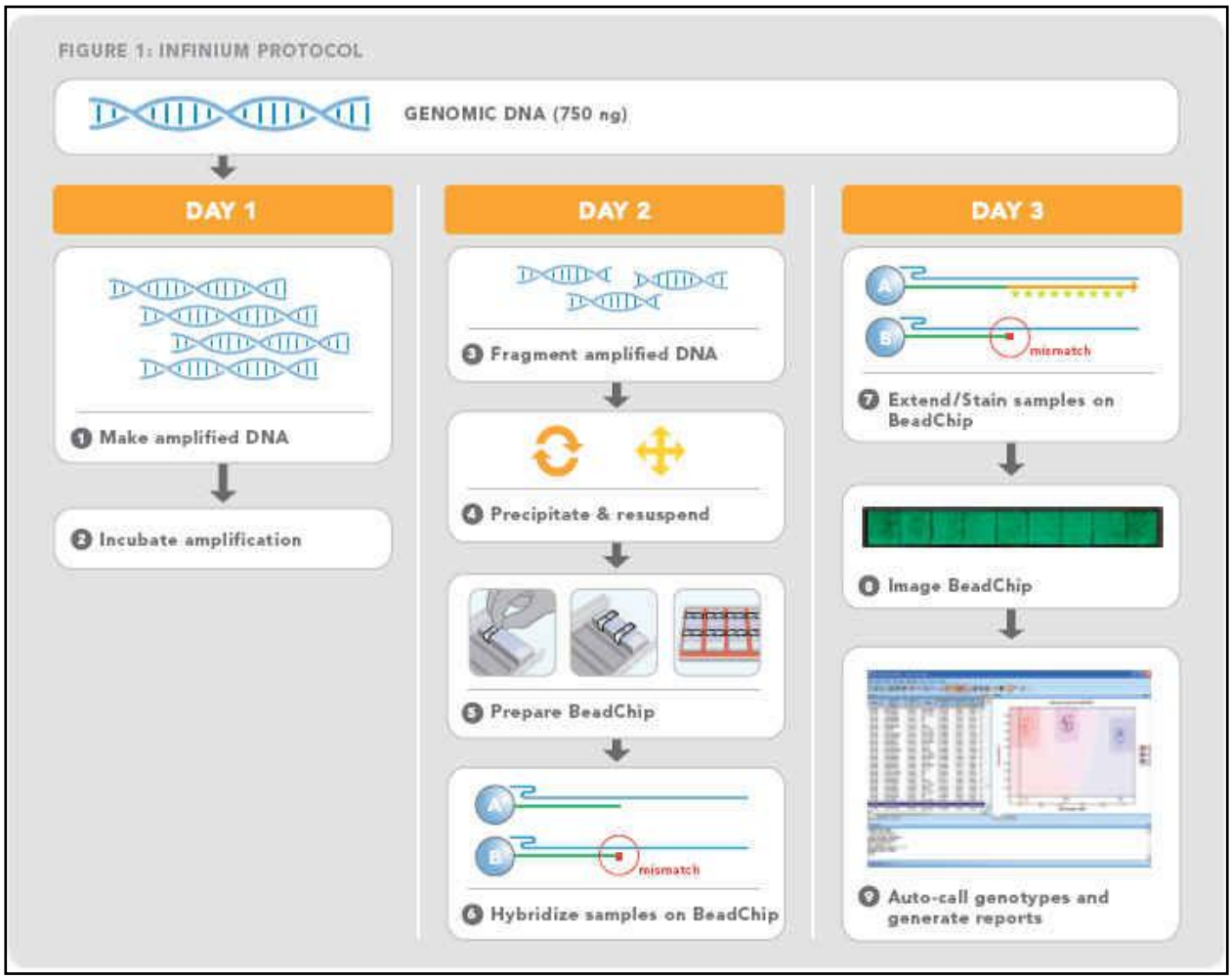

Figure 2.1 Protocol workflow for the Illumina Infinium™ assay.

Source: www.illumina.com. Image courtesy of Illumina, Inc.

Infinium is a registered trademark of Illumina.

2.5.4.2 Genotype calling and genotyping quality control

For this project, the samples genotyped in Duke IGSP were processed in batches of 200-250 each. Genotyping calling and genotyping quality control were performed using Illumina BeadStudio v3 software.

The data for each SNP are represented after normalization as a scatterplot of signal intensities for allele A against allele B; each point represents one subject. Normally, individuals can be separated into three clusters, representing the three possible genotypes, AA, AB and BB (Fig. 2.2).

Visually inspecting genotype calls for all markers would be an unrealistic task. Instead, the calling of these variants is carried out by cluster analysis, under an expectation of one, two, or three clusters - for the up to three genotype categories (Neale and Purcell 2008). Samples were clustered using in-house generated Illumina cluster files and genotype-calling algorithm. After clustering, all samples with call rates lower than 98% were removed. All SNPs with call frequencies less than 100% were then re-clustered. As this can introduce errors, the file is then manually evaluated. All re-clustered SNPs with HetExcess value between -1.0 to -0.1 and 0.1 to 1.0 and all SNPs with cluster separation values between 0 and 0.3, were deleted.

To avoid non-random missingness and false positives, a “1%” rule was applied: all SNPs for which >1% of samples were either not called or had “ambiguous calls” were deleted.

Finally, the raw Illumina output files are translated into files appropriate to be used as input files for the genetic statistics software packages used in the subsequent steps of the analysis.

As with any statistical procedure, errors of the clustering technique are a potential pitfall of this method for genotype calling. Therefore, in post-association analysis, for any SNP showing a significant association signal, the actual intensity plots are visually inspected one by one, as a necessary quality control step,and the SNPs where the plots show problems with the clustering are excluded from further analysis (Neale and Purcell 2008).

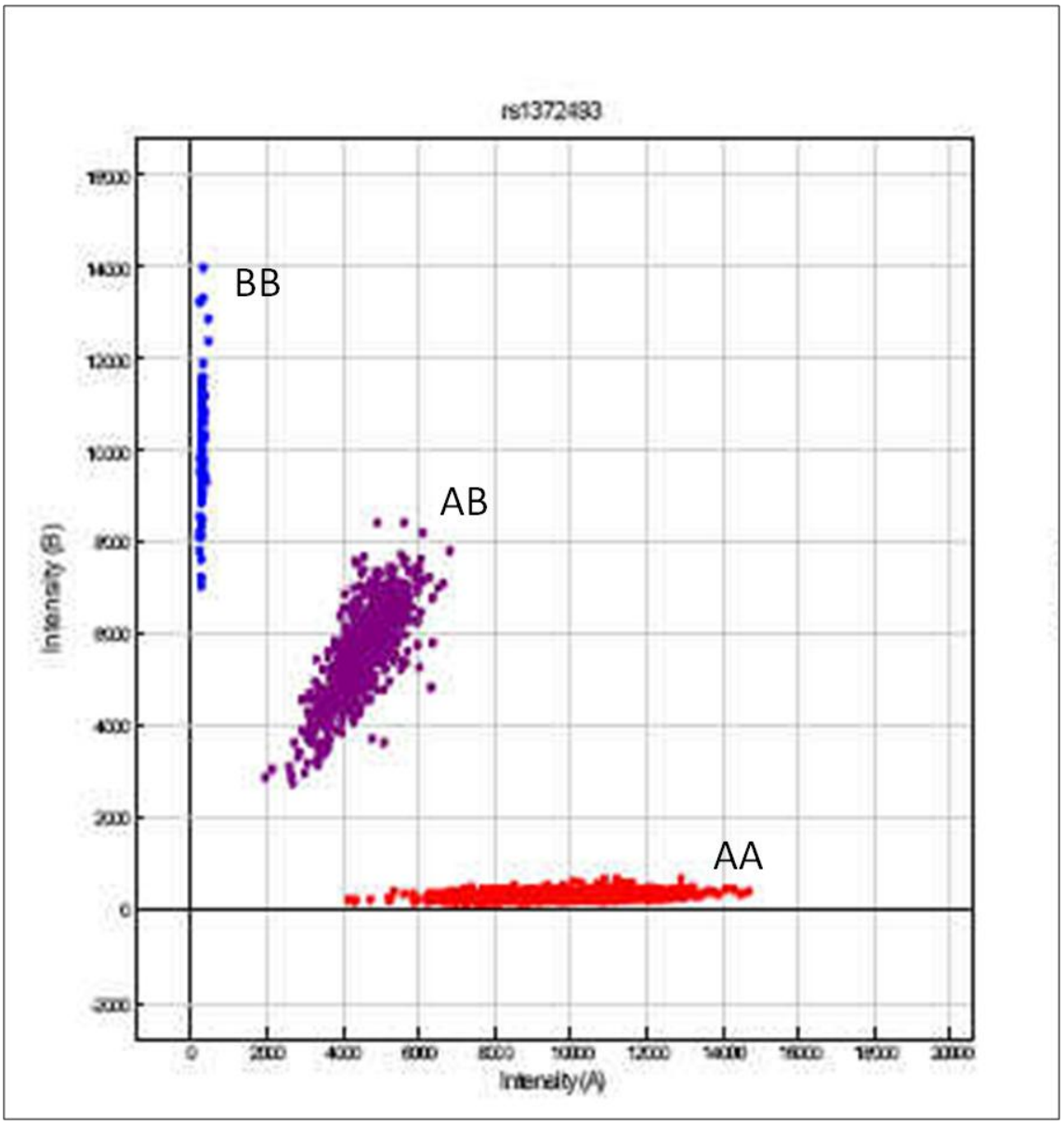

Figure 2.2 Cluster plot depicting genotyping calls for one SNP. This step was performed using Illumina BeadStudio v3 software. The data for each SNP is represented after normalization as a scatterplot of signal intensities for allele A against allele B, with each point representing one subject. Normally, individuals can be separated into three clusters, representing the three possible genotypes AA, AB and BB, with the middle cluster representing the heterozygotes (AB).

2.6 Data analysis

2.6.1 Software used for the data analysis

The genotype data were stored at the Duke University IGSP cluster and remotely accessed via SSH. Some steps of the analysis are computationally intensive and cluster computing was used.

In the Unix operating system-environment, the analysis was performed using the following software programmes:

a. R statistical package (R Development Core Team 2006), www.R-project.org;

b. PLINK (Purcell et al., 2007), http://pngu.mgh.harvard.edu/~purcell/plink;

c. EIGENSOFTplus, an R package to curate EIGENSTRAT analysis (Weale 2010).

The WGAViewer software (Ge et al., 2008) was used during the post-association analysis.

2.6.2 Power calculations

Power is a statistical term which means the probability of identifying a difference between two groups in a study in which that difference truly exists (Pearson and Manolio 2008). For the GWA study of partial epilepsy, power calculations were performed using the PGA Power Calculator software (Menashe et al., 2008), assuming a conservative estimate of disease prevalence of 0.05%, the additive genetic risk model and r^2 0.9 between a causal variant and a genotyped marker.

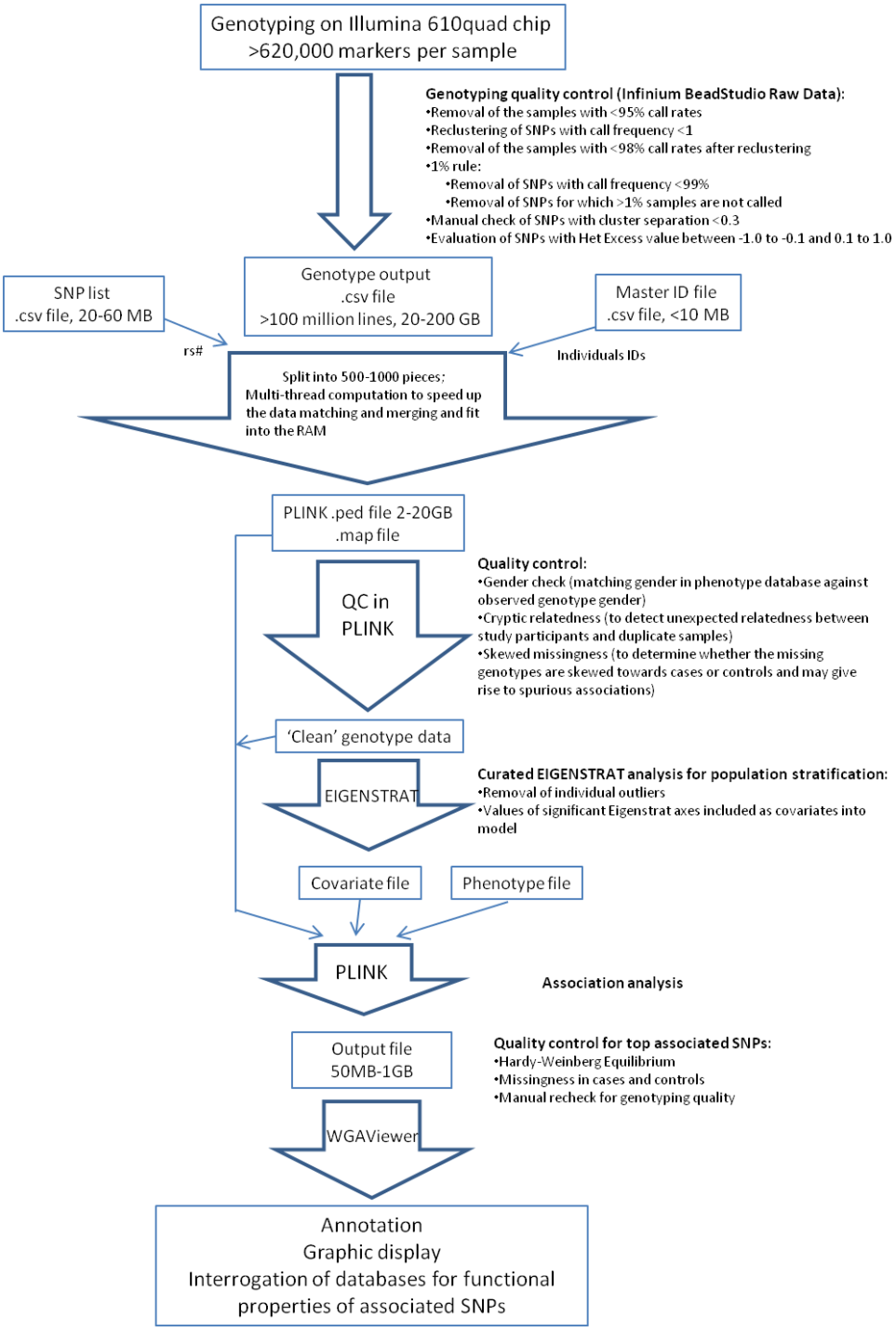

Figure 2.3 Flow chart describing the pipeline for data analysis used in the GWA studies included in this thesis.

This diagram shows several of the quality control steps used, including population stratification detection and correction, using a modified EIGENSTRAT analysis (Price et al., 2006), with Eigensoft Plus; test of association, with PLINK (Purcell et al., 2007); and post-association analysis of the top hits, using the WGAViewer software (Ge et al., 2008).

2.6.3 Statistical analysis

Statistical analysis was performed using the data analysis pipeline schematically shown in Fig. 2.3.

2.6.3.1 Quality control steps for the GWAS data

Quality control (QC) steps were used in the GWA studies to filter misclassified or unclassified SNPs or individuals, thereby excluding them from the downstream association analysis and avoid the possible impact of introducing false positives and/or false negatives.

a) "Gender" mismatches

"Gender" checks were performed for all samples. These are useful as a sanity check of the alignment of genetic and phenotypic data and also to identify and remove subjects who present as "gender" mismatches from downstream analyses. The "gender" check analysis was performed using PLINK (Purcell et al., 2007). X-chromosome data are used for this: as males have no heterozygous genotypes, their X chromosome SNPs will depart from Hardy-Weinberg equilibrium (HWE), while female X-SNPs will be approximately in HWE. The measure used by PLINK to test for "gender" mismatches is the inbreeding coefficient F, which measures the grade of departure from HWE.

Causes of "gender" mismatch include rare medical conditions, for example Turner syndrome (X0 karyotype), Klinefelter syndrome (XXY karyotype) or androgen insensitivity syndrome, X-chromosome mosaicism in females; and can also include labelling errors. One female patient was excluded because of "gender" mismatch, who had received a blood marrow transplant from her brother after an aplastic anaemia.

For the London cohort, all case notes were reviewed for the subjects who were classified as "gender" mismatches and some of these could be kept in the analysis if it could be proven from the demographic data there had been a labeling error of sex in the phenotype database. Individuals were excluded from further analysis when they had an intermediate "gender" call and when the demographic data did not match the "gender" call as calculated in PLINK.

b) Duplicates and cryptic relatedness

Subjects who are closely related will introduce a correlation structure that may lead to false negatives or false positives, particularly if there are widely different proportions of relatives in the cases and controls. This should, therefore, be accounted for and these individuals need to be excluded during the quality control phase.

Cryptic relatedness was checked among all pairs of samples in the study, using identity-by-descent (IBD) in PLINK (Purcell et al., 2007) and excluding one of each pair of individuals when the estimated IBD score was over 0.125. The excluded subject in a pair had the lower genotyping call rate (when individuals in the pair were both cases or both controls), or was the control subject if the pair was discordant for case-control status.

Linkage desiquilibrium-pruning

In preparation for the cryptic relatedness check and the population stratification correction procedures, LD pruning was performed, with the objective to select a smaller set of SNPs with minimal LD among them. Advantages include a shorter time of computer processing and the fact that cryptic relatedness quality control procedures work best under the assumption of no LD among SNPs.

A multi-stage procedure was used. Firstly, large-scale high-LD regions were excluded (Price et al., 2006), as these could create their own principal component (PC) axes in PC analysis. These include a 4Mb inversion on chromosome 8 (Tian et al., 2008), the extended MHC region (8Mb) on chromosome 6, with the full list of genomic regions at high-LD excluded from the EIGENSTRAT analysis listed in Table 2.7. The next step addressed small-scale LD, using a "sliding windows approach" in PLINK. The final step served to detect and correct for residual LD effects, by examining the distribution of SNP coefficients from PC analysis of the LD-pruned data (Weale 2010).

c) Skewed missingness

Checks were done for skewness in missing genotype data with respect to phenotype. SNP missingness is an obligatory quality control step, as there is a strong correlation between missingness and SNP quality and this has possible impact in creating false positives and false negatives.

d) Detection and correction for population stratification

Generating false positives due to hidden population stratification has been regarded as one prominent challenge in the methodology of GWA studies. Methods have now been developed that largely solve this issue. Use of the GWA data itself provides the basis for powerful solutions devised to detect and correct subtle population structure effects (McCarthy & Hirschhorn 2008).

Modified EIGENSTRAT method

The EIGENSTRAT method for correction for population stratification in GWA studies was first described in 2006 in two papers by Reich's group in Harvard (Patterson et al., 2006;Price et al., 2006); http://genepath.med.harvard.edu/~reich/Software.htm. The modified EIGENSTRAT method is used to detect and correct for population

stratification. This method has been already applied in several successful genome-wide association studies (for example (Fellay et al., 2007)) and involves principal component analysis of SNP data.

The modified EIGENSTRAT method is based on PC analysis of SNP genotype data, with projection of subjects onto PC axes and using the significant axes as covariates in the subsequent association analyses (Fellay et al., 2007).

Firstly, data were restricted to a linkage disequilibrium (LD)-pruned SNP set. This means that high LD regions of the human genome were excluded (Table 2.7) and SNPs "thinned" according to a LD criterion based on r^2. Next, each SNP is regressed onto the previous five SNPs and the residual entered into the PC analysis; outlier SNPs and/or outlier individuals are iteratively removed.

With the software program gPipeCMD, version 1.0, the EIGENSTRAT PC axes are plotted with the "ethnicity labels" ("self-described" ethnicity), for each subject and can be analyzed by visual inspection. Any outlier identified along any top-ranking PC axis is examined and removed. Note that the outliers identified by PC analysis will not automatically be population outliers, as they may be due to cryptic relatedness or poor quality genotyping.

PC analysis is performed again after outlier removal, in an iterative process. Lastly, PC axes can be tested statistically (axes are nominated based on Tracy-Widom statistics) to determine the best number of axes to take forward (Patterson et al., 2006) and use as covariates in the subsequent association analysis.

"Self-described" ethnicity data were recorded for most of the samples in this study. The majority of the study population is geographically and ethnically of European origin.

The combination of the EIGENSTRAT modified method (Price et al., 2006) and self-identified ancestry methods (Fellay et al., 2007;Kasperaviciute et al., 2010) allowed to identify individuals of European ancestry and correct for hidden population substructure.

Chr	Start position (NCBI build 36)	End position (NCBI build 36)
1	48060567	52060567
2	85941853	100407914
2	134382738	137882738
2	182882739	189882739
3	47500000	50000000
3	83500000	87000000
3	89000000	97500000
5	44500000	50500000
5	98000000	100500000
5	129000000	132000000
5	135500000	138500000
6	25500000	33500000
6	57000000	64000000
6	140000000	142500000
7	55193285	66193285
8	8000000	12000000
8	43000000	50000000
8	112000000	115000000
10	37000000	43000000
11	46000000	57000000
11	87500000	90500000
12	33000000	40000000
12	109521663	112021663
20	32000000	34500000

Table 2.7 Genomic regions at high-linkage disequilibrium excluded from the modified EIGENSTRAT analysis.

Abbreviations: chr, chromosome. In: Kasperaviciute D., Catarino C.B., et al., Common genetic variation and susceptibility to partial epilepsies: a genome-wide association study, Brain, 2010, vol. 133, no. Pt 7, pp. 2136-47, by permission of Oxford University Press.

2.6.3.2 Association analysis

The three most common analytic techniques for GWA case-control analysis are the chi-squared test of allele counts, trend tests (with a multiplicative model being assumed for the regression based on genotype category, coded as 0, 1 and 2) and a 2-degree of freedom genotypic model (one genotype category as baseline and the effects of the other two categories are modelled) (Neale and Purcell 2008).

For a diallelic locus with alleles *A* and *a*, there are three possible genotypes *AA*, *Aa* and *aa*. Taking the genotype *aa* as reference, the genotype relative risks (GRR) are defined as:

- $\theta_{AA} = GRR_{AA}$ = risk for AA genotype / risk for aa genotype;
- $\theta_{Aa} = GRR_{Aa}$ = risk for Aa genotype / risk for aa genotype;
- $\theta_{aa} = 1$,

Taking allele *a* as reference, the allelic relative risks (ARR), ARR_A and ARR_a, are defined by the multiplicative model as follows:

- $\theta_{AA} = (ARR_A)^2$;
- $\theta_{Aa} = ARR_A$.

Logistic regression was used for the association analysis of the case-control data, focused on single-marker genotype-trend tests of the quality control-passed SNPs, using an additive genetic model. Gender and significant EIGENSTRAT axes (as given by Tracy-Widom statistic with $p < 0.05$) were included in the model as covariates. Fig. 2.4 illustrates the generic logistic regression equation for genome-wide association case-control data.

$$Yi = \frac{exp(\beta 0 + \beta cov * covariates + \beta addXij)}{1 + exp(\beta 0 + \beta cov * covariates + \beta addXij)}$$

Where for Yi: 0 = control, 1 = case
for Xi: 0 = aa, 1 = aA, 2 = AA

Figure 2.4 Generic logistic regression equation for GWA case-control study data.

For the analysis stratified per country cohorts, the Cochran-Mantel-Haenszel test was used.

PLINK software (Purcell et al., 2007) was used for the association analysis; http://pngu.mgh.harvard.edu/~purcell/plink/.

The significance of allelic contingency tables was assessed using Pearson's chi-squared tests. In case of tables with cell counts lower than five, the Fisher's exact test was used instead.

2.6.3.3 Dealing with multiple testing

The threshold of genome-wide significance in genome-wide association studies, which is currently widely accepted, is $5x10^{-8}$ (McCarthy et al., 2008;Panagiotou & Ioannidis 2012).

Bonferroni correction is recognized as a conservative approach to correction for multiple testing. The initial genome-wide significance level obtained is divided by the number of tests, that is, the number of SNPs in the final analysis.

2.6.3.4 Post-association analysis

a) Post-association analysis annotation

For the post-association analysis annotation, the WGAViewer software was used (Ge et al., 2008). WGAViewer is a JAVA software package for annotation, graphic display and visualization of SNP-by-SNP association results, presented in the context of genomic information such as gene structure, with ready incorporation of information from other databases such as linkage disequilibrium data from the HapMap project. It also allows interrogation of databases for functional properties of SNPs and expression data from the GENEVAR database. The output from WGAViewer is displayed in both tabular and graphic formats.

Quantile-quantile (Q-Q) plot

In the context of GWA studies, Q-Q plots summarise the distribution of the test statistics, for each SNP included in the final analysis. Test statistics are ordered from lowest to highest and plotted against expected values under the null hypothesis. Q-Q plots have several uses, among them estimating the genomic inflation factor, λ and it is a "diagnostic plot" (McCarthy et al., 2008). This test of the genome-wide distribution of the test statistics compared with the expected null distribution is critical, and Q-Q plots are a useful visual tool to mark deviations of the observed distribution from the expected. True associations are seen as prominent departures from the null in the extreme tail of the distribution. Inflated λ values or residual deviations in the Q-Q plot may point to uncorrected population stratification, or undetected sample duplications or systematic technical bias (de Bakker et al., 2008).

Manhattan plot

Manhattan plots are a graphic display of the significance for all SNPs in the final analysis of the GWA study, relative to their genomic positions. An example is given in Fig. 2.5.

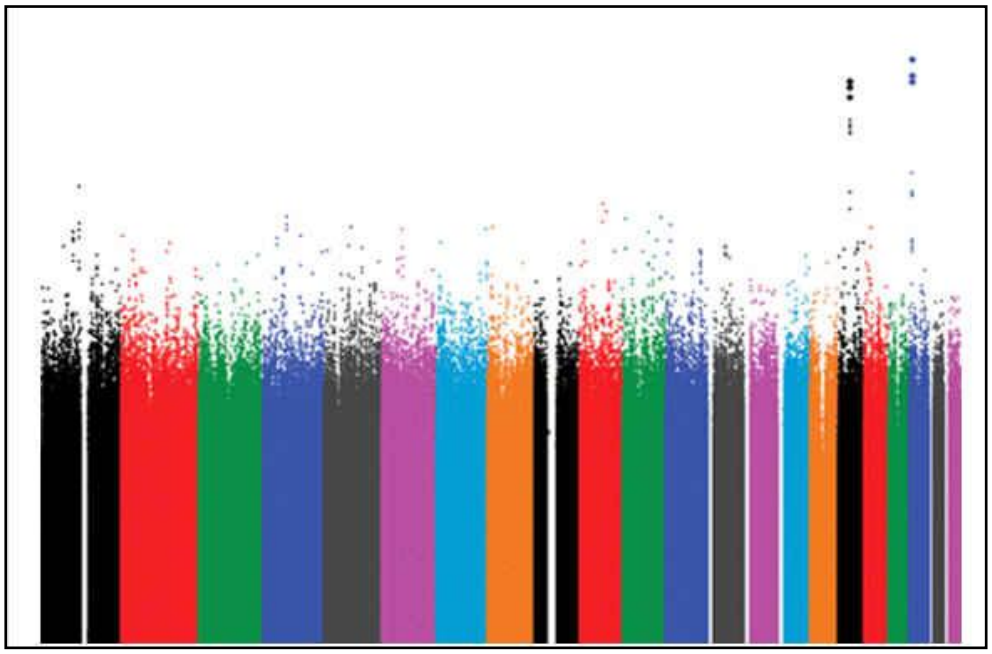

Figure 2.5 Example of Manhattan plot, from a recent GWA study for neutrophil count in a Japanese cohort (Okada et al., 2011). Chromosomal positions are plotted in the horizontal axis and the $-\log_{10}$ (*p*-values) are shown in the vertical axis. In this study, two genetic loci showed genome-wide significant ($p < 5 \times 10^{-8}$) association to the phenotype.

b) Post-association analysis quality control
Hardy-Weinberg equilibrium

When inspecting the SNPs with the lowest *p*-values, which appear as hits, checks are needed for deviation from Hardy-Weinberg equilibrium (HWE) in controls, as a necessary data quality control.

Only controls are checked for HWE, as positive association may confound the HWE test (Neale and Purcell 2008;Sham 1997).

If the relative frequency of alleles *i*, *j* are f_i and f_j, the relative frequency of genotype *ij* under Hardy-Weinberg equilibrium is: $2f_if_j$ if $i \neq j$ $(f_i)^2$ if $i = j$

Another way of representing this important concept is: if major allele (A)/minor allele (a) frequency are given by *p* and *q*, then, under HWE, P(Aa) = 2*pq* and P(AA) = p^2.

The Hardy-Weinberg assumption implies that the two chromosomes of each subject are sampled independently from the population. A sample of *N* independent subjects can then be treated as a sample of 2*N* independent chromosomes. To test for HWE, a Pearson goodness-of-fit test (χ^2; chi-squared) was used. The χ^2 approximation can be poor with low genotype counts, when Fisher's exact test is preferred.

Departures from HWE can be due to problems with genotype calling, where extreme departures from HWE may be expected. Visual inspection of cluster plots of those SNPs is the next step, to check for bad clustering, with subsequent exclusion of any SNP for which problems with genotype calling are confirmed.

Manual recheck of genotyping quality

A follow-up inspection is required for the SNPs found to have a low *p*-value and for the SNPs with departures from HWE. This is done using the Illumina Beadstudio software, by visual inspection of the original signal intensity (cluster) plots, to confirm that the SNPs were indeed called correctly, as otherwise genotype miscalls could give rise to false positives.

The signal intensity plots are cluster plots, with the genotyping raw data plotted along two axes, one for each allele, defining for each SNP clusters of data corresponding to the three genotypic groups. Rechecks were done of raw and normalised genotype data for the SNPs that were found to have a significant association with the phenotype and for those with departures from HWE.

For the WTCCC data, the cluster plots were reviewed using the software Evoker_0.4.3 (http://en.sourceforge.jp/projects/sfnet_evoker/).

Annotation of top hits

Further analysis of the WGAViewer output also helps to answer important questions in the immediate post genome-wide association phase, which include whether the top hits are located in or close to any gene, the distance from the closest exon, the distance from the closest gene, functional relevance of the SNPs, or whether they are non-synonymous coding or splice changing. This is part of the output available for review.

The notion that GWA signals are typically located close to underlying functional elements is supported by their frequent proximity to candidate genes associated with related Mendelian conditions or identified by pathway analyses (Anderson et al., 2011b).

The genetic markers in association with the phenotype of interest can cause non-synonymous changes in the coding region of a gene; this has been the case in less than 50% of the reported GWA results (Ioannidis et al. 2009). These can also be in non-coding regions and even in gene deserts (regions without any known protein-coding genes).

The affected genes can be near to or in the wider vicinity of the marker, or even on other chromosomes. Most of the regulatory variation that influences a gene is probably close to the gene, but long-range trans-acting regulatory variation has been documented (Ioannidis et al. 2009). All this has important implications on how both this analysis and its significance are approached and how to plan the follow-up efforts.

3 Chapter Phenotyping

3.1 Introduction

The goal of genetic epidemiological research is to locate and identify the genetic determinants contributing to risk of diseases or traits.

The choice of the phenotype to be studied is key to the success of genetic studies (Johnson 2011). Finding genetic variants associated with a disease or trait hinges on asking the right question, which then needs to be translated into an appropriate selection and definition of the phenotype.

A phenotype that is determined by a genetic component should be chosen, but also one that can be measured with accuracy and independently of observer. This task is challenging, but necessary for any study looking for genetic variants leading to susceptibility to a disease or trait.

Phenotyping, i.e. the gathering of the phenotypical data, is the next essential part, equally necessary for the success of any genetic study.

This chapter will review the issues of phenotype definition and phenotyping, in population-based genetic association studies, describing and discussing how these tasks have been tackled in the genome-wide association studies of the common epilepsies I describe in this thesis.

3.1.1 Definition of "phenotype"

Wilhelm Johannsen coined the term "*phenotype*" (Johannsen 1909), which derives from the Greek "phainein", meaning "to show". One more recent definition describes "*phenotype*" as "the observable expression of an individual's genotype" (Rao 2008). "*Phenome*" is a term which encompasses the "totality of all traits of an organism" (Mahner & Kary 1997).

3.1.2 Ideal properties of phenotype and phenotyping

The power of a genetic study is dependent on several factors: two of the most important factors are choice of phenotype and correct and accurate phenotyping methodologies.

The choice of phenotype should be strict and specific, to minimize misclassification. The phenotype should make biological sense and should reflect an underlying genotype to be discovered. The definitions of the chosen phenotypes must be agreed upon based on the best evidence and it should include information from epidemiological, biological, molecular and computational methods, in order to group signs and symptoms in such a way that it is possible to analyse the genetic influences on the phenotype (Winawer 2006). It should meet high standards of reproducibility, validity and quality (Schulze & McMahon 2004;Zondervan and Cardon 2007). It is very important to have homogeneous phenotyping within and between groups - otherwise the risk increases of missing a positive signal, i.e. of false negatives (Wojczynski & Tiwari 2008).

There are many challenges encountered when phenotyping complex diseases: heterogeneity of phenotypes; phenotypes that are dynamic and change with time; variance in phenotypes accounted for by interactions between genes and interaction between genetic and environmental factors. Phenotyping that fails to take into account all these issues may lead to false positive results or, on the contrary, may result in loss of power, diluting the effect of a true association (Wojczynski and Tiwari 2008).

Phenotyping quality is critical to success and it is therefore mandatory to apply quality control to phenotypical data and to introduce scientific principles and standards into phenotyping. Having objective measures, such as a test or a biological marker would be ideal, but this is not always possible. Phenotyping may consist of grouping clinical signs and symptoms, in conjunction with epidemiological, biological, molecular and computational data (Winawer 2006), with the goal of effectively analysing the genetic influences on the phenotype.

3.1.3 Phenotyping in GWA studies

Well-chosen and well-characterized phenotypes are a major requirement and a critical component of GWA studies.

As an example of the importance of phenotype definition, a successful GWA study of hypertension used as study phenotype the continuous variable “blood pressure” and this study allowed the identification of genetic variants associated with the risk of hypertension. It has been suggested that these genetic variants would not have been

identified had the authors used a dichotomous variable identifying “hypertension” (Newton-Cheh et al., 2009).

It is important to reflect on the underlying biology of the disease or trait to study and try to understand the relationships and inter-dependences between phenotypes. Also, it is helpful to think in terms of pathways and which variables can be confounders, mediators and effect-modifiers. In practice, the phenotype may be distant from the underlying gene; for example, gene(s) code gene product(s), which lead to physiological changes, which are risk factors, leading to a disease state and finally to clinical endpoints (Rao 2008).

3.1.3.1 Common, “complex” diseases

For the common “complex” diseases, there are specific challenges regarding phenotyping. These often include clinical heterogeneity. Phenotypes may be dynamic and change with time. Some of the variance in the phenotype may be expected to be accounted for by interactions between genes, or interactions between genetic and environmental factors, or by environmental factors alone. Possibly several genetic variants of relatively small individual effect may be contributing to disease susceptibility.

3.1.3.2 Multicentre studies

For a multicentre study, standardization of both phenotypic definitions and phenotyping methodology is required for each centre and between centres. The definitions of phenotypes must be agreed upon based on the best evidence, across the collaborating sites and it is essential the phenotypes and the phenotyping are homogeneous across cohorts (Sisodiya & Goldstein 2007). Also for replication and meta-analysis efforts, cross-site

phenotype consistency must be ensured, as differences between phenotypical definitions may make it more difficult to ensure power and validity of the study or replication.

3.2 Phenotyping the epilepsies

To design a GWA case-control study, one important first step is to ensure the definition of "case" is made and operationalized as accurately and specifically as possible (Zondervan and Cardon 2007).

The common epilepsies and more specifically, the phenotypes chosen to be studied, present several challenges in this regard, which will be the topic of this section.

3.2.1 General challenges of phenotyping the epilepsies

3.2.1.1 Clinical heterogeneity of the epilepsies

In epilepsy, there is significant clinical heterogeneity, with a diverse range of epilepsy syndromes described. Epilepsy is not a single disorder (Berg and Scheffer 2011), but a heterogeneous group of disorders.

In epilepsy research, the value of the epilepsy syndromic diagnosis has been strengthened by recent findings from genetic studies, which have provided some understanding of the underlying mechanisms of specific forms of epilepsy syndromes (Berg 2007).

To produce results that are valid, the principles of good epidemiological design need to be followed, the study case participants need to be representative of the disease

cases in the population; cases and controls need to be comparable (Pearson and Manolio 2008).

3.2.1.2 Diagnostic issues

There is no single diagnostic test for epilepsy. Making the diagnosis of epilepsy is based on the patient's history, supported by the results of a number of different investigations.

It has been estimated that around 20-30% of patients with refractory epilepsy who attend tertiary referral centres do not in fact have epilepsy (Chadwick & Smith 2002;Lesser 1996;Smith et al., 1999). The misdiagnosis of epilepsies by non-specialists is higher than for neurologists (mistake rate 18.9% versus 5.6%) (Leach et al., 2005). Psychogenic seizures (Lesser 1996) and syncope (Smith et al., 1999) are frequent causes of misdiagnosis. Rarer causes include, for example, long QT-syndromes (MacCormick et al., 2009), or cardiac arrhythmias (Neligan et al., 2009).

In the studies described in this thesis, the diagnosis of epilepsy across all cohorts was made by experienced epileptologists and clinical data and results of investigations confirmed, in order to minimize issues of misclassification attributable to misdiagnosis.

3.2.2 Classification issues of epilepsies and epilepsy syndromes

The phenotype in genomic studies of the epilepsies needs to be defined using somewhat subjective assessments, with use of diagnostic criteria. The ILAE classifications of seizures and of the epilepsies and epilepsy syndromes, affords an uniform framework for this type of research. The currently accepted classification of the epilepsies and epilepsy syndromes (ILAE Commission on Classification and Terminology 1989) constitutes the

basis for the phenotyping in the GWA studies described in this thesis. This classification may be incomplete and inaccurate, with criticisms including "most epilepsies are inadequately characterized, with imprecise terminology and suboptimal classification" (Berg and Scheffer 2011). This is, nevertheless, the most widely used classification in clinical practice and still the approved and accepted classification.

However, any classification of the epilepsies has limitations, as there are areas of uncertainty and classifications are dynamic and change with time; all this creates challenges for phenotyping in genomic studies of the epilepsies.

Sometimes it is not possible to classify a patient by epilepsy syndrome with the data available for review: it may not be possible to reliably localize the epileptogenic zone on the basis of ictal semiology and scalp EEG alone, which is sometimes the only information available. Furthermore, patients may move from one syndrome to another as part of the natural evolution of their epilepsy (ILAE Commission on Classification and Terminology 1989). This has been documented, for example, for children with childhood absence epilepsy who later develop juvenile myoclonic epilepsy (Medina et al., 2005).

Information on early history of a patient may be crucial for the definition of the epilepsy syndrome, for example in Dravet syndrome, where the age at seizure onset and the history during the first year of life are essential to make the diagnosis. This information may not always be accessible, if the parents are not available for interview and the paediatric notes are not available for review, which is often the case in adult epilepsy clinics.

The solutions that have been implemented included performing the studies in tertiary referral centres, where the patients are evaluated by experienced epileptologists, often over decades. In this study, all available data were reviewed. The criteria for each phenotype were discussed by all members of the consortium at the study design phase.

3.2.2.1 Partial epilepsies - Phenotyping

Partial epilepsies (also called "localization-related" or "focal") can be defined as "epilepsies with partial seizures" (ILAE Commission on Classification and Terminology 1989).

It is not always straightforward to classify the epilepsy syndrome. A proportion of the patients in the London cohort (about 5%) remained unclassified as to whether they had partial or generalised epilepsy, even after all the available data had been reviewed (Table 3.4). Also the borders between generalised epilepsy and partial epilepsy may not be as clear as previously thought (O'Muircheartaigh & Richardson 2012), which may mean that, with the current knowledge, patients may be misclassified. This could have diluted the ability to detect a true positive signal in the GWA study of the partial epilepsies, but is unlikely to have played a significant role.

3.2.2.2 MTLEHS - Phenotyping

Without objective measures, a symptom cluster would not necessarily define a homogeneous disorder. This would be problematic, as the same diagnosis could then be applied to patients with disease resulting from different pathophysiological pathways, which could imply underlying heterogeneity at the biological and genetic levels (Schulze et al., 2006;Wojczynski and Tiwari 2008). The 1989 report of the ILAE Commission on Classification and Terminology states that "a syndrome does not necessarily have the

same aetiology and the same prognosis" (ILAE Commission on Classification and Terminology 1989).

MTLEHS is a coherent clinical entity. Studying MTLEHS with a GWA study has the advantage that there is a consensus "definition" of the electroclinical criteria (Wieser 2004), with objective and quantitative diagnostic criteria. These include MRI data and may include histopathology data in those patients who underwent resective surgery. Patients with a compatible electroclinical syndrome, who had presurgical evaluation and those who had quantitative MRI proving unilateral HS, present an advantage of being more thoroughly phenotyped, with less potential for misclassification.

3.2.2.3 Histopathologically-proven MTLEHS

The gold standard for the diagnosis of MTLEHS is neuropathological confirmation, in a patient with the appropriate electroclinical and imaging profile (Wieser 2004). Pathologically, HS involves neuronal loss and reactive gliosis in the CA1 to CA4 regions of the hippocampus, dentate gyrus and subiculum. Ammon's horn sclerosis involves only CA1 to CA4. The expression "mesial temporal sclerosis", coined by Cavanagh and Meyer (1956), may also involve the amygdala and parahippocampal gyrus. The general heading of "HS" used throughout this thesis includes not only the more strictly defined HS, but also Ammon's horn sclerosis, endfolium sclerosis (Kim et al., 1990) and mesial temporal sclerosis (Falconer et al., 1964).

3.2.2.4 MTLEHS characteristics on MR imaging

MR imaging can depict the morphology, size, and some internal architecture of the hippocampal formation, amygdala and parahippocampal gyrus. The diagnosis of HS is made when there is hippocampal atrophy (detected with MRI in 90-95% of cases in

which HS is found in resected tissue) and hyperintensity on FLAIR and T2-weighted images (T2 increase in 80-85%); loss of internal architecture is seen in 60-95% of cases (Wieser 2004).

Imaging protocols have been optimized to evaluate the anatomy of the mesial temporal lobe structures (Duncan 2010;Woermann and Vollmar 2009). Given the complex orientation of the hippocampal formation, it is important that adequate imaging protocols are in place, to acquire images perpendicular to the long axis of the hippocampus, which allows comparison between the two sides and limits some of the volume averaging problems. A high resolution 3D-gradient echo acquisition, providing T1-like contrast, has the advantages of thinner slices, more rectangular slice profiles and capability to reconstruct along any plane to compensate for slight rotation of the head or variability of slice orientation, which permits a more accurate comparison between the two sides by visual inspection. Coronal or oblique coronal double echo images can demonstrate the signal abnormalities in these structures if present. Quantitative T2 measurements may improve the sensitivity: hippocampal volumetry can identify unilateral or bilateral hippocampal damage and may be used in the presurgical imaging work-up (Duncan 2010).

The sensitivity of the radiological diagnosis of HS by "expert" neuroradiologists in epilepsy specialised centres with adequate epilepsy protocols, is significantly higher than diagnosis by other "non-expert" neuroradiologists or with "standard" MRI (von Oertzen et al., 2002;Woermann and Vollmar 2009). This is not expected to have been a major factor in these GWA studies, because they were performed in tertiary referral

centres, where epilepsy MR protocols are applied across the cohorts, with review by expert neuroradiologists.

Patients with an initial MRI-negative epilepsy may later be found to have visible lesions when the MRI is reanalyzed after confirmation of the localization of the probable epileptogenic zone (Bien et al., 2009). Endfolium sclerosis, a neuropathological subtype of HS, is less readily identified by presurgical imaging studies (Van Paesschen et al., 1997).

3.2.3 Febrile seizures

The correct phenotyping of childhood febrile seizures presents a challenge in adult patients, where retrospective data collection is necessary, with the risk of recall bias. The paediatric hospital records may not be accessible for review and frequently the parents are not available for questioning, which would be effective ways to overcome this issue.

A recent study on the validity of the report of febrile seizures by parents showed the question posed in clinic "Did your child have a febrile seizure?" has high sensitivity and may be appropriate as screening instrument, but has low positive predictive value, with a second stage of evaluation necessary to identify true FS cases (Visser et al., 2013).

Further, the information on FS available in the case notes is frequently a dichotomic yes/no; less frequently, further data are available to describe the FS, such as duration, number, age at onset, presence or absence of lateralizing features, which would

then allow the classification of FS into simple FS, prolonged FS or febrile status epilepticus.

3.2.4 Family history of epilepsy and febrile seizures

The accuracy of the family history recording in epilepsy varies widely between case notes (Ottman et al., 2011).

The details of family history available from case notes and obtained in clinic, have a wide spectrum, depending on a number of factors. This may be a simple yes/no to the question "do you know of any case of epilepsy in your family" and have a wide spectrum up to detailed pedigrees with information collected on seizure history from the patient and his/ her affected and unaffected relatives.

3.3 Methods - Building the London EPIGEN database

The London dataset consists of adults with epilepsy recruited from epilepsy clinics at the National Hospital for Neurology and Neurosurgery and National Society for Epilepsy, consented for population-based genetic studies of epilepsy since 2001. The minimal inclusion criteria were: definite diagnosis of epilepsy, age 18 years or older and signed informed consent.

The starting point for the collection of phenotypical data was to organize and file all patients' consents collected since 2001 and simultaneously include in a London Epigen database all relevant data from the consent sheets, after rechecking in the case

notes or hospital databases, when available. All patients recruited for whom no consent was filed, were identified from all possible information sources, such as previous existing databases, both electronic and hardcopy, the database of the Neurogenetics laboratory of the Institute of Neurology, case notes and electronic hospital databases.

Cross-checking of all data across these different sources was systematically performed, including all relevant demographic and clinical information. The database served as a compilation of demographic data of all patients included in the study and also had different sections serving several purposes.

The purposes of the database were to collect all recruited patients; check if consents were archived for all patients; check all DNA samples available for each patient; and organize the collection of DNA samples in the laboratory, keeping a record also of all samples sent to the laboratory for genotyping; compile all relevant phenotypical information from all relevant sources; and optimization of patient recruitment in the clinics.

The different sections of the database are listed in Table 3.1 and include patient identifier, demographic data, information on consents, information on DNA samples, sources of clinical information and phenotypical data.

The database was then anonymised, the list of names of the patients kept in a safe hardcopy, together with the code, the patient identifier number, which identified the patient in the database.

3.3.1 Data included in the database

All subjects in the London cohort have been evaluated by experienced consultant epileptologists. Case notes and clinical data in electronic databases were reviewed and all detailed phenotyping entered in the London master database, created specifically for the GWA studies.

Demographic variables included in the London EPIGEN database are listed in Table 3.1.

Section of the database	Information contained in each section
Patient identifier	Patient identifier code
Information on patients' consents	Confirmation of filing of patient consent; version of consent filed
Information on DNA samples	DNA numbers Location of DNA samples
Information on genotyping status	Genotyping status Type of chip
Demographic data	Sex Date of birth Self-reported ethnicity
Phenotypical data	As described in Table 3.2.
Sources of data	List of data sources reviewed; and if any, list of discrepancies encountered and how they were resolved

Table 3.1 Main sections of the information included in the London EPIGEN master database.

Table 3.2 defines the main phenotypical variables included in the London EPIGEN database, which included, for each patient:

a) Partial epilepsy, generalised epilepsy, uncertain whether partial or generalised epilepsy, or partial and generalised epilepsy;

b) Presumed aetiology and more specific epilepsy syndrome, if possible;

c) MRI findings;

d) Febrile seizures;

e) Age at onset of habitual seizures;

f) Family history of epilepsy or febrile seizures.

The 1989 ILAE Classification of epilepsies and epilepsy syndromes was used to classify the patients according to epilepsy syndrome (ILAE Commission on Classification and Terminology 1989).

For all patients with partial epilepsy, phenotypical variables included:

g) Temporal lobe epilepsy;

h) Mesial temporal lobe epilepsy;

i) Hippocampal sclerosis;

j) Mesial temporal lobe epilepsy with hippocampal sclerosis;

k) Mesial temporal lobe epilepsy, not MTLEHS;

l) Secondary generalization.

For patients with MTLEHS, further phenotyping included:

m) Information on whether the patient had or not resective epilepsy surgery;

n) Histopathology findings of the surgical specimen;

o) Seizure outcome after resective epilepsy surgery.

	Definition
Partial epilepsy[a]	Defined according to the 1989 ILAE classification: "seizure semiology or findings at investigation disclose a localized origin of seizures" (ILAE Commission on Classification and Terminology 1989).
TLE[a]	Defined according to the 1989 ILAE classification (ILAE Commission on Classification and Terminology 1989).
HS[a]	Radiological diagnosis: criteria include hippocampal atrophy and hyperintensity on T2 and/or FLAIR sequences. Volumetric MRI data considered, if performed. Patients who had resective surgery and histopathological diagnosis of HS, would be considered as having HS, even if the MRI appeared normal.
MTLE[a]	Criteria as described in (Wieser 2004).
MTLEHS[a]	Criteria as described in (Wieser 2004).
Febrile seizures[a]	Defined according to the ILAE definition (ILAE Commission on Epidemiology and Prognosis 1993). Requires original paediatric hospital data or parental witness accounts. Data were also collected for patients where only the patient's account was recorded in case notes, but these were not included in the analysis.
Aetiology	Aetiology is recorded, with the indication of degree of probability ("possible", "probable").
MRI findings	Main MRI brain findings and year of the MRI study.
Age at onset of habitual seizures	Age at onset of habitual seizures in years, or less exact description ("infancy", "early childhood") when no exact age is available on case notes.

FH of epilepsy or FS[a]	When FH is known, details on any affected relative (degree of relatedness, data on epilepsy or FS, age at onset) are recorded.
Resective epilepsy surgery	Resective epilepsy surgery Yes/No and which type of surgery.
Epilepsy surgery outcome	Written description of seizure outcome after surgery and whether AEDs were tapered. ILAE outcome classification also used.

Table 3.2 Epilepsy phenotypes collected for the London cohort and their definitions.

Abbreviations: FH, family history; FS, febrile seizures; HS, hippocampal sclerosis; MTLE, mesial temporal lobe epilepsy; MTLEHS, mesial temporal lobe epilepsy with hippocampal sclerosis; TLE, temporal lobe epilepsy.
a For these phenotypes, possible categories were yes/no/unclear.

Mesial temporal lobe epilepsy with hippocampal sclerosis

MTLEHS was defined according to the diagnostic criteria included in the 2004 report of the ILAE workshop on MTLEHS (Wieser 2004). This definition encompasses a compatible electro-clinical syndrome, MRI findings and/or histopathological confirmation when resective surgery has been performed. As no single sign or symptom is specific of MTLEHS, it is the analysis of signs, symptoms, spatiotemporal sequence of the seizure, EEG, neuropsychology findings and imaging, taken together, which help to define it.

The criteria used to define MTLEHS were adapted from Wieser et al. (2004):

1) Seizure semiology: Features a. and/or b. are essential, with or without c.

a. "Aura": epigastric aura (ascending and substernal rising sensation); or non-specific aura; fear and anxiety and other emotional auras; déjà-vu, illusion of familiarity and strangeness; vegetative aura (widened pupils, palpitation, arrhythmia, goose-flesh pimples); olfactory aura; gustatory aura.

b. "Complex partial seizure". This may follow the aura; consists of arrest, alteration of consciousness (including amnesia) and automatisms. Frequent features include oral and appendicular automatisms; contralateral hand dystonia; pupillary dilatation; impaired consciousness; and some postictal dysfunction (cognitive impairment, memory deficits, mood changes, language deficits);

c. "Secondarily generalised seizures" may or may not occur.

2) Scalp EEG: Interictal EEG with nonspecific interictal temporal slowing; interictal epileptiform discharges maximal in anterior temporal, or sphenoidal electrodes; ictal EEG: rhythmic, crescendo-like theta activity with decreasing frequency and increased amplitude is the typical pattern; it may be "normal"; "flattening", diffuse

or over one temporal region.

3) MRI brain: criteria for HS, predominantly unilateral, include hippocampal atrophy and hyperintensity on T2 and/or FLAIR sequences. If performed, volumetric MRI data were considered.
4) Histopathology of the surgical specimen (anterior temporal lobectomy with hippocampectomy, or amygdalohippocampectomy), with criteria for HS (Blumcke et al., 2007).

For a patient to be classified as having MTLEHS, criteria 1) and 3) and/or 4) are essential; and criterium 2) is not essential (Wieser 2004).

Additional criteria could consolidate the diagnosis, if present; this included history of febrile seizures, family history of epilepsy or febrile seizures, existence of a "latent period", existence of a "silent period", or a progressive course.

Exclusion criteria included: MRI evidence of bilateral HS or of dual pathology; seizures beginning with primary visual, auditory or focal somatosensory auras; violent bilateral motor ictal behaviours; evidence of diffuse brain damage on neuroimaging, EEG and/or neurocognitive testing and focal neurological findings other than memory deficit.

All phenotypical information from the MTLEHS cases in the London cohort was reviewed by me and entered by me in the database. The MTLEHS diagnosis was independently reviewed for all cases by one senior adult epileptologist. Any discrepancy noted in the phenotypical classification was resolved by consensus after reviewing the clinical information from case notes, with stringent criteria for study inclusion.

Number of patients (%)	GWA study of PE		GWA study of MTLEHS	
	Genotyped	**Included in the analysis**	**Genotyped**	**Included in the analysis**
UK	1422 (33%)	1185	331 (28%)	265
Ireland	670	607	148	147
Belgium	580	418	77	67
USA	780	393	97	71
Finland	428	410	116	116
Switzerland	235	231	182	182
Norway	212	201	70	70
Austria	0	0	166	165
Total	**4327**	**3445** (80%)	**1187**	**1083** (91%)

Table 3.3 Numbers of cases genotyped for the discovery phase of the GWA studies, for each subcohort.

Abbreviations: GWA, genome-wide association; MTLEHS, mesial temporal lobe epilepsy with hippocampal sclerosis; NA, not applicable; PE, partial epilepsies.

3.4 Results

3.4.1 Descriptive statistics

2,910 patients with epilepsy had been phenotyped in the London cohort, as of March 2011. Phenotyping was ongoing for any new recruited patients and for any patient already included in the study for whom more clinical data became available for review, also as preparation for the planned replication efforts.

Table 3.3 provides an overview of the numbers of people with partial epilepsy and MTLEHS, who were genotyped and who were included in the final analysis, for all cohorts included in the discovery phase of the genome-wide association studies.

For the discovery phase of the GWA study of partial epilepsy and the GWA study of MTLEHS, 1,812 unique DNA samples of people with epilepsy from the London cohort were genotyped, 1,422 with partial epilepsies and 331 with MTLEHS.

Descriptive statistics of the phenotypical data for the London EPIGEN cohort are shown in Table 3.4. Of 2,910 patients wth epilepsy already phenotyped in the London database in March 2011 (53% female), mean age at habitual seizure onset was 16 years, approximately 80% had partial epilepsy and 5% had unclassified epilepsy.

Putative epilepsy aetiologies are presented in Table 3.5 and available MRI findings in Table 3.6, for the 3,445 patients included in the GWA study of the partial epilepsies, with 41% having cryptogenic or idiopathic partial epilepsy and 27% with MTLEHS.

Clinical variables	Patients with epilepsy*	Patients with PE genotyped for the discovery phase of GWA studies**
Total	n=2,910 [March 2011]	n=1,422
Sex, female (%)	1545 (53% of total)	704 (50% of total)
Age at habitual seizure onset, y; mean ±SD	16±12	17±13
Epilepsy syndrome		
• Partial epilepsy	2,240 (~80% of total)	1,422
• Generalised epilepsy	467	NA
• Uncertain whether partial or generalized, or both partial and generalised	146 (5% of total)	NA
• Temporal lobe epilepsy	1,091	698
• Mesial TLE, any aetiology	574	378
• MTLEHS	459	313
Hippocampal sclerosis	626	380
History of febrile seizures . MTLEHS and FS . PEnotMTLEHS and FS	274/1240[a] 151 97	190/759[a] 128 62
Seizure-related family history	187/536[a]	111/360[a]

Table 3.4 Descriptive statistics of the phenotypes in the London EPIGEN database.

a Number of patients with retrieved phenotypical data.

Number of cases (% of total)	**UK**	**Ireland**	**Belgium**	**USA**	**Finland**	**Switzerland**	**Norway**	**Total**
"Genetic" or "unknown cause"	517 (44%)	192 (32%)	168 (40%)	190 (48%)	257 (63%)	9 (4%)	96 (48%)	**1429 (41%)**
"Structural-metabolic"	668	415	250	203	153	222	105	**2016**
MTLEHS	265 (22%)	148 (24%)	67 (16%)	71 (18%)	116 (28%)	182 (79%)	70 (35%)	**919 (27%)**
MCD	141	38	21	16	12	12	1	**241**
Tumour	42	62	42	48	3	23	2	**222**
Infection	36	16	9	8	1	0	6	**76**
Trauma	32	60	22	21	2	0	7	**144**
Vascular malformation	34	26	27	9	4	0	7	**107**
Perinatal insult	34	12	8	2	9	0	9	**74**
Stroke	32	27	33	9	0	0	1	**102**
Neurocutaneous syndromes	1	3	3	3	0	0	0	**10**
Other structural-metabolic cause	51	23	18	16	6	5	2	**121**
Total	**1185**	**607**	**418**	**393**	**410**	**231**	**201**	**3445**

Table 3.5 Putative aetiologies in the GWA study of partial epilepsy.
Abbreviations: MCD, malformations of cortical development; MTLEHS, mesial temporal lobe epilepsy with hippocampal sclerosis.
In: Kasperaviciute D., Catarino C.B., et al., Common genetic variation and susceptibility to partial epilepsies: a genome-wide association study, Brain, 2010, vol. 133, no. Pt 7, pp. 2136-47, by permission of Oxford University Press.

MRI phenotype / No. cases (% of total)	UK	Ireland	Belgium	USA	Finland	Switzerland	Norway	Total
Normal MRI	487 (42%)	134 (22%)	94 (23%)	146 (37%)	209 (51%)	8 (4%)	81 (40%)	**1159 (34%)**
Unilateral HS	277	140	65	66	118	182	72	**920**
Bilateral HS	12	6	3	5	4	1	0	**31**
MCD	138	38	17	14	12	12	1	**232**
CVD	27	12	30	11	0	0	1	**81**
Perinatal injury	34	1	7	1	8	0	9	**60**
Other acquired injury	59	29	27	20	3	0	8	**146**
Vascular malformation	34	25	25	18	4	0	7	**113**
Tumour	42	60	42	50	3	23	2	**222**
Other	39	23	41	6	31	4	18	**162**
Incidental MRI findings	26	26	39	20	11	1	2	**125**
MRI NA; CT abn	10	15	10	0	0	0	0	**35**
Brain imaging data NA	0	98	18	36	7	0	0	**159**
Total	**1185**	**607**	**418**	**393**	**410**	**231**	**201**	**3445**

Table 3.6 MRI findings for the various cohorts in the genome-wide association study of partial epilepsy.

Abbreviations: abn, abnormality; CT, computed tomography; CVD, cerebrovascular disease; HS, hippocampal sclerosis; MCD, malformation of cortical development; MTLEHS, mesial temporal lobe epilepsy with hippocampal sclerosis; NA, not available.
In: Kasperaviciute D., Catarino C.B., et al., Common genetic variation and susceptibility to partial epilepsies: a genome-wide association study, Brain, 2010, vol. 133, no. Pt 7, pp. 2136-47, by permission of Oxford University Press.

3.5 Discussion

3.5.1 Limitations

3.5.1.1 Misclassification

Hippocampal sclerosis

In the genome-wide studies, only patients with confirmation of HS in the MRI were considered for inclusion as a MTLEHS case, except in the rare cases with normal MRI and a confirmed neuropathological diagnosis of HS. Not only hippocampal atrophy, but also unilateral hyperintensity of the hippocampus in T2 and FLAIR sequences were required for inclusion in the study, minimizing the risk of misclassification.

Patients with bilateral HS and dual pathology were excluded in order to have a more homogeneous phenotype for the analysis, to use a definition of MTLEHS that could minimize causal heterogeneity (Zondervan and Cardon 2007).

The interpretation of the MRI scan may vary with the experience of the neuroradiologist and the quality of the scan (Woermann and Vollmar 2009), but also in a single patient with MTLE the MRI findings may change with time, due to a dynamic evolution of the pathological process. Other factors may also play a role, such as developments of imaging technology or the sensitivity of the method used, with MR imaging at 7-Tesla comparatively more sensitive than previous techniques for detecting hippocampal atrophy (Henry et al., 2011).

Epilepsy syndromic classification

Patients who, for any reason, such as drug refractoriness or epilepsy severity, had more detailed investigations, or those who had repeated investigations over time, have a higher chance of having had more recent MRI studies and more prolonged EEG studies such as video-EEG telemetry. Such a re-evaluation may lead to a change in epilepsy syndromic classification in some cases, from generalised to partial epilepsy, or from temporal lobe to extra-temporal epilepsy. Nevertheless, this is probably a minor source of misclassification in the context of the studies described in this thesis, as the patients were included in tertiary referral centres.

The symptomatogenic zone has been defined as the cortical region giving rise to the symptoms of an epileptic seizure (Lüders and Awad 1992). This concept is important, because a propagation of the epileptic activity may sometimes lead to misclassification of the type of epileptic seizures and consequently of the epilepsy syndrome, particularly if the EEG and the MRI brain scan are normal or non-specific, or if they have misleading results (Remi et al., 2011) or incidental findings.

For some patients, it is almost impossible to reliably localise the epileptogenic zone on the basis of ictal semiology and scalp EEG alone, which are the only data available for many patients in these studies. Sometimes misclassification is impossible to avoid, even if all available sources of information are scrutinised. A good example is a frontal lobe onset seizure, particularly if the seizure onset zone lies in the medial posterior orbitofrontal cortex, which may mimic a “temporal lobe seizure” and the scalp EEG findings may be similar to a temporal onset seizure or inconclusive, not helping to make a

correct diagnosis. If the MRI is normal in such a situation, the potential for misclassification is very high (Engel, Jr. et al., 2008).

Other possible extra-temporal sources of "limbic-like" seizures include the occipital region (Palmini et al., 1993;Salanova et al., 1992;Usui et al., 2008;Williamson et al., 1992) and the insula (Isnard et al., 2000;Isnard et al., 2004). A quick propagation to the mesial temporal lobe is possible from these onset regions and the seizure semiology may mimic that of a seizure with mesial temporal lobe onset.

Misclassification may also derive from the data collection methods, both from individual classification errors, because of missing data or typographical errors, or from intrinsic errors or omissions in the classification systems of the epilepsies.

There are challenges when phenotyping MTLEHS and even if there are agreed-upon diagnostic criteria, these have an accuracy of about 75%, with further work needed to determine how to weigh these diagnostic criteria with a higher level of confidence (Wieser 2004).

The phenotype of one patient may change with time for other reasons. One patient previously characterized as having an epilepsy "of unknown cause", or "cryptogenic" or "probably symptomatic", in the 1989 ILAE classification (ILAE Commission on Classification and Terminology 1989) may show, in a later imaging study, evidence for unilateral HS. Later, this same patient may have surgery and the histopathological examination may reveal either dual pathology, or another pathology. One such patient,

with medically refractory epilepsy and presumed MTLEHS, was excluded from the analysis, as the neuropathology diagnosis was in fact a hamartoma and not HS.[6]

Febrile seizures

Data collection on febrile seizures was made, whenever possible, from interviewing parents in the clinic, or reviewing paediatric records. A note was also made when the available FS data from case notes did not fulfill these requirements. The retrospective nature of the data collection was a limitation of the study: the patients may not always recall details from childhood and parents or paediatric records are not always available, making recall bias possible. To deal with this limitation, we classified as "unknown" any case where paediatric records or direct account from parents were not available and excluded these from the analyses on FS. Further, for a significant proportion of the cases reviewed, it was not possible to classify the FS using the available data and the analysis was not possible on subtypes of febrile seizures - for example, it would be interesting to look at the subset of prolonged FS only.

3.5.2 Next steps

An important "next step" to improve the power of the GWA studies described in this thesis is to increase the sample size.

Further, optimizing the phenotype definition and ascertainment (Evangelou et al., 2011) can be helpful. This could be done, for example, by using a more specific diagnosis of MTLEHS, including in the analysis only individuals with "classical HS" at

[6] This case was included in the series of patients with large microdeletions and MTLE (Catarino et al., 2011a).

histopathology (Blumcke et al., 2007), or using only quantitative MRI data to evaluate hippocampal atrophy.

It is also worthwhile to invest in retrieving more data on FS, not only to increase the sample size of patients with FS in the study, but also to collect data that would allow for classification of the FS and allow the study of prolonged FS and febrile status separately from simple FS. A recent twin study including data on subtypes of FS (Eckhaus et al., 2013) has provided further support for the relevance of this "next step".

4 Chapter Genome-wide association study of partial epilepsy

4.1 Introduction

Partial (or focal) epilepsies are the most frequent type of epilepsy (Banerjee et al., 2009) and have a substantial heritability (Berkovic et al., 1998).

Familial partial epilepsies have been described and genes identified for some of the rare monogenic partial epilepsy syndromes, as discussed in Chapter 1. The genetic contribution for common partial epilepsies remains, however, largely unknown.

Some of the partial epilepsies have known putative aetiologies, which range from trauma, stroke or tumours, to infrequent aetiologies, such as rare point mutations (Kalachikov et al., 2002;Steinlein et al., 1995). Even when an association is known between partial epilepsy and a structural lesion, genetic variants may also contribute to susceptibility to recurrent seizures, as in "symptomatic" partial epilepsies as after traumatic brain injury: patients with a family history of epilepsy have a significantly higher risk of epilepsy after mild (OR 5.8; 95% CI 4.6 to 7.3) and severe brain injury (OR 10.9; 95% CI 4.2-24.3), suggesting that genetic factors contribute to the risk of post-traumatic epilepsy (Christensen et al., 2009).

The aim of the genome-wide association study of partial epilepsies was to look for common genetic variation associated with increased susceptibility to seizures, shared across all partial epilepsy syndromes.

4.2 Methods

4.2.1 Ethics

Informed consent was obtained from study participants and the study was approved by the ethics committee at each recruitment site according to national standards.

4.2.2 Recruitment and inclusion criteria

Patients with partial epilepsy were recruited in seven countries (Table 4.1) during clinical appointments.

The 1989 International League Against Epilepsy definition for partial epilepsy was used (ILAE Commission on Classification and Terminology 1989). Partial epilepsy was defined as epilepsy characterized by seizures of focal origin, as disclosed by the semiology or investigations, including ictal EEG when available. Patients were not selected by syndrome other than partial epilepsy, nor by known structural abnormality, if any. The diagnosis of partial epilepsy was made after reviewing the patient's case notes and subsequently reviewed by a consultant epileptologist who was part of this study and who had access to clinical history and available investigation results.

4.2.3 Subjects

4,327 patients with partial epilepsy and 8,085 controls, in a total of 12,412 study participants, 53% female (Table 4.2), were included in the study.

The controls included: (i) 285 controls from Switzerland and 288 controls from Finland, in a total of 573 controls, were people without neurological disorders recruited and genotyped for this study; (ii) 469 population controls from Finland, all 85 years or over old at the time of recruitment, originally from the Vantaa85+ study (Myllykangas et al., 2005;Peuralinna et al., 2008); (iii) 5667 population controls from the Wellcome Trust Case Control Consortium (Wellcome Trust Case Control Consortium 2007) phase 2, September 2009 data release; (iv) 1165 USA controls from the Duke Memory study (Cirulli et al., 2010;Need et al., 2009a), who consented to participate in epilepsy genetics research; 84% of control participants filled in a questionnaire about history of neurological conditions and anyone who reported antecedents of seizures was excluded; and (v) 211 Irish neurologically-normal controls from the Study of Irish Amyotrophic Lateral Sclerosis (Cronin et al., 2008).

4.2.4 DNA extraction and genotyping

DNA was extracted from blood samples using standard procedures.

All patients with epilepsy and the controls from the Switzerland, Finland (not Vantaa-85+) and USA cohorts, were genotyped at the Institute for Genome Sciences and Policy Genotyping Facility, Duke University.

The majority of these in-house genotyped patients (93.4%) and controls (77.4%) were genotyped using Human610-Quadv1 chips. The list of genotyping chips used in the GWA studies are listed in table 4.3.

4.2.5 Genotype calling and genotyping quality control

Genotype calling and quality control were performed using Illumina Beadstudio v3 software.

All samples were processed in batches of 200-250. Genotyping quality control measures were standardized across all batches. Samples were clustered using in-house generated Illumina cluster files. After clustering, all samples that had call rates <98% were deleted. All SNPs that had call frequencies <100% were then re-clustered.

The re-clustering steps may create SNP calling errors, therefore all re-clustered SNPs with HetExcess values between -1.0 to -0.1 and 0.1 to 1.0 and all SNPs with cluster separation values <0.3 were deleted. Next, to avoid false association resulting from non-random missingness, a "1%" rule was applied: all SNPs for which >1% of samples were not called, were deleted. These procedures resulted in deletion of 1% to 2.5% of SNPs in different batches and in genotype call rates of 99.93% to 99.96% for the remaining samples and SNPs. 34 duplicate samples were genotyped and the concordance rate for duplicate genotyping was >99.99%.

Finnish control data from the Vantaa85+ study were received in Beadstudio files and processed using the same protocol.

	UK	Ireland	Belgium	USA	Finland	Switzerland	Norway	Total
				Patients				
Number genotyped	1422	670	580	780	428	235	212	4327
Number included in the analysis	1185	607	418	393	410	231	201	3445
				Controls				
Number genotyped	5667	211	0	1165	757	285	0	8085
Number included in the analysis	5116	209	0	605	746	259	0	6935

Table 4.1 Patients and controls included in the GWA study of partial epilepsy: numbers for each sub-cohort.

Abbreviations: NA, not applicable; PE, partial epilepsy.

Sex, n female /total (%)	**Patients**	**Controls**
UK	605/1185 (51%)	2535/5116 (50%)
Ireland	310/607 (51%)	98/209 (47%)
Belgium	222/418 (53%)	0 (NA)
USA	217/393 (55%)	344/605 (57%)
Finland	242/410 (59%)	544/746 (73%)
Switzerland	116/231 (50%)	147/259 (57%)
Norway	112/201 (56%)	0 (NA)
Total	**1824/3445 (53%)**	**3668/6935 (53%)**

Table 4.2 Sex distribution for the various cohorts of patients and controls included in the analysis of the genome-wide association study of partial epilepsies.

Abbreviations: NA, not applicable.

Chips / No.	UK	Ireland	Belgium	USA	Finland	Switzerland	Norway	Total
Patients								
Human610-Quadv1	1018	562	418	393	410	231	201	**3233**
HumanHap550v3	167	0	0	0	0	0	0	**167**
HumanHap300v1	0	45	0	0	0	0	0	**45**
Total	**1185**	**607**	**418**	**393**	**410**	**231**	**201**	**3445**
Controls								
Human610-Quadv1	0	0	0	347	277	259	0	**883**
HumanHap550v3	0	209	0	81	0	0	0	**290**
HumanHap300v1	0	0	0	0	0	0	0	**0**
Human1-2M-Duo Custom	5116	0	0	0	0	0	0	**5116**
Human1M-Duov3	0	0	0	0	104	0	0	**104**
Human1Mv1	0	0	0	6	0	0	0	**6**
HumanHap550v1	0	0	0	171	0	0	0	**171**
HumanCNV370-Quadv3	0	0	0	0	171	0	0	**171**
HumanCNV370v1	0	0	0	0	194	0	0	**194**
Total	**5116**	**209**	**0**	**605**	**746**	**259**	**0**	**6935**

Table 4.3 Genotyping chips for the various cohorts of patients with partial epilepsy and controls included in the analysis of the GWA study of partial epilepsy.
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Quality control procedures were applied to the Wellcome Trust Case Control Consortium control dataset in the following order, listed below.

a) All individuals listed as "individual exclusions" in the data release documentation were excluded;

b) Any remaining individuals with >2% missing data were removed;

c) SNPs with more than 1% missing data were removed;

d) SNPs with Hardy-Weinberg equilibrium *p* below $1x10^{-10}$ were removed;

e) Allele frequencies in the 1958 Birth cohort and National Blood cohort subsets were compared using a χ^2 test and SNPs with *p*-values below $1x10^{-10}$ were removed;

f) To check for possible plate effects, principal component (PC) analysis was performed on the remaining data, using a subset of unlinked SNPs. Plate effects were suspected in two cases (shown in Fig. 4.1) and these samples were removed.

The Irish control genotype data were downloaded from the dbGaP database (http://www.ncbi.nlm.nih.gov/gap), dbGaP accession number phs000127.v1.p1. SNPs with call rates below 0.98 and cluster separation values below 0.3, as provided in the data release documentation, were removed. Then a check was made that none of the individuals had over 2% missing data.

4.2.6 "Gender" checks and relatedness checks

"Gender" checks were performed for all samples. The "gender" of the individuals was imputed using X-chromosome data as implemented in PLINK (Purcell et al., 2007). X-chromosome homozygosity was estimated for each sample. A male call was assigned if homozygosity exceeded 80%, female if below 20%. Imputed sex was compared with sex in the phenotype database and 20 mismatched samples (17 cases and 3 controls) were removed.

Relatedness checks were performed for all samples. Identity-by-descent (IBD) was estimated among all pairs of samples as implemented in PLINK, using 65,415 independent SNPs, generated using PLINK option "--indep-pairwise 1500 150 0.2". Where estimated IBD was higher than 0.125, one sample from the pair of individuals was removed. If related individuals were concordant for case-control status, the subject with the lower genotyping call rate was removed; if discordant, the control subject was removed from the analysis.

4.2.7 Population ancestry and stratification analysis

A combination of self-identified ancestry and EIGENSTRAT principal components methods (Price et al., 2006) was used, to identify individuals of European ancestry and correct for population stratification.

4.2.7.1 Modified EIGENSTRAT method to control for population stratification

A modified EIGENSTRAT method was used to correct for correlations among genetic variants, which arise because of population ancestry rather than due to association with the phenotype / disease (Price et al., 2006). All the genotype data were subjected to principal components analysis and the resulting significant PC axes were used as covariates in the subsequent association analysis.

Correlations among SNPs may also be due other reasons and some PC axes may be created because of these other sources of correlation, different from population ancestry. This may happen due to: a) long linkage disequilibrium (LD) regions; b) sample processing problems, such as batch effects; or c) genotype calling differences, such as genotyping chip differences or different genotype call algorithms.

a) To correct for LD effects and to ensure that EIGENSTRAT axes reflected only effects that applied equally across the whole genome: (i) known high-LD regions (Table 4.4) were excluded and (ii) the SNP dataset was "thinned" using PLINK option "--indep-pairwise 1500 150 0.2" (such that all SNPs in a window size of 1500 were required to have $r^2<0.2$), which resulted in a set of 65,415 independent SNPs . This set of SNPs was used in the EIGENSTRAT analysis. Each SNP was regressed on the previous 5 SNPs and the residual entered into the PC analysis, as previously described (Patterson et al., 2006).

Chromosome	Start position (NCBI build 36)	End position (NCBI build 36)
1	48060567	52060567
2	85941853	100407914
	134382738	137882738
	182882739	189882739
3	47500000	50000000
	83500000	87000000
	89000000	97500000
5	44500000	50500000
	98000000	100500000
	129000000	132000000
	135500000	138500000
6	25500000	33500000
	57000000	64000000
	140000000	142500000
7	55193285	66193285
8	8000000	12000000
	43000000	50000000
	112000000	115000000
10	37000000	43000000
11	46000000	57000000
	87500000	90500000
12	33000000	40000000
	109521663	112021663
20	32000000	34500000

Table 4.4 Regions at high linkage desequilibrium in the human genome, excluded from the modified EIGENSTRAT analysis.

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The PC axes with *p*-value below 0.05, as assessed by the Tracy-Widom method (Patterson et al., 2006), were considered statistically significant. To further ensure that no axes were dominated by a single high LD region of the genome, the SNP loadings ("gamma" coefficients of Price et al. (2006)) were inspected for all significant PC axes, using the Q-Q plots.

b) Correlations among individuals may be due to laboratory processing error, batch effects and plate effects. The EIGENSTRAT axes in the PC plots were inspected for both batch effects and plate effects. This is done by attributing different colour labels to the samples in the PC plot according to their different batches or plates, which is followed by visual inspection of the plot, with subsequent exclusion of any suspect samples, as in the example given in Fig. 4.1.

c) As correlations among SNPs may also be due to genotype calling differences, such as genotyping chip differences, all EIGENSTRAT axes were inspected for these effects and suspect samples were removed.

Similarly, the 31 SNPs discordant between the HumanHap550 and Human610-Quadv1 chips, were removed from the analysis. Only SNPs present in both Illumina Human610-Quadv1 and Human1-2M-Duo Custom chips were included in the analysis.

Only SNPs with minor allele frequency (MAF) of 1% and above were included in the analysis. This frequency cut-off was chosen because common variants were being targeted and the study was underpowered to detect associations with lower allele frequencies. Genotypes of SNPs with MAF <1% were less reliably called across the different cohorts.

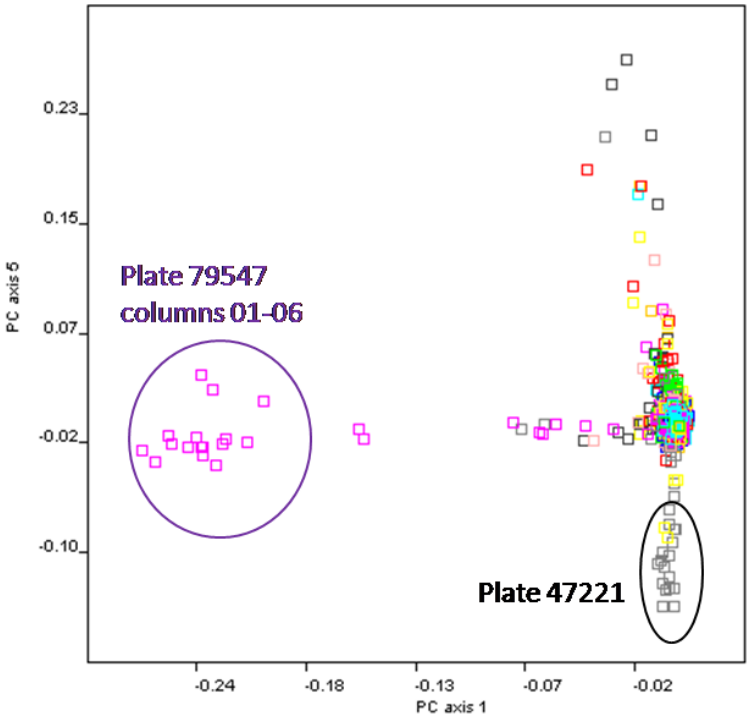

Figure 4.1 Principal components plot of WTCCC samples (UK controls).

Samples genotyped on different plates shown in different colours. Two plate effects were suspected (plates 47221 and 79547) and the samples were removed from the analysis.

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4.2.8 Association analysis

Association analysis was performed using PLINK (Purcell et al., 2007). Firstly, logistic regression was used, assuming a genetic additive model, including gender and all significant EIGENSTRAT axes, as assessed using the Tracy-Widom statistic with *p*-values below 0.05, as covariates into the model.

Patients of all ethnicities were recruited and genotyped, but only patients of European ancestry were included in the final genome-wide association analysis, to minimize confounding by population structure.

3,445 patients with partial epilepsy and 6,935 controls (10,380 study participants) were included in the analysis. 528,745 SNPs were included in the analysis.

4.2.8.1 Analysis excluding the Finnish cohort

As the Finnish cohort separates strongly from the other cohorts using PC analysis, this correction for population structure may not have been adequate to correct for differences between Finnish and other European cohorts. The data analysis was therefore repeated after excluding the Finnish cohort in both GWA studies and the results compared to the analysis including all cohorts.

4.2.8.2 Stratified analysis

Further, a stratified analysis using the Cochran-Mantel-Haenszel test was performed. For this analysis, seven strata were used, each corresponding to the recruitment country. To ensure homogeneity within each stratum, PC analysis for population stratification

analysis and correction was first performed within each stratum separately and the outliers removed.

4.2.9 Manual inspection of top associated SNPs

The cluster plots of the top associated SNPs were reviewed using Evoker_0.4.3 software, for the WTCCC data (http://en.sourceforge.jp/projects/sfnet_evoker/). Illumina Beadstudio v3 software was used for the in-house genotyped samples.

4.2.10 Pathway analysis (gene ontology analysis)

Pathway analysis was performed using the ALIGATOR method for gene ontology analysis (Holmans et al., 2009), for testing the SNPs with low (although not genome-wide significant) *p*-values in the GWAS of partial epilepsy for over-representation of pathways obtained from gene ontology categories.

To apply ALIGATOR, a GWAS p-value threshold needs to be specified, each pathway is then scored by counting the number of genes containing one or more SNPs witb p-value below the specified threshold, after which the score obtained is tested for significance by permutation.

These SNP sets were investigated using two thresholds, $p < 0.0001$ and $p < 0.001$. Only SNPs located within genes were included (based on NCBI SNP build 129 and NCBI sequence build 36.3). One SNP per gene, with the lowest p-value, was included in the ALIGATOR analysis, using 20,000 simulated replicate gene lists and 5,000 simulated replicate studies.

4.2.11 Power calculations

Power calculations were performed using the PGA Power Calculator software (Menashe et al., 2008), assuming a disease prevalence of 0.5%, the additive genetic risk model and r^2 0.9 between a causal variant and a genotyped marker (Fig. 4.2).

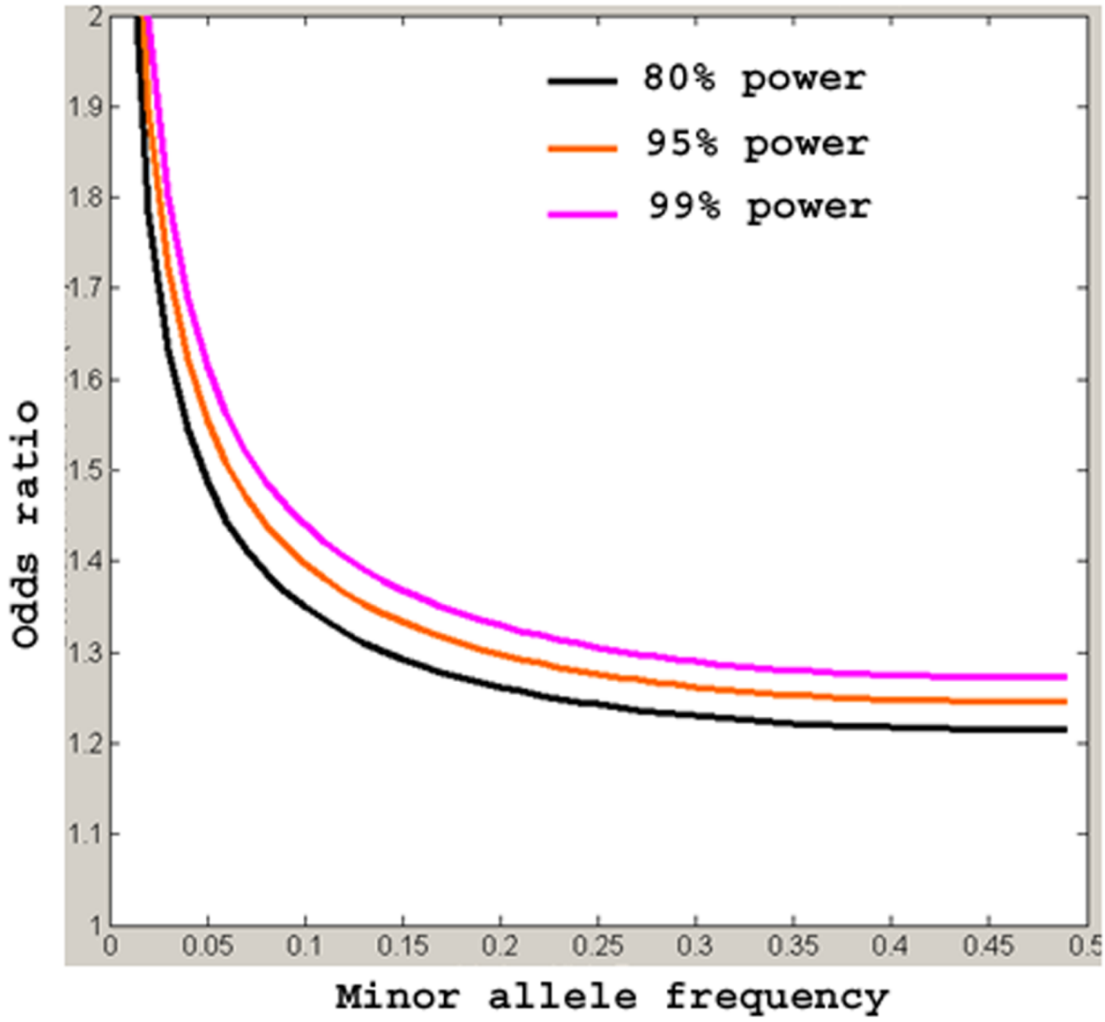

Figure 4.2 Lower detectable odds ratio at *p*-value 5 x 10^{-8} for different power levels in the GWA study of partial epilepsy.

Power calculations were performed assuming a disease prevalence of 0.5%, the additive risk model and r^2 = 0.9 between a causal variant and a genotyped marker.

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4.3 Results

4.3.1 Patients

Phenotypic details of the epilepsy syndromes and aetiologies of the patients for each cohort are shown in Table 4.5, using the scheme adapted from the 2010 ILAE proposal of organisation of the epilepsies (Berg et al., 2010), and lists the putative aetiologies for each patient included in the study. Table 4.6 lists the findings of the MRI brain scans for the patients in each cohort.

4.3.2 Quality control steps

4,514 study participants were specifically genotyped in the study: 3,941 patients with partial epilepsies and 573 controls. Of those, 4,383 study participants (97.1%), 3,816 patients and 567 controls, passed genotyping quality control filters.

After application of quality control procedures, the average genotyping call rate was 99.96% for subjects genotyped on Human610-Quadv1 and 99.93% for subjects genotyped on HumanHap550v3 chips. 34 known duplicate samples were genotyped. Genotype concordance rate was >99.99% regardless of whether samples were genotyped on the same chip type or on different chips. 20 subjects (0.4%, 17 patients and three controls) were excluded because sex mismatch was detected between phenotype and genotype data. One sample was removed because the same patient was found to have been recruited independently in two cohorts (UK and Ireland) and 48 subjects (27 patients and 21 controls) were removed due to relatedness to other study participants.

Epilepsy syndrome / No of patients (% of total)	**UK**	**Ireland**	**Belgium**	**USA**	**Finland**	**Switzerland**	**Norway**	**Total**
"Genetic" and "unknown cause"	517 (44%)	192 (32%)	168 (40%)	190 (48%)	257 (63%)	9 (4%)	96 (48%)	**1429 (41%)**
"Structural-metabolic causes"	668	415	250	203	153	222	105	**2016**
MTLEHS	265 (22%)	148 (24%)	67 (16%)	71 (18%)	116 (28%)	182 (79%)	70 (35%)	**919 (27%)**
MCD	141	38	21	16	12	12	1	**241**
Tumour	42	62	42	48	3	23	2	**222**
Infection	36	16	9	8	1	0	6	**76**
Trauma	32	60	22	21	2	0	7	**144**
Vascular malf	34	26	27	9	4	0	7	**107**
Perinatal insult	34	12	8	2	9	0	9	**74**
Stroke	32	27	33	9	0	0	1	**102**
Neurocutaneous syndromes	1	3	3	3	0	0	0	**10**
Other structural-metabolic causes	51	23	18	16	6	5	2	**121**
Total	**1185**	**607**	**418**	**393**	**410**	**231**	**201**	**3445**

Table 4.5 Aetiologies for the patients included in the genome-wide association study of partial epilepsy, for the various cohorts.

Abbreviations: malf, malformations; MCD, malformations of cortical development; MTLEHS, mesial temporal lobe epilepsy with hippocampal sclerosis.

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MRI phenotype / N patients (%)	**UK**	**Ireland**	**Belgium**	**USA**	**Finland**	**Switzerland**	**Norway**	**Total**
Normal MRI	487 (42%)	134 (22%)	94 (23%)	146 (37%)	209 (51%)	8 (4%)	81 (40%)	1159 (34%)
Unilateral HS[7]	277	140	65	66	118	182	72	920
Bilateral HS	12	6	3	5	4	1	0	31
MCD	138	38	17	14	12	12	1	232
CVD	27	12	30	11	0	0	1	81
Perinatal injury	34	1	7	1	8	0	9	60
Other acquired injury	59	29	27	20	3	0	8	146
Vascular malformation	34	25	25	18	4	0	7	113
Tumour	42	60	42	50	3	23	2	222
Other	39	23	41	6	31	4	18	162
Incidental MRI findings	26	26	39	20	11	1	2	125
MRI not available; CT scan shows abn	10	15	10	0	0	0	0	35
Brain imaging data not available	0	98	18	36	7	0	0	159
Total	**1185**	**607**	**418**	**393**	**410**	**231**	**201**	**3445**

Table 4.6 MRI findings for the various cohorts from the genome-wide association study of partial epilepsy.

Abbreviations: abn, abnormality; CVD, cerebrovascular disease; HS, hippocampal sclerosis; MCD, malformations of cortical development.
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[7] Please note that the information on table 4.6 "unilateral HS" is a radiological diagnosis, while the "MTLEHS" category on table 4.5 is a syndromic diagnosis taking into account all clinical, electrophysiological and radiological data.

The resulting dataset was merged with the quality-controlled control datasets from the Duke Memory study, Wellcome Trust Case Control Consortium, Vantaa85+ and Study of Irish Amyotrophic Lateral Sclerosis and a further three related controls were removed.

After correction for population structure, 3,445 patients with partial epilepsies and 6,935 controls of European ancestry were included for genome-wide association analysis. 528,745 SNPs were included in the analysis. For the SNPs present only on the chips Human610-Quadv1 and Human1-2M-Duo Custom, but not on the other types of chips, the sample size was smaller. The smaller sample size was 3,233 patients and 5,999 controls, if a SNP was not present on any other type of chip.

4.3.3 Association analysis

Firstly, association analysis using logistic regression was performed, assuming an additive genetic model and including gender and the fifteen significant EIGENSTRAT axes as covariates.

The quantile-quantile (Q-Q) plot is shown in Fig. 4.3 and showed a slight departure of the observed distribution of test statistics from the expected. The genomic inflation factor λ was 1.05, suggesting adequate correction for population stratification.

The Manhattan plot of the genome-wide association analysis of the partial epilepsies shown in Fig. 4.4A summarizes the results of the logistic regression analysis.

The *p*-values for all SNPs, resulting from the GWA study of partial epilepsies are publicly available online, at: http://www.ion.ucl.ac.uk/departments/epilepsy/themes/genetics/PEvsCTRL.

4.3.3.1 Stratified association analysis

Unequal sample sizes from different European subpopulations can bias the analysis of the population structure based on PC analysis, by overemphasizing variation within the largest cohorts and the PC-based correction of population structure may have over-compensated.

To deal with any possible residual population stratification and check the robustness of the association results, a stratified association analysis was performed using the Cochran-Mantel-Haenszel test. The results are graphically summarized in the Manhattan plot of the stratified analysis of the GWAS of partial epilepsy, seen in Fig. 4.4B, while the Q-Q plot in Fig. 4.5 shows a slight excess of low *p*-values, with a genomic inflation factor, λ, of 1.02, indicating adequate correction for population structure.

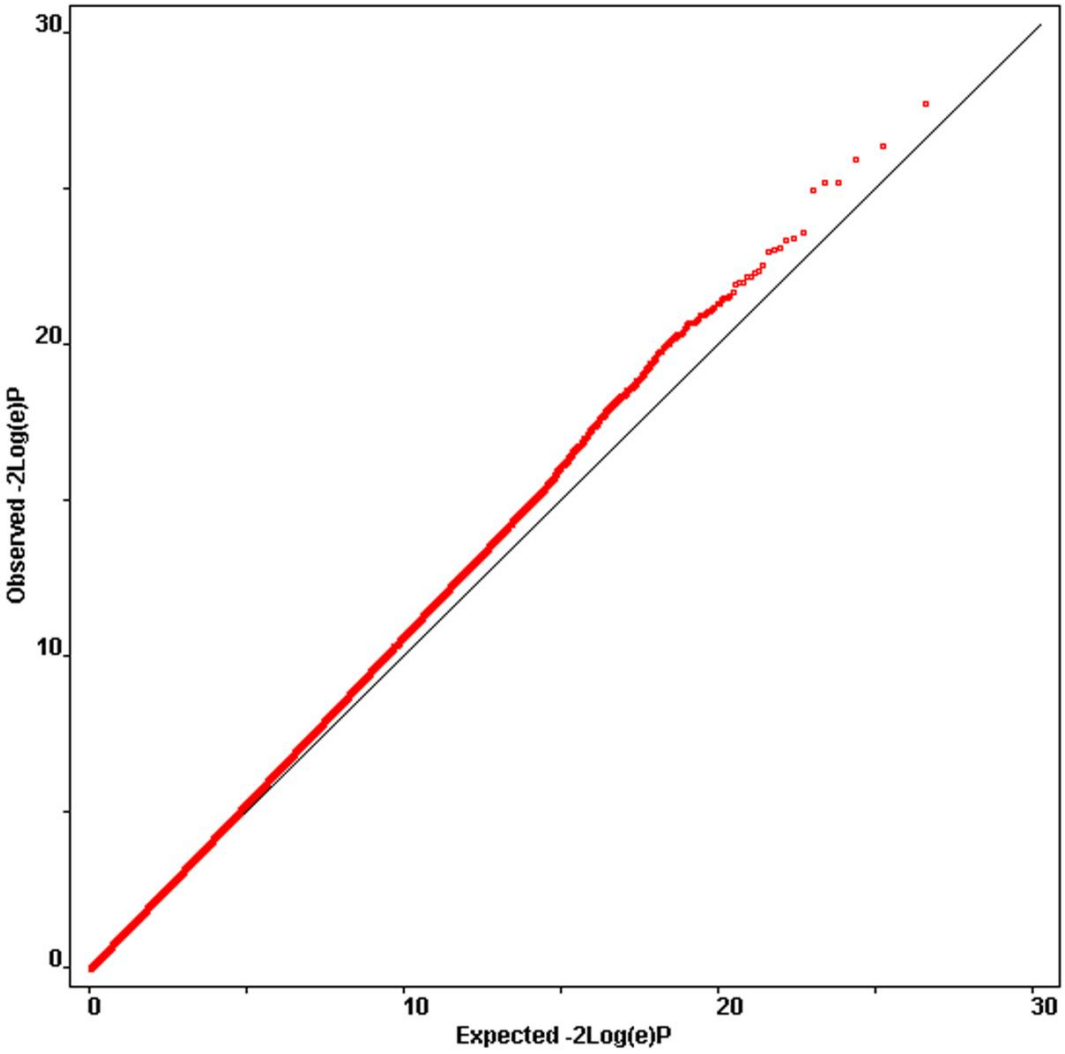

Figure 4.3 Quantile-quantile plot for the GWA study of partial epilepsy, based on the *p*-values from logistic regression tests. The genomic inflation factor (λ) is 1.05.

Figure generated in WGAViewer (Ge et al., 2008).

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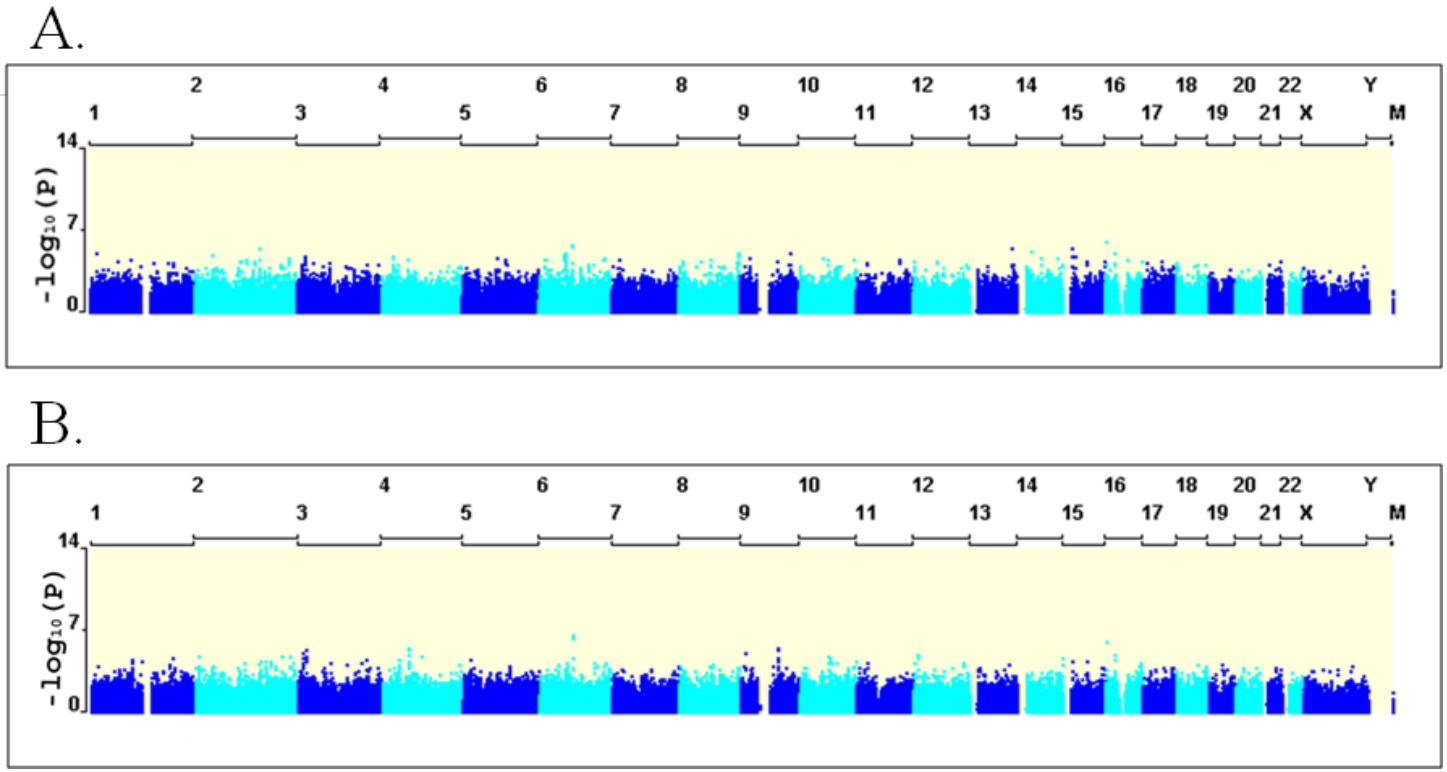

Figure 4.4 Manhattan plots for the GWA study of partial epilepsy: (A) logistic regression and (B) Cochran-Mantel-Haenszel test. Transformed -$\log_{10}$ (*p*-values) for the SNPs that passed quality-control are plotted against SNP positions on each chromosome. Chromosomes are shown in alternating colours. No genome-wide significant association has been identified between a genetic marker and common partial epilepsy.

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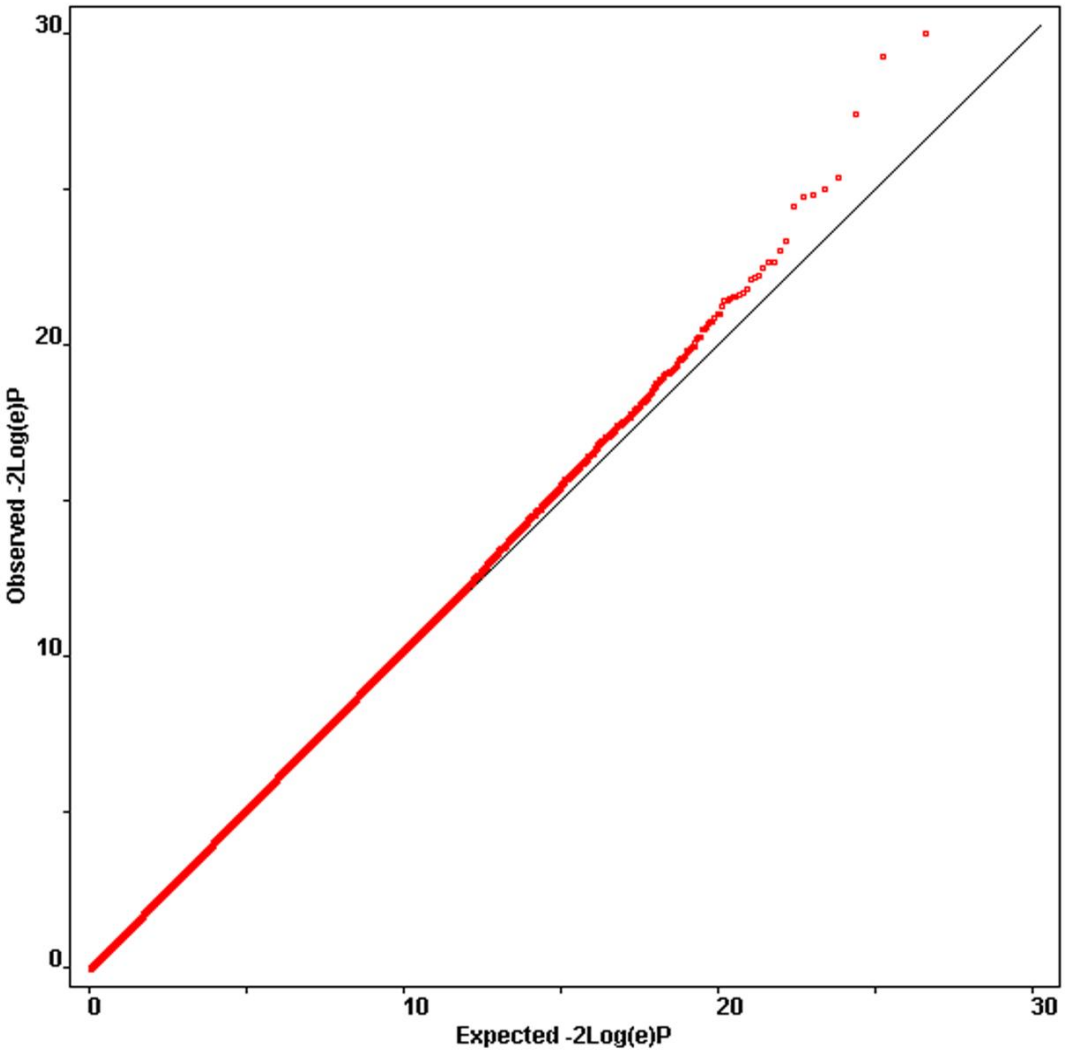

Figure 4.5 Q-Q plot of the stratified analysis of the GWA study of partial epilepsy, based on the *p*-values from the Cochran-Mantel-Haenszel tests.

The genomic inflation factor λ is 1.02.

Figure generated in WGAViewer (Ge et al., 2008).

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4.3.3.2 Top SNPs

Table 4.7 shows the SNPs with *p*-values below $5x10^{-5}$ in either the logistic regression or Cochran-Mantel-Haenszel tests.

None of the *p*-values in the study reaches the widely-accepted genome-wide significance threshold in association studies of $5x10^{-8}$ (McCarthy et al., 2008), nor the more conservative Bonferroni correction threshold of $9.5x10^{-8}$, which takes into account all 528,745 tests performed.

The top SNP, rs346291 ($p = 3.3x10^{-7}$), is located on chromosome 6p, within a predicted pseudogene and at 95kb and 116kb from the closest known genes, *SH3BGRL2* and *ELOVL4*, respectively. There is little LD in the region.

The second top SNP, rs9341799 ($p = 4.8x10^{-7}$), located on chromosome 6p, is in only moderate LD with rs346291 ($r^2 = 0.34$ in the dataset) (Fig. 4.6). To test the independence of association signals for these two SNPs, logistic regression analysis for rs9341799 was performed conditional on the genotype of rs346291, by incorporating this genotype as a covariate in the model. A residual association was detected ($p = 0.0102$), suggesting that these two signals are not completely independent.

Other top associated SNPs lie within interesting candidate genes and may warrant further investigation. The third in rank of the top associated SNPs, rs2601828 ($p = 1.2x10^{-6}$) is an intronic SNP located on chromosome 16, in the *ADCY9* gene, which encodes adenylate cyclase 9, which catalyzes the formation of cyclic AMP from ATP and

is involved in neuronal signalling. The *PRKCB* gene, which encodes protein kinase C, also involved in neuronal signalling, is another interesting candidate.

In the list of top SNPs, rs54331, located on chromosome 2q, with $p = 4.6 \times 10^{-5}$, is intronic to the *SCN1A* gene[8]. Although the association has not reached genome-wide significance, this warrants follow-up in a larger study, given its biological plausibility (McCarthy et al., 2008).

The top associated SNP rs12570947 did not pass post-association quality control, as the cluster plots revealed "bad" clustering of genotype calls in the EPIGEN and WTCCC datasets (Fig. 4.7). Consequently, rs12570947 was removed from the results.

4.3.4 Pathway analysis (gene ontology analysis)

Pathway analysis (gene ontology analysis) testing the results of the GWAS of partial epilepsy has provided evidence for association of susceptibility to partial epilepsy and genes coding for ion channels, including sodium channels, glutamate receptors, transmembrane transport, postsynaptic membrane (Tables 4.8 and 4.9). These results are very interesting, the pathways involved (ion channels, synaptic activity, transmembrane transport) have significant biological plausibility, and the genes included in these categories are interesting candidates for further research with follow-up analyses in the partial epilepsies.

[8] The neurological disorders associated with the gene *SCN1A* are reviewed in Chapter 1, section 1.7.

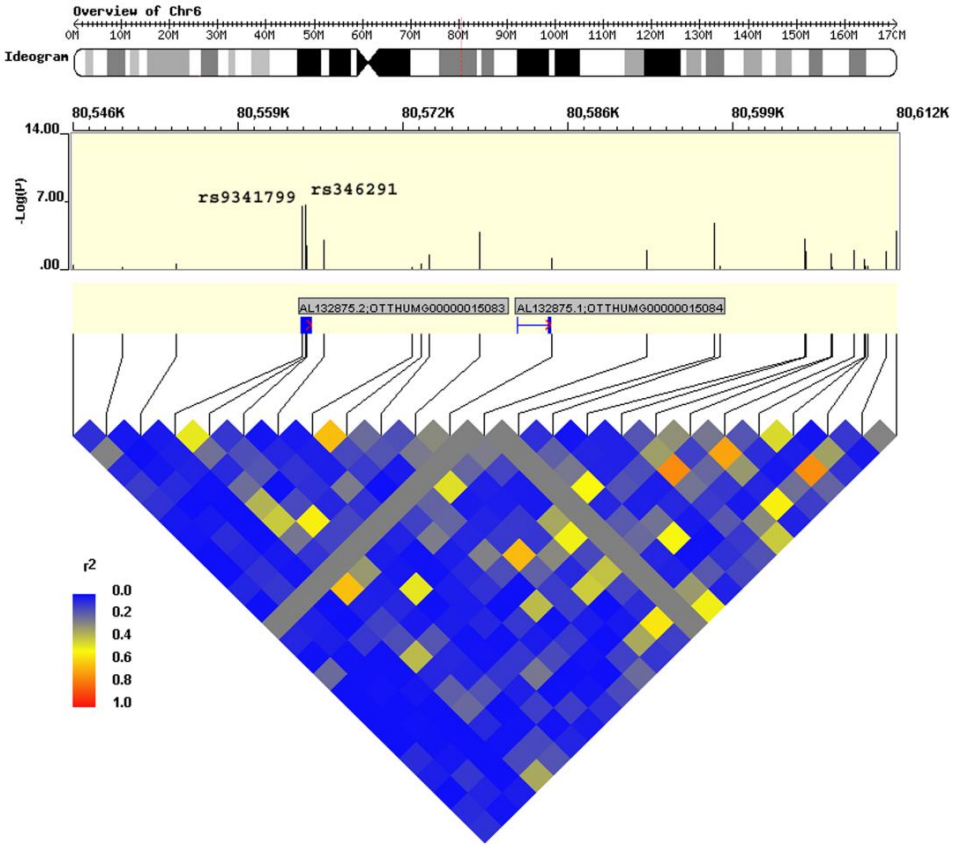

Figure 4.6 Overview of the region on chromosome 6 with the top associated SNPs.

(A) Results for the stratified analysis: CMH test, $-\log_{10}$ *p*-values, each bar represents one SNP. (B) Pairwise linkage disequilibrium (r^2) diagram for this region in HapMap CEU samples shows this is a region of low LD.

Figure generated in WGAViewer (Ge et al., 2008).

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SNP	Ch	Position (NCBI build 36)	Type	Closest gene	*p*-value (CMH)	*p*-value (LR)	OR (95% CI, CMH)	Minor allele	MAF in pts	MAF in ctrls	Genotype counts in pts	Genotype counts in ctrls
rs346291	6	80564836	within pseudo-gene	*AL132875.2*	$3.3x10^{-7}$	$2.5x10^{-6}$	0.8 (0.8-0.9)	A	0.335	0.366	384/ 1538/ 1523	950/ 3180/ 2802
rs9341799	6	80564519	within pseudo-gene	*AL132875.2*	$4.8x10^{-7}$	$2.1x10^{-6}$	1.2 (1.1-1.3)	G	0.405	0.373	569/ 1617/ 1215	943/ 3005/ 2617
rs2601828	16	4103871	intronic	*ADCY9*	$1.2x10^{-6}$	$1.0x10^{-6}$	1.2 (1.1-1.3)	A	0.253	0.222	200/ 1342/ 1903	349/ 2380/ 4206
rs1490157	3	21719246	intronic	*ZNF385D*	$5.3x10^{-6}$	$2.4x10^{-5}$	0.8 (0.8-0.9)	G	0.229	0.261	163/ 1229/ 2004	444/ 2538/ 3572
rs1989647	16	23959420	intronic	*PRKCB*	$1.3x10^{-5}$	$8.9x10^{-6}$	1.2 (1.1-1.3)	A	0.351	0.312	423/ 1536/ 1438	654/ 2791/ 3122
rs1320292	3	21701712	intronic	*ZNF385D*	$1.6x10^{-5}$	$1.8x10^{-5}$	0.8 (0.8-0.9)	A	0.208	0.240	140/ 1127/ 2116	361/ 2434/ 3772
rs951997	2	223567016	intronic	*MOGAT1*	$2.0x10^{-5}$	$4.5x10^{-5}$	1.2 (1.1-1.2)	A	0.476	0.443	796/ 1690/ 959	1354/ 3441/ 2138
rs1942006	10	67653901	intergenic	*CTNNA3*	$2.1x10^{-5}$	$4.1x10^{-5}$	1.2 (1.1-1.3)	A	0.300	0.274	306/ 1451/ 1687	538/ 2726/ 3666

rs1387822	3	21686466	intronic	*ZNF385D*	$2.9x10^{-5}$	$2.5x10^{-5}$	0.9 (0.8-0.9)	G	0.298	0.326	294/ 1462/ 1688	725/ 3070/ 3137
rs1396626	1	96025546	within known processed transcript	*AL683887.1*	$3.4x10^{-5}$	$3.3x10^{-5}$	1.2 (1.1-1.3)	A	0.318	0.288	351/ 1487/ 1607	585/ 2823/ 3522
rs16834756	2	154745009	intronic	*GALNT13*	$4.9x10^{-5}$	$3.7x10^{-6}$	0.7 (0.6-0.8)	G	0.030	0.046	6/ 190/ 3205	9/ 582/ 5973
rs545331	**2**	**166913962**	intronic	***SCN1A***	**$4.6x10^{-5}$**	**0.001**	NA	**A**	**0.254**	**0.280**	**223/ 1285/ 1893**	**519/ 2642/ 3408**

Table 4.7 Top SNPs with *p*-values below $5x10^{-5}$, for the GWAS of partial epilepsy (logistic regression and Cochran-Mantel-Haenszel tests).

Abbreviations: Ch, chromosome; CI, confidence interval; CMH, Cochran-Mantel-Haenszel; ctrl, control; LR, logistic regression; MAF, minor allele frequency; OR, odds ratio; pts, patients.

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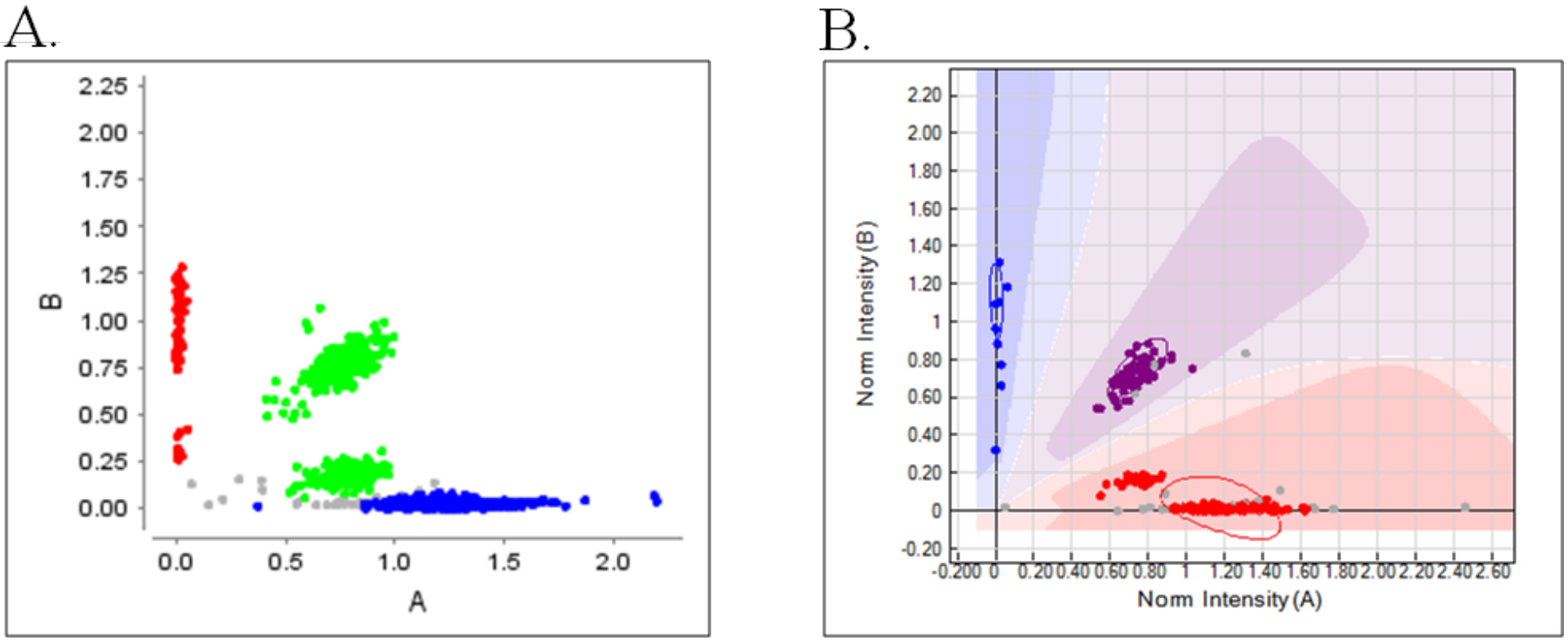

Figure 4.7 Cluster plots of rs12570947, one of the top associated SNPs, for the (A) WTCCC 1958 birth cohort and the (B) UK cohort of patients with partial epilepsy. This SNP rs12570947 showed bad clustering, resulting in different genotype calls in EPIGEN and WTCCC datasets and was, therefore, removed from the results.The cluster plots of the top associated SNPs were reviewed using Evoker_0.4.3 software for the WTCCC data (http://en.sourceforge.jp/projects/sfnet_evoker/) and Beadstudio software for the in-house genotyped samples.
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Gene ontology number	Type	Total number genes in category	Number of genes on list	Expected number of genes on list	*p*-value	Biological function
GO:0005230	FUNCTION	69	3	0.3	0.0032	**extracellular ligand-gated ion channel activity**
GO:0005234	FUNCTION	19	2	0.2	0.016	**extracellular-glutamate-gated ion channel activity**
GO:0004970	FUNCTION	18	2	0.2	0.016	**ionotropic glutamate receptor activity**
GO:0015276	FUNCTION	112	3	0.58	0.019	**ligand-gated ion channel activity**
GO:0022834	FUNCTION	112	3	0.58	0.019	**ligand-gated channel activity**
GO:0005231	FUNCTION	46	2	0.23	0.021	**excitatory extracellular ligand-gated ion channel activity**
GO:0004888	FUNCTION	832	6	2.28	0.022	**transmembrane receptor activity**
GO:0044248	PROCESS	839	4	1.22	0.032	cellular catabolic process
GO:0046982	FUNCTION	125	2	0.29	0.032	protein heterodimerization activity
GO:0045211	CELLULAR	115	3	0.72	0.035	**postsynaptic membrane**
GO:0022836	FUNCTION	277	4	1.25	0.035	**gated channel activity**
GO:0016788	FUNCTION	547	4	1.27	0.036	hydrolase activity, acting on ester bonds
GO:0000122	PROCESS	148	2	0.31	0.039	negative regulation of transcription from RNA polymerase II promoter
GO:0008066	FUNCTION	29	2	0.32	0.040	**glutamate receptor activity**
GO:0005529	FUNCTION	145	2	0.32	0.041	sugar binding
GO:0009056	PROCESS	952	4	1.41	0.049	catabolic process

Table 4.8 Results of the pathway analysis (gene ontology analysis) for partial epilepsy- associated SNPs with *p*-values below 0.0001 (CMH test); gene ontology categories with enrichment *p*-values < 0.05.

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Gene ontology number	Type	Total number genes in category	Number of genes on list	Expected number of genes on list	*p*-value	Biological function
GO:0005272	***FUNCTION***	***30***	***5***	***0.71***	***0.00005***	***sodium channel activity***
GO:0001518	***CELLULAR***	***12***	***3***	***0.2***	***0.0007***	***voltage-gated sodium channel complex***
GO:0034706	***CELLULAR***	***12***	***3***	***0.2***	***0.0007***	***sodium channel complex***
GO:0005248	***FUNCTION***	***15***	***3***	***0.22***	***0.0008***	***voltage-gated sodium channel activity***
GO:0022836	***FUNCTION***	***277***	***15***	***7.17***	***0.0043***	***gated channel activity***
GO:0030324	PROCESS	58	4	0.72	0.0051	lung development
GO:0005882	CELLULAR	85	3	0.38	0.0058	intermediate filament
GO:0045111	CELLULAR	86	3	0.39	0.0061	intermediate filament cytoskeleton
GO:0046873	FUNCTION	282	14	6.88	0.0062	**metal ion transmembrane transporter activity**
GO:0006368	PROCESS	35	2	0.13	0.0068	RNA elongation from RNA polymerase II promoter
GO:0030323	PROCESS	60	4	0.81	0.0075	respiratory tube development
GO:0006354	PROCESS	38	2	0.14	0.0079	RNA elongation
GO:0005216	***FUNCTION***	***341***	***16***	***8.38***	***0.0084***	***ion channel activity***
GO:0022838	***FUNCTION***	***349***	***16***	***8.41***	***0.0087***	***substrate specific channel activity***
GO:0015267	***FUNCTION***	***355***	***16***	***8.42***	***0.0088***	***channel activity***
GO:0022803	FUNCTION	355	16	8.42	0.0088	**passive transmembrane transporter activity**
GO:0006213	PROCESS	8	2	0.23	0.0091	pyrimidine nucleoside metabolic process
GO:0048286	PROCESS	10	2	0.16	0.0094	alveolus development
GO:0008266	FUNCTION	5	2	0.2	0.0097	poly(U) binding

Table 4.9 Results of the pathway analysis (gene ontology analysis) for partial epilepsy- associated SNPs with *p*-values below 0.001 (CMH test); gene ontology categories with enrichment *p*-values < 0.01.

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4.4 Discussion and conclusions

To my knowledge, this study was the first published genome-wide association study in epilepsy.

No genome-wide significant association was identified between a common genetic marker and common partial epilepsy. Genome-wide significance was not achieved for any SNP in the study, yet a few SNPs may warrant follow-up in a larger study.

Suggestive evidence for association was found with two SNPs on chromosome 6p, rs346291 and rs9341799 (r^2 of 0.34), with p-values of 3.3×10^{-7} and 4.8×10^{-7}, respectively.

In the list of top SNPs, rs54331 is intronic to *SCN1A,* with *p*-value of 4.6×10^{-5}, which is not genome-wide significant. Nevertheless, this SNP would also be interesting to follow-up, given the biological plausibility of association and prior knowledge of the contribution of *SCN1A* to susceptibility to epilepsy.

It could be that common genetic variants with effect sizes above a modest odds ratio of about 1.3, for a single variant, were not significant contributors to genetic susceptibility shared across the partial epilepsies. An alternative explanation is that the study may be underpowered and that common genetic variation may have a role in predisposition to the partial epilepsies when considered across syndromes in individuals of European descent; it would be interesting to pursue this further.

The pathway analysis showed evidence of association with susceptibility for partial epilepsy, of genes included in the categories/ pathways ion channels, neurotransmission, synaptic activity, transmembrane transport (Tables 4.8 and 4.9), all of highly significant biological plausibility. This provides a route for further research, by exploring the genes included in these pathways in follow-up studies of partial epilepsy.

4.4.1 Limitations

Given the design of the study and its purpose, the study encompasses marked clinical heterogeneity (Table 4.5). "Partial epilepsy" is a heterogeneous label, which covers a large range of electroclinical syndromes, with different underlying pathologies and probably differences in the contribution of genetic variants. For example, the idiopathic focal epilepsies, such as autosomal dominant lateral temporal lobe epilepsy, are likely to have a genetic basis that does not completely overlap with the genetic variants contributing to a "symptomatic" epilepsy, such as the post-traumatic epilepsies and this may complicate this method of trying to look for genetic factors that predispose to the partial epilepsies.

The differentiation of partial and generalised epilepsies is based on a classification agreed upon by experts, but does not always have clear borders and there are cases which defy classification. Further, the dichotomy of generalised and partial may be reconsidered in the future, with some patients now classified as having generalised epilepsy possibly having focal origin of seizure activity (O'Muircheartaigh and Richardson 2012). This issue is not expected to have contributed for significant misclassification in this study, as all patients included have evidence for focal origin of seizure activity and stringent electroclinical criteria were used in the study.

Steps were taken to minimize misclassification, with all patients' case notes being reviewed independently for the diagnosis of epilepsy and epilepsy syndrome by one researcher and one consultant epileptologist.

Consistency of phenotypes and phenotyping between centres is paramount for the success of any GWA study. This important issue was discussed at study design phase among the members of the consortia, and ensured by previously agreeing on inclusion and exclusion criteria, by using the same classification scheme for every phenotype across the tertiary epilepsy referral centres. A limitation of this study was that no check of consistency of phenotyping was performed for all centres involved. This should be done in future studies, for example, by using cases for all participating clinicians to classify, thereby assessing interrater consistency and allowing to correct for any divergences that may be recognised.

The controls used for many of the cohorts were shared controls used in previous genomic studies. This is a well-established approach in genome-wide association studies (Wellcome Trust Case Control Consortium 2007). A small degree of misclassification is always possible. In some cases the inclusion criteria for control status involved the use of a questionnaire. The prevalence of febrile seizures and seizures in the general population (Hauser & Beghi 2008;Sadleir & Scheffer 2007) may mean that a few controls could have had childhood FS, childhood epilepsy in remission, an isolated unprovoked seizure, or an acute symptomatic seizure. There is also the possibility of misdiagnosis, with a control having seizures without a diagnosis of epilepsy - for example if the individual only has auras or nocturnal seizures that have thus far remained undiagnosed - but that should be exceedingly rare in this group.

There is a possibility of false negatives in GWA studies, given the stringent thresholds used for significance.

4.4.2 Next steps

To increase the power of the GWA study, one strategy is to increase the sample size. Larger cohorts may be required to capture a signal of association and a meta-analysis would also be helpful.

It may be also that the genetic model to explain genetic susceptibility to the epilepsies has to take into account the great level of clinical and genetic heterogeneity of the partial epilepsies, accepting, for example, that possible dilution of the signal from the inclusion of "symptomatic" epilepsies may not improve power.

To analyze only patients with partial epilepsy classified as "idiopathic" or "cryptogenic" (44% of the total of partial epilepsies in our cohort, Table 3.5), excluding epilepsies with a clear epileptogenic lesion, could theoretically increase the homogeneity of the study cohort, thereby increasing power. Nevertheless, the aim of the current study was to look for common genetic variants associated with increased susceptibility to partial epilepsies, whatever the possible "cause" of the epilepsy in each patient, assuming genetic variants that contribute to susceptibility to epilepsy across the partial epilepsy syndromes.

To analyze both partial and generalised epilepsies, looking for genetic variants associated with risk of epilepsy (predisposition for recurrent epileptic seizures), independently of any classification, is also a feasible next step; this again would not be able to answer the main question posed by this study, i.e., to look for genetic variation contributing to susceptibility to partial epilepsy transversally across any possible subtypes of partial epilepsies.

5 Chapter Genome-wide association study of mesial temporal lobe epilepsy with hippocampal sclerosis

5.1 Introduction

5.1.1 Definition and relevance of MTLEHS

Mesial temporal lobe epilepsy with hippocampal sclerosis (MTLEHS) is a common and frequently refractory epilepsy type (Semah et al., 1998). In selected cases, it can be amenable to resective surgery, with good results (de Tisi et al., 2011;Engel, Jr. et al., 2008;Wiebe et al., 2001) and it is in fact the most frequent substrate leading to resective surgery in adults with refractory TLE (Falconer et al., 1964).

MTLEHS is defined by a set of criteria agreed upon by a 2004 ILAE expert panel, which include clinical, neurophysiology, neuroimaging and/or neuropathology findings (Wieser 2004).[9]

Historically, HS was seen as a "single entity" and thought to be the cause of seizures in patients with MTLE who had surgery and showed neuropathological evidence of HS (Falconer et al., 1964;Meldrum 1997).

More recently, there is accumulating evidence from neuropathology, neuroimaging, animal models and other, that MTLEHS is heterogeneous (Thom et al., 2010b). Furthermore, even if HS is believed to be the epileptogenic lesion for the patients with MTLEHS who undergo resective surgery and become seizure free as a result, the

[9] A review of the accepted criteria for the diagnosis of MTLEHS is detailed in Chapter 3, section 3.3.

underlying “cause” is unknown and most probably is thought to be multifactorial, including the contribution of genetic factors.

5.1.2 Relationship between MTLE, HS and FS

Prolonged febrile seizures are frequently found as “initial precipitating injury” in the personal and familial antecedents of patients with MTLEHS (Mathern et al., 1995;Menzler et al., 2011). The relationships between MTLE, HS and FS (Cendes 2004;Harvey et al., 1995) are currently a hot research topic (Hesdorffer et al., 2012;Stafstrom 2011). Genetic factors are known to play an important role in the susceptibility to FS (Colosimo et al., 2007;Escayg et al., 2000b;Kobayashi et al., 2002;Mantegazza et al., 2005;Scheffer et al., 2007), MTLE and HS (Cendes 2004). It is likely that complex interactions between genetic factors and environmental factors are involved in the association of FS, MTLE and HS (Cendes 2004).

5.1.3 Suitability of MTLEHS for GWA studies

Genome-wide searches for causal genetic variants in MTLE(HS) have been identified as a major priority in epilepsy research (Baulac and Pitkanen 2008).

MTLEHS is, despite its known heterogeneity, very interesting to study using the GWA approach, because it is common and discrete (Engel, Jr. et al., 2008) and has a set of diagnostic criteria – both clinical, electroencephalographic, imaging and neuropathological (Wieser 2004), which include objective and quantitative data. Furthermore, many patients have very detailed phenotypical data, as they are frequently refractory to treatment and undergo extensive assessment for suitability for epilepsy surgery. This contributes to an increase of specificity and accuracy of the diagnosis, which are critical for genetic studies.

There are data suggesting that MTLEHS has a genetic contribution, but no accurate estimates of heritability. Even if heritability of MTLEHS is not substantial, other common disorders thought to have low heritability have had good results in GWA studies, with several genetic variants confirmed or discovered. There are several examples of common diseases that were previously thought to be mostly "sporadic" and not to have a significant genetic component - for example, Parkinson's disease, for which many genes and genetic variants contributing to risk have now been identified. Thus, investigating the genetics of MTLEHS is worthwhile and the GWA methodology is appropriate.

5.2 Methods

A multicentre genome-wide association study of MTLEHS was performed, looking for genetic variants associated with increased susceptibility to developing MTLEHS.

The rationale and main steps of the methodology of the GWA studies have been described in Chapter 2 and in Chapter 4, Section 4.2. In this section, the focus will be on the specificities of the GWA study of MTLEHS.

5.2.1 Subjects

Participating groups in the GWAS of MTLEHS included the EPIGEN consortium (UK, US, Belgium, Ireland), the GenEpA consortium (Switzerland, Finland, Norway), as was also the case for the GWA study of partial epilepsies, plus another group from Vienna, Austria (Table 5.1).

Phenotypical definitions were previously agreed across consortia and thoroughly discussed on Chapters 2 and 3. Inclusion criteria in the study included diagnosis of MTLEHS, as identified in the 2004 ILAE workshop report (Wieser 2004). All cases had histopathology and/or imaging confirmation of hippocampal sclerosis. For patients with definite MTLE and evidence of HS, exclusion criteria included bilateral hippocampal sclerosis and dual pathology.

1,187 patients with MTLEHS and 8,423 healthy controls – for a total of 9,610 study participants, were genotyped and included in this study. This included 166 patients

with MTLEHS and 338 controls from the Austrian cohort, who had not been included in the GWAS of partial epilepsy.

5.2.2 DNA extraction and genotyping

DNA was extracted using standard protocols, from a blood sample and 63 people with MTLEHS had DNA extracted from brain tissue obtained during resective surgery.

Genotyping was performed using Illumina chips and genotyping technology, as described in Chapter 2 Methods. The patients and controls of the Austrian cohort were genotyped with the Illumina HumanHap300v1 chip, with around 317,503 probes.

5.2.3 Quality control steps

The quality control procedures for the GWA study of MTLEHS were performed as described in Chapters 2 and 4.

For the GWA study of MTLEHS, 166 patients with MTLEHS and 338 healthy controls from a group from Austria-Vienna, were also included, together with the cohorts included in the GWA study of the partial epilepsies: UK-London, US-Duke, Belgium-Brussels, Ireland-Dublin, Switzerland-Zurich, Norway-Oslo, Finland-Helsinki and Finland-Kuopio.

The datasets used in the analysis were the genotype files from the Austrian cohort, received in PLINK binary format and the dataset used for the genome-wide association study of partial epilepsies from the EPIGEN and GenEpA consortia.

	UK	Ireland	Belgium	USA	Finland	Switzerland	Norway	Austria	Total
Patients with MTLEHS									
genotyped	331	148	77	97	116	182	70	166	**1187**
included in the final analysis	265	147	67	71	116	182	70	165	**1083**
Controls									
genotyped	5667	211	0	1165	757	285	0	338	**8423**
included in the final analysis	5118	210	0	605	747	264	0	338	**7282**

Table 5.1 Number of participants (patients with MTLEHS and healthy controls) genotyped and included in the analysis, for each subcohort in the GWA study of MTLEHS.

The first step was to merge the genotype files[10] in PLINK binary format, after confirming there were no asymmetric SNPs and SNPs with "-" minor alleles, no problematic SNPs allocated to different chromosomes and no SNPs where the data were on opposite strands between datasets. 306,611 SNPs were shared between both datasets.

A check was made for "gender" mismatches, as previously described in Chapters 2 and 4. Twenty samples were excluded in total, with no sample from the Austrian cohort excluded.

The following step was to check for cryptic relatedness and duplicates, after performing LD pruning, which resulted in 33,996 SNPs. Over 20,000 SNPs are considered sufficient for an accurate prediction of the population stratification axes, as suggested by simulations published by Price and colleagues (2006). The threshold for the IBD estimate ($\hat{\pi}$, pi-hat) used for the cryptic relatedness checks, was set at 0.125.

Detection and correction for population stratification was then performed, using principal component (PC) analysis with the modified Eigenstrat method. This step was later repeated for each cohort separately after removal of any outliers seen in the joint analysis of all patients and controls. The Austrian patients and controls as a group could not be visually separated from the other clusters. No chip effect was found from the analysis of the PC plots. One Austrian case and no Austrian control were removed.

After the pre-association analysis quality control checks, the cleaned dataset to bring forward consisted of 1,083 MTLEHS patients and 7,282 controls.

[10] A description on how to merge genotype files in PLINK binary format, is available from (Weale 2010).

5.3 Results

5.3.1 Power calculations

Calculations to estimate the power of detecting a significant hit for the GWA study of MTLEHS were performed using the software Genetic Power Calculator (Purcell et al., 2003), which applies variance-components models (Sham et al., 2000) and is available at http://pngu.mgh.harvard.edu/~purcell/gpc.

The power calculations estimated a sample size of at least 1,000 cases needed to have 80% power to detect an association with a risk genotype, considering 0.05% prevalence, MAF 0.3, odds ratio 1.3, control: case ratio 7, using the additive genotype risk model, at an alpha level of 5×10^{-8}.

5.3.2 Genome-wide analysis of MTLEHS versus controls

1,083 patients with MTLEHS and 7,282 controls, all of European ancestry, were included in the final analysis (Table 5.1). 531,164 SNPs were included.

Association analysis was performed using logistic regression and the main findings are summarised in Table 5.2. The corresponding Manhattan plot is shown in Fig. 5.1.

Three top SNPs are close (in the case of rs7587026 and rs580041) or intronic (in the case of rs11692675) to the *SCN1A* gene (Fig. 5.2). The corresponding *p*-values of association with the phenotype MTLEHS are 1.6×10^{-7}, for rs7587026, 3.1×10^{-7}, for rs11692675 and 4.8×10^{-7}, for rs580041. The *p*-values between 5×10^{-7} and 10^{-7} are suggestive evidence for association, just outside a "borderline" genome-wide significance (Panagiotou and Ioannidis 2012). The three top SNPs are in LD with each other, with r^2 of 0.7 between rs7587026 and rs11692675 (Fig. 5.3).

These three top SNPs passed the post-association quality control steps. Visual checks of the genotyping clustering graphs show that the clustering is adequate (Fig. 5.4). The genotype counts show a consistent trend of association across all cohorts. The analysis of the Q-Q plot (Fig. 5.5) indicates a slight excess of low *p*-values, with evidence for true associations revealed as prominent departures from the null in the extreme tail of the distribution, and with the genomic inflation factor, λ, of 1.02, indicating an adequate correction for population structure.

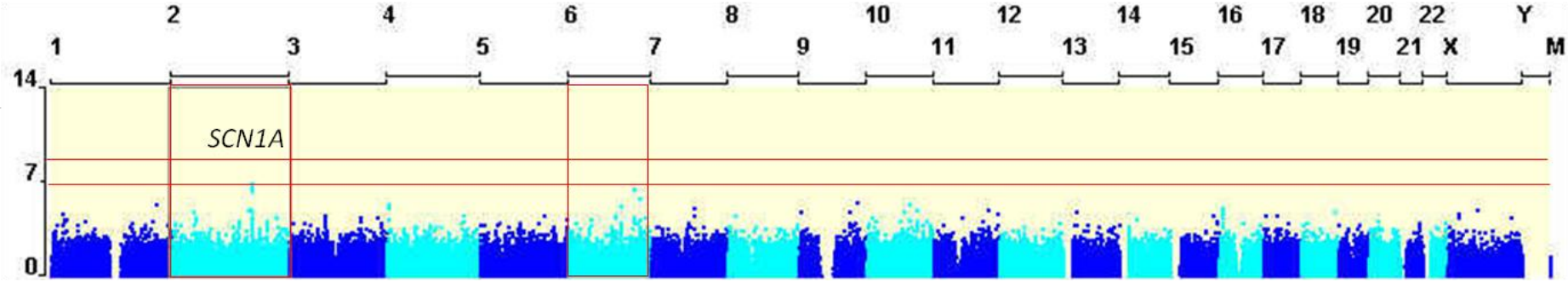

Figure 5.1 Manhattan plot summarizing the results of the genome-wide association study of MTLEHS.

No hit is found with *p*-value below 5 x 10^{-8}, which means that no SNP has reached genome-wide significance.

Three top hits on chromosome 2, located close to the *SCN1A* gene, are "borderline" genome-wide significant for the association with MTLEHS, with *p*-values between 1 x 10^{-7} and 5 x 10^{-7}.

On chromosome 6 there was one other SNP which had a *p*-value close to "borderline" genome-wide significance, but it did not pass post-association quality control and was therefore excluded from further consideration.

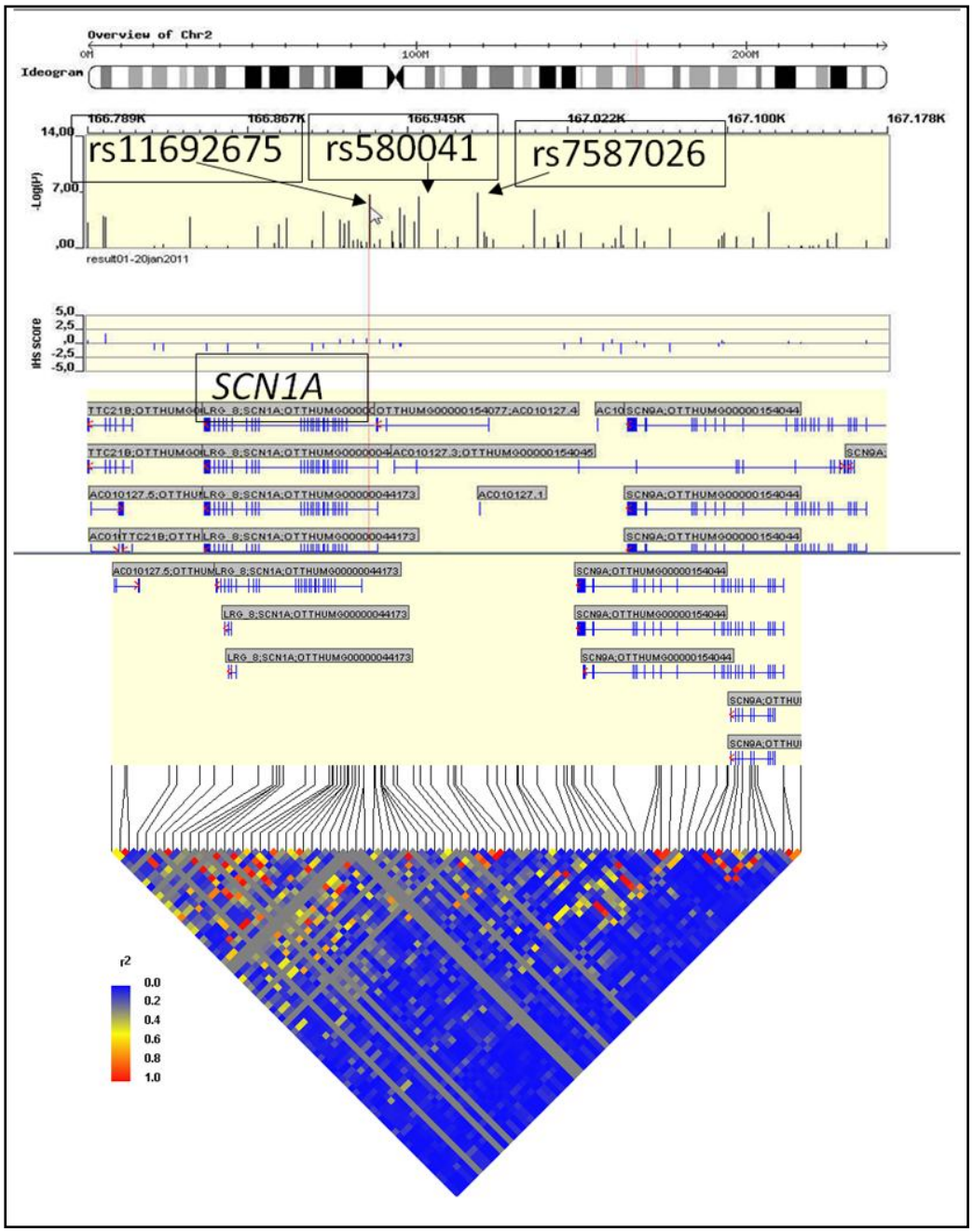

Figure 5.2 The three top SNPs of the GWAS of MTLEHS are located in a region of chromosome 2, close (rs580041, rs7587026) or intronic (rs11692675) to the *SCN1A* gene. (A) Overview of chromosome 2, which includes the *SCN1A* gene. (B) The $-\log_{10}$ (*p*-values) from the GWAS of MTLEHS are graphically shown for each genotyped SNP in chromosome 2 and the three top SNPs are highlighted. (C) Graphical representation of the genes contained in the region (*SCN1A*, highlighted). (D) Pairwise linkage disequilibrium diagram of the region containing the top associated SNPs of the GWAS of MTLEHS. This figure was drawn using WGAViewer software (Ge et al., 2008).

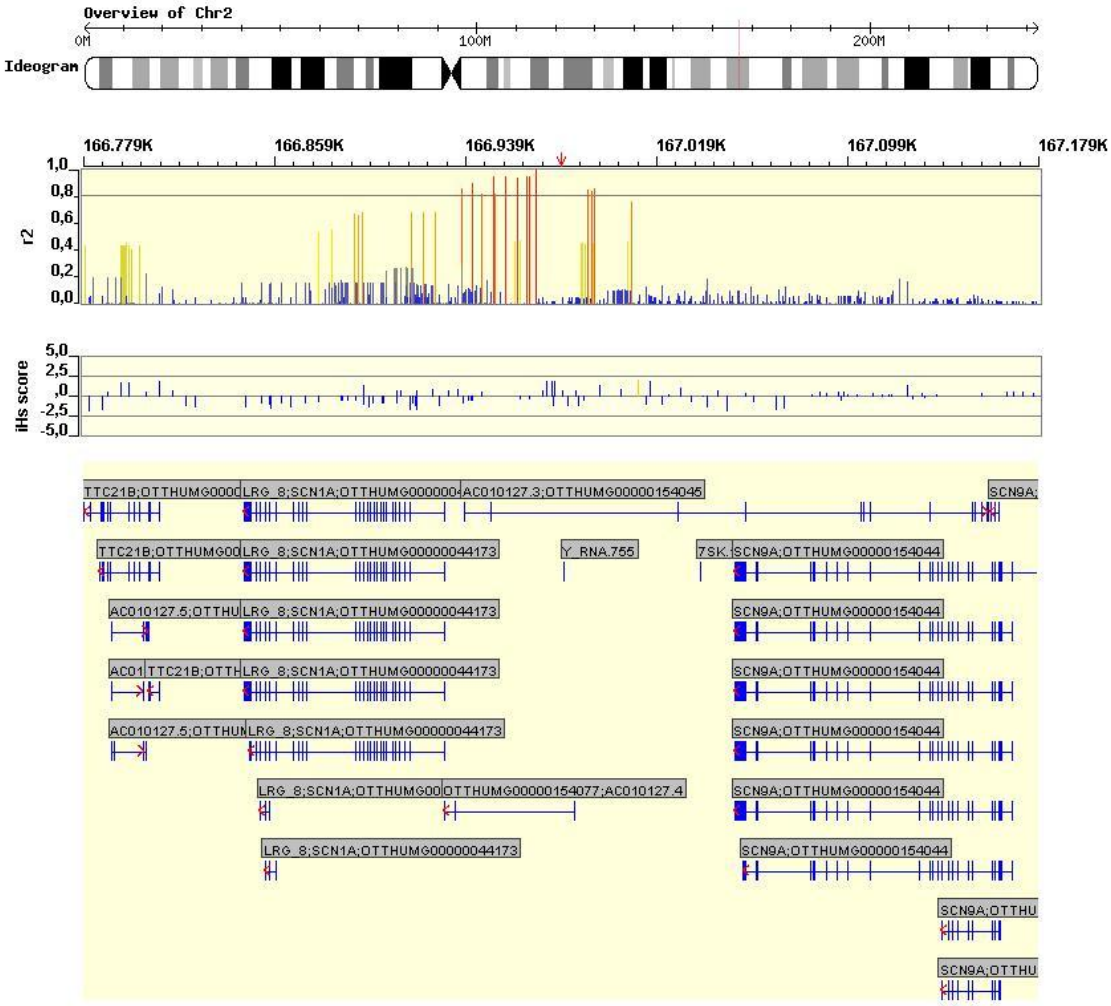

Figure 5.3 Graphical representation of chromosome 2, with the genes included and plot of the regional linkage desequibrium structure (r^2 plotted in the x-axis and physical location in the y-axis), showing one block of high LD, with several SNPs at $r^2 \geq 0.8$ around the region that includes the *SCN1A* gene.
Screenshot from output of WGAViewer software (Ge et al., 2008).

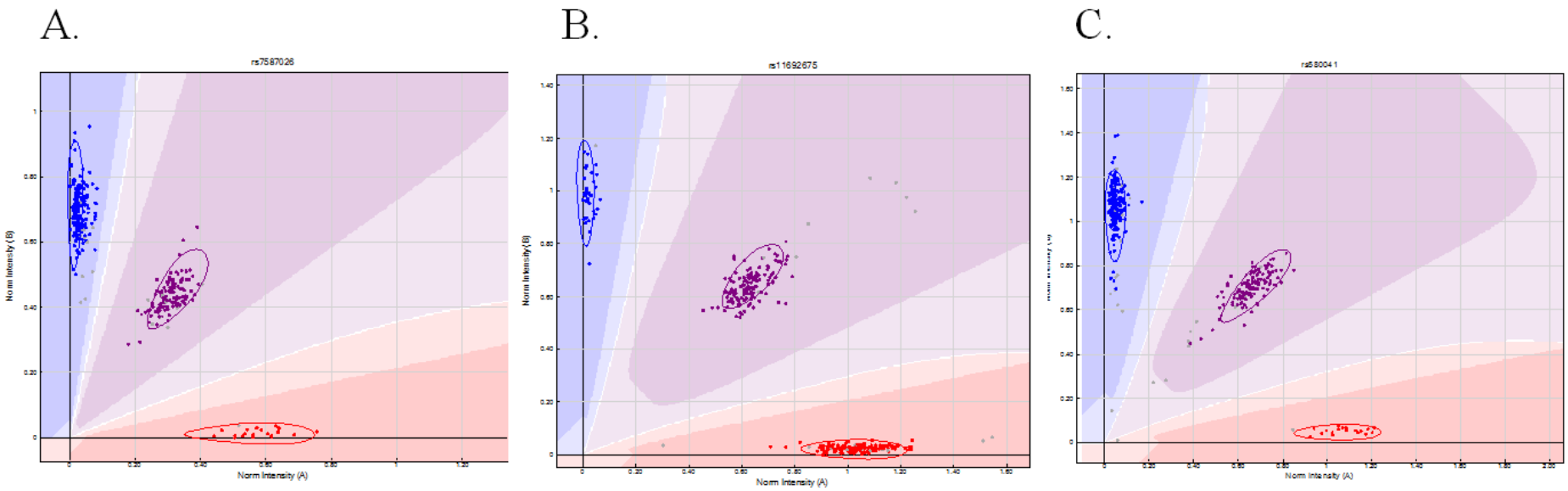

Figure 5.4 Cluster plots showing appropriate genotyping calls for the three top associated SNPs from the GWA study of MTLEHS. (A) rs7587026, (B) rs11692675 and (C) rs580041.

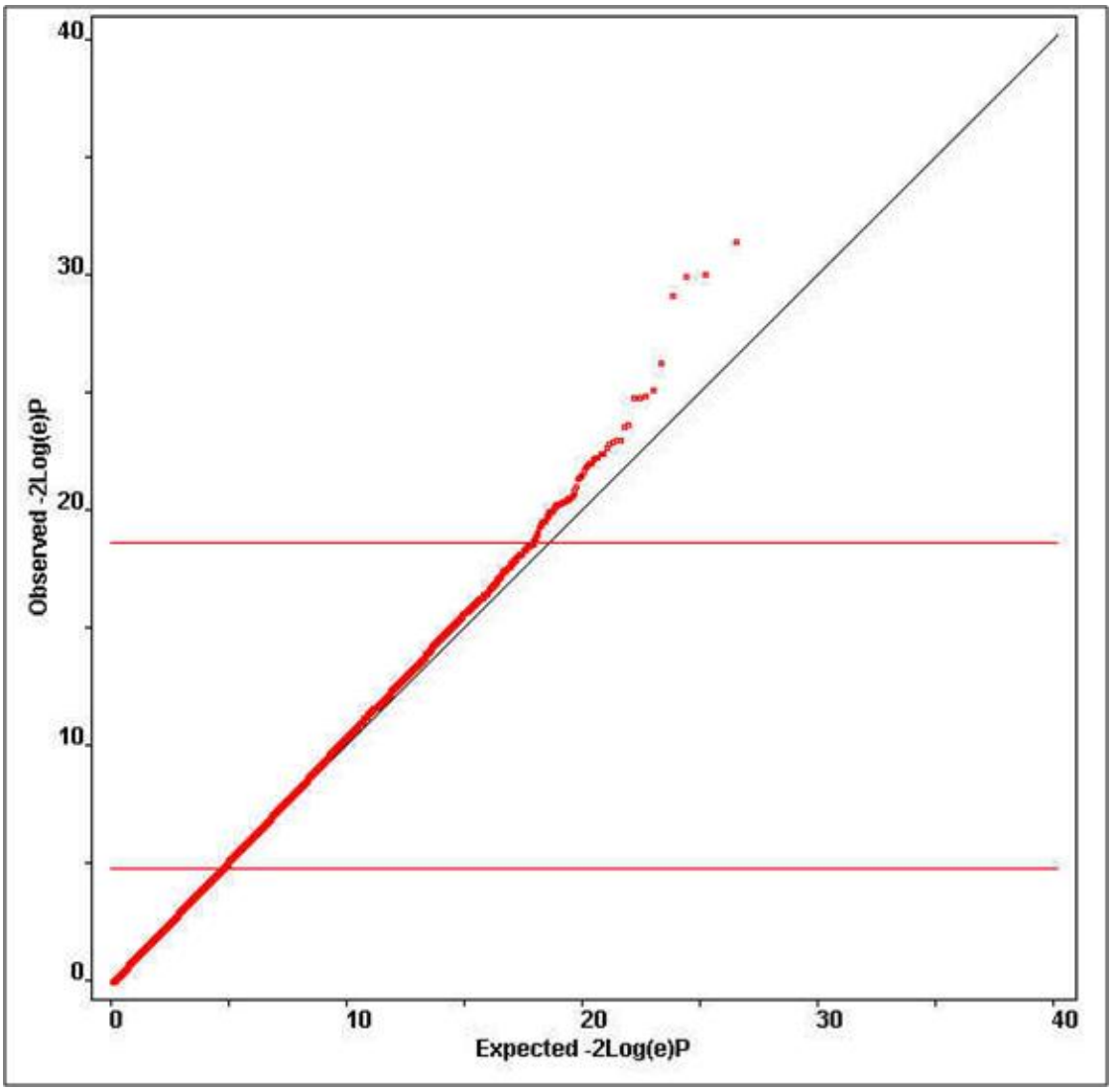

Figure 5.5 Quantile-quantile (Q-Q) plot for the GWA study of MTLEHS (logistic regression, MTLEHS versus population controls), with the observed *p*-values versus expected *p*-values (in log scale) distribution for all polymorphic SNPs typed in the GWA study of MTLEHS and included in the final analysis.

The deviation from the expected distribution under the null hypothesis means there are several SNPs with possible true association with the phenotype.

Inflation of observed statistics due to potential population structure can be estimated; the genomic control inflation factor or lambda (λ) is 1.02, suggesting an adequate correction for population structure.

SNP	Chr	Position	Type	Minor allele	Closest gene	*p*-value	OR (95%CI)	MAF in cases	MAF in controls
rs7587026	2	166978750	within pseudogene	A	***SCN1A***	**1.6×10^{-7}**	**1.31 (1.18-1.45)**	0.32	0.26
rs11692675	2	166926428	intronic	G	***SCN1A***	**3.1×10^{-7}**	**1.29 (1.17-1.42)**	0.38	0.32
rs580041 [a]	2	166950510	within pseudogene	A	***SCN1A***	**4.8×10^{-7}**	**1.30 (1.17-1.43)**	0.31	0.26
rs2786180	6	147512336	within pseudogene	G	-	2.1×10^{-6}	1.28 (1.16-1.42)	-	-
rs1857454	10	85741293	intergenic	A	-	4.1×10^{-6}	1.25 (1.13-1.37)	-	-
rs1203764	4	2437290	within pseudogene	G	-	4.2×10^{-6}	1.24 (1.13-1.36)	-	-

Table 5.2 Top hits of the GWA study of MTLEHS (logistic regression, MTLEHS versus population controls) with *p*-value below 1×10^{-6}.

Three top SNPs have *p*-values below 5×10^{-7} and are located on chromosome 2, intronic or close to the *SCN1A* gene. The corresponding odds ratio is about 1.3 (95% confidence interval, 1.2-1.4). SNP rs11692675, with $p = 3.1 \times 10^{-7}$, is intronic to the *SCN1A* gene. The SNPs rs7587026 and rs580041 are in linkage desequilibrium with rs11692675 and are located close to *SCN1A*, with *p*-values at 1.6×10^{-7} and 4.8×10^{-7}, respectively.

Abbreviations: Chr, chromosome; CI, confidence interval; MAF, minor allele frequency; OR, odds ratio; QC, quality control.

a rs580041 is in perfect LD with rs7587026 in white Europeans, $r^2=1$.

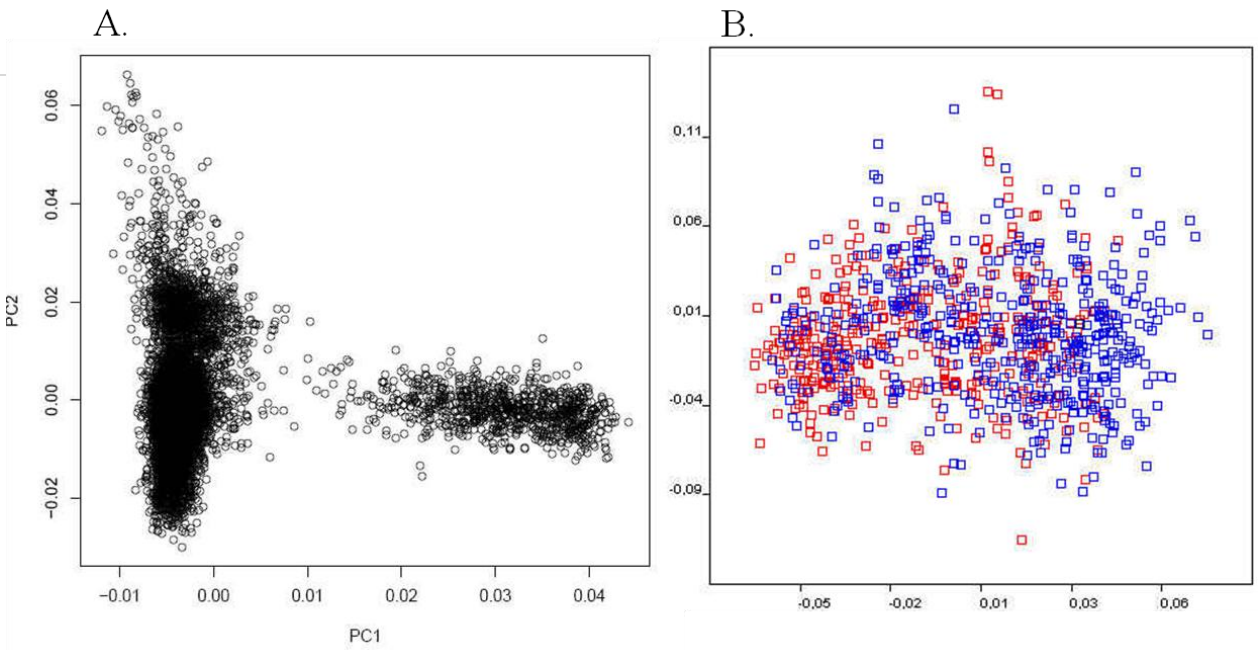

Figure 5.6 Finnish cohort - graphical representations of PC analysis, presenting a statistical summary based on PC1 and PC2. Plot (A) includes all individuals from all cohorts, who were kept in the analysis after correction for population stratification. The Finnish individuals are seen as a cluster on the right of the plot, which can be separated from the other cohorts, seen as a cluster on the left. As this could suggest the correction for population stratification was not ideal, the analysis was repeated after exclusion of the Finnish cohort, but the results were not significantly different from the primary analysis. The plot in (B) depicts the Finnish controls only, showing that the two sub-cohorts of Finnish controls, labelled GSK-Finns (red) and Vantaa-85+ (blue), cannot be separated, as expected, since they contained Finns only.

Each small circle or square depicts one individual.

5.3.3 Analysis of MTLEHS versus controls, excluding the Finnish cohort

After the correction for population stratification using the modified EIGENSTRAT method, the Finnish cohort could still be separated from the other cohorts using principal component analysis (Fig. 5.6).

To check whether this finding had any implication, or whether the correction for population structure had been adequate, the data were re-analysed, this time including in the analysis all cohorts with the exclusion of the Finnish cohort. In this analysis, 968 patients with MTLEHS and 6,519 controls, of European ancestry, were included. The results found were similar to the results of the primary analysis, which included all cohorts. The top three SNPs were the same, with similar *p*-values: rs7587026, 5.7×10^{-7}; rs11692675, 4.4×10^{-7}; and rs580041, 1.3×10^{-6}.

5.3.4 Exploring the role of febrile seizures in the results of the GWAS of MTLEHS

There is an epidemiological association between febrile seizures, MTLE and HS[11].

The *SCN1A* gene has been linked to febrile seizures, in the context of simple FS (Mantegazza et al., 2005), GEFS+ (Escayg et al., 2000b), Dravet syndrome - included within the GEFS+ spectrum (Depienne et al., 2009b) and familial temporal lobe epilepsy with febrile seizures (Colosimo et al., 2007).

[11] See Chapter 1, section 1.6, "Relationship between MTLE, HS and FS", for a literature review on the association between febrile seizures, mesial temporal lobe epilepsy and hippocampal sclerosis.

An association between an *SCN1A*-splicing SNP and febrile seizures has been published (Schlachter et al., 2009), although these results are still inconclusive (Le Gal et al., 2011;Petrovski et al., 2009).

Given the epidemiological association between febrile seizures and MTLEHS and the connections between febrile seizures and the *SCN1A* gene, a secondary analysis was performed, to check whether the association between the *SCN1A*-associated SNPs and MTLEHS is modified by the phenotype febrile seizures.

For phase one of the GWA studies, data on FS were available for review for 516 patients with MTLEHS, 234 MTLEHS with FS and 282 MTLEHS without FS, respectively. There were data on FS for the cohorts from London-UK, Duke-USA, Brussels-Belgium and Dublin-Ireland (Table 5.3).

From the available data on febrile seizures, the percentage of patients with MTLEHS included in the GWAS, who also had antecedents of febrile seizures, varied from 34% in the Duke-USA cohort to 52% in the Brussels-Belgium cohort.

	MTLEHS with FS	MTLEHS no FS
UK- London	106	101
Belgium-Brussels	47	26
USA-Duke	27	65
Ireland-Dublin	54	90
Total	**234**	**282**

Table 5.3 Data on febrile seizures available for analysis, for each cohort included, in the discovery phase of the GWAS of MTLEHS.

The Austrian, Finnish, Norwegian and Swiss cohorts had not provided information on febrile seizures at this stage of the study.

	MTLEHS+FS versus CTRL	MTLEHSnoFS versus CTRL	MTLEHS+FS v MTLEHSnoFS
n of patients / n of controls	205 / 6513	255 / 6513	205 / 255
rs7587026, *p*-value	1.2×10^{-5}	0.16	0.025
rs11692675, *p*-value	2.0×10^{-4}	0.04	0.17
rs580041, *p*-value	2.1×10^{-5}	0.25	0.021
rs922224[a], *p*-value	0.075	0.080	0.94

Table 5.4 Exploring the role of febrile seizures on the association of MTLEHS and the top hits resulting from the GWA study of MTLEHS.

The association of the top three associated SNPs is still significant for the patients with MTLEHS who had febrile seizures in childhood, even if not genome-wide significant; this result is as expected, given that the numbers are smaller.

No association was seen in patients with MTLEHS without antecedents of childhood febrile seizures and the top SNPs of the GWA study of MTLEHS.

Abbreviations: CTRL, controls; FS, febrile seizures; PE, partial epilepsy; +, with.

a rs922224 was used as a proxy for the SNP rs3812718, which has been reported in the literature to be associated with febrile seizures[12] (Le Gal et al., 2011;Schlachter et al., 2009).

[12] See Chapter 1, section 1.5.5: "Genetic studies of febrile seizures".

	(All PE)+FS versus CTRL	**(All PE)+FS versus (All PE)noFS**	**(PEnotMTLEHS)+FS versus CTRL**
n of patients / n of controls	313 / 6521	313 / 1512	107 / 6521
rs7587026, *p*-value	4.3×10^{-5}	0.0025	0.37
rs11692675, *p*-value	0.001	0.014	0.63
rs580041, *p*-value	9.9×10^{-5}	0.0047	0.41
rs922224[a], *p*-value	0.23	0.90	0.61

Table 5.5 Analysing the role of febrile seizures on the association of partial epilepsy and the top hits resulting from the GWA study of MTLEHS.

Abbreviations: CTRL, controls; FS, febrile seizures; PE, partial epilepsy; +, with.

a rs922224 was used as a proxy for the SNP rs3812718, which has been reported in the literature to be associated with febrile seizures (Le Gal et al., 2011;Schlachter et al., 2009).

5.3.4.1 Analysis of MTLEHS with FS versus controls and MTLEHS without FS versus controls

The top SNPs from the GWA study of MTLEHS show suggestive association in the association analysis of MTLEHS with FS versus population controls, but the association does not reach genome-wide significance. This is, however, not unexpected, because the power of this subgroup analysis is lower, given the smaller sample size (Table 5.4). Definite conclusions will necessitate larger sample sizes, replication and meta-analysis.

In the association analysis of MTLEHS without personal antecedents of FS versus population controls, the top SNPs of the GWA study of MTLEHS do not show evidence of association with the phenotype (Table 5.4), despite similar sample size.

5.3.4.2 Looking at an *SCN1A* splice site variant previously associated with FS

The SNP rs3812718 is a *SCN1A* splice site variant that has been suggested in the literature to be associated with FS (Schlachter et al., 2009). As rs3812718 was not included in the chips used in these GWA studies, a proxy was used: the SNP rs922224 was included in the study chips and is in "perfect" linkage disequilibrium with rs3812718, with r^2 of 1.

A conditional analysis of rs922224 was performed on the top SNPs from the GWAS of MTLEHS. The conditional analysis of rs922224 on top SNP rs7587026 yielded a *p*-value of 0.3, with OR 0.95 (95% CI 0.9-1.1). This result suggests that the effect of the top SNP rs7587026 is not independent of the effect of the SNP rs922224, which is intronic to the *SCN1A* gene.

5.3.4.3 Exploring the association between the top SNPs of the GWAS of MTLEHS and FS, in the cohort of partial epilepsies

The *a priori* knowledge suggested that the results of the GWA study of MTLEHS, with association to *SCN1A*, could be related to the febrile seizures phenotype and further exploration was therefore necessary. This was also done for the cohort of patients included in the GWAS of partial epilepsy (Kasperaviciute et al., 2010).

The results suggest that the association found between three SNPs intronic or close to the *SCN1A* gene on chromosome 2q and MTLEHS may be specific for patients with MTLEHS with personal antecedents of childhood FS and is not present for patients with MTLEHS and no antecedents of childhood FS, nor for patients with other partial epilepsies (Table 5.5). Interestingly, conditional analysis showed that the effect of the top SNP was not independent from the effect of rs922224, a SNP intronic to *SCN1A* and previously found to be associated to FS. The sample size may, however, still not be large enough to ensure definitive conclusions, as no strict genome-wide significant association was found, and future studies with larger samples and replication are needed to confirm these results.

5.4 Discussion

To the best of my knowledge, this has been the first genome-wide association study of mesial temporal lobe epilepsy with hippocampal sclerosis and also the first to explore a possible association of the childhood febrile seizures phenotype with MTLEHS and the partial epilepsies in the context of a large genomic study.

The main findings of the GWA study of MTLEHS are summarised in Tables 5.1 and 5.2. We found evidence for association between MTLEHS with personal history of childhood FS and common genetic variation - three SNPs intronic or close to the *SCN1A* gene, on chromosome 2q.

The associations have not reached genome-wide significance as currently defined, (genome-wide significance threshold is set at $p \leq 5 \times 10^{-8}$), but were "borderline" genome-wide significant (Panagiotou and Ioannidis 2012), with *p*-values between 5×10^{-7} and 1×10^{-7}. Nevertheless, the biological plausibility establishes a high *a priori* likelihood for this association (Sisodiya et al., 2005), which, while not establishing a definitive nexus of causality, is strong evidence to warrant follow-up studies.

Previous GWA studies of several complex disorders have "rediscovered" genes that had been shown by decades of work to be important in the pathophysiology of those diseases (Hirschhorn 2009). This now also seems to be the case with epilepsy. An association has been shown between *SCN1A* and epilepsy syndromes[13] (Abou-Khalil et al., 2001;Claes et al., 2001;Freilich et al., 2011;Harkin et al., 2007;Okumura et al.,

[13] See 1.7 for a discussion on the association between *SCN1A* and epilepsy syndromes and between *SCN1A* and febrile seizures.

2007;Zucca et al., 2008) and between *SCN1A* and familial febrile seizures (Escayg et al., 2000b;Mantegazza et al., 2005).

Follow-up studies, most importantly replication and meta-analysis, will be central to confirm the relevance of the findings of the GWA studies presented in this thesis.

Given the epidemiological data suggesting a link between MTLEHS and febrile seizures, we have explored whether the result of the GWA study of MTLEHS could be driven by an association with FS. The sample size was, however, not large enough to definitely disentangle the role of FS in the association between MTLEHS and the *SCN1A*-associated SNPs. Available data on FS for the MTLEHS cases were limited and only available for a few cohorts. More febrile seizure data were missing for the people with partial epilepsy "not-MTLEHS" included in the GWA study of partial epilepsy. Although no definite conclusions can be drawn after this discovery phase GWAS, there is a trend pointing to a significant influence of the FS phenotype in the association found between the *SCN1A*-associated top SNPs and MTLEHS. This warrants further exploration in a larger cohort.

5.4.1 Limitations

Limitations of this study include the small sample size of patients with information on febrile seizures. For more than 90% of the cases where robust information was available regarding the presence or absence of febrile seizures, we knew only whether a patient had febrile seizures in childhood or not, but for the majority of patients included in the study, the information required for classifying the FS was not available, such as age at first FS, age at last FS, total number, duration, presence or not of lateralizing features. Information

on other characteristics, such as temperature causing FS, need or not of hospitalization, treatment required, or examinations performed to exclude intracranial infection, also proved hard to retrieve in our cohorts of adult patients.

Another limitation regards the available information on hippocampal sclerosis, which was insufficient to help disentangle the role of hippocampal sclerosis in the association between MTLEHS with FS and the top associated SNPs in the *SCN1A* gene region. The London cohort had a few patients with reliable information on patients with MTLE without HS. These data were, however, not available for the majority of cohorts and any analysis would therefore, be underpowered with the currently available data.

Consistency of phenotypes between all tertiary referral centres involved in this study was ensured by study design and choice of phenotypes, with homogeneous classification scheme used and phenotyping criteria across cohorts, but the consistency of phenotyping has not been rechecked across all centres, which is a limitation of this study. This can be done using, for example, cases for all participating clinicians to classify.

5.4.2 Next steps

5.4.2.1 Imputation

Imputation is the next logical step, to try to fine-tune the localisation of the signal of association, which has been found to be located in a region of chromosome 2q in close proximity and intronic to the *SCN1A* gene. As there are other genes for sodium channels in the vicinity, it is important to confirm a precise localization of the association signal.

5.4.2.2 Replication in an independent sample and Meta-analysis

Future steps should include preparing a replication cohort for the GWAS of MTLEHS. This is already under way and a number of samples are already available for replication (120 cases from the London cohort). Cases are available from Dublin,Ireland and Utrecht, The Netherlands and collaboration with more groups is being discussed.

5.4.2.3 Studies to clarify the role of febrile seizures

To definitely answer the question of which role febrile seizures play in the association between MTLEHS and the top associated SNPs intronic or close to *SCN1A*, more data should be collected on febrile seizures. Larger numbers of cases with febrile seizures will translate into more power to evaluate whether the association can be confirmed for only those with MTLEHS with history of FS or all people with MTLEHS.

To investigate further the role of febrile seizures in the association found between MTLEHS and the *SCN1A* gene, paediatric cohorts of patients with febrile seizures could be genotyped. In these cohorts, the phenotypical information on febrile seizures is expected to be more accurate. A prospective study will be more helpful, as it will allow to check who will go on to develop chronic epilepsy.

5.4.2.4 Studies to clarify the role of hippocampal sclerosis

More research is needed looking into the role of HS in the associations found in this study. One possible study would be to look for association in an adequately-sized group of patients with MTLE in the absence of HS (“cryptogenic” MTLE, or MTLE due to other causes).

5.4.2.5 Studies using a more stringent phenotypical definition of MTLEHS

In the GWA study of MTLEHS, patients with a compatible electro-clinical syndrome of MTLEHS were included, even without histopathological confirmation of HS. Patients were included, therefore, who did not have epilepsy surgery, because of responsiveness to AED, patient refusal, co-morbidities, lack of social support, or other reasons.

Phenotyping was stringent, with exclusion of patients with auditory auras or any other clinical, neuropsychological, imaging or electrophysiological data that could point to an epileptogenic zone outside the mesial temporal lobe. Patients were excluded if there was bilateral HS or dual pathology, widespread damage on neuropsychological evaluation or other evidence of a larger epileptogenic zone than the ipsilateral mesial temporal lobe.

Using in the GWA studies a more "homogeneous" phenotypical definition of MTLEHS could lead to a higher specificity of diagnosis and be beneficial in terms of the power of the GWA studies to detect a true association. Possible ways to decrease heterogeneity within MTLEHS cohorts could include the use of histopathology data, including in the final analysis only patients with MTLE and histopathological confirmation of HS, or only patients with "classical HS" on the study of the surgical specimen. Another way could be to include only patients for whom quantitative MRI data are available confirming the hippocampal atrophy. Disadvantages of both these methods would be the decrease in sample size, with the need to establish a wider network of collaborating centres.

5.4.3 Conclusions

The results of the GWA study of MTLEHS suggest an association between common sporadic MTLEHS and common genetic variation in three SNPs on chromosome 2q, intronic or close to the *SCN1A* gene, with a more robust result in the group of patients with MTLEHS with antecedents of childhood febrile seizures, and no evidence for association for MTLEHS without antecedents of childhood FS.

These results provide support for *SCN1A* contribution to increased susceptibility to MTLEHS with FS. The *a priori* biological plausibility of the association between *SCN1A* and epilepsy has been established by many studies, as reviewed in Chapter 1.

Also in other common sporadic neurologic diseases, such as Parkinson's disease, the results of the GWA studies have included association to genes previously known to be linked to "familial" disease, such as alpha-synuclein (*SNCA*) and tau (*MAPT*) (Simon-Sanchez et al., 2009). This also happened in the GWA study of MTLEHS and can be viewed as adding to the validation of the GWA study design as appropriate to studying of common neurologic diseases.

The experience of the GWA studies of partial epilepsy and of MTLEHS, support the feasibility of the GWA study design in epilepsy research, for the identification of novel candidate genes contributing to the risk of common epilepsies and for validation of previously known associated genes. In the near future, use of larger cohorts with GWA methodology can be expected to help disentangling the genetic architecture of the common epilepsy syndromes.

6 Chapter Microdeletions and mesial temporal lobe epilepsy

6.1 Introduction

Mesial temporal lobe epilepsy with hippocampal sclerosis (MTLEHS) is the most common form of partial epilepsy in adults (Engel, Jr. et al., 2008).

It has objective diagnostic criteria (Wieser 2004), but there is heterogeneity recognized between patients. It is controversial whether MTLEHS is a single disorder, an epilepsy syndrome, or rather a group of disorders or different syndromes resulting from a "final common pathway" by which patients with different disorders develop MTLE (Berg 2008;Berg et al., 2010;Wieser 2004). In the past, some have considered MTLEHS as an epilepsy syndrome and it was so classified in the 1989 ILAE classification of the epilepsies and epilepsy syndromes (ILAE Commission on Classification and Terminology 1989). The 2004 ILAE expert workshop on MTLEHS discussed the questions: "How well defined is the syndrome of MTLEHS?" and "How homogeneous is it?" and has not considered it an epilepsy syndrome nor a disease (Wieser 2004). The 2010 ILAE classification revision has also proposed MTLEHS should not be considered an epilepsy syndrome (Berg et al., 2010).

Genetic factors are thought to play a role in susceptibility to MTLE, "not as an unitary process" (Wieser 2004), with the observed phenotypic heterogeneity possibly reflecting in part genetic heterogeneity in a "complex" multifactorial disorder.

6.2 Heterogeneity of MTLEHS

Heterogeneity of MTLEHS is manifest in several domains, including clinical, electroencephalographic, imaging and neuropathological.

MTLE is often associated with antecedents of an "initial precipitating injury" (IPI), often before the age of 6 years, which may include febrile seizures (FS), trauma, hypoxia or an intracranial infection. Onset of unprovoked seizures is usually during adolescence or adulthood, after a "latency period". An early good response to AEDs may be followed by refractoriness after a variable period of time (Engel, Jr. et al., 2008).

Clinical presentation varies between patients, with presence or absence of IPI(s), "latency period" and variable age at onset of habitual seizures. Retrospective studies and surgical series have shown a high prevalence of IPIs in patients with MTLE (Mathern et al., 1995). Limitations of the studies on IPIs include the fact that exhaustive histories are not always available and incidental IPIs could be listed (Wieser 2004). IPIs could be proximate triggers of MTLE, or of HS, in some cases, but are not always present.

6.2.1 Drug response

MTLEHS is frequently refractory to medication and the majority of cases in literature are recruited from tertiary epilepsy centres. Response to AEDs, however, varies between medically refractory MTLE and "benign" MTLE, where patients respond well to medication (Labate et al., 2011).

"Benign" MTLE has been recognised as possibly underdiagnosed and most often recognized outside tertiary referral centres (Labate et al., 2011). Sporadic "benign" MTLE is often similar, both electroclinically and in neuroimaging, to familial MTLE, with clinical and genetic evidence supporting a "complex" inheritance model for familial MTLE (Labate et al., 2011).

6.2.2 Imaging findings

Brain imaging findings may vary from unilateral HS, hippocampal atrophy without apparent signal change, bilateral signal changes and presence or not of dual pathology. HS is the most common lesion found in patients with MTLE, often diagnosed by MRI, an essential part of the preoperative evaluation (Duncan 2011;Jackson et al., 1990;Kuzniecky et al., 1987;Walczak et al., 1990).

The role of HS in the development of MTLEHS and in the development of medically refractory epilepsy, is not fully understood (Labate et al., 2006). HS may be the epileptogenic lesion in some cases, but for most patients with MTLEHS, the aetiology is not known. Although HS and TLE may both appear after a brain injury, they need not necessarily be causally related (Thadani et al., 1995). Prolonged FS during childhood have been associated with damage to temporomesial structures (Cendes et al., 1993), but the role of FS in the development of MTLE remains controversial (Cendes 2004;Shinnar 2003;Tarkka et al., 2003).[14]

[14] A review of the discussion on the relationships between temporal lobe epilepsy, febrile seizures and hippocampal sclerosis is included in Chapter 1, section 1.6.

HS on MRI does not necessarily imply medical intractability, as asymptomatic family members of people with familial MTLE may have MRI evidence of HS, suggesting that in certain families the hippocampal abnormalities themselves might be inherited and do not necessarily lead to epilepsy (Aguglia et al., 1998;Labate et al., 2006;Labate et al., 2011).Whether the presence or absence of visually recognizable HS in itself signifies a different type of epilepsy is not established. Labate et al. (2011) argue that these distinct phenotypes "lie along a biological continuum" and are "not necessarily (…) a distinct type of epilepsy".

It is known that a proportion of patients with MTLE present dual pathology on MRI. Interpretation of studies on dual pathology is not always straightforward, as its definition is sometimes vague and it is not always clear whether both abnormalities are epileptogenic (Fauser & Schulze-Bonhage 2006;Mathern et al., 2008). Possible pathologies encountered together with MTLE and HS include cortical dysplasia (Eriksson et al., 2004), heterotopia (Lopez et al., 2010), cavernomas and low grade tumours.

6.2.3 Neuropathology findings

HS is the most frequent neuropathological finding in TLE (Falconer et al., 1964). Neuropathologically, it is characterized by segmental neuronal loss, gliosis (Blumcke 2008) and reorganization (Thom et al., 2009b), in the cornu ammonis 1 (CA1), CA3 and CA4 regions of the hippocampus.

Heterogeneity is apparent in MTLE and also in HS (Thom et al., 2010b). The different neuropathology patterns of HS led to the proposal of a clinico-pathological

classification of HS into: classical HS (neuronal loss in CA1 and CA4/hilus and relative neuronal preservation in CA2, dentate gyrus and subiculum); endfolium sclerosis (neuronal loss in the hilar region); and CA1-predominant HS (neuronal loss only in CA1) (Blumcke et al., 2007).

A proportion of patients with TLE have HS and other histopathological findings, such as increase in heterotopic neurons in the subcortical white matter (Thom et al., 2001) and variable associated histopathology in the limbic system or other parts of the brain.

6.2.4 Surgical outcome of MTLEHS

Resective surgery has a long history in TLE. More than 50 years ago, it was reported that, in the absence of mass lesions, good seizure control could be obtained after temporal lobectomy, when the underlying pathology was HS (Falconer et al., 1964;Falconer & Cavanagh 1959;Falconer & Taylor 1968;Jensen & Klinken 1976;Penfield & Flanigin 1950;Rasmussen 1983;Thadani et al., 1995).

Since then, resective surgery became the preferred treatment for intractable TLE (Dasheiff 1989;Glaser 1980), when the findings from presurgical evaluation are concordant (Duncan 2011). A randomized clinical trial provided evidence that surgery is more effective in stopping disabling seizures than AEDs alone: only 8% of patients were seizure free on medication alone, compared to 58% after temporal lobe resection (Wiebe et al., 2001). Surgery has also been suggested to improve longevity (Choi et al., 2008) and quality of life (Zupanc et al., 2010).

Some patients are considered not to be good candidates for surgery after the presurgical evaluation (Duncan 2011). Of the patients with MTLEHS who undergo surgery, one third fail to become seizure-free (Wiebe et al., 2001), with this proportion increasing to 40% not seizure-free at longer-term follow-up (de Tisi et al., 2011).

Predictors of good surgical outcome in MTLE are proposed at group level and include clinical, imaging, electrophysiological and histopathological findings (Engel, Jr. et al., 2003;Janszky et al., 2005;Radhakrishnan et al., 1998;Spencer and Huh 2008;Spencer et al., 2005;Tonini et al., 2004;Wyler et al., 1995).

Absence of secondarily generalised seizures (Hennessy et al., 2001;Jeong et al., 2005;McIntosh et al., 2001) and unilateral temporal interictal epileptiform discharges (Hennessy et al., 2001), have been shown to be associated with good surgical outcome in MTLEHS and these results have been replicated.

Positive predictors also include presence of unilateral HS on MRI; concordance of findings in the presurgical evaluation between clinical, imaging and neurophysiological findings; and histopathologically-confirmed HS.

The extent of surgical resection was suggested to correlate positively with seizure outcome (McIntosh et al., 2001). Incomplete resection may be a contributing factor for seizure persistence after temporal lobectomy, with post mortem studies confirming in some cases the extension of HS to the caudal portion of the hippocampus outside the typical surgical resection (Thom et al., 2010a;Thom et al., 2010b).

The finding of non-“classic” neuropathological patterns of HS (Blumcke et al., 2007), which correspond to 4-10% of cases, has also been associated with poorer outcome (de Lanerolle et al., 2003;Thom et al., 2010b;Van Paesschen et al., 1997).

The literature regarding outcome and prognostic factors after anterior temporal lobectomy can be at times contradictory. An extensive review showed this is partly due to small sample sizes, no information on the proportion of patients lost to follow-up and diverse study design and methodology (McIntosh et al., 2001). Many factors identified as predictors of favourable surgical outcome in TLE lose their predictive value when the subgroup of MTLEHS is examined (Hardy et al., 2003), suggesting these factors may be, in fact, predictors of MTLEHS. For some putative prognostic factors, such as younger age at surgery (Jeong et al., 2005;McIntosh et al., 2001), or time between seizure onset and epilepsy surgery (Janszky et al., 2005), failed attempts of replication followed a positive study of association. Further, factors associated with short-term postsurgical outcome are not always predictors of long-term outcome and are not predictive factors anymore when seizure freedom is defined as absolute freedom from all seizures (Aull-Watschinger et al., 2008).

6.3 Microdeletions in epilepsy

Genomic microdeletions have been identified recently in common sporadic epilepsies. Large, recurrent microdeletions in chromosome 16p13.11, 15q11.2 and 15q13.3 have since been established as risk factors for epilepsy (Mulley & Mefford 2011;Scheffer & Berkovic 2010). Initially found to underlie about 3% of the genetic/ idiopathic generalised epilepsies (de Kovel et al., 2010;Helbig et al., 2009), these microdeletions have also been found in a range of focal epilepsies (Heinzen et al., 2010;Mefford et al., 2010).

A broad phenotypic spectrum has been associated with large recurrent microdeletions at 16p13.11 and 15q11.2 (Mulley and Mefford 2011) and this can be extended to patients with MTLE and histopathologically-proven HS (Catarino et al., 2011a;Liu et al., 2012).

These microdeletions have also been found in people without epilepsy or family history of epilepsy, manifesting incomplete penetrance and variable expressivity (Sisodiya & Mefford 2011). Data supporting their role in the pathogenicity of the epilepsies include alterations in gene expression found with 16p13.11 microdeletions; involvement of several known epilepsy genes, such as *KCNA1*, *GABRA1*, *GABRG2*; among other evidence (Heinzen et al., 2010).

Additional recurrent and non-recurrent CNVs have been found in the epilepsies and more are expected to be found with the widening use of genomic approaches in large cohorts of people with epilepsy, promising to lead to discovery of novel genes and

genomic regions associated with increased susceptibility to seizures (Mulley and Mefford 2011). 7.9% of 315 children with epileptic encephalopathies were found to carry rare CNVs, including recurrent deletions at 7q21 and 16p11.2, with half of the variants being considered likely pathogenic (Mefford et al., 2011b).

Finding a rare, novel, recurrent or non-recurrent CNV is naturally followed by evaluation of its likely pathogenicity, including searches for any previous association of a gene involved in the deletion with known diseases or traits, in the literature and in patient and genetic databases; study of gene expression patterns; and functional studies of the genes involved (Mefford et al., 2010).

The role of CNVs in susceptibility to epilepsy is likely integrated in a polygenic profile, also influenced by other genetic, epigenetic and environmental factors, contributing to the susceptibility to seizures in any given person (Mulley and Mefford 2011).

6.3.1 Hypothesis and aims

The detection of a microdeletion including genes in some cases expressed ubiquitously in the brain could theoretically raise concern about its potential influence on outcome measures following epilepsy surgery, with regard to seizure control or other domains. It could be hypothesized that the presence of a microdeletion could represent widespread brain involvement, similar to cognitive impairment or secondary generalised tonic-clonic seizures, both of which reduce the chances of good outcome across various domains after resective epilepsy surgery (Malmgren et al., 2008;Spencer and Huh 2008). On the other

hand, these genetic variants might not affect outcome, because of spatial variability in gene expression (Hardy et al., 2009), among other factors.

The aim of this study was to document the clinical characteristics and surgical outcome of a series of patients with MTLE, where large microdeletions were found.

6.4 Methods

6.4.1 Ethics approval

This work was approved by the relevant local research ethics committees. All phenotypic data and samples were collected in accordance with the ethical standards set forth by the Joint Research Ethics Committe of the National Hospital for Neurology and Neurosurgery, London, UK; Duke University Institutional Review Board, Durham, NC, USA; Ethics Committee of the Erasme Hospital and Ethics Committee Gasthuisberg, Brussels, Belgium; Kantonale Ethik-Kommission, Zurich, Switzerland; Beaumont Hospital Ethics Committee, Dublin, Ireland; and the Advisory Board of Health Care Ethics, Sub-Committee on Medical Research Ethics, Helsinki, Finland (Heinzen et al., 2010). All patients provided written informed consent.

6.4.2 Subjects and inclusion criteria

A multicentre collaboration was established for recruitment, phenotyping and genotyping of people with epilepsy. A genome-wide screen was performed to identify CNVs in a large cohort of 3,812 patients with epilepsy. More than 90% of the patients included had focal epilepsies. Recurrent microdeletions at 16p13.11, 15q11.2 and other large recurrent and non-recurrent microdeletions, were present in 0.8% of the patients with epilepsy included in the study, both in generalised epilepsies and focal epilepsies, while the 15q13.3 microdeletion was only found in patients with generalised epilepsies and not in patients with partial epilepsy (Heinzen et al., 2010).

Patients with MTLE, who had resective surgery for their medically refractory seizures and who were found to have genomic microdeletions larger than 1Mb, or large 16p13.11 microdeletions (0.8 Mb), were included in this study.

6.4.3 Phenotyping and data analysis

All available clinical data were evaluated, including all pre-surgical investigations: MRI brain scan, video-EEG telemetry, neuropsychometry, neuropsychiatric assessment. The type of epilepsy resective surgery was noted. The histopathology of the surgical specimen was reviewed by an expert neuropathologist (Dr Maria Thom, UCL Division of Neuropathology).

Post-surgical outcome data were evaluated in terms of seizure control, at one year and at last follow-up, using the ILAE outcome classification (Wieser et al., 2001). Postsurgical changes in AED therapy and neuropsychological, psychiatric and employment outcomes after surgery, were reviewed.

6.5 Results

Of 3,812 patients with epilepsy, who had a genome-wide screening for CNVs (Heinzen et al., 2010), ten patients with medically refractory MTLE and resective epilepsy surgery, were found to have large microdeletions at 16p13.11, 15q11.2, or other microdeletions larger than 1Mb and were included in this study.

The flowchart in Fig. 6.1 details the inclusion and exclusion criteria. Patients were excluded if the epilepsy syndromic classification was not TLE, or if the TLE could not be classified as mesial TLE. Patients who were seizure-free on AEDs or were otherwise not candidates for epilepsy surgery, were also excluded.

The demographic and clinical data of the patients included are summarised in Table 6.1. Details on type of surgery and post-operative outcome across several domains, including seizure control, are provided in Table 6.2.

Post-surgical follow-up time varied between 10 to 156 months (median 48 months). Seven patients had anterior temporal lobectomy, two selective amygdalo-hippocampectomy and one neocorticectomy with amygdalectomy. Eight patients (8/10) were rendered seizure free by surgery.

The two patients, who were not seizure-free after surgery: **a)** one, had neocorticectomy and amygdalectomy, with non-specific histopathology findings and was, at last follow-up, in ILAE outcome class 3, after seven years of postsurgical seizure

freedom; and **b)** the other, had a temporal lobectomy, with a hamartoma on neuropathology and was in ILAE outcome class 5 since the surgery.

The full range of microdeletions is listed in Table 6.3, with the list of genes included in Table 6.4. The results of the histopathology review of the surgical specimen are provided in Table 6.5.

Of the 23/3,812 patients, who had 16p13.11 deletions larger than 100 Kb (Fig. 6.2), three had had surgery for medically refractory MTLE and shared an identical 800 Kb deletion, comprising seven genes. One of these had MTLEHS with histopathologically-proven classical HS; another, a hamartoma and no evidence of HS (Fig. 6.3) and the third, normal MRI brain imaging and non-specific findings on histopathology (Table 6.5).

Eight patients with large microdeletions had MTLEHS with histopathologically-proven HS, all classifiable as classical HS. From these, four had antecedents of febrile seizures in childhood.

A family history of epilepsy or febrile seizures was documented in one (1/3) of MTLE patients and 16p13.11 microdeletion and one (1/2) with 15q11.2 microdeletion. A personal history of febrile seizures was present in one (1/3) patient with 16p13.11 microdeletion and one (1/3) with 15q11.2 microdeletion.

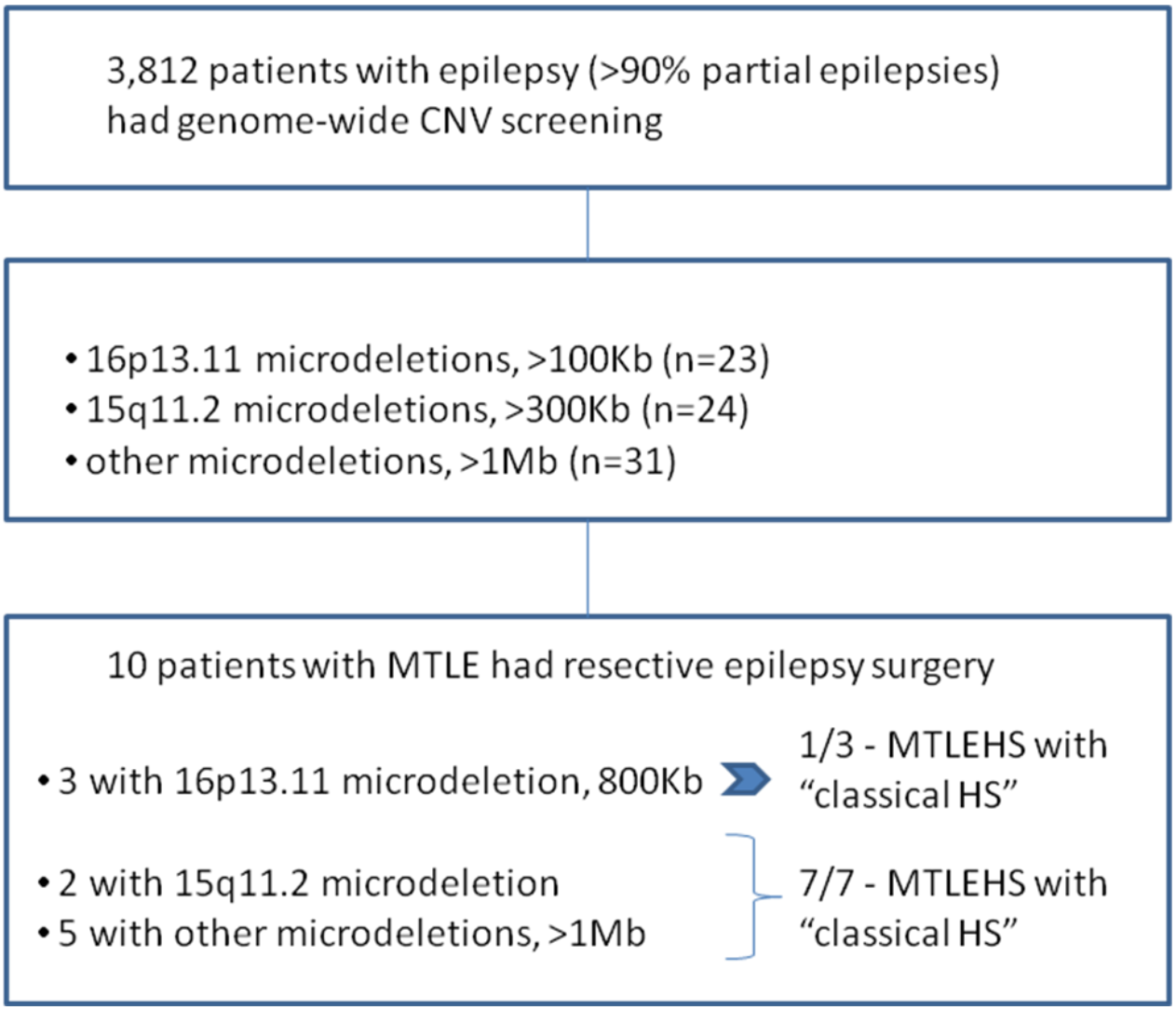

Figure 6.1 Flowchart with inclusion and exclusion criteria, from the initial cohort of 3,812 patients with epilepsy screened for CNVs, to the ten patients with MTLE, resective epilepsy surgery and large microdeletions, who were included in this study.

ID	Sex / age	Descent	Age at onset of habitual szs (y)	Febrile szs	Sz types	Sz-related family history	Video-EEG interictal findings, IED (and slow activity)	Video-EEG ictal findings (at sz onset)	MRI brain
1	F /40	Brazilian	4	YES	CP, SGTC	NO	L ant-mid temporal	L temporal	L HS
2	M/42	White British	28	NO	SP, CP, SGTC	YES – 2 maternal sibs	R temporal	R temporal	R HA
3	M/NA	Irish	28	Not known	CP, SGTC	NA	R temporal (and R temporal slow)	R temporal	Normal
4	F/25	Pakistani	1	NO	SP, SGTC	NO	L temporal (and L temporal slow)	L temporal	L HS
5	F/NA	Swiss	first decade	YES	SP, CP, SGTC	YES	L temporal (and bilateral slow)	L temporal	L HS
6	M/42	White British	7	NO	CP	NO	L ant-mid temporal	No seizures recorded	L HS
7	M/40	White British	2.5	YES	SP, CP, SGTC	NO	L ant-mid temporal	Not lateralised	L HS
8	M/41	White British	34	YES	CP, rare SGTC	YES – brother	L temporal IED (and L temporal slow)	L temporal	L HS
9[a]	M/30	White British	11	NO	SP, CP	NO	R temporal	R temporal	R HS
10	F/53	Swiss	6	NO	SP, CP	NO	NA	R temporal [b]	R HA

Table 6.1 Demographic and clinical data, including results of the preoperative investigations, for the patients with MTLE included in this study. Abbreviations: ant-mid = antero-mid; CP = complex partial; F = female; HA = hippocampal atrophy; HS = hippocampal sclerosis; IED = interictal epileptiform discharges; L = left; M = male; NA = not applicable or not available; R = right; SGTC = secondary generalised tonic-clonic; SP = simple partial; sz = seizure; y = years.

a) Case 9 has been reported in (Kasperaviciute et al., 2011); b) intracranial recording with depth electrodes.

ID	Duration epilepsy to surgery (y)	Surgery type	Neuropath	Postop follow-up (mo)	ILAE outcome class	Current no. / Preop no. AEDs	Post-surgical outcome - Cognitive	Post-surgical outcome - Psychiatric	Post-surgical outcome - Employment
1	34	L ATLx	Classical HS	36	ILAE class 1	2 / 3	no significant changes on psychometry	reactive depression	works
2	8	R ATLx	Hamartoma	76	ILAE class 5	1 / 1	good outcome	no psychiatric issues	works
3	8	R neocortic and Ax	Non-specific findings.	156	ILAE class 1 7yrs, then class 3	2 / 3	good outcome	no psychiatric issues	no change
4	24	L ATLx	Classical HS	10	ILAE class 1	1 / 2	good outcome (verbal recall and visual memory improved	no psychiatric issues	NA
5	32	L sAHx	Classical HS	36	ILAE class 1	1 / 2	awaits postop psychometry	no change	no change
6	41	L ATLx	Classical HS	36	ILAE class 1	1 / 2	no significant changes on psychometry	no psychiatric issues	works
7	30	L ATLx	Classical HS	96	ILAE class 1	3 / 3	some verbal memory problems, non-verbal memory improved	reactive depression resolved	works full-time
8	3	L ATLx	Classical HS	60	ILAE class 1	1 / 2	good outcome	no psychiatric issues	works full-time
9[a)]	18	R ATLx	Classical HS	15	ILAE class 1	1 / 2	improved attention span and verbal memory, slight decline of visual memory	no psychiatric issues	works
10	40	R sAHx	Classical HS	72	ILAE class 1	0 / NA	NA	NA	NA

Table 6.2 Type of surgery, neuropathology results and post-surgical outcome, for the patients with MTLE included in this study.

Abbreviations: AEDs=antiepileptic drugs; Ax = amygdalectomy; ATLx = anterior temporal lobectomy with amygdalo-hippocampectomy; L = left; mo = months; NA = not applicable or not available; no. = number of; postop = postoperative; R = right; sAHx = selective amygdalo-hippocampectomy.

a) Case 9 reported in (Kasperaviciute et al., 2011).

ID	Cytoband	Breakpoints	Size (Mb)	Gene list
1	**16p13.11**	chr16:15387380-16225138	0.8	*MPV17L, C16orf45, NDE1, MYH11, C16orf63, ABCC1, ABCC6*
2		chr16:15387380-16225138		
3		chr16:15387380-16198600		
4	**15q11.2**	chr15:18285782-20868229	1.3	*OR4N4, OR4M2, A26B1 (POTEB), NIPA2, NIPA1, TUBGCP5, CYFIP1*
5		chr15:18822307-19852603	1.0	*OR4N4, OR4M2, A26B1*
6	7q31.32-31.33	chr7:123252578-126117199	2.9	*HYAL4, SPAM1, LOC136157, GPR37, POT1, GRM8*
7	17p12	chr17:14040467-15411904	1.4	*COX10, CDRT15, HS3ST3B1, PMP22, TEKT3, CDRT4, FAM18B2*
8	4q32.3	chr4:167446375-168643447	1.2	*SPOCK3*
9	17q12	chr17:31922987-33333394	1.4	*ZNHIT3, MYO19, PIGW, GGNBP2, DHRS11, MRM1, LHX1, AATF, ACACA, C17orf78, TADA2L, DUSP14, AP1GBP1, DDX52, HNF1B, LOC284100*
10	4q35.2	chr4:189052964-190737252	1.97	*AC093909.2, AC020698.4, TRIML2, TRIML1, ZFP42*

Table 6.3 List of genes included in the recurrent microdeletions at 16p13.11 and 15q11.2 and non-recurrent microdeletions larger than 1Mb, found in patients with MTLEHS, who had resective surgery, in the genome-wide CNV study of epilepsy.

In: Catarino C.B., et al., Epilepsia, Genomic microdeletions associated with epilepsy: not a contraindication to resective surgery, vol. 52, no. 8, pp. 1388-1392, DOI 10.1111/j.1528-1167.2011.03087.x (Catarino et al., 2011a).

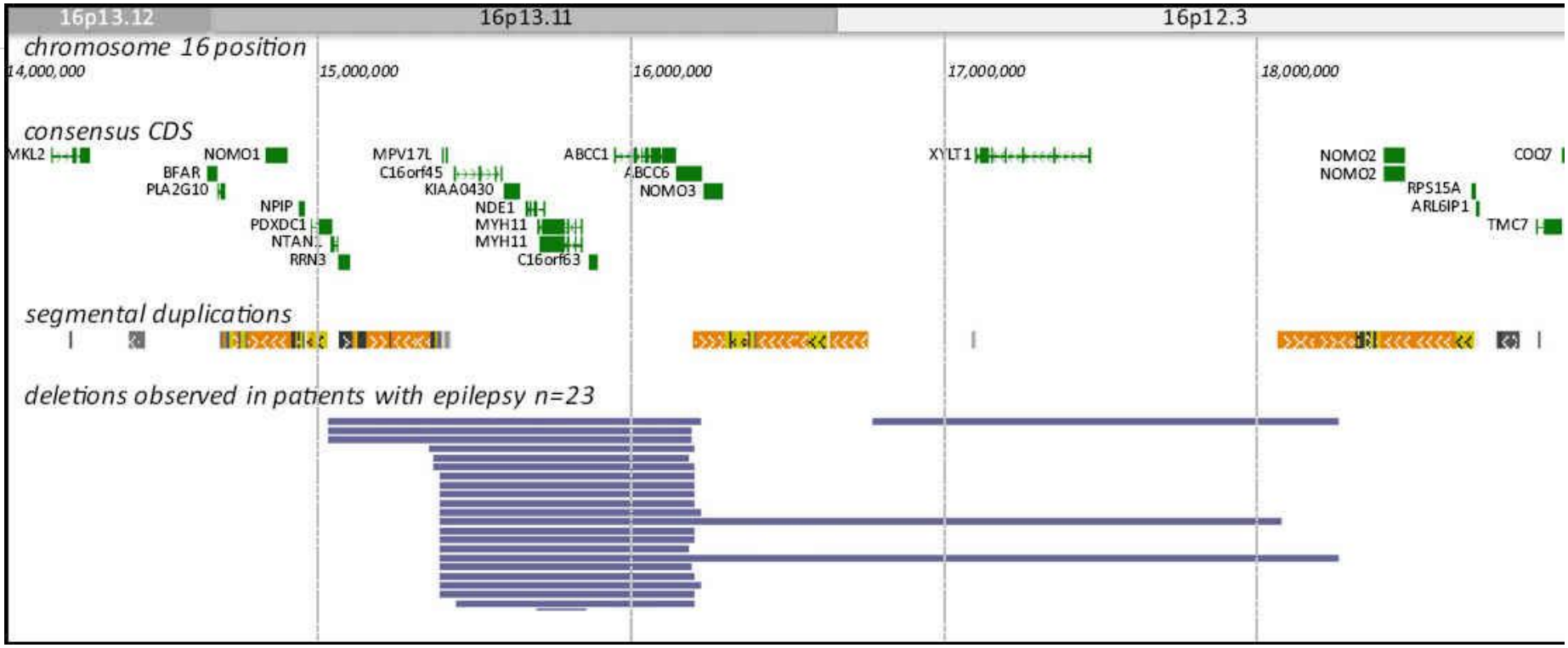

Figure 6.2 Physical location on chromosome 16, and genes included in the region, of the 16p13.11 microdeletions found in 23 of the 3,812 patients with epilepsy screened for CNVs. Three of these patients had resesctive epilepsy surgery for medically refractory MTLE, and were therefore included in this study.

Reprinted from American Journal of Human Genetics, Vol. 86, Heinzen EL et al., Rare deletions at 16p13.11 predispose to a diverse spectrum of sporadic epilepsy syndromes, Pages No. 707-718, Copyright (2010), with permission from Elsevier (Heinzen et al., 2010).

ID	Deletion	Gene list	Gene name	Gene function
1 **2** **3**	16p13.11	***NDE1***	***nudE nuclear distribution gene E homolog 1***	Centrosome duplication and formation and function of the mitotic spindle. Essential for cerebral cortex development. May regulate neuronal production by controlling orientation of the mitotic spindle during division of cortical neuronal progenitors.
		ABCC1	MRP1	ATP-binding cassette transporter.
		ABCC6	MRP6	
		MPV17L	MPV17 mitochondrial membrane protein-like	Participates in reactive oxygen species metabolism.
4 **5**	15q11.2[a]	*OR4N4**	olfactory receptor, family 4, subfamily N, member 4	Potential odorant receptor.
		*OR4M2**	olfactory receptor, family 4, subfamily M, member 2	
		A26B1(POTEB	POTE ankyrin domain family, member B	NA
		NIPA2	non imprinted in Prader-Willi/ Angelman syndrome 2	Selective Mg2+ transporter.
		NIPA1	non imprinted in Prader-Willi/ Angelman syndrome 1	
		TUBGCP5	tubulin, gamma complex associated protein 5	Microtubule nucleation at the centrosome.
		CYFIP1	cytoplasmic FMR1 interacting protein 1	Translational repression.
6	7q31.32-31.33	*GPR37*	G protein-coupled receptor 37	Possible functional role in the central nervous system.
		POT1	protection of telomeres 1 homolog	Telomere maintenance.
		GRM8	glutamate receptor, metabotropic 8	Receptor for glutamate.
7	17p12	*COX10*	COX10 homolog, cytochrome c oxidase assembly proten	Component of the mitochondrial respiratory chain.
		TEKT3	tektin 3	Structural component of ciliary and flagellar microtubules.
		FAM18B2	family with sequence similarity 18, member B2	NA
8	4q32.3	*SPOCK3*	sparc/osteonectin, cwcv and kazal-like domains proteoglycan 3	May participate in steps of neurogenesis.
9	17q12	*ZNHIT3*	zinc finger, HIT-type containing 3	Thyroid receptor interacting proteins.

		PIGW	phosphatidylinositol glycan anchor biosynthesis, class	Biosynthesis of GPI-anchor.
		GGNBP2	gametogenetin binding protein 2	May be involved in spermatogenesis.
		DHRS11	dehydrogenase/reductase SDR family member 11	NA
		MRM1	mitochondrial rRNA methyltransferase 1 homolog	Mitochondrial ribosomal RNA methyltransferase.
		LHX1	LIM homeobox 1	Transcription factor.
		AATF	apoptosis antagonizing transcription factor	Possible inhibitor of histone deacetylase HDAC1.
		ACACA	acetyl-Coenzyme A carboxylase alpha	Long-chain fatty acid synthesis.
		TADA2L	transcriptional adaptor 2A	Histone acetyltransferase activity. Role in chromatin remodeling.
		DUSP14	dual specificity phosphatase 14	Involved in the inactivation of MAP kinases.
		SYNRG	synergin, gamma	May play a role in endocytosis.
		DDX52	Asp-Glu-Ala-Asp box polypeptide 52	Probable ATP-dependent RNA helicase.
		HNF1B	HNF1 homeobox B	Transcription factor.
10	4q35.2	*TRIML2*	tripartite motif family-like 2	E3 ubiquitin-protein ligase, role in blastocyst development.
		TRIML1	tripartite motif family-like 1	
		ZFP42	zinc finger protein 42 homolog	May be involved in trancriptional regulation.

Table 6.4 List of genes and their functions, included in the recurrent 16p13.11 and 15q11.2 microdeletions and in the non-recurrent microdeletions larger than 1Mb, in patients with medically refractory MTLE, who had resective surgery and were included in the genome-wide CNV study.

* The genes marked with asterisk were included in the 15q11.2 microdeletions of both patient 4 and 5.

Source: Genecards: www.genecards.org [Last accessed: 29/08/2012].

All eight patients with MTLEHS and histologically-proven classical HS (Table 6.5) had displayed clinical features considered "typical" for MTLEHS (Wieser 2004) (Table 6.1). All were seizure-free after epilepsy surgery, corresponding to ILAE outcome class 1 (Table 6.2). In all but one, AEDs had been decreased in number or daily dose during the long-term postsurgical follow-up and two were already off AEDs.

At post-surgical follow-up, there were no patients with unexpected findings in the cognitive, psychiatric and employment domains (Table 6.2).

ID	Type of surgery	Main pathological findings	Hippocampus	Temporal neocortex
1	ATLx	Classical HS (Fig. 6.3 C).	Neuronal loss and gliosis in CA1 and CA4. Mild GCD.	Focal neuronal loss and gliosis in superficial cortex in pole (TLS) (Thom et al., 2009a).
2	ATLx	Hamartoma (Fig. 6.3 A-B). No HS; specimen incomplete.	Neuronal loss not seen in CA1. Only CA1 available for analysis.	Small glio-neuronal hamartoma in middle temporal gyrus white matter.
3	NCxAx	Non-specific findings.	NA (Amygdala included, but hippocampal structures not present in specimen.)	Patchy laminar reactive astrogliosis.
4	ATLx	Classical HS.	Neuronal loss and gliosis in CA1 and CA4. Mild GCD and some depletion of GC.	Numerous corpora amylacea in white matter.
5	sAHx	Classical HS.	Neuronal loss and gliosis in CA1 and CA4.	NA
6	ATLx	Classical HS.	Neuronal loss and gliosis in CA1 and CA4. Moderate GCD.	No pathology.
7	ATLx	Classical HS.	CA1 neuronal loss and gliosis. GCD.	Gliosis only. No dysplasia.
8	ATLx	Classical HS.	Neuronal loss and gliosis in CA1 and CA4. Moderate GCD.	Patchy cortical and white matter gliosis.
9	ATLx	Classical HS.	Neuronal loss and gliosis in CA1 and CA4.	Normal cortex.
10	sAHx	Classical HS.	Moderate to marked astrogliosis.	NA

Table 6.5 Main pathological findings of the temporal lobectomy specimen.

Abbreviations: ATLx = anterior temporal lobectomy; GC(D) = Granule cell (dispersion); NA = not applicable; NCxAx = neocorticectomy and amygdalectomy; sAHx = selective amygdalo-hippocampectomy; TLS = temporal lobe sclerosis (Thom et al., 2009a).
In: Catarino C.B., et al., Epilepsia, Genomic microdeletions associated with epilepsy: not a contraindication to resective surgery, vol. 52, no. 8, pp. 1388-1392, DOI 10.1111/j.1528-1167.2011.03087.x (Catarino et al., 2011a).

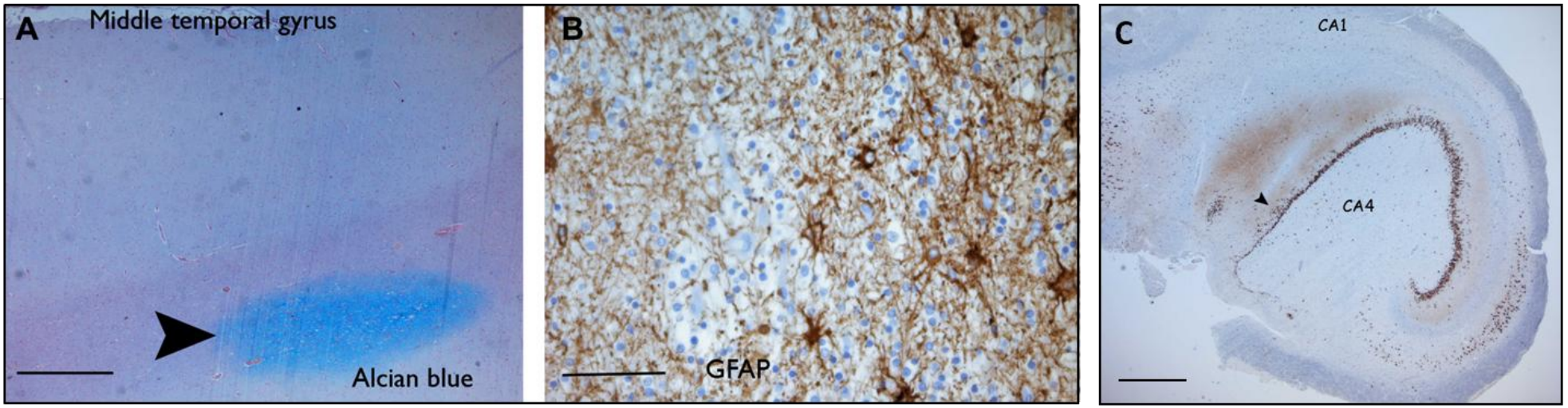

Figure 6.3 Histopathology of the surgical specimen in two patients with MTLE and a 16p13.11 microdeletion.

(A, B) Hamartoma (Case 2): (A) Small glio-neuronal hamartoma in the white matter of the middle temporal gyrus. Alcian Blue stain, at low magnification (objective x 2.5) showing abnormal matrix. Scale bar = 500 µm. (B) The hamartoma was mainly comprised of GFAP-negative, CD34-negative and focally synaptophysin-positive small round cells. There was no mitotic activity and no evidence on adjacent sections of more extensive cortical dysplasia or tumour. Scale bar = 30 µm.

(C) Classical hippocampal sclerosis (Case 1). Neuronal loss in CA1 and CA4, with mild focal granule cell dispersion (arrowhead, NeuN staining). Scale bar = 1000 µm.

6.6 Discussion and conclusions

MTLE is a common and heterogeneous phenotype, likely influenced by several genetic variants, both common and rare, including copy number variation. This study shows that recurrent microdeletions at 16p13.11 and 15q11.2 and other large non-recurrent microdeletions, can be found in patients with MTLEHS and not exclusively in patients with "idiopathic"/"cryptogenic" epilepsies. Some patients in this series have an electro-clinical picture of "typical" MTLEHS, including some with histopathologically-proven diagnosis of "classic HS". Some had personal antecedents of febrile seizures.

Microdeletions at 16p13.11 and 15q11.2 have been associated with other partial epilepsies (Heinzen et al., 2010), idiopathic generalised epilepsies (de Kovel et al., 2010) and epileptic encephalopathies (Mefford et al., 2011b). It is probable these large microdeletions are associated with increased susceptibility to seizures. Interesting candidate genes in the 16p13.11 deletion, for example, include the *NDE1* gene. Functional studies and further research are necessary to look for "causal" relationships and disentangle pathogenic pathways and their significance in seizure susceptibility.

These large microdeletions have been found in patients with distinct epilepsy syndromes. Further, the phenotypical heterogeneity of MTLEHS is probably a function of genetic heterogeneity, with contribution of several genes. More research is needed, therefore, to establish which genetic variants, if any, have a role in determining the MTLEHS phenotype and what is the importance and inter-relationship of genetic variants in influencing susceptibility to seizures, TLE, MTLEHS, febrile seizures and/ or HS.

The detection of a microdeletion including genes in some cases expressed ubiquitously in the brain could theoretically raise concern about its potential influence on outcome measures following epilepsy surgery, with regard to seizure control or other domains. It could be hypothesized that the presence of a microdeletion could represent widespread brain involvement, similar to cognitive impairment or secondary generalised tonic-clonic seizures, both of which reduce the chances of good outcome across various domains after resective epilepsy surgery (Malmgren et al., 2008;Spencer and Huh 2008). On the other hand, such microdeletions might not affect outcome, because of spatial variability in gene expression (Hardy et al., 2009), among other factors.

Importantly, from the 10 patients in this series with MTLE who met criteria for resective surgery and who harbour large microdeletions, 8/8 with MTLEHS had an excellent post-surgical outcome (ILAE class 1). Seizure-free patients had microdeletions at 16p13.11, 15q11.2 and five different non-recurrent microdeletions.

Patients with MTLE, who are otherwise good candidates for surgery, as reported for other MTLE cohorts in the literature (Dunlea et al., 2010;McIntosh et al., 2001;Spencer and Huh 2008), can have a good surgical outcome, even if putatively pathogenic microdeletions are found.

Whilst this observation must be tempered by the small size of the series and requires further confirmation, these results suggest that finding large microdeletions, some proven to have a pathogenic role, in patients with refractory partial epilepsy, does not necessarily preclude a good prognosis following epilepsy surgery, in case surgery is a reasonable option (Duncan 2011).

The psychiatric outcome varied, with presurgical psychiatric comorbidity common in cases with postsurgical psychiatric "issues", as previously reported in the literature (Kanner et al., 2009).

There are examples in the literature of patients with epilepsy with probable or definite genetic basis, who have good outcome after resective epilepsy surgery. In GEFS+ families with *SCN1B* mutations, excellent outcome was reported after anterior temporal lobectomy in selected individuals with refractory MTLEHS (Scheffer et al., 2007). A small series was published of four patients with tuberous sclerosis complex and mutations in *TSC1* or *TSC2*, who were seizure-free on AEDs at last follow-up, after resection of discrete brain epileptogenic lesions (Hirfanoglu & Gupta 2010). One child with early-onset epileptic encephalopathy had epilepsy surgery at 18 months, with focal cortical dysplasia type Ia on histopathology and a *STXBP1* mutation was found post-operatively; a 95% reduction of seizure frequency was noted immediately after surgery, but no data were published on long-term surgical outcome (Weckhuysen et al., 2013).

Further studies will be important to firmly establish a relationship between specific large microdeletions and increased susceptibility to seizures and/or increased risk of partial epilepsy, generalized epilepsy, MTLE, HS. As genetic variants are uncovered that contribute to increased susceptibility to seizures, it will be important to look into pathogenic pathways and mechanisms, looking for the possible impact on the clinical management and prognosis of patients with epilepsy.

7 Chapter Dravet syndrome

7.1 Introduction

7.1.1 Definition and classification of Dravet syndrome

Dravet syndrome (Severe Myoclonic Epilepsy of Infancy, SMEI; MIM 607208), first described around thirty years ago, is a severe epilepsy with onset in infancy (Dravet 1978;Dravet et al., 2005). DS includes SMEI and severe myoclonic epilepsy of infancy-borderland (SMEB), where one or two cardinal features of SMEI may be missing (no myoclonic seizures, moderate development delay) (Mullen & Scheffer 2009).

DS is characterised by onset of recurrent febrile and/or afebrile hemiclonic or generalised seizures, or status epilepticus, in a previously healthy infant, followed by appearance of multiple seizure types, generally resistant to antiepileptic drugs (AEDs), with developmental arrest or regression (Dravet et al., 2005;Jansen et al., 2006;Wolff et al., 2006). Onset may occur at up to 15 months of age (Depienne et al., 2009b). Mortality may be up to 15% by 20 years (Dravet et al., 2005).

7.1.2 Epidemiology of Dravet syndrome

DS is comparatively uncommon, with an estimated incidence of <1/40,000 children (Dravet et al., 2005;Hurst 1990). More common than initially thought, DS is estimated to account for 8% of seizures in infancy (Dravet et al., 2005). The prevalence in adults in not well known.

It is important to diagnose DS because it is considered, at least in part, an epileptic encephalopathy, though other factors may contribute to outcomes (Ragona et al., 2011): seizures and frequent epileptiform activity seen on EEG are held in part responsible for cognitive, behavioural and other impairments (Dravet et al., 2005); both seizures and interictal discharges are potentially treatable with the correct edication, but may be worsened by a few antiepileptic drugs and their control might improve outcomes in DS (Scheffer et al., 2009). Sometimes it is difficult to distinguish between SMEI and cryptogenic partial epilepsy (Sarisjulis et al., 2000). Other diagnostic difficulties, particularly in adults, have to do wirh lack of availability of sufficient information in the first years of life.

7.1.3 Dravet syndrome in adults

It is now recognised that DS is under-diagnosed and under-reported in adulthood (Scheffer et al., 2009). In childhood, DS has been well studied, but the place of DS in adults with epilepsy is less well-understood. A literature review of the published studies which include adults with DS is summarised in Table 7.1.

For adult patients with chronic epilepsy who are long-standing attenders at clinic, details of the early history may become obscured and the diagnosis of DS may not be considered. The long-term course of DS has therefore not been fully characterised, particularly in patients aged 40 years and over.

Authors, year	No. adults in study (total no. patients in study)	Age range in study (median), in years	Dravet syndrome subtypes	*SCN1A* structural variation
Only adults in the study				
Present study (Catarino et al., 2011b)	22	20-66 (median 39)	SMEI & SMEB	12 / 20 mutations (60%)
(Genton et al., 2011)	24	20-50	SMEI & SMEB	Not mentioned
(Andrade et al., 2010)	2	19, 34	SMEI	Not mentioned
(Marini et al., 2009)	2	26, 30	SMEI	one duplication exon 26; one amplification exon 26
(Akiyama et al., 2010)	31	18-43 (median 22)	14 SMEI & 17 SMEB	25 / 31 mutations (80%)
(Zucca et al., 2008)	1	28	SMEI	1 / 1 deletions
(Fujiwara 2006)	2	19, 19	SMEI	Not mentioned
(Depienne et al., 2006)	4	23-40	SMEI	4 / 4 mutations
(Berkovic et al., 2006a)	2	17.5, 47	1 SMEI & 1 SMEB	2 / 2 mutations
(Jansen et al., 2006)	14	18-47 (median 26.5)	SMEI & SMEB	10 / 14 mutations (70%) (+ 1 *GABRG2* mutation)
Mostly children in the study				
(Ragona et al., 2010)	Not specified (37)	0.5-28 (mean 16)	SMEI	37 / 37 mutations
(Kassai et al., 2008)	Not specified (64)	3-20	SMEI	Not mentioned
(Striano et al., 2007a)	Not specified (28)	3-23 (mean 9.4)	SMEI	Not mentioned
(Striano et al., 2007b)	Not specified (58)	0.3-25	SMEI	Not mentioned
(Dravet et al., 2005)	Not specified (105)	2.5-33.6 (median 11.5)	SMEI & SMEB	Not mentioned
(Dravet et al., 1992)	Not specified (63)	3-27	SMEI	Not mentioned
(Rossi et al., 1991)	Not specified (15)	9-24 (mean 15)	SMEI	Not mentioned

Table 7.1 Published studies which include adults with Dravet syndrome. Abbreviations: SMEB = severe myoclonic epilepsy of infancy-borderland; SMEI = severe myoclonic epilepsy of infancy. In: Catarino, C.B., et al., Dravet syndrome as epileptic encephalopathy: evidence from long-term course and neuropathology, Brain, 2011, vol. 134, no. Pt 10, pp. 2982-3010, by permission of Oxford University Press (Catarino et al., 2011b).

7.1.4 Genetics of Dravet syndrome

Initial discovery of de novo *SCN1A* mutations in DS was in 2001 (Claes et al., 2001). Around 80% of DS cases are caused by *SCN1A* mutations, ninety percent of which occur de novo (Depienne et al., 2009b;Mullen and Scheffer 2009). Haploinsufficiency is thought to be the mechanism underlying most cases (Depienne et al., 2009b;McArdle et al., 2008;Mullen and Scheffer 2009). Of the 20-30% of DS cases with no detectable *SCN1A* mutations (Harkin et al., 2007), 10-15% have pathogenic copy number variants (CNVs) (Depienne et al., 2009b;Marini et al., 2007;Marini et al., 2009;Mulley et al., 2006;Wang et al., 2008a).

SCN1A encodes the protein $Na_v1.1$, the voltage-gated sodium channel α1 subunit. Voltage-gated sodium channels are found throughout the brain and are essential for the initiation and propagation of action potentials (Vacher et al., 2008;Whitaker et al., 2001). Specifically, $Na_v1.1$ are expressed on neuronal cell bodies, proximal dendrites and some axons of cortical pyramidal cells; hippocampal granule cells, pyramidal cells and interneurons; and cerebellar Purkinje cells, in the adult human brain (Inda et al., 2006;Westenbroek et al., 1989;Whitaker et al., 2001).

Genetic heterogeneity, genetic modifiers (Meisler et al., 2010) and environmental factors probably contribute to the variable expressivity of the phenotype of patients with *SCN1A* mutations.

A mutation in the *GABRG2* gene, which encodes GABA receptor subunit gamma-2, a member of the gamma-aminobutyric acid $(GABA)_A$ receptor family, was identified in a family with genetic epilepsy with febrile seizures plus (GEFS+), where one individual

had DS (Harkin et al., 2002). The *SCN1B* gene has also been shown to be involved in DS (Patino et al., 2009), but *SCN1B* mutations are not a common cause of DS (Kim et al., 2013).

Mutations in the *SCN2A* gene (Kamiya et al., 2004;Shi et al., 2009) and deletions involving the sodium channel gene cluster on chromosome 2q (Davidsson et al., 2008;Lossin 2009;Meisler et al., 2010;Pereira et al., 2004) have been reported in DS-like syndromes. Mutations in *PCDH19*, which encodes protocadherin-19, has been associated with a DS-like syndrome in females (Depienne et al., 2009a;Dibbens et al., 2008).

Potential genetic modifiers of DS include *SCN9A* (Singh et al., 2009), *CACNA1A* (Ohmori et al., 2013) and *CACNB4* (Ohmori et al., 2008b).

7.1.5 Animal models of Dravet syndrome

Scn1a knock-out or knock-in animal models of DS, heterozygous mice for either a null or truncated *Scn1a* allele, respectively, have severe phenotypes, manifesting spontaneous seizures, motor deficits, ataxia and premature death (Kalume et al., 2007;Martin et al., 2010;Ogiwara et al., 2007;Tang et al., 2009;Yu et al., 2006).

A mouse model with a deletion of $Na_v1.1$ sodium channels in the inhibitory interneurons alone also causes seizures and premature death (Cheah et al., 2012).

In heterozygous mice for either a null or truncated *Scn1a* allele, whole-cell sodium currents are significantly reduced in inhibitory (GABAergic) interneurons in both hippocampus and cortex, but less so in hippocampal pyramidal cells (Ogiwara et al.,

2007;Yu et al., 2006). In a knock-in mouse model of DS carrying a truncating *Scn1a* mutation identical to a human *SCN1A* mutation, with absence of $Na_v1.1$ in their brains, inhibitory GABAergic interneurons were shown to have impaired sodium channel activity, while excitatory cortical pyramidal neurons were mostly unaffected (Martin et al., 2010). Reduced sodium currents in hippocampal and cortical GABAergic interneurons lead to altered firing patterns and hyperexcitability (Catterall et al., 2008;Martin et al., 2010;Tang et al., 2009;Yu et al., 2006).

The reduced expression of $Na_v1.1$ in Purkinje cells, leading to abnormal sodium flux, may contribute to the ataxia observed in animal models (Yu et al., 2006). Further parallels between animal models and human DS include sensitivity to body temperature elevation and age-dependence of seizure frequency and severity (Oakley et al., 2009).

7.1.6 Inflammation in Dravet syndrome

Inflammation and immune-inflammatory mediators have received attention in epileptogenesis, febrile seizures and some chronic epilepsies (Ravizza et al., 2008;Vezzani 2008;Vezzani & Granata 2005). The associated inflammation has been shown to be critical in leading to increased excitability after a febrile seizure (Reid et al., 2013). DS may provide a model to advance understanding of inflammation and fever in seizure susceptibility and epileptogenesis (Baulac et al., 2004;Oakley et al., 2009).

7.2 Aims

One important aim of this study was to gather clinical information on DS throughout adulthood, with a view to inform management of adult patients with DS. This was an observational study, not intended to be a systematic study of prevalence in adults with severe epilepsy.

Another major aim was the genetic characterization of the cohort of adult DS patients, by screening the *SCN1A* gene for mutations and deletions, in the patients and their parents when possible and systematically looking for genotype-phenotype correlations.

The third major aim was a detailed and systematic neuropathology investigation of DS, which had not been done before.

7.3 Methods

7.3.1 Ethics approval

This project was approved by the Joint Research Ethics Committee of the National Hospital for Neurology and Neurosurgery and the UCL Institute of Neurology and the relevant local Human Research Ethics Committees. All individuals had appropriate consent, or assent from relatives or legal guardians in the case of minors or adults with intellectual impairment, for genetic testing. Era-appropriate consent was obtained for post mortem examination and retention of brain tissue for research purposes.

7.3.2 Patient ascertainment and inclusion criteria

All people with a diagnosis of Dravet syndrome at the specialized adult epilepsy clinics of the National Hospital for Neurology and Neurosurgery, at both the Queen Square and the Chalfont Centre sites, were identified through clinic letters. For each DS patient identified, I reviewed all relevant clinical information.

I screened the clinical data for all adults with chronic epilepsy who were residents at the Chalfont Centre for Epilepsy, died between 1988 and 2008, had a post mortem examination and for whom medical records were available. I identified all patients with a possible or probable diagnosis of DS in this previously undiagnosed group of the historical cohort of 235 peple who were in residential care at the Chalfont Centre.

Patients were included in the study if they had clinical criteria compatible with the diagnosis of DS as defined in the 1989 ILAE classification of epilepsy syndromes (ILAE

Commission on Classification and Terminology 1989) (Table 7.2). Patients with SMEB were also included.

7.3.3 Phenotyping

All available clinical and investigational data were reviewed. Early history, detailed seizure records, follow-up records, medication and family history were available for review, as were serial electroencephalography reports, imaging data, neuropsychometry studies and neuropathology reports.

Electroclinical characteristics	**Features of Dravet syndrome**
Age at seizure onset	Seizure onset during the first year of life[a].
Seizure types	Generalised and/or hemiclonic seizures at onset; later, myoclonic seizures[b] and often partial seizures.
Development	Normal development before seizure onset. Psychomotor development delay from the second year of life.
EEG	Generalised spike-waves and polyspike-waves, early photosensitivity and focal abnormalities.
Other neurological signs	Ataxia, pyramidal signs and interictal myoclonus may appear.
Drug response	Epilepsy "very resistant to all forms of treatment".
Family history	Family history of epilepsy or febrile seizures possible.

Table 7.2 Electroclinical characteristics of Dravet syndrome according to the 1989 ILAE classification of epilepsy syndromes (ILAE Commission on Classification and Terminology 1989).

a Onset up to 15 months has been considered to be compatible with a diagnosis of DS (Depienne et al., 2009b);
b Absence of myoclonic seizures in a patient with a clinical picture otherwise compatible with SMEI is considered as DS and called SMEB (Mullen and Scheffer 2009).

7.3.4 Genetic testing

7.3.4.1 Extraction of DNA

For the adult post mortem DS cases, frozen material was available in one case. DNA was extracted from 25mg of frozen brain tissue according to manufacturers' instructions for the "mousetail protocol" in the Wizard® Genomic DNA purification Kit (Promega). For all patients ascertained from clinics, DNA was extracted from blood samples using standard protocols. For all children, DNA was extracted from blood samples.

7.3.4.2 Molecular analysis of *SCN1A* gene

DNA sequencing and Multiplex Ligation-dependent Probe Amplification (MLPA) of *SCN1A* gene were undertaken using standard methods. Parents of patients with a mutation also had genetic testing where possible, with direct sequencing or MLPA of the *SCN1A* region found to have a mutation or a deletion in the proband.

Sequencing of *SCN1A*

The coding region and intron/exon boundaries of *SCN1A* were sequenced using 30 primer pairs (details on request) and Big Dye Terminator Sequencing chemistry version 1.1 (Applied Biosystems) on a 3730xl capillary sequencer (Applied Biosystems). Sequencing analysis was performed using SeqScape v2.5 software (Applied Biosystems). The translation initiator Methionine is numbered as +1 (NCBI accession number: NM_006920; NP_008851).

Dosage Analysis by MLPA

MLPA analysis was undertaken when an *SCN1A* mutation was not detected by sequencing. MLPA using the *SCN1A* kit (SALSA P137-A2; MRC-Holland, Amsterdam, The Netherlands) was carried out according to manufacturers' instructions; amplified

fragments were analysed using a 3730xl capillary sequencer and GeneMarker v1.80 software (SoftGenetics).

Individual peaks corresponding to each exon (*SCN1A* as well as control genes) are identified based on the difference in migration relative to the size standards (LIZ 500, Applied Biosystems). The GeneMarker analysis software uses the MLPA ratio method to determine the deviation of each allele peak, relative to the average deviation of all peaks. This method standardizes the data so that the median point within the data set is considered to be 1 and calculates the deviation of each peak from this median as a ratio. A peak with a ratio of less than 0.75 indicates a deletion while a peak with a ratio of greater than 1.25 indicates a duplication.

7.3.4.3 Molecular analysis of other genes

Two female patients, negative for *SCN1A* mutations or deletions, were tested for *PCDH19* mutations by gene sequencing. This was done in a laboratory in the Royal Children's Hospital, University of Melbourne, in Victoria, Australia.

7.3.5 Genotype-phenotype analysis

The cohort was divided into living adults with DS and living children with GEFS+; and children with DS with death before 12 years and adults with DS who died after the age of 45 years. Each of these groups was analysed for type of *SCN1A* mutations and distribution of *SCN1A* missense mutations.

Further, I looked for clinical differences between the group of adults with a *SCN1A* mutation and the adults without a *SCN1A* mutation.

7.3.6 Neuropathology

The whole brain of three adults with DS who had post mortem study ("adult DS cases", PM1-PM3), three adult controls with no known neurological disease who had post mortem examination ("PM controls", Controls 3-5) and two adult disease controls with HS who had post mortem study ("HS controls", Controls 1-2), were studied. Adults with disease were former residents at the Epilepsy Society (National Society for Epilepsy, Chalfont Centre) (Sander et al., 1993).

Four children with DS who had post mortem study ("paediatric DS cases") were studied as comparators. One anterior temporal lobectomy specimen from a child with intractable epilepsy with generalised tonic-clonic seizures, left hippocampal sclerosis (HS) and an *SCN1A* mutation ("*SCN1A*+ surgical case") (Livingston et al., 2009) and one post mortem brain from a child with severe febrile seizures in the GEFS+ spectrum, were also studied. A brain biopsy was obtained in childhood from an individual ascertained as an adult (Case 4) and was also available for study.

Routine histological staining with Haematoxylin and Eosin (H&E) and Luxol Fast Blue (LFB) and immunohistochemistry (IHC) with a range of neuronal, interneuronal, inflammatory, vascular markers and markers of neurodegeneration, were performed.

7.3.6.1 Macroscopic examination

Studies were undertaken to look for subtle malformations, hippocampal sclerosis (using standard qualitative, quantitative and immunohistochemical examination), cortical neuronal loss (qualitative examination), loss of specific cell populations (qualitative and semi-quantitative immunohistochemistry for interneurons), abnormalities of brainstem nuclei or tracts, distribution and quantitation of cells labelled with antibodies to $Na_v1.1$ and for evidence of inflammatory and other disease processes, with examination with antibodies to HLA-DR and connexin-43.

Formalin-fixed post mortem whole brains were sliced coronally along the anteroposterior axis and each slice was carefully re-examined for macroscopic abnormalities.

Systematic histological sampling using blocks of 5mm thickness were taken from several regions where possible: frontal (F1/F2), parietal, temporal and occipital cortex, insula, cingulate gyrus, cerebellum, hippocampus, amygdala, thalamus, basal ganglia, midbrain, pons, medulla and spinal cord at the cervical level. For two adult DS cases (PM1 and PM3), additional blocks were taken from medial and orbital frontal cortex (Brodmann areas 6, 8, 11) and insula. Surgically-resected temporal neocortex and hippocampal tissue was available for the *SCN1A*+ surgical case. All blocks were processed in alcohol then xylene and embedded in paraffin within one week of sampling.

7.3.6.2 Routine histological stains and Immunohistochemistry

Routine histological stains, Haematoxylin and Eosin (H&E) and LFB, were performed on sections from all regions.

Immunohistochemistry (IHC) was performed on the post mortem hippocampal, frontal cortical (F1/F2), cerebellar, pontine, medullary and spinal cord sections and the surgically-resected hippocampal and temporal neocortical sections.

5 µm sections were dewaxed in xylene and dehydrated in graded alcohol and brought to distilled water. Sections were then incubated in 3% hydrogen peroxide for 15 minutes and microwaved in antigen retrieval buffer (Vector, CA, USA) at 800W for 15 minutes. Sections were left to cool for 20 minutes before primary antibodies were applied overnight at 4 ºC. Primary antibodies were diluted in Dako Diluent. On the following day, Dako Envision horseradish peroxide solution was applied on the sections for 30 minutes. Chromogen activation was performed using Dako Envision diaminobenzidine and substrate buffer for 2-5 minutes. Labelled sections were then counterstained in Haematoxylin solution, dehydrated in graded concentrations of alcohol, defatted in xylene and coverslipped (TissueTek, Sakura, The Netherlands). Sections were washed in phosphate buffer solution between each step.

For double-labelled immunofluorescence, sections were incubated with anti-$Na_v1.1$ diluted in Dako Diluent (1:50) overnight. Dako Envision horseradish peroxide solution was applied for 30 minutes on the following day. Fluorescein isothiocyanate (FITC)-conjugated tyramide in 1x amplification buffer (1:500, Perkin-Elmer, UK) was applied for 8 minutes. The sections were then incubated in anti-NPY 1:5000, anti-

glutamic acid decarboxylase 1:1000 (GAD, Chemicon, USA) or anti-parvalbumin 1:300 (PV, Swant, Switzerland) overnight before species-specific Alexa Fluor 546-secondary antibodies diluted in Dako Dilutant 1:100 (Molecular probes, Invitrogen, USA) were applied for 3 hours at room temperature.

Negative controls, with omission of primary antibodies or addition of blocking peptides, showed no positive labelling. Immunofluorescent-labelled sections were viewed under a confocal laser scanning microscope (Zeiss LSM610 Meta). Images were taken using a 63X oil-immersion objective lens at 1 µm interval through the Z-axis of the section. Images were processed with LSM Image Browser software (Zeiss, Germany).

Further panels of antibodies were used as markers of neurodegenerative processes. AT8-immunolabelling in the hippocampi of all adult DS cases was analysed according to Braak staging scale (Braak & Braak 1991).

7.3.6.3 Quantitative analysis

For the adult DS cases and controls, stereological quantification of hippocampal pyramidal cells and interneurons (CA1 and CA4) and of $Na_v1.1$-immunopositive cells in the hippocampus and frontal cortex, was performed.

20µm sections were cut from the left hippocampus and stained in 0.02% cresyl violet solution for 30 minutes at 60 ºC. For each section, the pyramidal cell density of CA1 and CA4 was estimated by stereology (Histometrix, Kinetic Imaging, UK), after delineating the pyramidal cell layer (Duvernoy 1988) at 2.5X magnification. Within each region, well-stained pyramidal cells were counted at 63X with an oil-immersion objective, using the optical dissector method (Williams & Rakic 1988) and a counting

box of 160μm^2 x 10μm. Results were expressed per mm^3. Systematic random sampling was used, with a sampling factor of 30-80% (mean number of fields per case was 128). This was carried out by one researcher blinded to pathology and clinical data.

For quantitation of the small, intensely-labelled $Na_v1.1$-immunopositive cells, the hippocampus and frontal cortex were first delineated on a slide and area measurements obtained using ImagePro Plus software (Media Cybernetics, Inc., USA) connected to a light microscope (Zeiss, Germany), at 2.5X. Results were expressed as number of cells per μm^2.

Counts for interneurons in CA1 and CA4 were done from sections immunolabelled against CB, CR, PV and NPY, using a semi-automated system (Histometrix, Kinetic Imaging, UK) (Thom et al., 2010a) and results expressed as number of cells per mm^2.

7.4 Results

Twenty-two adults with Dravet syndrome were included, ten female (45%), median age at last follow-up 39 years (range 20-66 years). Demographic data are summarised in Table 7.3.

7.4.1 Clinical findings

The clinical data of the patients included in the study are summarised in Tables 7.3 to 7.6. For the adults with Dravet syndrome, the data include the clinical evolution into adulthood and a timeline is presented in graphical form in Fig. 7.1.

7.4.1.1 Seizure history

From onset in infancy, there was no significant period of seizure freedom recorded. In two patients, recognition of a false "seizure-free period" in childhood led to AED cessation, but increased seizure severity and frequency led to recommencement of AEDs and in retrospect the parents recognized that subtle seizures had never ceased to occur.

There was an evolution of seizure semiology and predominance of certain seizure types with time (Table 7.4). There was no single pattern for seizure evolution for all patients.

All patients had multiple seizure types in adulthood (Table 7.4). For ten patients seizures were mostly nocturnal and comprised brief tonic or tonic-clonic seizures. Seizures were recorded in video-EEG telemetry for ten adults; seizures observed were complex motor, dyscognitive, tonic, or secondarily generalised, with focal EEG onset

pattern or no recognizable EEG change. Myoclonus was not prominent in adulthood, though its frequency may have been under-reported. No adult in the series had documented absences: all “absence-like” (dyscognitive) seizures recorded in adulthood had focal EEG onset or no EEG change documented. Fever sensitivity persisted into adulthood, with even minor variations of temperature sufficient to trigger seizures in nine patients. No patient had any meaningful seizure-free period. Non-convulsive status epilepticus was documented with EEG on at least one occasion in seven patients. Triggers included intercurrent infections and slight increases in body or ambient temperature.

All patients had multiple AEDs, with differential control of different seizure types (Table 7.5), but none had complete seizure freedom.

ID	Sex	Age at F/U or at death[d] (y)	Age at sz onset (mo)	Seizure-related family history	*SCN1A* mutation or deletion
PM1	F	46[d]	3	no	+
PM2	M	66[d]	11	no	NA
PM3	M	46[d]	18	no	NA
4	M	39	6	no	-
5	M	25	10	mother, FS	+
6	M	60	12	maternal uncle, epilepsy	+
7	M	41	9	two maternal first cousins, epilepsy	+
8	F	43	12	no	+
9	F	27	8	no	+
10	M	20	7.5	paternal uncle, FS; paternal first cousin, epilepsy	+
11	F	29	7	no	+
12	M	43	7	mother, childhood epilepsy in remission	-
13	M	21	12	no	-
14	F	40	15	MZ twin sister, epilepsy	-
15	M	31	6	no	-
16	F	48[d]	2.5	no	-
17	M	21	3	father, epilepsy	-
18	F	26	3	maternal aunt, epilepsy	-
19	F	44	6	no	+
20	F	39	10	sister, childhood epilepsy; 2 brothers, mother and maternal aunt, FS	+
21	F	23	4.5	no	+

22	M	33	4	no	+
PM23	M	2[d]	5	no	+
PM24[a]	F	10[d]	2	no	+
PM25[a]	M	11[d]	8	mother and maternal grandmother, FS	+
PM26[e]	F	11[d]	10	no	-/NA
PM27[b]	M	5[d]	18	no	+
28/*SCN1A*+	M	12	10	sister, FS; mother, FS and epilepsy	+

Table 7.3 Demographic features and seizure-related family history of 22 adults (PM1-PM3 and 4-22) and four children (PM23-PM26) with Dravet syndrome and two other children with other epilepsy syndromes, who carry a *SCN1A* mutation (PM27 and 28/*SCN1A*+surgical).

Abbreviations: F = female, FS = febrile seizure, F/U = follow-up; ID = intellectual disability, M = male, mo = months, MZ = monozygotic, NA = not available, sz = seizure, y = years.
a Described in (Wallace et al., 2003). *b* Described in (Deng et al., 2007;Harkin et al., 2007). *c* Described in (Livingston et al., 2009).
d Age at death. *e* MLPA results not available (PM26).

In: Catarino, C.B., et al., Dravet syndrome as epileptic encephalopathy: evidence from long-term course and neuropathology, Brain, 2011, vol. 134, no. Pt 10, pp. 2982-3010, by permission of Oxford University Press (Catarino et al., 2011b).

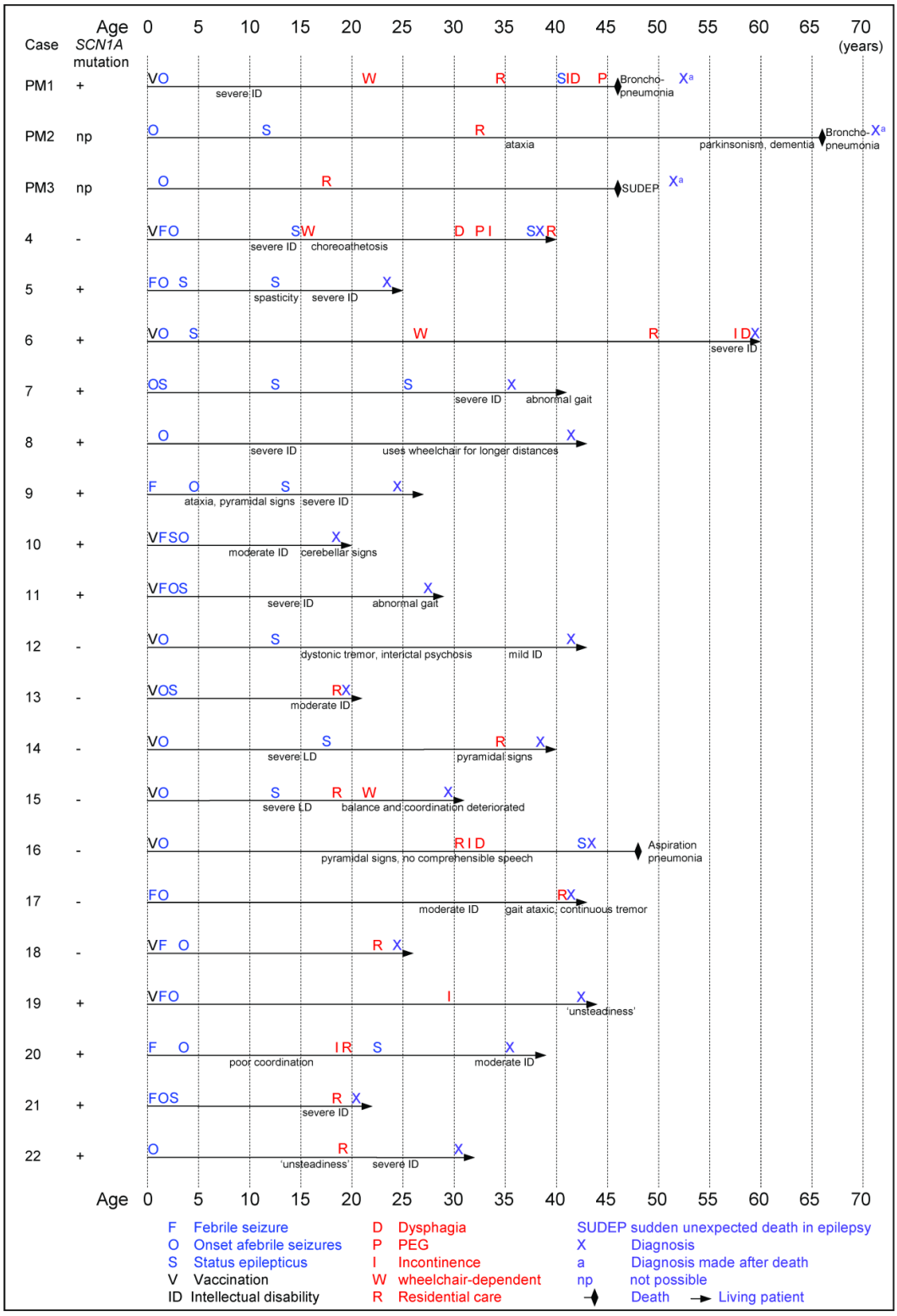

Figure 7.1 Timeline of clinical milestones in a cohort of adults with Dravet syndrome.

In: Catarino, C.B., et al., Dravet syndrome as epileptic encephalopathy: evidence from long-term course and neuropathology, Brain, 2011, vol. 134, no. Pt 10, pp. 2982-3010, by permission of Oxford University Press (Catarino et al., 2011b).

ID	Age and sz type at onset	Triggers at sz onset	Sz types in childhood	Sz types in adulthood	Development / Autistic features / Behavioural problems	Psychometry data	Intellectual outcome, last follow-up[d]	Other neurological signs	Functional outcome, last follow-up
PM1	3mo, GTC	vaccination (no further details)	GTC, CP	GTC, My, Fo, At, SE, NCSE	development delayed after sz onset / no / behavioural problems	no formal neuropsychometry	severe ID	pyramidal	deceased
PM2	11mo, GTC	none	My, GTC, NCSE	GTC, My, At, NCSE	development delayed after sz onset / no / behavioural problems	progressive cognitive decline, dementia from 55yrs	severe ID	progressive ataxia, parkinsonism, dementia, cerebellar	deceased
PM3	18mo, GTC	none	My, GTC, Fo	GTC, My, Fo	development delayed after sz onset / autistic features / behavioural problems	10yrs, FSIQ 77, 17yrs, FSIQ 57	severe ID	cognitive slowing, dysarthria, ataxia	deceased
4	6mo, nd	vaccination (whooping cough, 24h)	GTC, My, dyscognitive, hemiclonic, SG	GTC, My, CP, At, dyscognitive, NCSE	development regression after sz onset / no / behavioural problems	no formal neuropsychometry	severe ID	extrapyramidal (choreoathetosis, dystonia), fixed contractures	no speech, institutionalized, full care, PEG, incontinent, wheelchair-bound
5	10mo, FS, hemiclonic	fever	GTC, hemiclonic, dyscognitive, SG, At, My	GTC, CP, At, My, dyscognitive, SE	development regression after sz onset / autistic features / no	no formal neuropsychometry; progressive, slow cognitive decline	severe ID	pyramidal (spasticity)	lives at home with parents, behavioural problems, minimal speech (only repeats words)
6	12mo, GTC	vaccination (whooping cough, 8h)	GTC, SG, hemiclonic, dyscognitive, At	GTC, CP, My, T, SE, NCSE	development regression from 6yrs/ autistic features / behavioural problems	at 6y in mainstream school; At 27yrs, VIQ 51, PIQ 58	severe ID	not documented	Recognises basic words, able to say the time, PEG, recurrent respiratory infections, wheelchair-bound, incontinent, institutionalized

7	9mo, GTC	slight increase of temperature	GTC, My, SE, dyscognitive	GTC, dyscognitive	development regression from 15mo/ no / behavioural problems	no formal neuropsychometry	severe ID	marked scoliosis, gait abnormality	walks unaided, with stooped posture and legs in semi-flexion; performs one-stage command
8	12mo, nd	no trigger documented	dyscognitive, My	CP, dyscognitive, My	development delayed after sz onset / autistic features / behavioural problems	no formal neuropsychometry	severe ID	not documented	Lives with parents; walks unaided, uses wheelchair for longer distances; speaks in short phrases, mainly sign language; eats unaided, with spoon; recurrent respiratory infections
9	8mo, FS	fever	GTC, CP, dyscognitive, CP, My, Fo, NCSE	GTC, dyscognitive, My, T	development delayed after sz onset / autistic features / no	no formal neuropsychometry, cognitive decline in adulthood	severe ID	truncal ataxia, pyramidal, hand tremor, wide-based gait	NA
10	7.5mo, FS	fever, vaccination (whooping cough, hours)	CP, SG	CP, GTC, dyscognitive, SE	development delayed after sz onset / autistic features / behavioural problems	5yrs: FSIQ 63. 12yrs: VIQ 55, PIQ 68. 16yrs: FSIQ 40; 20yrs: moderately impaired learning range, limited expressive language, very poor comprehension, very weak working memory, unable to carry out two-step commands	moderate ID	cerebellar, truncal and gait ataxia, action and postural tremor	Lives with parents; needs constant one-to-one care
11	7mo, Feb SE	Fever, whooping cough infection	GTC, CP, SE, At	GTC, dyscognitive, CP, T, SE, NCSE	development delayed after sz onset / autistic features / no	no formal neuropsychometry	severe ID	abnormal gait, pyramidal (hyperreflexia)	Lives with parents; requires help for ADL; able to walk unaided; single words

12	7mo, GTC	vaccination (whooping cough, timeline not documented)	GTC, CP	GTC, CP	development delayed after sz onset / no / no	at 42y, MMSE=20/30	mild ID	extrapyramidal (dystonic tremor, hypomimia, bradykinesia)	Lives with parents; self-caring with some help
13	12mo, nd	vaccination (third dose of triple vaccination, 12 hours)	My, GTC	GTC, dyscognitive, CP	development regression after sz onset / autistic features / behavioural problems	at 19yr, MMSE=13/30	moderate ID	none documented	Residential care; behavioural problems, may become paranoid.
14	15mo, GTC	vaccination (measles vaccination, several days)	GTC, My, dyscognitive	GTC, My, At, dyscognitive	development regression after sz onset / autistic features / behavioural problems	no formal neuropsychometry	severe ID	kyphosis, pyramidal	Residential care; speaks one or two words, performs simple orders, walks unaided
15	6mo, GTC	vaccination (triple vaccine, 9days)	GTC, dyscognitive, NCSE, My	GTC, dyscognitive, My	development regression after sz onset / autistic features / behavioural problems	no formal neuropsychometry, but gradual decline	severe ID	gait ataxia	Nursing home; minimal communication, walks with help
16	2.5mo, hemiclonic	vaccination (triple vaccine, two days)	hemiclonic, CP, My, GTC	GTC, My, hemiclonic, At, T, NCSE	development delayed after sz onset / no / behavioural problems	no formal neuropsychometry, but gradual decline	severe ID	pyramidal	deceased
17	3mo, FS	fever	My, GTC, dyscognitive, At, My	GTC, My, dyscognitive, SE, NCSE	development delayed after sz onset / no / no	no formal neuropsychometry	moderate ID	action tremor, extrapyramidal	Residential care; does basic domestic chores with prompting
18	3mo, FS	fever, vaccination (no details)	My, CP, At, T	GTC, T, CP	development delayed after sz onset / autistic features / behavioural problems	no formal neuropsychometry	severe ID	intention tremor	Institutionalised
19	6mo, FS	fever vaccination (pertussis, 2	GTC, dyscognitive	GTC, CP	development regression after sz onset / autistic	no formal neuropsychometry	severe ID	gait ataxia	Lives with parents, has carers; entirely dependent, doubly

		days)			features / behavioural problems				incontinent
20	10mo, FS	fever	GTC, At, My	GTC, My, At, T, SE	development delayed after sz onset / autistic features / behavioural problems	at 40y, MMSE=14/30	moderate ID	none documented	Institutionalised; feeds herself, requires help with domestic chores
21	4.5mo, Feb SE	fever	dyscognitive, At, hemiclonic; At, My	GTC, T, CP	development delayed from 9mo / autistic features / behavioural problems	no formal neuropsychometry	severe ID	kyphosis	Institutionalised; speech limited to one or two phrases, able to walk independently
22	4mo, GTC	no trigger documented	CP, GTC, My	T, GTC, My	development delayed from 3y / autistic features / behavioural problems	no formal neuropsychometry	severe ID	wide-based gait	Institutionalised; no speech, walks with help, requires help with all ADL
PM23	5mo, Afebrile GTC	no trigger documented	GTC, My. No FS.	NA	development delayed from 18mo	no formal neuropsychometry	mild global cognitive delay. Limited expressive language	none documented	deceased
PM24 [a]	2mo, Feb SE	fever	FS, My, CP, Abs, GTC, SE, At, hemiclonic	NA	development never normal, regression at 5y	no formal neuropsychometry	severe ID (nonverbal)	crouch gait	deceased
PM25 [a]	8mo, SE	no trigger documented	GTC, recurrent SE, My, At, T, My Status, Fo	NA	developmental regression with sz onset/ autistic features/ behavioural problems	no formal neuropsychometry	severe ID	ataxia and spasticity	deceased
PM26	10mo, FS	fever	FS, Abs, My, GTC, SE, CP, hemiclonic	NA	developmental slowing from 10 mo / no / behavioural problems	no formal neuropsychometry	severe ID	ataxia and tremulous	deceased

PM27 [b]	18mo, Feb SE	fever	FS, Fo-SG, SE	NA	normal development / no / no	no formal neuropsychometry	normal	none	deceased
28/ *SCN1A*+ surgical[c]	10mo, FS	fever	nocturnal GC, CP, hemiclonic, SG	NA	development delayed from 3y / autistic features / behavioural problems	no formal neuropsychometry	moderate ID	none documented	In a special school

Table 7.4 Clinical features of 22 adults and four children (PM23-PM26) with Dravet syndrome and two children with other epilepsy syndromes, who carry an *SCN1A* mutation (PM27 and 28/*SCN1A*+surgical case).

Abbreviations: Abs = absence, ADL = activities of daily living, At = atonic, CP = complex partial, Feb SE = febrile status epilepticus, Fo = focal, FS = febrile seizure, FSIQ = full-scale IQ, GC = generalised clonic, GTC = generalised tonic-clonic, HS = hippocampal sclerosis, ID = intellectual disability, IED = interictal epileptiform discharges, mo = months, My = myoclonic, NCSE = non-convulsive status epilepticus, nd = undetermined seizure type, PEG = percutaneous endoscopic gastrostomy, PIQ = performance IQ, SE = convulsive status epilepticus, Sz = seizure, T = tonic, VIQ = verbal IQ, WM = white matter, Yrs = years.
a Described in Wallace et al. (2003). *b* Described in Deng et al. (2007) and Harkin et al. (2007). *c* Described in Livingston et al. (2009).
d Classification of intellectual outcome as described by McIntosh et al. (2010).

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7.4.1.2 Co-morbidities

At last follow-up, the oldest living patient is 60 years. Sixteen patients are in residential care; the others live at home with support.

Neurological deterioration continued throughout life in all patients (Fig. 7.1), with further impairment of speech, mobility and ability for daily activities (Table 7.4). Kyphoscoliosis was documented in six patients. Cerebellar signs were found in five patients, pyramidal signs in seven and extrapyramidal in four patients. Non-ictal urinary incontinence occurred late in the evolution.

The majority (18/22) of adults with DS had, at last follow-up, severe intellectual disability, as classified in McIntosh et al. (2010). Behavioural problems or "autistic-like" features were features at some time in the evolution in most (20/22) patients in this series, who were adults at last follow-up (Table 7.4).

As for systemic co-morbidities, recurrent respiratory infections were documented in six patients. Dysphagia emerged as a late feature in five patients, documented in or after the fourth decade of life, leading eventually to percutaneous endoscopic gastrostomy. One adult died during the follow-up period, from repeat aspiration pneumonia (case 16). No post mortem brain tissue was available for review from this case.

7.4.1.3 Vaccination history

For eleven adult patients with DS, a close temporal relation of seizure onset with vaccination (Table 7.4) was documented. A relationship between vaccine encephalopathy,

SCN1A mutations and DS has been previously described (Berkovic et al., 2006a;McIntosh et al., 2010).

7.4.1.4 Family history

A family history of epilepsy and/or febrile seizures was recorded for nine adults with DS (Table 7.3) and another DS adult patient had a sibling who had had one isolated seizure. Case 20 comes from a family with GEFS+. Another adult with DS (Case 6) has a 15-year-old sister with microcephaly, quadriparesis, profound cognitive impairment and spasms, who is on chronic AED therapy, but does not have a syndromic or molecular genetic diagnosis, she does not carry the *SCN1A* mutation found in her brother, nor an *SCN1A* deletion or duplication.

7.4.1.5 Anti-epileptic drugs and non-pharmacological treatment

A list of the current and past antiepileptic drug therapy is presented in Table 7.5, which also includes any changes to the AEDs made after the diagnosis of Dravet syndrome and their impact on seizure control, cognitive function and quality of life.

At last follow-up, most patients were on AED polytherapy. No patient was seizure-free, but in several cases secondarily generalised seizures were controlled with medication.

Seven adult patients with DS have already had drug changes instituted following the diagnosis. Only in three patients has sufficient follow-up elapsed to evaluate an effect of the medication changes and in these three cases there was improvement in seizure control, even after years of drug resistance. In two of these three patients, a significant additional improvement in cognition and quality of life has already been recorded. As for

the other four patients who had had drug changes but only a short period of follow-up, some early indication of benefit for some seizure types is apparent in three, while the one patient with no or minor change in seizure frequency has not yet started any new AED after diagnosis.

Table 7.5 also includes the non-pharmacological therapy used in the adults with DS in this series. Ketogenic diet has been trialled in five adult patients, with significant improvement reported in at least one. Epilepsy surgery has been performed in one patient, who had an anterior thalamotomy (Fig. 7.2) several decades ago, with no reported decrease in seizure frequency.

ID	AED changes after diagnosis of DS	Improvement with AED changes after diagnosis	AED history	Other treatment	Improvement[a] (seizure types)	Documented worsening[a] (seizure types)
PM1	NA	NA	CBZ, CLB, GBP, LTG, PB, PHT, VPA	-	PHT (GTC), VPA	PHT (My)
PM2	NA	NA	CBZ, CLB, PB, PHT, PRM, VPA	-	-	CBZ, PHT
PM3	NA	NA	ACZ, CBZ, CLB, PB, PHT, PRM, VGB, VPA	-	-	-
4	No new AED started	NA	CBZ, CLB, CNZ, LTG, PHT, PRM, SLT, VGB, VPA	-	PB, VPA	-
5	Stopped CBZ; reintroduced VPA; started STP+VPA; decreased LTG	sz control improved cognition NA	CBZ, GBP, LEV, LTG, OXC, PHT, STP, TGB, TPM, VGB, VPA	-	PHT (GTC), STP+VPA	CBZ, OXC (“drops”)
6	Started LEV	sz control improved cognition improved	CBZ, GBP, PB, PHT, PRM, SLT, VGB, VPA	Stereotactic anterior thalamotomy, mephenytoin, phenacemide, benuride	LEV (GTC), PRM,VPA	-
7	Stopped CBZ	sz control unchanged cognition NA Short follow-up	CBZ, CNZ, DZP, LEV, PB, PHT, PRM, VPA	VNS	CNZ	-
8	No changes made	NA	PRM, TPM, VPA	-	PRM, VPA	-

9	No new AED started	NA	CBZ, CLB, LEV, LTG, NTZ, OXC, PB, TPM, VGB, VPA	KD	CLB, KD, VPA	-
10	Increased ZNS: suggested STP, not yet started	NA	CBZ, CLB, ESX, LEV, LTG, PB, PGB, PHT, TPM, VGB, VPA, ZNS	-	VPA, ZNS	LTG (“drops”), PGB
11	No new AED started	NA	ACZ, CBZ, ESX, GBP, LEV, LTG, NTZ, PB, PHT, PRM, VGB, VPA	ACTH, corticosteroids, VNS, KD, GOS exclusion diet	TPM	LTG
12	No new AED started	NA	ACZ, CBZ, CNZ, LEV, LTG, PB, PHT, PRM, SLT, VGB, VPA	-	SLT, VPA	-
13	No new AED started	NA	CLB, LEV, LTG, OXC, VGB, VPA	Prednisolone	VPA, LEV (stopped GTC)	-
14	NA	NA	CBZ, CNZ, DZP, ESX, LTG, NTZ, PHT, VGB	KD, ethotoin	-	-
15	No new AED started	NA	CBZ, CLB, CNZ, ESX, LTG, NTZ, PB, VPA	-	CLB, ESX (Dyscognitive), LEV, VPA	-
16	NA	NA	CBZ, CLB, DZP, LEV, LTG, NTZ, OXC, PB, PGB, PHT, VPA	-	CBZ, VPA	OXC (My)
17	No new AED started	NA	CLB, CNZ, DZP, ESX, LEV, LTG, TPM, VPA,	pyridoxine, biotin	VPA (GTCS)	-

			PIR			
18	Started VPA	sz control unchanged Short follow-up	CBZ, CLB, GBP, LEV, LTG, TPM, VPA	-	VPA	-
19	No new AED started Stopped LCM; suggested STP, not yet started	NA	CBZ, CLB, LCM, LEV, LTG, VPA, TPM, ZNS	-	-	LCM[a]
20	No new AED started	NA	CBZ, CLB, CNZ, ESX, LEV, LTG, NTZ, PB, PHT, PIR, TPM, VGB, VPA	KD	-	-
21	Started STP (+CLB), later stopped. tapered RUF; restarted VPA.	sz control unchanged Short follow-up	CBZ, CLB, CNZ, GBP, LEV, LTG, PB, PHT, RUF, STP, TGB, TPM, VGB, VPA	pyridoxine	TPM, VPA, STP	RUF[a], VGB[a], CBZ[a], LTG[a]
22	Stopped PGB; started ZNS	sz control improved cognition improved	ACZ, CBZ, CLB, CNZ, DZP, GBP, LEV, LTG, NTZ, PGB, PIR, VGB, VPA, ZNS	-	CBZ (GTC), CLB, LEV, PIR (My), VPA, ZNS	CBZ (My), GBP (My), LTG[a], PGB[a]
PM23	NA	NA	VPA	-	VPA (My)	-
PM24	NA	NA	CLB, CNZ, LEV, LTG, STP, TPM, VPA	pyridoxine	STP	LTG
PM25	NA	NA	CBZ, CNZ, DZP, LTG, PB, PHT, STP, TPM,	steroids, VNS, KD	STP, VNS	-

			VGB, VPA			
PM26	NA	NA	CBZ, CNZ, GBP, LTG, TPM, VPA	None	LTG	GBP
PM27	NA	NA	LTG, VPA	None	-	-
28/*SCN1A*+ surgical	NA	NA	No data available	Ant TLx	-	-

Table 7.5 Antiepileptic drug history and non-pharmacological therapy.

Abbreviations: Abs = absences; ACZ = acetazolamide; AED = antiepileptic drug; Ant TLx = anterior temporal lobectomy with amygdalo-hippocampectomy; CBZ = carbamazepine; CLB = clobazam; CNZ = clonazepam; DZP = diazepam; ESX = ethosuximide; GBP = gabapentin; GOS = Great Ormond Street; GTC = generalised tonic-clonic; KD = ketogenic diet; LCM = lacosamide; LEV = levetiracetam; LTG = lamotrigine; My = myoclonic; NA = not available; NTZ = nitrazepam; OXC = oxcarbazepine; PB = phenobarbital; PGB = pregabalin; PHT = phenytoin; PIR = piracetam; PRM = primidone; RUF = rufinamide; SLT = sulthiame; STP = stiripentol; TGB = tiagabine; TPM = topiramate; VGB = vigabatrin; VNS = vagal nerve stimulator; VPA = sodium valproate; ZNS = zonisamide.
a Data on which specific seizure types improved or worsened are not always available for every antiepileptic drug.

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7.4.1.6 Mortality

Mortality is known to be high during early childhood in Dravet, at about 15-18% (Dravet et al., 2005). Sakauchi et al. (2011) recently identified high-risk age periods in childhood with respect to specific causes of mortality, observing that the incidence of sudden death is highest at 1-3 years and acute encephalopathy with status peaks at 6 years; the risk of mortality seems to decline sharply after the age of 12 years, though very long term follow-up was not part of their report. As this study is not a prospective or longitudinal study, caution must be taken in considering mortality in Dravet syndrome: the distribution in this series may appear bimodal, with one peak before 12 years and another peak after the 4th decade, but this may simply reflect study design.

Causes of death in this adult series (Fig. 7.1; Table 7.6) include three cases of bronchopneumonia and one case of sudden unexplained death in epilepsy (SUDEP). In the paediatric DS group, three patients died of SUDEP and one had global ischaemic brain injury; it is unclear for the latter case whether there was a seizure followed by cardiorespiratory arrest.

ID	Age at death (years)	Cause of death
PM1	46	Bronchopneumonia + recurrent NCSE
PM2	66	Bronchopneumonia
PM3	46	SUDEP
16	48	Aspiration pneumonia
PM23	2	SUDEP
PM24	10	SUDEP during a 46 degree Celsius day
PM25	11	SUDEP
PM26	11	Global ischaemic brain injury
PM27	5	Convulsive status epilepticus

Table 7.6 Causes of death and age at death for the four deceased adults with DS (PM1-PM3 and case 16) and five children (PM23-PM27) included in this study.
Abbreviations: NCSE = non-convulsive status epilepticus; SUDEP = sudden unexplained death in epilepsy.

7.4.1.7 Case report (PM1): Adult with Dravet syndrome and *SCN1A* mutation; diagnosis made at post mortem

Female patient, with onset of seizures at 3 months of age, following vaccination; the type of vaccination and time interval between vaccination and seizure onset are unknown. At onset, she had hemi-clonic seizures and generalised tonic-clonic seizures. Later, myoclonic jerks and atonic seizures were also recorded. The seizures were refractory to several AEDs (Table 7.5). She had several episodes of status epilepticus, both convulsive and non-convulsive. Fever sensitivity persisted in adulthood. No family history of epilepsy or febrile seizures was documented. She walked at 18 months, with abnormal gait and was later unable to walk from her early twenties. Her schooling was in the special sector. She was admitted to the Epilepsy Society Chalfont Centre. Serial routine EEGs showed slow background activity and multifocal interictal epileptiform discharges. MRI brain scan, performed in her forties, showed mild cerebral and cerebellar atrophy and no other changes. She was noted to be progressively more dependent and at age 40 was fully dependent and doubly incontinent. She developed dysphagia, requiring PEG for feeding. Recurrent chest infections led to frequent admissions to hospital in status epilepticus. She died at 46 years of age, of bronchopneumonia, complicated with recurrent non-convulsive status epilepticus.

ID	EEG, age at study, findings	Neuroimaging, age at study, findings
PM1	~40y: slow background activity, IED multifocal, in both posterior quadrants	MRI, ~40y: mild cerebral and more severe cerebellar atrophy
PM2	~30y: slow background, bifrontal IED	MRI, ~60y: diffuse cerebral atrophy, gross cerebellar atrophy
PM3	~30y: slow background, multifocal IED	CT, 46y: cerebellar atrophy
4	2y: normal; 16y: slow background, multifocal IED, >L posterior quadrant; 21y: slow background, frequent bilateral IED; 38y: no photosensitivity, moderate bi-hemispheric cortical dysfunction, multifocal bilateral IED, > R	CT, 16y: normal; CT, 35y: generalised loss of cerebral and cerebellar volume, incidental arachnoid cyst; MRI, 38y: cerebral and cerebellar atrophy
5	7y: generalised spike-wave discharges; 14y: normal background, IED both hemispheres; 17y: slow background, bifrontal IED; 22y: generalised IED, more abundant during overnight sleep; 26y, V-T (Fig 3): bilateral diffuse slow activity, frontocentral and bifrontal IED; complex motor seizures, with onset R posterior temporal/temporo-parietal; complex motor szs and electrographic szs, with non-lateralised fronto-central onset	MRI, 23y: normal
6	12y: generalised abnormality, frequent paroxysmal features, variable laterality; 14y: generalised slow, slow activity L frontal>R; 17y: slow background, generalised slow; 29y: generalised slow activity anteriorly; 41y: slow background, slow activity and IED L frontal>R, IED L parasagittal	CT, ~30y: minimal cortical atrophy MRI, ~50y: no significant abnormality apart from thalamotomy lesion and its associated track; MRI, 59y: more significant cerebral atrophy
7	20mo: normal; 34y: slow background, multifocal spikes, bilateral independent, R-sided emphasis; one dyscognitive sz, no EEG change	MRI not done
8	NA	MRI not done
9	25y: slow background, frequent bilateral frontocentral IED, R>L; one SG seizure, with non-lateralised fronto-central onset	CT, 3y: normal
10	4y: moderate abnormality with slow activity posterior; generalised or posterior IED, R>L;	MRI, 20y: normal

	13y: normal; 20y: bifrontal, R frontocentral and infrequent L temporal IED. Seven szs captured, non-lateralised frontocentral onset	
11	10mo: normal; 11mo: burst of slow activity R>L; 2y: brief R and L-sided bursts of spike and wave; 13y: diffusely slow background, frequent bilateral IED; 21y: background slowing, IED bifrontal	CT, 3y: normal; 21y: normal
12	25y: slow background, bilateral IED; 32y: bilateral fronto-temporal IED; 37y: bursts of bitemporal slow with no consistent lateralisation; IED, R-sided>L	MRI, 31y: few conspicuous WM lesions in both cerebral hemispheres; 42y: no change.
13	1y: normal background, generalised spike wave; 15y: excessive slowing L>R, bilateral parieto-temporal IED, L>R; 19y: persistent L sided slow activity, IED both posterior hemispheric regions, parietal and posterior temporal, R>L	MRI, 19y: old infarct in R striatum
14	39y: several frontal szs, > during nocturnal sleep, possibly mesial frontal, not lateralised	MRI, 39y: microcephaly
15	22y: Diffuse slowing over both hemispheres, frequent runs of generalised spike-wave, with shifting lateralisation	MRI not done
16	NA	MRI not done
17	12y: multifocal epileptiform activity, bilateral frontotemporal and R occipito posterior temporal cortex; NCSE 19y: diffuse slow activity in the background, abundant generalised spike wave discharge, particularly in the morning soon after waking	MRI, 19y: non-specific WM changes, longstanding; no other abnormality
18	20y: bilateral and multifocal discharges R>L esp in temporal region 26y: one brief tonic sz, no EEG change recorded. Bifrontal slowing; spike-slow waves over L anterior or frontocentrotemporal; multifocal spikes-sharp waves;	CT,1y: normal; MRI, 26y: normal
19	NA (EEG not tolerated)	CT, 43y: normal
20	6y: multifocal IEDs, L>R 36y: two SG sz and several subtle or subclinical seizures during sleep;	MRI, 30y: normal; 36y: mild cerebellar atrophy, thick cranial vault, particularly in the frontal region

	multifocality, generalised and lateralised IED involving both hemispheres, suggests extratemporal epilepsy	
21	15y, V-T: >30 seizures of frontal lobe semiology in 48h, most from sleep; slow background activity, IED, independent R and L frontal, bilateral frontocentral, rare during wakefulness, prominent during sleep. 21y, V-T: 5 seizures from sleep, tonic and TC; slow background activity, IED over posterior quadrants	MRI, 22y: L hippocampal sclerosis
22	24y: slow background activity, no IED; 32y: slow background; slow fronto-central bilateral with side to side fluctuation, no IED	MRI, 24y: normal
PM23	6mo: normal background, brief generalised IED, no photosensitivity.	MRI, 7mo: normal
PM24	5m: Normal 9m: generalised spike-wave activity with myoclonic seizures elicited during photic stimulation, multifocal epileptiform activity	MRI, 2y: normal
PM25	6y: very active multifocal and generalised IED	MRI: normal
PM26	12m: minor asymmetrical background 2y: R-sided slowing 4y: multifocal IED, diffuse slowing	CT, 1y: normal MRI, 5y: normal
PM27	2y: infrequent focal IED from R central region, Ictal: SG sz beginning in the R posterior quadrant	MRI: normal
28/*SCN1A*+ surgical	10y: bifrontal discharges, L frontotemporal IED	MRI, 10y: L hippocampal sclerosis

Table 7.7 Serial electroencephalographic and imaging data.

Abbreviations: EEG, electroencephalography; IED = interictal epileptiform discharges; L = left; NA = not available; NCSE = non-convulsive status epilepticus; R = right; SG = secondary generalised; Sz = seizure; V-T = video-EEG telemetry; y = years.

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7.4.2 Neuroimaging findings

Brain imaging with or without light sedation was successful in all but four of the adults with DS. Most frequently, brain imaging was normal, or showed non-specific findings, including cerebral and cerebellar atrophy, or cerebellar atrophy alone (Fig. 7.2A). One adult with DS and *SCN1A* mutation (Case 21) had unilateral hippocampal sclerosis on MRI performed at 22 years (Fig. 7.2B). Evidence of the anterior thalamotomy performed at the age of 16 years was seen for Case 6 (Fig. 7.2C-D).

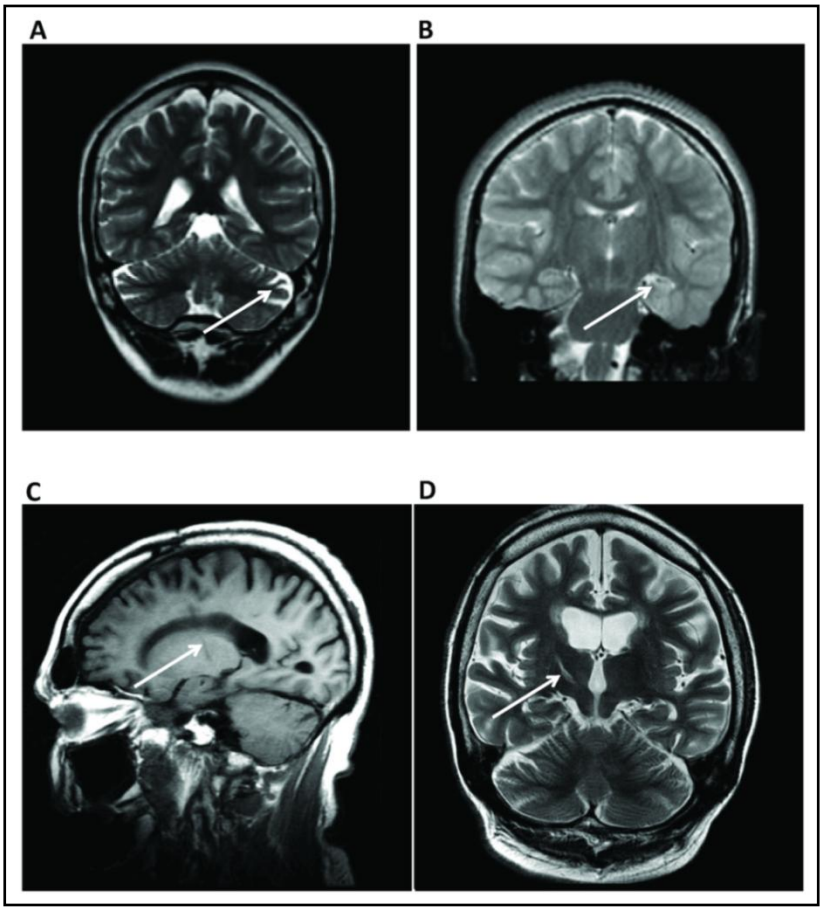

Figure 7.2 MRI brain findings in adults with Dravet and *SCN1A* mutation. Cerebellar atrophy (A, sagittal T1, case 6) was seen in some cases. Case 21 was the only adult with Dravet in our series with hippocampal sclerosis (left) evident on MRI (B, coronal T2). Case 6 had a stereotactic thalamotomy at 16 years (C, sagittal T1/D, coronal T2). Arrows show the location of the main abnormalities in each figure.

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7.4.3 Electroencephalographic findings

Serial electroencephalography (EEG) data were available for twenty-one adults with DS. At least one seizure was recorded with video-EEG for ten patients; seizure types recorded included tonic, focal motor, dyscognitive and secondarily generalised. Focal EEG features (Fig. 7.3A-D) were recorded in seventeen of the adult cases. Ictal EEG onset was maximal in the fronto-central regions in four cases (Fig. 7.3C)

Interictal EEG in all adult cases with DS showed slow background activity. For ten adults, childhood EEG data were available: four had one previous EEG in early childhood with generalised epileptiform discharges. No generalised epileptiform discharges were seen in the EEGs done in adulthood, but focal features were seen, including focal or multifocal interictal epileptiform discharges, or focal ictal discharges (Table 7.7).

Non-convulsive status epilepticus was documented on prolonged video-EEG monitoring in two patients, for whom subtle seizures with predominant impairment of consciousness had previously been confused with behavioural problems.

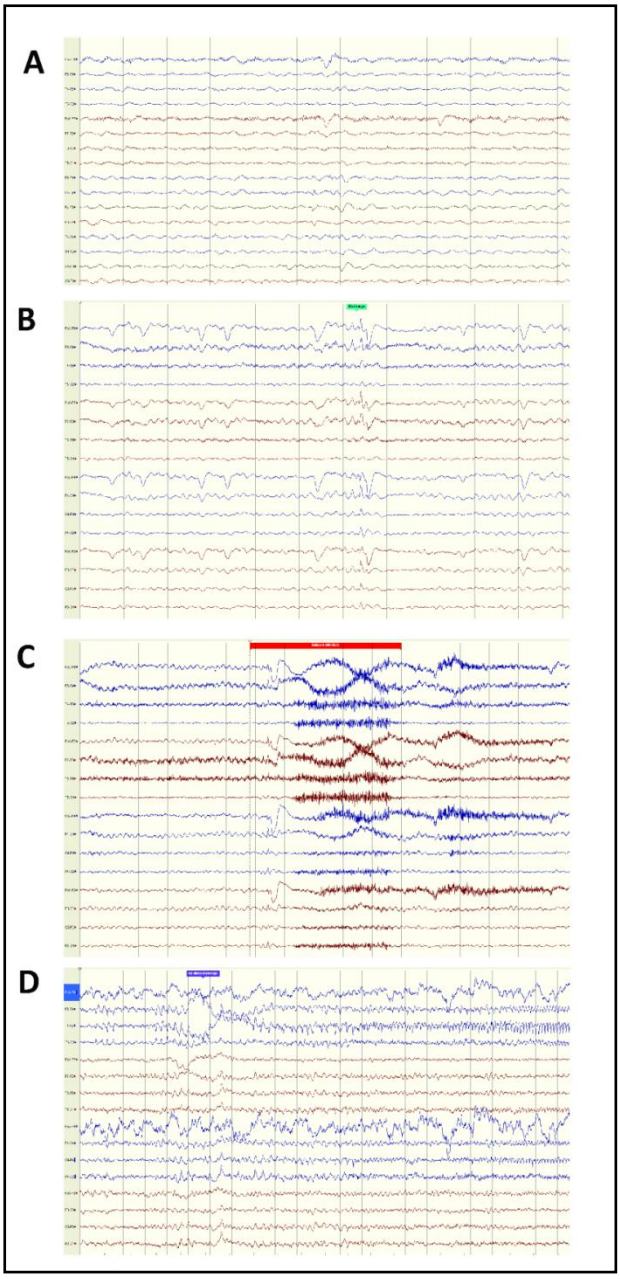

Figure 7.3 Scalp electroencephalographic findings in adults with Dravet syndrome. Routine EEG, Case 6: A (bipolar montage), slow background activity, 3-5 Hz, rare bi-frontal IEDs. Video-EEG, Case 5, 26 years: B (bipolar longitudinal), bihemispheric cortical dysfunction and bi-frontal IED; C (combined longitudinal and transverse bipolar), complex motor seizures with fronto-central EEG onset, non-lateralised; and D (bipolar longitudinal), electrographic seizures with right posterior temporal EEG pattern.

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7.4.4 Genetic findings

Twenty adults with DS had genetic analysis and *SCN1A* mutations were found in twelve (60%). The *SCN1A* mutations were all different (Table 7.8; Fig. 7.4) and all but one patient had novel mutations. One patient (Case 21) was found to have three *SCN1A* mutations, which to my knowledge had not been previously described in the literature.

For four adults with DS with a *SCN1A* mutation, both parents were available and agreed to be tested for the mutation found in the proband. In these four cases, the mutations were found to be *de novo*.

For one of the three adult postmortem DS cases (PM1), it was possible to extract DNA from the available frozen post mortem brain tissue and screen for *SCN1A* mutations. A novel missense mutation, c.677C>A, p.Thr226Lys was detected in exon 5 of *SCN1A*. This change results in the substitution of a highly-conserved, uncharged polar threonine residue with a charged polar lysine residue (Grantham distance: 78) at position 226. This amino acid falls within the S4 voltage sensing transmembrane region of domain 1 (D1) of the $Na_v1.1$ channel and as such is highly likely to be pathogenic. The change p.Thr226Lys had been previously unreported, but a different mutation in the same location, the p.Thr226Met mutation, had been reported in two unrelated cases, one with SMEB and the other with cryptogenic generalised epilepsy (Harkin et al., 2007).

DNA of adequate quality could not be extracted from the available formalin-fixed paraffin-embedded brain tissue for the other two adult cases (PM2 and PM3).

Of the four paediatric post mortem DS cases included in the study, two had an *SCN1A* mutation, one had a whole *SCN1A* gene deletion and one was not found to have a mutation but had not yet been checked for deletions. The two other paediatric cases, one surgical case with ICE-GTC and one post mortem case in the GEFS+ spectrum, both had *SCN1A* mutations previously documented (Table 7.8).

For two adult female patients without an *SCN1A* mutation or deletion, the screening of the *PCDH19* gene was also negative. Other genes known to be involved in DS or DS-like syndromes were not screened for mutations in this series.

ID	Nucleotide changes	Exon/ Intron	Mutation type	Inheritance	Amino acid change	Protein domain	Variation in the same position on the *SCN1A* variant database
PM1	c.677C>A	Exon 5	Missense	Nd (parents NA)	p.Thr226Lys	DI-S4	c.677C>T,p.Thr226Met, de novo (Harkin et al., 2007)
5	c.4913T>C	Exon 26	Missense	De novo (parents and one sister analysed)	p.Ile1638Thr	DIV-S4	none in that position; one c.4911_4914delGATC,p.I1638VfsX11 (Depienne et al., 2009b)
6	c.992delT	Exon 7	Truncating	Nd (no parent analysed)	p.Leu331X	DI-S5-S6	two: c.992dupT,p.Leu331fs, de novo; 992[T]993ins,L331fsX339 (Mancardi et al., 2006)
7	c.264+3delAGTG	Intron 1	Splice donor, deletion	Nd (no parent analysed)	p.?	-	one c.264+5G>A, de novo (Mancardi et al., 2006)
8	c.5639G>A	Exon 26	Missense	Nd (one parent analysed, mother negative)	p.Gly1880Glu	COOH terminal	none found in this position
9	c.3797A>C	Exon 19	Missense	De novo	p.Glu1266Ala	DIII-S2	none found in this position
10	c.603-2A>G	Intron 4	Splice site	De novo	p.?	-	none found in this position
11	c.4384T>C	Exon 23	Missense	De novo	p.Tyr1462His	DIII-S6	one c.4385A>G,p.Tyr1462Cys (Zucca et al., 2008)
19	c.2792G>A[a]	Exon 15	Missense	Nd	p.Arg931His	DII-S5-S6	(Löfgren & DeJonghe, 2010)
20	c.4568T>C	Exon 24	Missense	Nd (no parent analysed)	p.Ile1523Thr	DIII-DIV	none found in this position
21	c.80G>C; c.3749C>T; c.3706-2A >G[b]	Intron 18	Missense; missense; one splice acceptor	Nd (no parent analysed)	p.Arg27Thr; p.Thr1250Met; aberrant splicing (p.?)	N-terminal; DIII- S2; -	none found in this position; none found in this position; c.3706-2A>G, inheritance not determined (Singh et al., 2009; Löfgren & DeJonghe, 2010)

22	c.2717_2727delinsAC	Exon 15	In-frame deletion	Not determined (no parent analysed)	p.Val906_Met909delinsAsp	DII-S5	none found in this position
PM23	NA	Whole *SCN1A* gene	Whole *SCN1A* gene deletion	De novo	NA	NA	(Depienne et al., 2009b;Marini et al., 2009)
PM24	c.5536_5539delAAAC	Exon 26	Truncation	De novo	p.Lys1846fsX1856	COOH terminal	case reported in (Wallace et al., 2003) (Claes et al., 2001;Depienne et al., 2009b;Harkin et al., 2007;Kearney et al., 2006; Löfgren & DeJonghe 2010;Mancardi et al., 2006;Zucca et al., 2008)
PM25	IVS22-14T>G	Intron 22	Splice site	De novo	p.?	DIIIS5-S6	case reported in (Wallace et al., 2003)
PM27	c.4970G>A	Exon 26	Missense	De novo	p.Arg1657His	DIV-S4	case reported in (Deng et al., 2007;Harkin et al., 2007)
28/*SCN1A*+ surgical	c.652T>C	Exon 5	Missense	Inherited (mother and sister)	p.Phe218Leu	DI-S4	case reported in (Livingston et al., 2009)

Table 7.8 *SCN1A* mutations and structural variants identified in this study.

Abbreviations: del = deletion; ins = insertion; dup = duplication; NA = not applicable or not available; ND = not determined.
Intronic changes nomenclature: ex. c.xx+1G>C refers to the +1intron position following coding base xx, with +/- sign denoting the intronic 5'-beginning or 3'-ending, respectively. p.? denotes an unknown effect on the protein, an effect is expected but difficult to predict.
All mutations are novel, except: a) c.2792G>A, previously reported by Löfgren and DeJonghe, 2010; and b) c.3706-2A >G (Singh et al., 2009).
Source: *SCN1A* variant database, available at http://www.molgen.ua.ac.be/SCN1AMutations.
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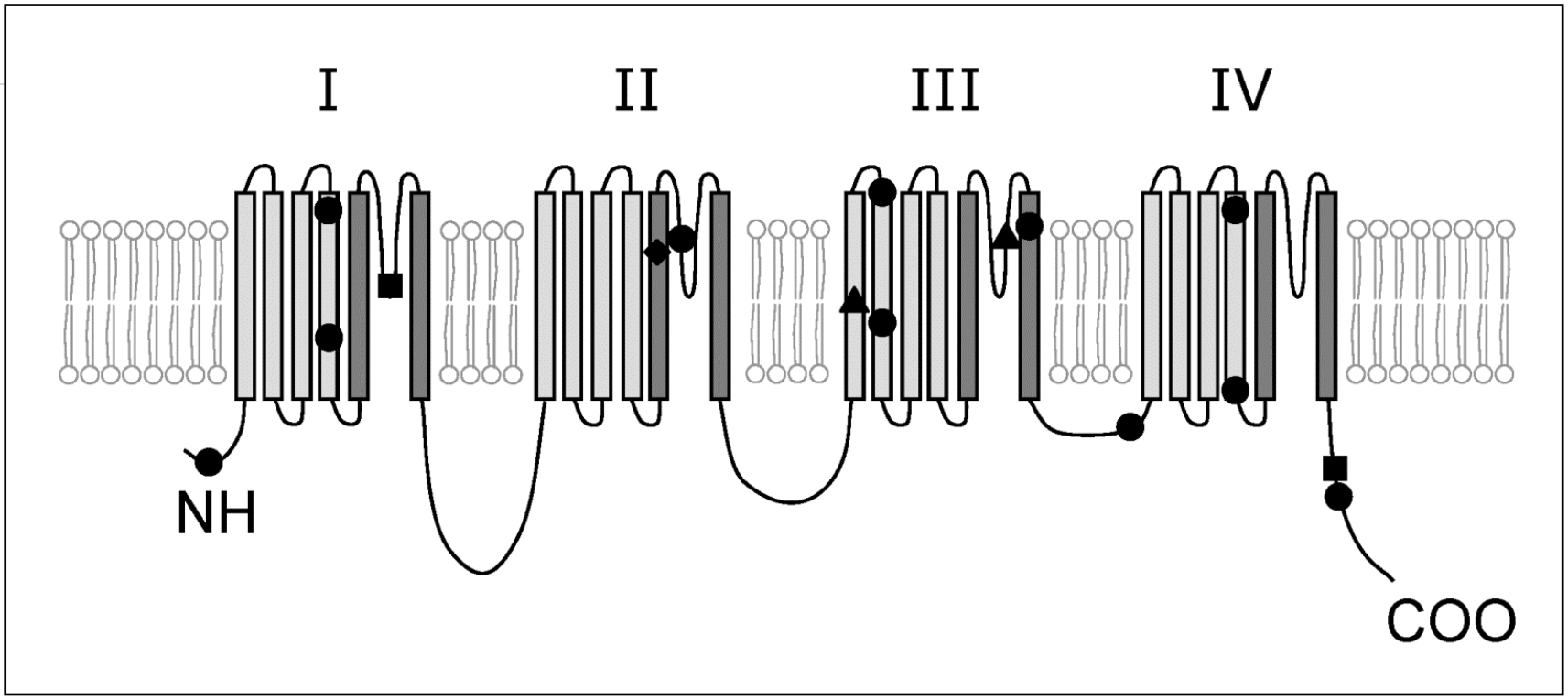

Figure 7.4 Schematic representation of the *SCN1A* mutations found in adults with Dravet syndrome in this study.

The protein has four domains, DI-DIV, each consisting of six transmembrane segments, S1-S6. Circle = missense; square = truncating; triangle = splice-site mutation; diamond = in-frame deletion. Positioning of the mutations within segments is approximate.
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SCN1A protein scheme adapted from Harkin, L.A., et al., The spectrum of SCN1A-related infantile epileptic encephalopathies", Brain, 2007, vol. 130, no. Pt 3, pp. 843-852, by permission of Oxford University Press (Harkin et al., 2007).

7.4.4.1 Genotype-phenotype analysis

Regarding the distribution of *SCN1A* missense mutations in this series, it is similar to previous descriptions in the literature (Zuberi et al., 2011); these are commonly located in the voltage sensor S4 - 2/2 children with GEFS+, 1/1 adult post mortem Dravet case with one missense mutation, 2/8 living Dravet adult cases with missense mutation(s); and the pore-forming S5-S6 and S6 segments of the SCN1A protein: 2/8 living Dravet adult cases with missense mutation(s) (Table 7.9).

Genotype-phenotype associations are summarised in Table 7.9. In the paediatric Dravet post mortem sub-group, no missense mutations were observed; in the adult Dravet deceased subgroup for whom genetic analysis was possible, 1/2 had an *SCN1A* missense mutation. Both children with GEFS+ phenotype had missense mutations. For the 17 adults living with DS tested, 8 had missense mutations (Table 7.9).

	Type of *SCN1A* mutation	Distribution of *SCN1A* missense mutations
Children with Dravet, death between 2 and 11 years (n=4, PM23-PM26)	Truncating - 1 Whole gene deletion - 1 Splice site - 1 No mutation, no result yet for deletion - 1[a]	No missense mutation found
Children with GEFS+, one alive, 12 years, one death at 5 years (n=2, 28 & PM27)	Missense - 2	S4 - 2
Adults with Dravet, death between 46 and 66 years (n=4, PM1-PM3 & 16)	Missense - 1 No mutation, no deletion - 1 No genetic analysis possible - 2[b]	S4 - 1
Adults with Dravet, alive, 20 to 60 years (n=18, Pts 4-15 & 16-22)	Missense - 8[c] Truncating deletion - 1 Splice site - 3[c] Insertion/deletion - 1 No mutation or deletion found - 7	S4 - 2 S5-S6 - 1 S6 - 1 Others - 4[c] – S2 - 2[c] – DIII-DIV - 1 – C-terminal - 1

Table 7.9 Genotype-phenotype analysis: *SCN1A* mutation type and distribution of *SCN1A* missense mutations.

Abbreviations: D = (*SCN1A* protein) domain; GEFS+ = genetic epilepsy with febrile seizures plus; S = (*SCN1A* protein) segment.
a For one child with Dravet, who died, the result was not available regarding the presence of deletion, after a negative mutation analysis.
b For two adults with Dravet, who died, it was not possible to perform genetic analysis on the post mortem material.
c Patient 21 had three *SCN1A* mutations found, two missense and one splice acceptor.

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7.4.5 Neuropathology

Macroscopic findings and results from histological and immunohistochemical studies are summarised in Tables 7.10 and 7.11, respectively.

7.4.5.1 Cerebral cortex

The cortex of the temporal, parietal and occipital regions showed normal cytoarchitecture, for all adult DS cases and PM controls.

The frontal cortex of two of the adult DS cases (PM1 and PM2) showed preserved architecture, with no neuronal cell loss, similar to the PM controls (Fig. 7.5A-B). In one adult DS case, PM3, the fronto-polar and frontal dorsal cortex presented a "micro-columnar" architecture, with exaggeration of the vertical alignment of cortical neurons (Fig. 7.5C), which did not fulfill criteria for focal cortical dysplasia type 1 (Blumcke et al., 2011).

a) IHC - Neuronal and interneuronal markers

Preserved cytoarchitecture of the frontal cortex and no neuronal loss, were shown with NeuN staining, CR, CB and PV staining and NPY (Fig. 7.6A-C), in the adult DS cases and PM controls.

b) IHC - Nav1.1-immunostaining

Nav1.1-immunostaining showed normal pyramidal cell count and distribution in the frontal cortex of the adult DS cases (Fig. 10A), PM controls and HS controls. A population of small, intensely-labelled Nav1.1 cells was seen in the lower cortical layers and white matter of the adult DS cases and PM controls (Fig. 7.7B). The number and

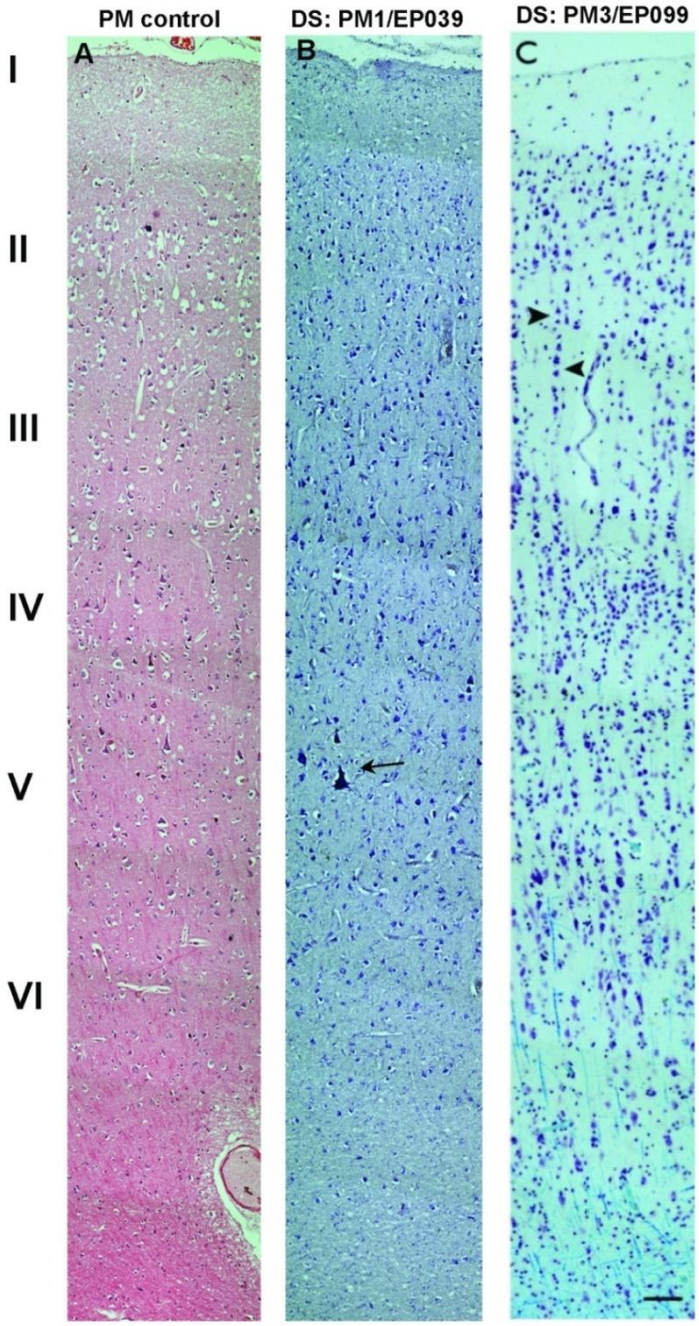

Figure 7.5 Frontal cortex: histological staining. (A) Normal frontal cortex from a PM control (H&E, 7 µm). (B) For the adult DS case PM1, CV staining of the motor cortex shows normal architecture, with good preservation of the cortical laminae and Betz cells (arrow). (C) In the adult DS case PM3, a focal "micro-columnar" appearance (arrowheads to columnar alignment; CV and LFB, 14 µm). Scale bar =100µm.

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location of these cells were similar between adult DS cases and PM controls or HS controls (Fig. 10C). These cells are likely inhibitory (GABAergic) cells, as shown by double-labelling with GAD, NPY and PV (Fig. 7.7D-F).

c) IHC - Connexin and inflammatory markers

The distribution and morphology of the Cx43-, GFAP- and HLA-DR-immunopositive cells in the frontal cortex of the adult DS cases were similar to the PM controls (Fig. 7.6). In both HS controls, HLA-DR immunopositive cells in the frontal cortex were larger, more intensely-labelled and formed clusters (Fig. 7.6).

d) IHC - Vascular markers

The number, appearance and distribution of vWF-immunopositive blood vessels were similar for the adult DS cases, PM controls and HS controls (Fig. 7.6).

ID	Macroscopic findings (brain weight post-fixation, g)	Cortex: frontal, parietal, temporal and occipital	Hippocampus, amygdala, thalamus, basal ganglia	Cerebellum	Brainstem and cervical spinal cord	Cause of death (age at death, in years)
PM1	Cerebellar atrophy, with preferential involvement of anterior lobe and vermis (1331)	Normal	Normal	Loss of Purkinje cells	Myelin loss in dorsal columns of spinal cord	Bronchopneumonia + recurrent NCSE (46)
PM2	Mild cerebellar atrophy; discolouration and loss of periventricular white matter; old fronto-basal contusion (1100)	Focal periventricular white matter and myelin loss	Normal	Mild Purkinje cells loss	Myelin loss in dorsal columns of spinal cord	Bronchopneumonia (66)
PM3	Cerebellar atrophy (1380)	Fronto-polar, dorsal frontal and occipital cortex, with "micro-columnar" architecture	Normal	Loss of Purkinje cells	Normal	SUDEP (46)
PM23	Normal. Some leptomeningeal congestion (1273)	Normal	Mild bilateral endfolium hippocampal gliosis. No mossy fibre sprouting.	Mild patchy gliosis but no discernable Purkinje cell loss.	Normal brainstem. Cord not available	SUDEP (2)
PM24	Normal (1062)	Frontal and occipital cortex: normal	Hippocampus (one side): no sclerosis, CA1 hyperconvoluted.	Purkinje cells preserved. Mild vacuolation of white matter noted.	Normal	SUDEP during a 46 degree Celsius day (10)
PM25	Swollen brain with herniation (1300[b])	Frontal and temporal: widespread ischaemic neurons. No MCD or evidence of chronic atrophy	Not all subfields available for histology. CA1 shows acute neuronal changes but no evidence of chronic sclerosis	Acute injury of Purkinje cells superimposed on mild chronic loss	No malformation. Ischaemic neurons noted in medulla	SUDEP (11)
PM26	Swollen brain (1245[b])	Frontal and temporal:	No sclerosis (mild	Autolytic changes; no	No histology	Global ischaemic brain

		No MCD; no atrophy	endfolium gliosis)	evidence of chronic atrophy		injury (11)
PM27	Leptomeningeal congestion and uncal grooving (1266)	Frontal cortex : normal architecture but pan cortical necrosis and reactive changes consistent with cerebral infarction of 10 days	Hippocampus (one side): no evidence of chronic HS; acute anoxic changes to end-folium neurons	Autolytic changes but no evidence of atrophy/Purkinje cell loss	Normal	Convulsive status epilepticus (5)
28/ *SCN1A*+ surgical[a]	Not applicable	Normal temporal neocortex	Pyramidal cell loss in left hippocampus	Not applicable	Not applicable	Not applicable
Control 1	Modest dilatation of lateral ventricles, L hippocampal formation signficantly smaller than right (1156)	Normal	Pyramidal cell loss in the left hippocampus	Loss of Purkinje cells	Normal	SUDEP (49)
Control 2	Not available	Cell loss in upper cortical layers of parietal and temporal cortices	Pyramidal cell loss in both hippocampi	Loss of Purkinje cells	Normal	Pulmonary oedema (74)
Control 3	Normal (1185)	Normal	Normal	Normal	Normal	Cardiac arrest (36)
Control 4	-	Normal	Normal	Normal	Normal	Not available (58)
Control 5	Normal (1540)	Normal	Normal	Loss of some Purkinje cells	Normal	Not available (57)

Table 7.10 Summary of neuropathology findings: macroscopic findings and results histological staining (H&E, LFB and CV).

Abbreviations: HS = hippocampal sclerosis; L = left; MCD = malformation of cortical development; NCSE = non-convulsive status epilepticus; SUDEP = sudden unexplained death in epilepsy.
a For the *SCN1A*+ surgical case, only the resected hippocampus and temporal neocortex were available for study.
b Pre-fixation brain weight (no post-fixation brain weight available for these cases).
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Antibody		Antigen	Type	Source	Working dilution
Anti-calbindin	CB	CaBP calbindin	polyclonal	Swant, Switzerland	1:10,000
Anti-calretinin	CR	CaBP calretinin	polyclonal	Sigma, USA	1:2,000
Anti-parvalbumin	PV	CaBP parvalbumin	polyclonal	Swant, Switzerland	1:6,000; 1:3,000
Anti-connexin 43	Cx43	connexin 43	monoclonal	Zymed, USA	1:150
Anti-von Willebrand Factor	vWF	von Willebrand Factor	monoclonal	Chemicon, USA	1:1,000
Anti-dynorphin	AbD	dynorphin	polyclonal	Serotec, UK	1:25
Anti-glial fibrillary acidic protein	GFAP	Intermediate filament glial fibrillary acidic protein	polyclonal	Dako, Denmark	1:1,500
Anti-human leucocyte antigen-DR	HLA-DR	human leucocyte antigen-DR	monoclonal	Dako, Denmark	1:100
Anti-neuronal nuclei	NeuN	Neuronal nuclear protein	monoclonal	Chemicon, USA	1:100

Anti-neuropeptide Y	NPY	neuropeptide Y	polyclonal	Sigma, USA	1:5,000
Anti-sodium voltage channel isotype 1.1	$Na_v1.1$	sodium voltage channel isotype 1.1 (461-481, intracellular loop between DI and DII)	polyclonal	Alomone Labs, USA	1:50
anti-alpha-synuclein	-	alpha-synuclein	monoclonal	Novocastra, USA	1:50
anti-AT8	AT8	phosphorylated tau protein	monoclonal	Innogenetics, Belgium	1:1,200
anti-neurofilament	-	neurofilament	monoclonal	Dako, Denmark	1:500
anti-ubiquitin	-	ubiquitin	polyclonal	Dako, Denmark	1:1,200
anti-glutamic acid decarboxylase	GAD	glutamic acid decarboxylase	monoclonal	Chemicon, USA	1:1,000

Table 7.11 Antibodies used in the immunohistochemistry studies of Dravet syndrome.

Abbreviations: CaBP = calcium-binding proteins.

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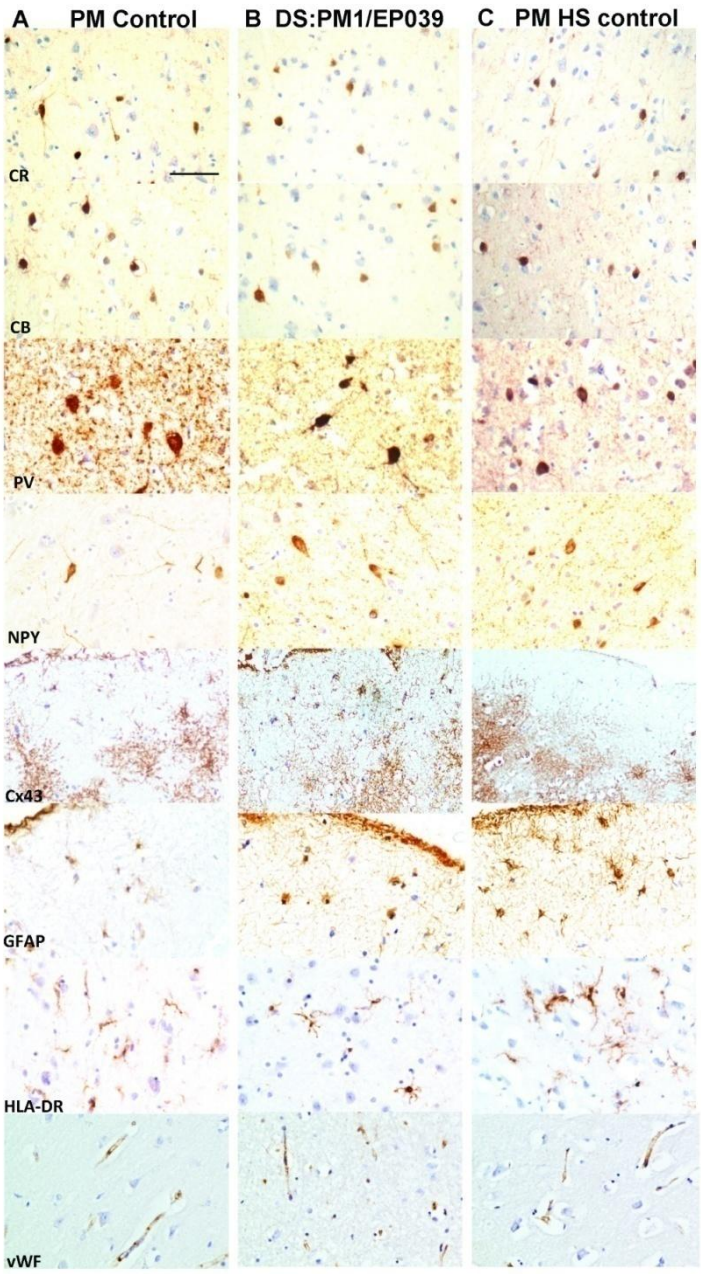

Figure 7.6 Frontal cortex: immunolabelling.

The distribution and morphology of the immunolabelled cells, for a panel of interneuronal, inflammatory and vascular markers, were not significantly different between post mortem controls (A), adult DS cases (B) and HS controls (C).
The images for Cx43 and GFAP are taken from layer I and for all other markers from layers II and III. Scale bar = 50µm.

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		NeuN	$Na_v1.1$	CR	CB	PV	NPY	GFAP	HLA-DR	Cx43	vWF	Dynorphin
PM1	Hippocampus	+	+	+	+	+	+	+	+	++	+	+
PM2		+	+	+	+	+	+	++	+	++	+	+
PM3		+	+	+	+	+	+	+	+	++	+	+
28/*SCN1A*+ surgical[a]		*loss	+	+	+	*loss	++	++	++	++	+	++
Control 1		*loss	+	+	+	+	++	++	++	++	+	+
Control 2		*loss	+	+	+	+	++	++	++	++	+	++
PM1	Cerebral cortex	+	+	+	+	+	+	+	+	+	+	nd
PM2		+	+	+	+	+	+	+	+	+	+	nd
PM3		+	+	+	+	+	+	+	+	+	+	nd
28/*SCN1A*+ surgical[a]		+	+	+	+	+	++	+	+	+	+	nd
Control 1		+	+	+	+	+	++	+	+	+	+	nd
Control 2		*loss	+	+	+	+	++	++	+	+	+	nd
PM1	Brainstem and cervical spinal cord	nd	nd	+	+	+	nd	nd	nd	nd	nd	nd
PM2		nd	nd	+	+	+	nd	nd	nd	nd	nd	nd
PM3		nd	nd	+	+	+	nd	nd	nd	nd	nd	nd
28/*SCN1A*+ surgical[a]		nd[a]	nd[a]	nd[a]	nd[a]	nd[a]	nd[a]	nd[a]	nd[a]	nd[a]	nd[a]	nd
Control 1		nd	nd	+	+	+	nd	nd	nd	nd	nd	nd
Control 2		nd	nd	+	+	+	nd	nd	nd	nd	nd	nd
PM1	Cerebellum	*loss	+	+	*loss	+	+	++	++	+	+	nd
PM2		+	+	+	+	+	+	+	+	+	+	nd
PM3		*loss	+	+	*loss	+	+	++	++	+	+	nd
28/*SCN1A*+ surgical[a]		nd[a]	nd[a]	nd[a]	nd[a]	nd[a]	nd[a]	nd[a]	nd[a]	nd[a]	nd[a]	nd
Control 1		*loss	+	+	*loss	+	++	+	+	+	+	nd
Control 2		*loss	+	+	*loss	+	+	++	++	+	+	nd

Table 7.12 Summary of immunohistochemistry results for the adults with Dravet syndrome, HS controls and *SCN1A*+ surgical case. Abbreviations: (++) increased, (+) similar, or (-) decreased immunolabelling compared to PM controls. *loss= cell loss; nd = not done.
a For the *SCN1A*+ surgical case, only the resected hippocampus and temporal neocortex were available for study.

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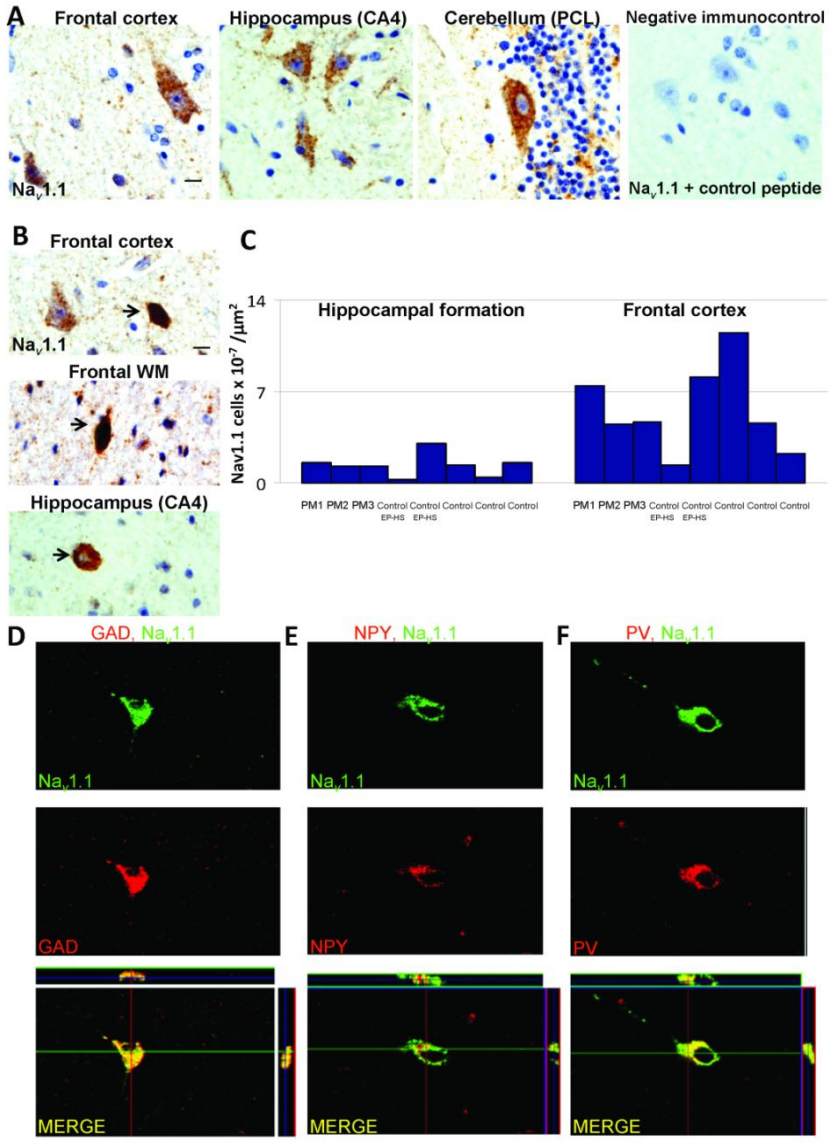

Figure 7.7 Na$_v$1.1-immunolabelling in frontal cortex, hippocampus and cerebellum. (A) Na$_v$1.1-immunoreactivity is seen in the cytoplasm of pyramidal cells in frontal cortex and hippocampus and of cerebellar Purkinje cells, in the adult DS cases. (B) A number of small, intensely-labelled Na$_v$1.1-immunopositive cells (arrows) are found in the frontal lower cortical layers and white matter and in CA4, but not in the cerebellum. (C) The number of small, intensely-labelled Na$_v$1.1-immunopositive cells in frontal cortex and hippocampus is not markedly different between adult DS cases, HS controls and post mortem controls with no known neurological disease. (D-F) Double-labelling shows these intensely-labelled Na$_v$1.1 cells co-express GAD (D), NPY (E) and PV (F). Scale bars = 10µm (A-B). CA, cornu Ammonis; PCL, Purkinje cell layer; WM, white matter.

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7.4.5.2 Hippocampus

The hippocampi of the adult DS cases showed neuronal preservation in all subfields, similar to the PM controls (Fig. 7.8A-B), in contrast with the neuronal loss and granule cell dispersion seen in the HS controls (Fig. 7.8C) and the *SCN1A*+ surgical case (Fig. 7.8D). This was confirmed by stereological quantification of CV-stained pyramidal cells in CA1 and CA4 (Fig. 7.8E). Only one paediatric DS case, PM23, showed mild bilateral endfolium gliosis (Table 7.10).

The interneuronal populations in the hippocampi, investigated using CB, CR, PV and NPY, showed normal appearance and localization, similar to the PM controls (Fig. 7.8E). Quantification of the interneurons (2D cell counts) in CA1 and CA4 was normal (Fig. 7.8F).

The amygdala, thalamus and basal ganglia were normal in the adult DS cases.

a) IHC - Neuronal and interneuronal markers

There was neuronal preservation in the hippocampi of the adult DS cases, with normal number and distribution of CR-, CB-, PV- and NPY-immunopositive cells, similar to PM controls. Immunoreactivity for dynorphin, a marker of mossy fibre sprouting (Thom et al., 2009b;Vezzani et al., 1999), was not seen in the molecular layer for the adult DS cases and PM controls. Both the HS controls and *SCN1A*+ surgical case showed neuronal loss in CA4 and mossy fibre sprouting (Fig. 7.9A-C).

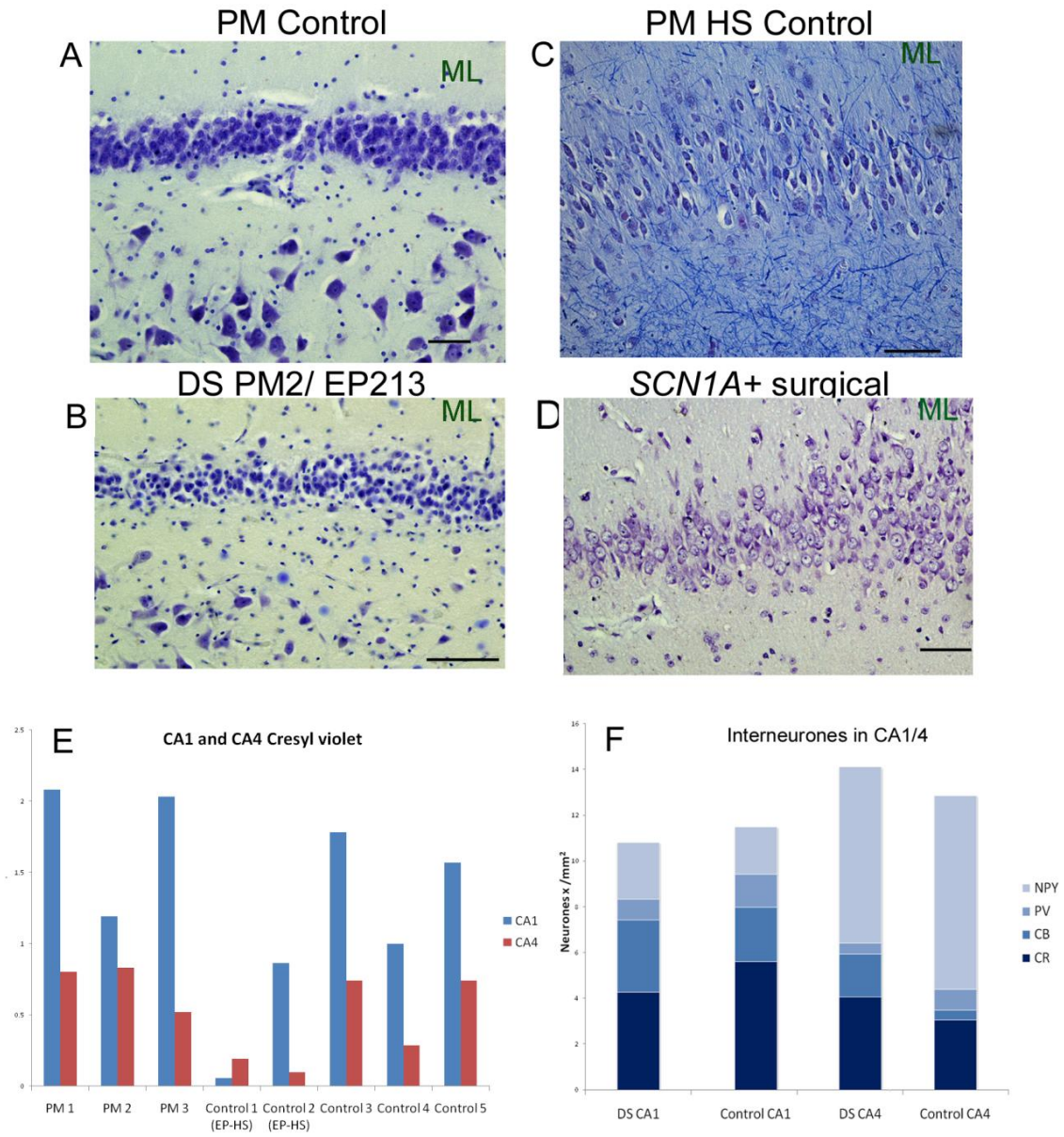

Figure 7.8 Hippocampus: histological staining and interneuronal cell counts.

The CV-stained hippocampus from post mortem controls (A) and the adult DS cases (B) are normal, while the HS control (C) and *SCN1A*+ surgical case (D) show pyramidal cell loss in CA4 and granule cell dispersion. (E) Stereological quantification of CV-stained neurons shows lower numbers of pyramidal cells in CA1 and CA4 for HS controls (Control 1-2 EP-HS) compared to adult DS cases (PM1-PM3) and post mortem controls with no known neurological disease (Controls 3-5). (F) Areal 2D counts of CB, CR, PV and NPY-immunopositive cells in CA1 and CA4 show a similar average number of hippocampal interneurons in the adult DS cases and post mortem controls with no neurological disease.
Scale bar = 50µm. ML, molecular layer.

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b) IHC - Nav1.1-immunostaining

$Na_v1.1$ labeling was seen in pyramidal cells and granule cells in the hippocampi, in the adult DS cases (Fig. 7.7B) and PM controls. A population of small intensely-labelled $Na_v1.1$-immunopositive cells was seen throughout the hippocampi of the adult DS cases, as in the PM controls (Fig. 7.7C).

c) IHC - Connexin and inflammatory markers

In contrast to the PM controls, where no Cx43-immunoreactivity was detected in the hippocampus (Fig. 7.9A), the adult DS cases had Cx43-immunopositive cells in CA4 and the granule cell layer border - this was similar to the findings of the HS controls (Fig. 7.9B-C) and the *SCN1A*+ surgical case. Scattered GFAP-immunopositive cells and some HLA-DR-immunopositive microglial cells, were seen in the hippocampi of the adult DS cases and the PM controls (Fig. 7.9A-B). In contrast, the HS controls and *SCN1A*+ surgical case showed GFAP-immunopositive cells, a dense matrix of GFAP-immunopositive fibres (Fig. 7.9C) and larger and clustered HLA-DR-immunopositive cells in the hippocampus (Fig. 7.9A-C).

d) IHC - Vascular markers

The number, appearance and distribution of vWF-immunopositive blood vessels were similar for the adult DS cases, PM controls, HS controls (Fig. 7.9A-C) and *SCN1A*+ surgical case.

e) IHC - Markers of neurodegeneration

The adult DS cases showed rare AT8-immunopositive neurons in the hippocampi (Braak stages 0-2). No neuronal inclusions or plaques were seen and all other markers of neurodegeneration showed similar labeling for the adult DS cases and PM controls.

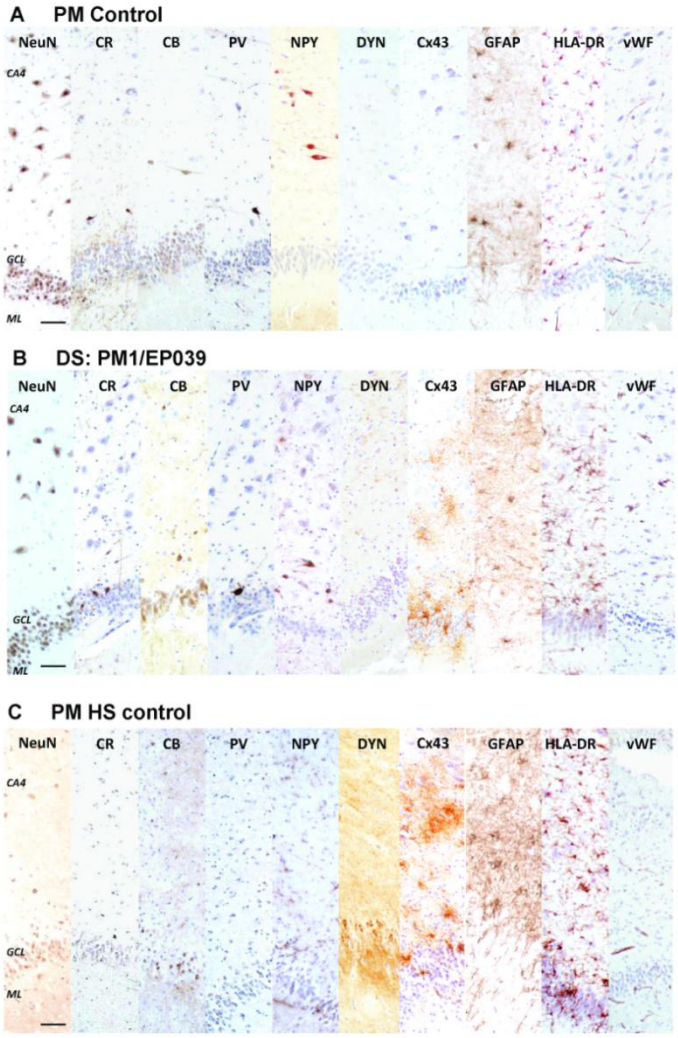

Figure 7.9 Hippocampus: immunolabelling with interneuronal, inflammatory and vascular markers.

The distribution and morphology of NeuN, CR, CB, PV and NPY-immunopositive cells in the hippocampus are similar between post mortem controls (A) and DS cases (B), while loss of these cells is seen in the HS controls (C). Dynorphin immunoreactivity is intense in the molecular layer of the HS controls, but not in the DS cases or post mortem controls. Cx43 immunoreactivity is higher in the hippocampus of the DS cases and HS controls, as compared to the post mortem controls with no neurological disease. Immunoreactivity to GFAP, HLA-DR and vWF is not different between DS cases and post mortem controls.

Scale bars = 50µm. CA = cornu ammonis, GCL = granule cell layer, ML = molecular layer.

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7.4.5.3 Cerebellum

Cerebellar atrophy was evident in the adult DS cases (Fig 7.7; Table 7.10) and also in one PM control (Control5) and both HS controls.

a) IHC - Neuronal and interneuronal markers

Focal loss of CB- and PV-immunopositive Purkinje cells and dendrites was seen in two adult DS cases, PM1 and PM3 (Fig. 7.10A-B), one PM control (Control 5) and both HS controls. For the other adult DS case, PM2, there was occasional loss of CB- and PV-immunopositive cells. CR-immunopositive cells were preserved in the Purkinje and granule cell layers for the adult DS cases and PM controls. NPY-immunoreactivity was not seen in the cerebellum in the adult DS cases or PM controls.

Nav1.1-immunopositive Purkinje cells were seen in all adult DS cases (Fig. 7.7A), as in PM controls, while no small intensely-labelled Nav1.1-immunopositive cells were seen in the cerebellum of the adult DS cases or PM controls.

b) IHC - Connexin and inflammatory markers

A few Cx43-immunopositive cells in the molecular layer and GFAP- and HLA-DR-immunopositive cells in the granule cell layer and white matter, were seen for adult DS cases and PM controls, with similar distribution and appearance.

c) IHC - Vascular markers

vWF-immunoreactivity was normal in the adult DS cases, similar to the PM controls.

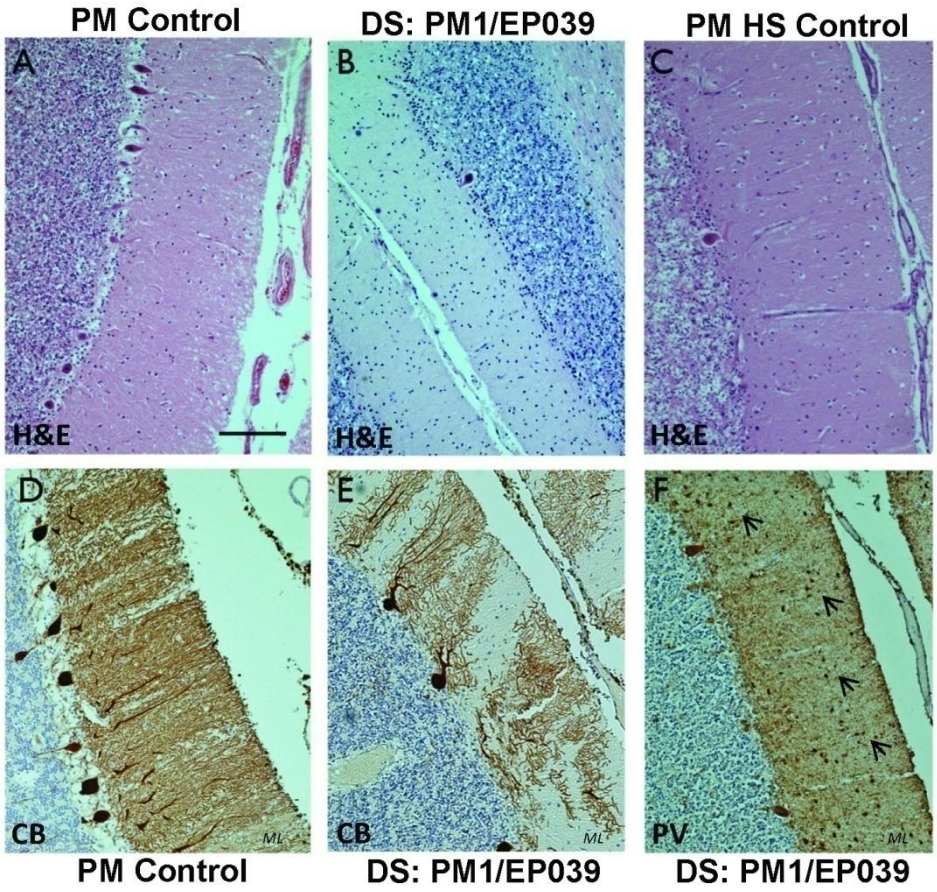

Figure 7.10 Cerebellum: histological staining and immunolabelling.

(A) H&E shows a normal cerebellum of a PM control. Purkinje cell loss in the cerebellum of the adult DS case, PM1 (B) and HS control (C). Purkinje cells and their processes normally extend into the molecular layer as seen in D for a PM control, is evident in CB- and PV- immunolabelled cerebellar sections from the adult DS case, PM1 (E, F). Small, PV-immunopositive cells are observed in the cerebellar molecular layer of the adult DS cases (F, arrows) and PM controls (not shown).

Scale bar = 100µm. ML = molecular layer.

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7.4.5.4 Brainstem and cervical spinal cord

The brainstem of the adult DS cases did not show significant pathology, except for loss of myelin in the dorsal columns of the medulla and cervical spinal cord of the adult DS cases, PM1 and PM2 (Fig. 7.11A; Table 7.11), with focal macrophage infiltration (Fig. 7.11B) and axonal swelling (Fig. 7.11C-D). Both these DS cases had dysphagia and ataxia. The immunohistochemistry studies of the brainstem, including CR, CB, PV; GFAP; ubiquitin, alpha-synuclein and non-phosphorilated neurofilaments, were normal.

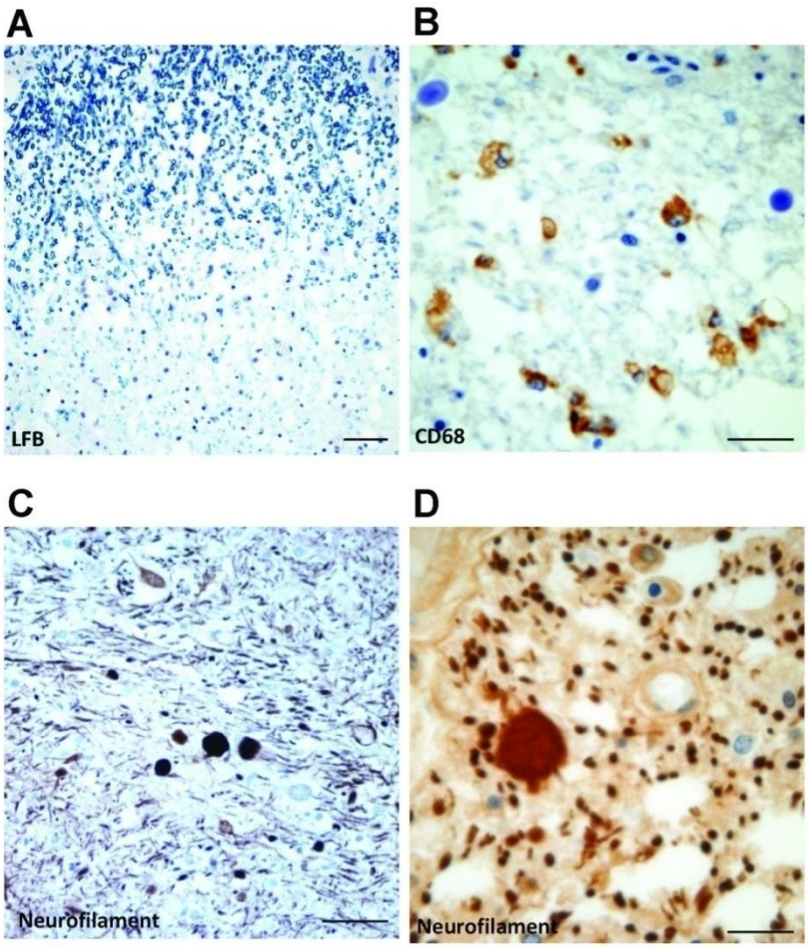

Figure 7.11 Brainstem (medulla) and cervical spinal cord: histological staining and immunolabelling.

For the adult Dravet cases PM1 and PM2, an area with myelin pallor is seen in the dorsal columns of the medulla (A, LFB), with infiltration of CD68-immunopositive macrophages (B, CD68); some axonal swelling is seen in the cervical spinal cord (C-D, neurofilament). Scale bar = 50µm (A,C); 25µm (B,D).
In: Catarino, C.B., et al., Dravet syndrome as epileptic encephalopathy: evidence from long-term course and neuropathology, Brain, 2011, vol. 134, no. Pt 10, pp. 2982-3010, by permission of Oxford University Press (Catarino et al., 2011b).

7.5 Discussion

DS is an important epilepsy syndrome, being amongst the first genetic epilepsy syndromes for which the molecular basis has been unravelled, enabling functional studies and animal models to reveal fundamental insights into the underlying pathophysiology (Catterall et al., 2008).

DS is thought to be underestimated in prevalence and underdiagnosed in adults (Scheffer et al., 2009). There are many gaps in the understanding of the clinical evolution of DS in later ages, particularly after the fourth decade of life, as for many years DS has been considered to be of the remit of the child neurologist. As children with DS were prospectively followed, it became clear that some did reach adulthood (Dravet et al., 2005). More recently, adult patient series have been characterized (Akiyama et al., 2010;Jansen et al., 2006), but the great majority of adults were under 35 years at last follow-up. Surviving adults over 35 with DS may have not been diagnosed, given that the syndrome was only described thirty years ago (Dravet 1978) and the diagnosis is often not considered in the adult neurology clinics.

This series shows that diagnosis even late(r) in life, in patients previously labelled as having drug-resistant epilepsy with intellectual disability of unknown cause, can carry important implications for affected patients: rational treatment changes can be instituted with possible benefit, even after years of drug resistance. In addition, recognition of the changes in language, cognition, swallowing and gait and determining whether specific patterns exist, may help to improve diagnostic and prognostic information and may reinforce a mandate for treatment changes.

DS is an important example of the value of study of an apparently rare epilepsy and the value of clinical acumen in syndrome discovery and clinical diagnosis.

Twenty-two adult DS patients were identified who had not been diagnosed in childhood. Two-thirds were over 39 years at last follow-up, a greater proportion than for other studies to date (Table 7.1). Two DS adult cases reached their sixties; survival to the seventh decade had not been previously reported.

This is not a systematic evaluation of the prevalence of DS or *SCN1A* mutation in adults with severe epilepsy, but an observational study of a highly-selected patient group from a tertiary referral centre. Together with the very detailed clinical records available and the neuropathology evaluation, this provided a unique opportunity for a study on the long–term follow-up and outcome of adult patients with DS.

7.5.1 Long-term evolution

Features of DS in adults include drug-resistant seizures, with a seizure repertoire that differs from that in childhood. Atypical absences and generalised interictal epileptiform discharges seen in childhood were not documented in this adult series. In many of the adult patients in this series, the predominant seizures are nocturnal, with focal semiological features and sometimes secondary generalisation; focal onset was often documented on ictal EEG. This concurs with the findings of Akiyama et al. (2010), whose recent series of adult DS showed 35/40 apparently generalised seizures had frontal origin, with or without secondary generalisation in the ictal EEG.

Another conclusion is that DS may be found in older and younger adults and is a diagnosis that needs consideration in this group, because it has management implications. That unexpected longevity is possible further mandates efforts at earlier diagnosis and prompt effective treatment.

Although long life is possible, long-term functional, seizure-related, cognitive and social outcomes appear unfavourable, with cognitive and physical decline, gait disturbance and later dysphagia, incontinence and increasing dependence for all activities of daily life. It is not possible to predict at this time how earlier recognition and treatment might influence these outcomes.

Dysphagia has emerged as a shared dysfunction in older people with DS. This is a novel observation in DS and not a feature of other chronic epilepsies, except some of the progressive myoclonic epilepsies, epilepsies associated with cerebrovascular disease and Lennox-Gastaut “syndrome” (Ogawa et al., 2001). Dysphagia may manifest with unexplained cough, or recurrent respiratory infections, which may lead to neurological deterioration and weight loss. Notably, for homozygous null *Scn1a*–/– knock-out mice, manual feeding extends survival (Yu et al., 2006). Awareness and early diagnosis of dysphagia may prevent complications, which include worsening of seizure control, poor nutrition and fluid intake, poor quality of life and life-threatening aspiration pneumonia. The neuropathological basis of the dysphagia is unclear, though visible changes in the brainstem were noted in two patients with DS and dysphagia.

The neuropathology of human DS has not been previously well characterized. This is the first systematic neuropathological study in DS. It included three adults and

four paediatric post mortem DS cases and two other *SCN1A*-mutation carrying paediatric cases with other syndromes. Several findings are of interest.

Seizure freedom was not attained in any of the adults, but seizure control was significantly improved in the three cases with sufficient follow-up after specific post-diagnosis AED changes, with use of appropriate drugs and withdrawal of others previously described as worsening control (such as lamotrigine, carbamazepine, vigabatrin (Guerrini et al., 1998;Perucca et al., 1998), phenytoin and oxcarbazepine (Table 7.5)), which may have different effects on different seizure types in Dravet syndrome. Even if the patient had had drug-resistant seizures for many years, the suppression of at least one seizure type was possible for at least several months, as also shown in a recent report (Akiyama et al., 2010). For the oldest living patient, at 60 years, rational drug changes proved possible once the clinical diagnosis, with confirmation from molecular genetics (which was important in this case given the lack of literature on long-term features of DS), gave carers confidence in such AED changes. A previous AED change had led to status epilepticus and strong reluctance to entertain further changes. Subsequent drug changes led to significant benefits, even after 60 years of drug-resistant seizures: convulsive seizures were controlled and the patient began speaking again for the first time for over five years.

Patients with DS often have autism-like behavioural features and autism spectrum disorder has been associated with seizures in the first year of life (Saemundsen et al., 2008). In a recent report, the neuropathological examination of one Dravet paediatric case, who died of SUDEP, showed multifocal micronodular dysplasia of the left temporal cortex and bilateral endfolium gliosis (Le Gal et al., 2010). No other subtle malformation,

as reported in abstract form by Hayashi et al. (2004), was found in this series. In one of the adults with DS, there was an exaggerated columnar architecture, or radial alignment of neurons involving frontal and occipital regions. This patient had a history of autistic spectrum disorder. Although studies in autism have also described abnormalities of cortical minicolumns (Casanova et al., 2010), neuropathological data in DS remain very limited; this is an observation, but general conclusions cannot be drawn from a single case.

7.5.2 Hippocampal sclerosis in Dravet syndrome

One of the 22 adults with DS and a *SCN1A* mutation had unilateral hippocampal sclerosis (HS) on an MRI brain scan performed in his 20s. His previous MRIs were not available for review. The *SCN1A*+ surgical case had unilateral HS.

A few previous studies have shown that in a small proportion of patients with DS and *SCN1A* mutations, HS is observed (Striano et al., 2007b) and this may not be present in the early childhood scans (Siegler et al., 2005). Also in GEFS+ patients with *SCN1A* mutations HS has been described (Bonanni et al., 2004). Prospective MRI studies in DS are required.

It is of note that even on quantitative analysis, there was no neuropathological evidence of neuronal loss in the post mortem adult DS cases, showing that DS per se and *SCN1A* mutation (one post mortem adult DS case), are not sufficient to cause hippocampal neuronal loss despite decades of drug-resistant seizures and recurrent episodes of status epilepticus.

Rarely, significant clinical and imaging changes have been reported in DS following status epilepticus (Chipaux et al., 2010;Sakakibara et al., 2009;Tang et al., 2011). There may be age-dependent vulnerability of the brain to injury induced by seizures (Haut et al., 2004), but it is difficult to separate out effects of seizures on the brain from the effects of the disease process per se and the effects of drugs and other factors.

It has been suggested that *SCN1A* mutation may protect hippocampal neurons (Auvin et al., 2008), but more research is needed to determine whether (and which, if any) *SCN1A* mutations (or other causes of DS) are actually neuroprotective and it should be noted that DS is not primarily a hippocampal epilepsy.

7.5.3 Genotype-phenotype analysis

Genotype-phenotype analyses are often complex (Kanai et al., 2009;Scheffer 2011;Zuberi et al., 2011). Caution is required in interpretation and more so in selected series.

No single clinical characteristic in this series allowed the distinction between *SCN1A* mutation-positive and mutation-negative adult cases, but the numbers are small for subgroup comparisons.

Considering the type of *SCN1A* mutations in the two extremes of age at death, a pattern may seem to emerge: in the four children with Dravet who died early, there were no missense mutations (Table 7.9); of the patients who died after age 45, out of the two in whom genetic analysis was possible, one had one *SCN1A* missense mutation and the other was found not to have a *SCN1A* mutation or deletion. No truncating mutations were found

in this group. Comparing with published data, there seem to be more missense than truncating *SCN1A* mutations in the older Dravet group.

Limitations include possible ascertainment bias, selection bias, small numbers and predominance of paediatric cases in published data, but one could hypothesise that missense mutations are more frequent in patients with longer survival, testable with a prospective longitudinal study.

7.5.4 Neuropathology of Dravet syndrome

Overall, no histopathological hallmark of DS was identified in this study. A striking finding was the preservation of neurons and interneurons, within the hippocampus and the cerebral cortex, despite decades of medically refractory poorly-controlled seizures. The paediatric post mortem cases showed extensive changes, but these were compatible with their agonal states. Therefore, in neither adult nor paediatric post mortem cases, at the levels examined with the blocks available for study, were there any pathological changes to explain the observed cognitive developmental arrest or decline.

No significant alterations were found in distribution and morphology of inhibitory interneuronal subsets in cerebral cortex, hippocampus, cerebellum or brainstem, in the adult DS cases. The findings of the quantitative analysis were similar to those of the post mortem controls with no neurological disease. The prevalence of small, intensely-labelled $Na_v1.1$-immunopositive cells was not different in adult post mortem DS cases and post mortem controls. This structural normality does not exclude putative functional

abnormalities in any of these cell types or their interactions, as reported for the mouse models of DS (Ogiwara et al., 2007;Yu et al., 2006).

The clinical association between seizures and febrile episodes was not underpinned by significant evidence of persistent excessive neuroinflammatory pathology in the DS cases. Connexin-43 (Cx43), GFAP and HLA-DR immunoreactivities in the frontal cortex were not different between adult post mortem DS cases and controls. In the hippocampus, higher numbers of Cx43-immunopositive cells in adult post mortem DS cases and HS controls were observed, compared to post mortem controls with no neurological disease, where no Cx43-immunolabelling was seen in the hippocampus. Previous studies have suggested that the upregulation of Cx43 in MTLEHS may facilitate seizure propagation (Fonseca et al., 2002;Kielian 2008). GFAP and HLA-DR immunoreactivities were similar between adult post mortem DS cases and controls with no neurological disease, in contrast with a greater expression in HS post mortem controls. In the cerebellum, Cx43-immunoreactivity was similar between adult post mortem DS cases and controls (low immunoreactivity). The immunoreactivity of GFAP and HLA-DR is mainly observed in the granule cell layer and white matter of adult post mortem DS cases and controls, with higher immunoreactivity in the molecular layer of cases, with loss of Purkinje cell and processes.

Cerebellar atrophy was a frequent finding in DS cases but did not differ, either in pattern or distribution, to that previously described in patients with chronic epilepsy without DS (Crooks et al., 2000). The exact mechanism of selective Purkinje cell loss, as well as the potential relationship to observed ataxia, requires further study. In contrast to a

previous post mortem report in a child with DS (Renier & Renkawek 1990), no cerebellar dysplasia was seen in any case in this series.

Vacuolar demyelinating myelopathy of the dorsal columns of the cervical cord was noted in two DS patients. This is not a typical finding in patients with epilepsy and although a toxic or metabolic cause remains possible, future studies in DS may elucidate whether this is frequent in DS. It is of interest that ataxia can be observed in DS. More data are required to establish whether the vacuolar myelopathy is related to it and whether such myelopathy could be prevented by better seizure control or modulation of $Na_v1.1$ function. Interestingly, $Na_v1.1$ channels are expressed in white matter astrocytes (Black et al., 1994) in close relationship with oligodendrocytes (Waxman & Black 1984).

7.5.5 Dravet syndrome as epileptic encephalopathy

DS has been considered an "epileptic encephalopathy" in the ILAE classification (Engel, Jr. 2001) and a syndrome carrying higher risk of epileptic encephalopathy in the 2010 organisation proposal (Berg et al., 2010). Controversy exists as to whether the seizures and interictal discharges themselves are responsible for the cognitive decline (Dravet et al., 2005).

The data presented in this study show DS is at least in part an epileptic encephalopathy. The neuropathology study has not shown any consistent cerebral structural abnormalities, neuronal loss or neurodegeneration; and clinically, even after many decades of drug-resistant seizures, medication changes may improve seizure control

and be associated with some cognitive improvement, with a positive impact on the quality of life.

7.5.6 Limitations of the study

Limitations of this study include that the fact that, although there is some longitudinal data, it is a cross-sectional study. It is, therefore, not possible to fully disentangle what is the natural history of DS and what may relate to chronic effects of AEDs or other factors. The initial description of the Unverricht-Lundborg disease, a progressive myoclonic epilepsy, for example, included a progressive neurological deterioration, but this was found later to be due in large part to the use of phenytoin (Eldridge et al., 1983); and avoidance of this antiepileptic drug has meant improved outcomes and life expectancy may indeed approach normal (Kalviainen et al., 2008).

DNA of sufficient quality was not possible to retrieve from two of the three adult post mortem cases, because no frozen tissue was available. Furthermore, most other genes previously implicated in DS or DS-like epilepsy syndromes were not screened in this study. Moreover, there may be more causal genetic variants involved in the aetiopathology of DS, which are yet to be discovered.

The numbers of post mortem cases are small. Neuropathological analyses with electron microscopy were not possible, as no appropriately fixed material was available. The pathological components of this study are cross-sectional; it was possible, however, to show that the neuropathological substrate, at least at the levels examined, appears largely intact.

7.5.7 Next steps

Long-term follow-up of newly-diagnosed infants and children with DS, who are appropriately treated, is necessary to determine formally whether effective control of seizures and interictal discharges prevents encephalopathy and other co-morbidities (Scheffer et al., 2009), including cognitive decline and additional features reported in this study and in the literature.

Prospective MRI studies in DS are also required. These studies would benefit from collaboration between groups, to increase the size of the study cohorts, as this will increase the power of the studies.

A promising next step in the genetics research of Dravet syndrome is the use of next generation sequencing in *SCN1A*-negative DS patients, to look for novel candidate genes (Mefford et al., 2011a).

8 Chapter Discussion and conclusions

8.1 Summary of the main findings

8.1.1 Genome-wide association study of partial epilepsy

The results of stage one/discovery phase of the GWA of partial epilepsy include in the list of top SNPs, a SNP intronic to *SCN1A* (rs54331), with a *p*-value of 4.6×10^{-5}. Although not reaching the threshold of genome-wide significance, this signal is nevertheless interesting to follow-up in a larger cohort, given the biological plausibility of association, with the prior knowledge of contribution of *SCN1A* to susceptibility to seizures (reviewed in Chapter 1) and the results of the GWA study of MTLEHS.

The sample size should be increased, a replication phase planned and a meta-analysis performed. If this is a true signal, then *SCN1A* could be found to be a susceptibility factor for common partial epilepsies and this warrants more research.

It is important to note that these studies have not included generalised epilepsy, which means conclusions cannot be drawn about generalised epilepsy, for which the GWA methodology is also promising, but which are outside the remit of the studies presented in this thesis. Recently, the result of the discovery phase of a GWAS on idiopathic generalized epilepsies was published (Steffens et al., 2012) and interestingly the top findings also include a common SNP close to *SCN1A*, although this signal did not reach genome-wide significance and has yet to be replicated.

8.1.2 Genome-wide association study of mesial TLE with HS

In the well-powered multicentre genome-wide association study of MTLEHS, "borderline" genome-wide statistically significant evidence was found for association between three SNPs intronic or located close to the *SCN1A* gene and MTLEHS, MTLEHS with a personal history of FS, but not MTLEHS without antecedents of FS.

The result of stage one/discovery phase showed an association with SNPs intronic or close to *SCN1A*, with "borderline" genome-wide significance, *p*-values between $1x10^{-7}$ and $5x10^{-7}$ (Panagiotou and Ioannidis 2012), which means it is suggestive of true association, especially when taking into account the prior knowledge of the involvement of *SCN1A* in the epilepsies, including familial MTLE.

This is a robust and promising first step, which now requires follow-up work. A plus of this work was the phenotyping, with classification of each patient's epilepsy in the cohort into specific ILAE epilepsy syndromes performed in a stringent way: when classification was not possible or unclear with the available data, the patient was not included in the study.

The findings need to be interpreted with caution, however, and additional data are required. Definite conclusions will follow only after the replication efforts in an independent collaborative sample. Meta-analysis of all available data will be then a necessary next step.

It is possible that:

a) *SCN1A* is associated with susceptibility to developing epilepsy, i.e. increased susceptibility to recurrent seizures;

b) *SCN1A* is associated with susceptibility to developing MTLEHS;

c) Another gene, close to *SCN1A*, may be the one truly associated;

d) *SCN1A* may be associated with febrile seizures, independently of whether or not the person has MTLEHS (the current study is underpowered to answer this question) or MTLE;

e) A sub-syndrome of MTLEHS (MTLEHS with FS) may be the one associated with *SCN1A* and not necessarily all MTLEHS;

f) *SCN1A* may predispose to febrile seizures, which could then lead to HS and MTLE;

g) *SCN1A* may predispose to a spectrum of seizure susceptibility, from febrile seizures alone, to febrile seizures plus, to MTLEHS, or other epilepsy syndrome, depending on modifier genes and environmental factors and their interactions;

h) More than one of these options may be possible at the same time.

Importantly, an association found in a GWA study between a genetic marker and a common trait or disease, even if genome-wide significant, does not prove causality and the leap from association to causality requires more research.

A possible role of febrile seizures in the association found between the *SCN1A*-associated SNPs and common MTLEHS was analyzed. A trend was found in favour of a possible role of febrile seizures when associated with MTLEHS. On the other hand, when the analysis was limited to the partial epilepsies excluding MTLEHS, with or

without febrile seizures, despite higher numbers, no association was found. This discovery phase of the GWA study is underpowered for definite conclusions on the role of febrile seizures mediating or confounding the association of MTLEHS and *SCN1A* and this question should be addressed with further studies, for definitive conclusions.

There are alternative possible scenarios to explain a role of febrile seizures in a probable association between genetic variants leading to increased susceptibility and MTLEHS.

a) The phenotype of febrile seizures may add to the specificity of the diagnosis of MTLEHS and therefore be a marker of the higher specificity in phenotyping leading to better power to find a true association;

b) It is possible that the trigger of the febrile seizure in early childhood is required to set off the mechanisms of epileptogenesis that later will lead to temporal lobe epilepsy in genetically predisposed individuals;

c) Febrile seizures may just be an age-specific clinical expression of susceptibility to seizures in the patients already predisposed to the MTLEHS syndrome (Annegers et al., 1987;Berg and Shinnar 1996).

8.2 Study limitations

GWA studies require genome-wide statistical significance at *p*-values below 5 x10^{-8} and large-scale replication efforts (Chanock et al., 2007;Ioannidis & Khoury 2011).

A study looking at evidence of association in the stage one/ discovery phase of GWA studies with "borderline" significance, was able to show that possibly the stringency of the currently adopted significance threshold could be relaxed (Panagiotou and Ioannidis 2012).

An association between MTLEHS (MTLEHS with FS) and three SNPs intronic or close to the *SCN1A* gene was found, with "borderline" genome-wide significance, *p*-values between 1 x 10^{-7} and > 5 x 10^{-8} (Panagiotou and Ioannidis 2012).

Specifically for the GWAS of MTLEHS (MTLEHS with FS), the knowledge that the *SCN1A* gene has been previously robustly associated with several "Mendelian" epilepsies and febrile seizures, should inform our interpretation.

To conclude, the findings of this study are suggestive of a true association between MTLEHS (MTLEHS with FS) and three SNPs close or intronic to the *SCN1A* gene, but will only be conclusive after the replication phase, with the underlying biology pointing to an increased *a priori* chance this is in fact a true association.

8.2.1 Potential limitations and methodological challenges

Limitations of the GWA approach include the modest effect sizes of the common genetic susceptibility variants and the need for stringent statistical thresholds (Zeggini et al., 2008).

The sample size in the studies described in this thesis may not be large enough. An important strategy to increase power for such studies to detect smaller effect loci is to increase the sample size, by increasing the number of cases and or controls in the analysis.

8.3 Next steps

8.3.1 Imputation

Another way to increase power is to extend SNP coverage through imputation of untyped SNPs, which is an in silico method of inferring missing genotypes, increasing the number od markers available for association testing. Genotype imputation is used in the analysis of GWA studies to increase power and fine-map associations (Marchini et al., 2007;Marchini & Howie 2010). Imputation also has an important role for the combination of results using meta-analysis (Marchini and Howie 2010).

8.3.2 Validation and replication in GWA studies

Before the GWA era, association studies used small samples, were underpowered to detect loci of realistic effect size and there were “over-liberal declarations of association” in the literature, which contributed to only a few of the claimed associations proving to be

real. This history and the high dimensionality of GWA studies, with "vulnerability" to errors and biases and modest anticipated effect sizes explain why replication is so essential in evaluating GWA findings- The aim of replication is to determine which of the findings arising from the primary GWA reflect true reproducible associations. Efforts at replication should focus not only on the signals for which the statistical evidence is strongest, but also on an efficient identification of additional susceptibility loci with more modest effect sizes but with biologically functional candidacy (McCarthy et al., 2008).

Robust replication is essential for the rigorous documentation of proposed associations in GWA studies. For the purpose of replication, the recommended approach is to examine the genetic variant of interest for association in diverse data sets, using the same analysis model (Ioannidis et al., 2009). The next step is, therefore, to pursue replication in adequately sized independent panels, for confirmation of the association signals found. Collaboration with other groups will be required to achieve this.

There may be differences in frequency of the causal genetic variant or differential interaction with environmental factors in different populations. To look for replication in other populations is important, in order to test whether the signals can be generalised across different populations (Ioannidis et al., 2009).

Extending the GWA to populations of non-European origin is an important step forward in this type of research. The GWA studies described in this thesis have focused on European-descent samples. In the future it would be relevant to extend large-scale discovery efforts to non-European populations, studying a wider range of ethnic groups, with cohorts who represent more diversity, both genetic and environmental. Expectations

may be that these studies could reveal additional population-specific loci, or have better power to detect loci, because of differences in allele frequency or differences in linkage disequilibrium patterns between populations influencing the odds for discovery (McCarthy 2008;McCarthy and Hirschhorn 2008).

8.3.3 Meta-analysis

In the search for additional association signals, meta-analysis of GWA data has proven to be an effective method to overcome the limitations of power that may compromise one individual study, as larger datasets improve power to detect loci of modest effect (Ioannidis et al., 2009;McCarthy and Hirschhorn 2008). Meta-analysis has proven to be effective, having yielded many additional risk loci for several common diseases, such as ulcerative colitis (Anderson et al., 2011a) or type 1 diabetes and coeliac disease (Wang et al., 2010) and many other common diseases.

Analysis of more specific and accurate phenotypes, instead of grouping all different forms of partial epilepsy as was done for the GWA study of partial epilepsy, may be expected to encompass less phenotypic and genetic heterogeneity. A possible attempt to solve this could be to include in the GWA analysis only specific epilepsy syndromes, as has been done in the GWA study of MTLEHS. Other possibility would be to include only the "idiopathic" and "cryptogenic" epilepsies, excluding the "symptomatic" epilepsies. In fact, it is possible that the inclusion of the "symptomatic" epilepsies with "idiopathic" and "cryptogenic" epilepsies may have contributed to a dilution of a possible true signal, increasing the risk of false negatives. The rationale for excluding the "symptomatic" epilepsies would be an attempt to try to decrease the heterogeneity expected from multiple diverse putative aetiologies. Given the smaller

numbers, power will necessarily be more limited when considering subgroups and therefore an important next step would be to increase numbers of the chosen subgroups, repeat the analysis and perform a meta-analysis on all available data.

In the 2010 proposal for revised terminology and organization concepts of seizures and epilepsies (Berg et al., 2010), the "epilepsies of unknown cause", previously called "cryptogenic or possibly symptomatic", are singled out as "the most fertile area for future research in (…) genetics" and the authors further state that "among these poorly differentiated epilepsies are likely to be additional genetic electroclinical syndromes" (Berg et al., 2010).

In order to increase specificity and accuracy in phenotyping in the cohort of MTLEHS, a possible next step could be to try to identify subsets of more clinically homogeneous MTLEHS patients, by use of quantitative measures, with reproducible and reliable quantification methods. This could encompass neuropathologically-proven hippocampal sclerosis cases only; or use of quantitative imaging measures, always in combination with the required strict electroclinical criteria, thereby defining the cases included in the GWAS of MTLEHS according to the most accurate and specific definition, while minimizing possible heterogeneity. However, numbers would be smaller and collaboration with more centres necessary, in order to achieve sufficient power.

8.3.4 Collection of more febrile seizures data

To collect more febrile seizures data on patients with MTLEHS and with other partial epilepsies, would increase the power to disentangle the role of FS in the genetic associations found. There was significant missing data on FS in some of the cohorts, which did not allow sufficient power to achieve definitive conclusions with this analysis.

Information was not available for the vast majority of patients on whether the FS were simple, complex/prolonged, or febrile status. It is known that the risk of developing epilepsy after simple FS is only mildly elevated in relation to the population risk (Annegers et al., 1987;Berg and Shinnar 1996;Nelson and Ellenberg 1976;Shinnar 2003;Verity and Golding 1991), while a history of prolonged FS or febrile status is associated with substantially increased risk of future epilepsy (Annegers et al., 1987;Berg and Shinnar 1996;Shinnar 2003). This means that analyzing the data on the characteristics of FS in these cohorts and classifying each patient with FS into either simple or prolonged FS could be expected to add important information and an increase in power would follow, by increasing the specificity of the relevant phenotyping.

A proportion of the controls used in the GWA studies was submitted to a health questionnaire as a selection procedure. Data were not available on whether they were specifically questioned on previous history of febrile seizures, acute symptomatic seizures, childhood epilepsy, or epilepsy in remission. This methodology involves a potential for recall bias, with the retrospective nature of the data collection. This means that a few controls may have been misclassified in this regard. This number is probably not significant, however and if it had any effect, it would be of diluting the signal and not of creating false positives.

In diabetes research, the effects of inherited variation in insulin action on glucose metabolism may be masked by the capacity of the normal pancreas to compensate (O'Rahilly 2009). For unstable phenotypes and genetic influences influencing seizure threshold, a similar thing could theoretically happen, if other compensatory mechanisms are at work, thereby "masking" the ability to find one such signal with this methodology. This may be an issue with genomics research of seizure susceptibility.

8.3.5 Common disorders and Mendelian diseases

In some common disorders, some genes found to be associated with "common" disease had already been known to cause "monogenic" forms of disease, for example, Parkinson's disease and the genes *SNCA* and *LRRK2* (Klein & Ziegler 2011).

A parallelism can be drawn with the GWA study of MTLEHS (MTLEHS with FS), which shows a suggestive association with SNPs close or intronic to *SCN1A*, a gene previously identified as causal for monogenic forms of epilepsy and febrile seizures. This adds not only to the biological plausibility, but also to the credibility of the data and the methodology (Klein and Ziegler 2011).

This points to "Mendelian" and "complex" forms of disease being not fundamentally different, but part of a spectrum of disease, sharing some genetic susceptibility factors.

8.3.6 Common and rare genetic variation in the "common" diseases

To explain the genetic architecture of the common disorders, the "common disease-common variant" model and the "common disease-rare variant" model have been proposed. The "common disease-common variant" model defends common genetic variation underlies the genetic susceptibility to common diseases, while, for the "common disease-rare variant" model, rare genetic variants are thought to contribute significantly to the susceptibility of common diseases.

These models are not mutually exclusive and both common and rare genetic variation probably contribute to susceptibility to the common epilepsies. An example of the relevance of rare variation for the common epilepsies is the large recurrent copy number variants in 15q13.3, 16p13.11 and 15q11.2, established as important risk factors for "common" epilepsy.[15]

A "shifting paradigm" (Gorlov et al., 2008) also to pursue the study of the role of rare variants in common disorders should actively be followed, as its importance becomes more evident (Gorlov et al., 2011). Also in the "Mendelian", "familial" disorders, there should be a demand for a shift in the approach, to include the study not only of rare variants, but also of common variation, particularly at regulatory sequences (Chakravarti & Kapoor 2012).

[15] For a more in-depth discussion of the role of copy number variation in the "common" epilepsies, see also Chapter 6.

In support of this approach for the genetic studies of the common diseases, is recent work in Crohn's disease, with next-generation sequencing of loci found by GWAS to be associated with susceptibility for the disease; the authors identified independent rare, some novel, genetic variants contributing to the risk of disease, confirmed by replication in an independent case-control study (Rivas et al., 2011).

8.4 Future work

Future directions for follow-up studies after the results of the discovery phase of the genome-wide association studies may include the studies described below.

8.4.1 Fine mapping and sequencing replicated top hits

To track down causal genetic variation after finding an association between a genetic variant and the phenotype of interest, an important step is to fine map and sequence the replicated top SNP loci. Imputation based on sequencing data may boost true GWAS signals and enable fine mapping of causal variants (Shea et al., 2011).

8.4.2 Analysis of gene-gene and gene-environment interactions

Environmental factors may have an effect modification on the interaction between the genetic variant and phenotype. A recent example is, for example, a study on stroke genomics, where population dietary folate and serum homocysteine were shown to modify the association between the *MTHFR* genotype and risk of stroke (Holmes et al., 2011).

8.4.3 Gene expression studies, expression quantitative trait locus analysis

Another important approach integrates genome-wide data on sequence variation with global transcript profiling information, studying the genetic basis of variation in expression levels. Gene expression can be used as a quantitative phenotype in the GWA study, which can help ascribe functional annotation to the associated loci (McCarthy and Hirschhorn 2008) and to identify genetic loci that control quantitative variation in gene expression, known as eQTLs (Geschwind & Konopka 2009).

8.4.4 Looking for "missing heritability"

For many complex traits or diseases, the variance accounted for by the genetic variants highlighted by GWA studies as being associated with the disease or trait, is only a fraction of the total genetic variance calculated from family studies and that is the gap behind the expression "missing heritability" (Maher 2008;Manolio et al., 2009).

There are many challenges around genomic studies of the "complex" diseases, as multiple genes with (supposedly) modest effect sizes will be involved and many reasons for the missing heritability have been given (Manolio et al., 2009). The "missing heritability" will be in rare genetic variants, not well captured by the existing GWA study platforms. It will also be in common alleles of minor effect, which are still difficult to detect with GWA methodology and the currently available sample sizes and resulting low power: genetic variants of small effect sizes may need substantially larger sample sizes to be detectable.

Among the other challenges to be faced, gene-gene interactions and gene-environment interactions are still largely ignored in data analyses. Epigenetic factors may play a role; and also disease heterogeneity may play a role: "what if the "disease" under study is actually dozens, hundreds, thousands of different diseases that all look the same?" (Manolio et al., 2009). Furthermore, the full effect of a gene may not be captured, for example if the gene effect is maximized in a certain age window of an individual (Rao & Gu 2008).

Definitive measures of the heritability of a "complex" disease may, however, be "unattainable", as "the precise extent to which inherited factors determine inter-individual differences in risk varies between populations and over time" (Manolio et al., 2009) and even in highly heritable conditions, non-genetic factors will also play a role (O'Rahilly 2009).

GWA studies have applications other than discovery of individual SNPs associated with common diseases: they can be used to identify other genetic variants associated with common diseases, such as copy number variants; to identify genetic variants associated with quantitative traits; to rank the relative importance of previously identified susceptibility genes – example, ApoE*ε4 in Alzheimer's disease (Coon et al., 2007); to demonstrate gene-gene interactions, or modification of the association of one genetic variant by another; to detect high-risk haplotypes; to identify SNPs associated with gene expression (Pearson and Manolio 2008).

8.4.5 Copy number variation

Genome-wide discovery of large copy number variants (CNVs) has been facilitated by advances in two technologies - array comparative genomic hybridization and SNP genotyping platforms (Cooper et al., 2011).

Dense SNP genotype data can be used to detect copy number variants and to evaluate their association to disease by SNP-based whole-genome association studies (McCarroll et al., 2006). Other methods to study copy number variation (Mulley and Mefford 2011) include Multiplex Ligation-dependent Probe Amplification (MLPA) (Mei et al., 2007;Schouten et al., 2002); array-Comparative Genomic Hybridization (array-CGH) (Galizia et al., 2012;Striano et al., 2012); and next generation sequencing (Cirulli & Goldstein 2010).

8.4.6 Methylation studies

Epigenetics studies the heritable factors, which can be transmitted to progeny cells during cell division but are not directly attributable to the DNA sequence. These include DNA methylation, which plays an important part in gene control and other mechanisms affecting the chromatin environment of a gene, such as histone modifications leading to alterations in chromatin structure, thereby influencing gene expression (Strachan and Read 1999).

Studies in animal models of MTLE suggest that epigenetic mechanisms may influence the interactions between genetics and environment during epileptogenesis (Kobow & Blumcke 2011), making it worthwhile to research its role in epilepsy. Huang

et al. (2002) showed increased expression of a transcriptional repressor factor, NRSF/REST, in the pilocarpine model of status epilepticus, with downregulation of mRNA and protein levels of the glutamate receptor GluR2. Tsankova et al. (2004) showed, in a kainate-induced status rat model, that inhibition of acetyltransferase activity of CREB-binding proteins suppressed histone hyperacetylation at gene promoter regions in the hippocampus, with a concomitant decrease of epilepsy severity. A review on epigenetic studies in epilepsy discussed the evidence for abundant DNA promoter methylation in human specimens from patients with TLE compared to controls (Kobow et al., 2009).

Further, epigenome-wide association studies are a promising tool for use in the epilepsies (Rakyan et al., 2011).

8.4.7 From association to causality

After finding an association between a disease or trait and one SNP corresponding to a particular locus in the genome, finding the genes associated with increased susceptibility to the disease or trait is the next step. It is still a major challenge to identify the few phenotypically causal variants among the many variants present in the human genome and this will be even more so for whole-genome and whole-exome sequencing results, especially for “complex” diseases or traits (Cooper & Shendure 2011).

8.4.8 Functional studies

Research efforts must be undertaken into the pathophysiology mechanisms through which the identified genetic variants influence disease risk. This will require multidisciplinarity, with contributions from cell biology, biochemistry, animal models, electrophysiology, and others (O'Rahilly 2009).

The challenge is to go from involved genetic variant to a better understanding of the pathogenesis of the epilepsies. Functional studies and animal models (knock-out, knock-in) will be key for translation of the genetic findings into more knowledge of the pathogenic pathways involved, with a view to translation into practical clinical use.

Combination of electrophysiological and anatomical phenotypes is required to relate molecular pathways operating at the synapse to cellular function and, subsequently, to complex circuits (Geschwind and Konopka 2009). Specific phenotypes probably result from cumulative effects or interactions of a few or several genes - the identified one may be a player among many. This may be the case not only for complex epilepsies but also in the "Mendelian" epilepsies. Recent studies suggested that defective protein trafficking and protein-protein interactions may modulate the effect of a mutation and underlie phenotypic variation in epilepsies related to channelopathies (Rusconi et al., 2007). The diversity of mutations that may cause similar phenotypes argues for points of physiological convergence giving rise to network hyperexcitability, which is then able to selectively cause specific phenotypes (Avanzini & Noebels 2009).

The induced pluripotent stem cells (iPSCs) technology allows neuronal cells to be generated from skin fibroblasts; this is a novel tool (Takahashi & Yamanaka 2006), which allows the assessment of effects of mutations in the context of all other genomic variants

present in the patient's genome. Furthermore, it allows electrophysiological recording from neuronal cells containing tissue-specific accessory proteins and splicing factors, otherwise not available in other test systems (Meisler et al., 2010).

8.4.9 Animal studies

The development of animal models will continue to help to research the pathophysiology mechanisms underlying neuronal hyperexcitability caused by genetic variation identified in the epilepsies.

It has been shown that the background genetics of a knockout strain can have a profound influence on any observed phenotype. This highlights the need to consider the role of individual genetic background in animal models of epilepsy (Schauwecker 2011).

There is functional heterogeneity among mutant ion channels and a complex relationship between clinical and biophysical phenotypes. There is no known unifying mechanism that would explain how the spectrum of observed functional effects of different epileptogenic mutations relates to the epilepsy syndrome seen in patients. Development of animal models and multielectrode array technology, will be useful in identifying epileptogenic networks able to selectively cause specific phenotypes. This is essential for a deeper understanding of epileptogenesis and may be helpful in designing novel therapeutic strategies (Avanzini and Noebels 2009).

8.5 Future translation to clinical practice: from GWA study to clinical application

The achievements of the past few years with the GWAS methodology have been substantial for many common diseases and quantitative traits, but much remains to be done.

After the results of the GWA study for any common disease or trait, initially only association signals, it is crucial to discover which gene(s) indeed predisposes to disease. Rarely a causal variant is revealed, sometimes it is not possible to say which is the causal gene.

Other important questions include if and how genes interact with each other to modify risk of disease; what proportion of disease is due to these variants; whether patients can be stratified on the basis of their genotype; and also, what is the role of epigenetics and of environmental factors (Bowcock 2007).

The work of moving from association signal to causal variant and from causal variant to understanding the molecular and cellular underpinnings of disease, is crucial for the translation of the GWAS results into clinical practice.

The expectation is that the biological insights will ultimately lead to new therapies, biomarkers and disease prevention. GWAS information can be used to support both development and use of pharmaceutical agents, for example, through the identification of mechanisms involved in the generation of adverse events.

8.5.1 Risk assessment

An important focus of research is on how to use the knowledge of association between genetic variants and disease to more practical uses, such as risk assessment, but there are many challenges ahead (Wei et al., 2009). Now that the cohort sizes for some diseases have reached the hundreds of thousands, with several dozens of common genetic variants associated, for example breast cancer (Michailidou et al., 2013), it appears to be closer the possibility of risk assessment from the knowledge of which common variants someone carries.

8.5.2 Changes in disease classification

More definite results of the GWA studies of the epilepsies and subsequent follow-up studies, are expected to have implications for the classification of the epilepsies, as molecular genetic findings are expected to help in tackling this important translational question. In its most recent classification proposal, the ILAE Commission on Classification and Terminology states that it will continue to pursue an incorporation of the new concepts in molecular cell biology and genetics, together with the new concepts from neuroimaging, neurophysiology, into the classification systems (Berg and Scheffer 2011). It can be expected that the results of genome-wide studies in epilepsy will have an impact on the classification of the epilepsies and epilepsy syndromes (Berg & Blackstone 2006;Berg and Scheffer 2011).

In other neurological diseases, for example, in facioscapulohumeral muscular dystrophy, the results of a large-scale population analysis showed that the genetic variation currently considered as the genetic signature of the disease is a common

polymorphism present in only half the patients, so the genetic basis of the disease needs to be revisited and this is expected to have implications in clinical practice and patient care (Scionti et al., 2012).

The classification of some epilepsy syndromes has already been influenced by some recent genetic findings, for example, the subclassification within the "benign" familial neonatal and infantile epilepsies has been rethought (Table 1.5) (Mulley et al., 2011a).

8.6 Next-generation sequencing-based association studies

With the increased use of whole-exome sequencing (Do et al., 2012;Kiezun et al., 2012) and whole-genome sequencing and the possibility for interrogating the whole genome with one affordable assay, more insights are expected into the genetic architecture of the epilepsies, including MTLEHS. New challenges, including in terms of phenotyping, can also be expected (Hennekam & Biesecker 2012).

Maps of genomic structural variants, including copy number variants, are now available as a resource for sequencing-based association studies (Handsaker et al., 2011;Mills et al., 2011). Most recently, the 1000 Genomes Project interim data became available, thereby offering high-density reference panels of rare genetic variation (Abecasis et al., 2012) and promising to introduce more tools to tackle the complex questions involved in the search for susceptibility genetic variants of the common "complex" epilepsies.

Appendix 1

Website addresses, software links and database links.

Website addresses

	Website URL
"1000 Genomes" project	http://www.1000genomes.org
Database of genomic variants	http://projects.tcag.ca/variation/
dbGaP database	http://www.ncbi.nlm.nih.gov/gap
EpiGAD (Epilepsy Genetic Association Database)	http://www.epigad.org
EPIGEN (EPIlepsy GENetics) Consortium	http://www.epilepsygenetics.eu
GeneCards	http:// www.genecards.org
Genetics Home Reference	http://ghr.nlm.nih.gov
GWAS Central	http://www.gwascentral.org
HapMap project	http://www.hapmap.org
Institute of Genome Sciences and Policy, Duke Center for Human Genome Variation	http:// www.genome.duke.edu
NCBI database of SNPs ("*db SNP"*)	http://www.ncbi.nlm.nih.gov/projects/SNP dbSNP build 135 is the latest NCBI database of SNPs has 30,443,455 SNPs and includes data from the "1000 Genomes" project
NHGRI Catalog of published genome-wide association studies	http://www.genome.gov/gwastudies Hindorff LA, MacArthur J (European Bioinformatics Institute), Wise A, Junkins HA, Hall PN, Klemm AK and Manolio TA. A Catalog of Published Genome-Wide Association Studies. Available at: www.genome.gov/gwastudies. Accessed [23/04/2012]
Online Mendelian Inheritance of Man® (*OMIM*®)	http://www.omim.org, Copyright® 1966-2014 Johns Hopkins University.
SCN1A variant database	http://www.molgen.ua.ac.be/SCN1AMutations
World Health Organisation (WHO), Epilepsy pages	http://www.who.int/topics/epilepsy/en/

Software links

	Website URL
Eigensoft Plus	R package to curate EIGENSTRAT analysis, by Mike Weale.
Evoker_0.4.3 software	http://en.sourceforge.jp/projects/sfnet_evoker/
Genetic Power Calculator (Purcell et al., 2003)	http://pngu.mgh.harvard.edu/~purcell/gpc/
PLINK	http://pngu.mgh.harvard.edu/~purcell/plink
R statistical computing and graphics software	http://www.r-project.org/
WGAViewer (Ge et al., 2008)	http://sourceforge.net/projects/wgaviewer/

Appendix 2

Information sheets and participant consent form of the population-based association genetic studies of epilepsy.

UCL INSTITUTE OF NEUROLOGY
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DEPARTMENT OF CLINICAL AND EXPERIMENTAL EPILEPSY

Head of Department: Professor D Kullmann MA, MB BS, FRCP

Director of the Institute: Professor A Thompson FRCP
Secretary of the Institute: Mr R P Walker BSc. (Econ.)

Version 6, 26.03.09

Information for patients

A POPULATION BASED GENETIC STUDY OF EPILEPSY

You are invited to participate in a study that is being carried out at The National Hospital for Neurology and Neurosurgery. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your doctor if you wish. Ask if there is anything that is not clear or if you would like more information. Take time to decide if you would like to take part.

This is a study to investigate the genetic pre-disposition to epilepsy and its treatment. Research in this field has increased our understanding of some of the genes that can cause epilepsy and determine response to some treatments. People with changes in these genes are more likely to develop epilepsy or have a different response to their treatment. By looking at a large population, we hope to identify the incidence of these gene changes in the UK and improve our understanding of epilepsy.

If you decide to participate, we will take a blood sample. We will need approximately 3 to 4 teaspoons of blood (15-20ml). This procedure should not cause any problems, but very occasionally can result in mild bruising or slight discomfort. We will isolate genetic material (DNA) from the blood. The DNA will be coded. Tests will then be performed to determine if there are any changes in the genes that may increase your susceptibility to epilepsy. By screening patients such as you, we may establish whether there are any common gene changes that are responsible for epilepsy in the UK. We may ask you for another sample of blood (3 to 4 teaspoons again) to examine another type of genetic material called mRNA. We may also ask family members if they would donate a blood sample also. We will not be notifying patients of their results, as this is a research study.

If you agree to take part in this project, we will collect information about your epilepsy. We may also ask for you to have a special photograph of the face as part of this collection of information. If we do ask, we will make arrangements for taking the photograph that are convenient to you. You may decline to have your photograph taken if you wish. All the information gained from this study will be held on computer at the Institute of Neurology in the Department of Clinical and Experimental Epilepsy, and only Dr Sisodiya and the research fellows undertaking the study will have access to it. This information will be studied to gain a better understanding of genetics in epilepsy. We also have a formal collaboration with the Institute of Genome Sciences and Policy (IGSP), Duke University, USA (Prof David Goldstein), and coded data will be transferred to IGSP for analysis under the terms of this formal agreement and other formal assurances that equivalent data protection measures will be upheld at IGSP. Dr Sisodiya will have sole responsibility for access to this information. Dr Sisodiya will maintain the anonymity of the information which will all be coded. At the end of the study, the information will be kept for further research, the nature of which will depend on our initial findings. The information and results we collect will be kept, maintaining anonymity, for at least five years, to uphold good research practice. Data Protection Act 1998: the information you give will be used only for medical research and will be stored securely and disposed of securely.

In line with current practice in the field of genetics, we plan to make overall results, which cannot be related back to individual people, openly available upon publication of the main findings from the study. Coded, individual genetic results, which also cannot be traced back to individual people, will be made available only to qualified researchers with strict agreements on use of the information in due course. This is to further enhance the value of the research and to maximise the potential of the findings for research into epilepsy. The data released will be coded. If you do not wish your data to be released in this part of the study you can ask us not to do so and this will not affect your participation in the rest of the study, or your care and management.

If something that is of clinical significance is found, a doctor in the team will discuss it with you and arrange further tests or clinical appointments if appropriate.

All proposals for research using human subjects are reviewed by a research ethics committee before they can proceed. This proposal was reviewed by the Joint Research Ethics Committee of the National Hospital for Neurology and Neurosurgery and the Institute of Neurology, University College London.

You do not have to take part in this study if you do not want to. It is up to you to decide whether or not to take part. If you decide to take part you may withdraw at any time without having to give a reason. Your decision whether to take part or not will not affect your care and management in any way. If you decide to take part you will be a given a copy of this information sheet to keep and be asked to sign a consent form, a copy of which you will also be given to keep. If you decide to take part you are still fee to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

You may ask for all information collected for this study to be destroyed at any time. All information regarding your medical records will be treated as strictly confidential and will only be used for medical and research purposes. Your medical records may be inspected by competent authorities and properly authorised persons but if any information is released this will be done so in coded form so confidentiality is strictly maintained.

If you have any further questions, please get in touch with Professor SM Sisodiya at The National Hospital for Neurology and Neurosurgery, Queen Square, London WCIN 3BG. Telephone 020 3108 0125. Fax: 020 3108 0115

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Head of Department: Professor D Kullmann MA, MB BS, FRCP

Director of the Institute: Professor A Thompson FRCP FRCPI
Secretary of the Institute: Mr R P Walker BSc. (Econ.)

Centre Number:
Study Number: 00/N081
Patient Identification Number for this trial:

CONSENT FORM

Title of project: A population based genetic study of epilepsy
Name of Researcher: SM Sisodiya

PLEASE INITIAL BOX

1. I confirm that I have read and understood the information sheet dated 26.03.2009 (version 6 for patients, or version 2 for relatives) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that sections of any of my medical notes may be looked at by responsible individuals or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
4. I agree to take part in the above study. ☐

Name of patient	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

Comments or concerns during the study
If you have any comments or concerns you may discuss these with the Investigator. If you wish to go further and complain about any aspect of the way you have been approached or treated during the course of the study, you should write or get in touch with the Complaints Manager, UCL Hospitals.

Please quote the UCLH project number at the top of this consent form.

1 copy for patient | 1 copy for Researcher | 1 copy to be kept with hospital notes

Appendix 3

Supplementary table, Chapter 4.

SNP	*P* (CMH)	*P* (LR)	Chr	Position (b36)	Type	Closest gene	Distance to gene	Minor allele	MAF in patients	Genotype counts in patients	MAF in controls	Genotype counts in controls
rs346291	$3.3x10^{-7}$	$2.5x10^{-6}$	6	80564836	Within non-coding gene	*AL132875.2*	0	A	0.335	384/1538/1523	0.366	950/3180/2802
rs9341799	$4.8x10^{-7}$	$2.1x10^{-6}$	6	80564519	Within non-coding gene	*AL132875.2*	0	G	0.405	569/1617/1215	0.373	943/3005/2617
rs2601828	$1.2x10^{-6}$	$1.0x10^{-6}$	16	4103871	Intronic	*ADCY9*	0	A	0.253	200/1342/1903	0.222	349/2380/4206
rs2172802	$3.3x10^{-6}$	0.001	4	62453209	Intronic	*LPHN3*	0	G	0.233	175/1255/2014	0.251	391/2602/3746
rs2841498	$4.0x10^{-6}$	0.0003	9	87930045	Intergenic	*AL354897.1*	155180	A	0.189	130/1044/2271	0.172	203/1977/4753
rs2814734	$4.4x10^{-6}$	$5.0x10^{-5}$	9	87992789	Intergenic	*AL451132.1*	-114705	A	0.266	250/1330/1863	0.241	419/2499/4013
rs10018746	$4.5x10^{-6}$	0.0016	4	62445246	Intronic	*LPHN3*	0	G	0.241	181/1274/1946	0.257	403/2565/3602
rs1490157	$5.3x10^{-6}$	$2.4x10^{-5}$	3	21719246	Intronic	*ZNF385D*	0	G	0.229	163/1229/2004	0.261	444/2538/3572
rs2475335	$9.3x10^{-6}$	0.0001	9	10260263	Intronic	*PTPRD*	0	G	0.192	116/1090/2239	0.173	205/1990/4740
rs3773282	$1.1x10^{-5}$	0.0003	3	13630307	Intronic	*FBLN2*	0	G	0.260	226/1341/1878	0.236	386/2495/4051
rs1989647	$1.3x10^{-5}$	$8.9x10^{-6}$	16	23959420	Intronic	*PRKCB*	0	A	0.351	423/1536/1438	0.312	654/2791/3122
rs2132074	$1.3x10^{-5}$	0.0002	4	62416499	Intronic	*LPHN3*	0	A	0.304	330/1423/1673	0.329	724/3003/3036
rs2418103	$1.4x10^{-5}$	0.0018	12	10856255	Intronic	*CSDAP1;CSDA*	0	G	0.184	116/1030/2291	0.203	305/2212/4417
rs1320292	$1.6x10^{-5}$	$1.8x10^{-5}$	3	21701712	Intronic	*ZNF385D*	0	A	0.208	140/1127/2116	0.240	361/2434/3772
rs6848888	$1.6x10^{-5}$	0.003	4	94572864	Intronic	*GRID2*	0	A	0.299	299/1460/1686	0.319	668/3089/3176
rs4556959	$1.7x10^{-5}$	0.0024	2	205366339	Within non-coding gene	*AC016903.1*	0	A	0.044	7/287/3107	0.036	11/454/6104
rs951997	$2.0x10^{-5}$	$4.5x10^{-5}$	2	223567016	Intronic	*MOGAT1*	0	A	0.476	796/1690/959	0.443	1354/3441/2138
rs1942006	$2.1x10^{-5}$	$4.1x10^{-5}$	10	67653901	Intergenic	*CTNNA3*	25818	A	0.300	306/1451/1687	0.274	538/2726/3666
rs6712604	$2.1x10^{-5}$	0.0003	2	8235239	Within non-coding gene	*C2orf46*	0	G	0.161	96/920/2428	0.180	245/2012/4675
rs1120229	$2.2x10^{-5}$	$6.1x10^{-5}$	6	80597212	Intergenic	*AL132875.1*	12885	A	0.401	557/1613/1231	0.369	903/3039/2626
rs2305849	$2.3x10^{-5}$	0.0022	12	10854537	Intronic	*CSDAP1;CSDA*	0	A	0.184	117/1031/2297	0.203	305/2206/4422
rs11819622	$2.3x10^{-5}$	0.0008	10	72287444	Intronic	*KIAA1274*	0	A	0.110	41/673/2731	0.129	121/1547/5261
rs10151805	$2.4x10^{-5}$	$9.3x10^{-5}$	14	105974781	Intergenic	*C14orf80*	9196	G	0.274	253/1380/1811	0.245	450/2497/3987
rs4072799	$2.4x10^{-5}$	0.003	9	90487924	Intergenic	*AL772337.1*	6719	C	0.161	77/957/2409	0.182	220/2086/4627
rs1018626	$2.6x10^{-5}$	0.0005	1	194269455	Intergenic	*AL513348.1*	-50618	G	0.095	33/587/2825	0.085	31/1110/5792

rs1387822	$2.9x10^{-5}$	$2.5x10^{-5}$	3	21686466	Intronic	*ZNF385D*	0	G	0.298	294/1462/1688	0.326	725/3070/3137
rs3773283	$2.9x10^{-5}$	0.0006	3	13626592	Intronic	*FBLN2*	0	G	0.281	270/1394/1781	0.257	467/2628/3836
rs4298061	$3.1x10^{-5}$	0.0001	3	21726363	Intronic	*ZNF385D*	0	C	0.224	170/1201/2073	0.249	416/2617/3902
rs9637779	$3.3x10^{-5}$	0.0005	5	19160015	Intergenic	*AC106744.1*	118520	G	0.240	192/1250/1959	0.219	322/2190/3952
rs1396626	$3.4x10^{-5}$	$3.3x10^{-5}$	1	96025546	Within non-coding gene	*AL683887.1*	0	A	0.318	351/1487/1607	0.288	585/2823/3522
rs10823320	$3.4x10^{-5}$	0.0003	10	70987060	Non-synonymous coding	*HKDC1*	0	G	0.031	3/206/3231	0.040	13/534/6380
rs3785392	$3.6x10^{-5}$	$6.6x10^{-5}$	16	23944483	Intronic	*PRKCB*	0	G	0.355	435/1545/1421	0.320	682/2834/3052
rs3806629	$3.7x10^{-5}$	0.0006	3	160283815	Within non-coding gene	*KRT8P12*	0	A	0.065	18/399/2944	0.049	20/624/6081
rs923665	$3.7x10^{-5}$	0.0006	4	38537708	Intergenic	*AC096739.1*	27952	G	0.233	190/1228/2027	0.258	461/2654/3819
rs2593018	$4.3x10^{-5}$	0.0003	9	88009220	Intergenic	*AL451132.1*	-98274	A	0.159	99/896/2446	0.144	139/1720/5072
rs986503	$4.3x10^{-5}$	$5.6x10^{-5}$	3	21714103	Intronic	*ZNF385D*	0	A	0.209	139/1141/2121	0.240	363/2424/3782
rs11580295	$4.4x10^{-5}$	0.0001	1	119836236	Within non-coding gene	*AL359915.1*	0	G	0.342	423/1508/1514	0.311	662/2984/3286
rs545331	$4.6x10^{-5}$	0.001	2	166913962	Intronic	*SCN1A*	0	A	0.254	223/1285/1893	0.280	519/2642/3408
rs16834756	$4.9x10^{-5}$	$3.7x10^{-6}$	2	154745009	Intronic	*GALNT13*	0	G	0.030	6/190/3205	0.046	9/582/5973
rs1565901	$5.0x10^{-5}$	$4.2x10^{-5}$	4	62407327	Intronic	*LPHN3*	0	A	0.141	73/816/2512	0.164	164/1829/4575
rs493517	$5.1x10^{-5}$	0.0037	1	97721227	Intronic	*BX908805.1*	0	G	0.394	508/1530/1194	0.417	1074/2943/2081
rs10152421	$5.3x10^{-5}$	$4.2x10^{-6}$	15	26985509	Intronic	*GABRB3*	0	A	0.289	293/1377/1731	0.255	445/2464/3660
rs17269978	$5.4x10^{-5}$	0.001	15	60374681	Intergenic	*FOXB1*	76326	G	0.092	23/589/2832	0.111	90/1352/5490
rs12267364	$5.5x10^{-5}$	0.0003	10	134554926	Intronic	*INPP5A*	0	A	0.074	16/471/2909	0.061	23/751/5795
rs1515308	$5.7x10^{-5}$	0.0002	2	180443444	Intronic	*ZNF385B*	0	G	0.214	164/1145/2135	0.238	381/2533/4017
rs2138196	$6.0x10^{-5}$	0.0004	2	180452188	Intronic	*ZNF385B*	0	G	0.198	151/1060/2233	0.221	329/2405/4198
rs4765723	$6.0x10^{-5}$	0.0013	12	3352543	Intronic	*TSPAN9*	0	C	0.244	183/1297/1921	0.224	310/2319/3938
rs495257	$6.1x10^{-5}$	0.0046	1	97721392	Intronic	*BX908805.1*	0	G	0.394	510/1529/1195	0.417	1075/2947/2086
rs17318292	$6.1x10^{-5}$	0.0002	10	87750258	Intronic	*GRID1*	0	A	0.507	878/1734/833	0.477	1591/3428/1914
rs2573555	$6.5x10^{-5}$	0.0017	10	78062571	Intronic	*C10orf11*	0	A	0.062	14/370/2850	0.045	13/523/5566
rs17239966	$6.7x10^{-5}$	$8.6x10^{-5}$	10	67671031	Intergenic	*CTNNA3*	8688	G	0.286	285/1396/1762	0.261	485/2644/3805
rs6790448	$7.0x10^{-5}$	0.0027	3	151075674	Intronic	*P2RY12*	0	G	0.407	526/1578/1125	0.379	883/2994/2395

rs11123348	$7.0x10^{-5}$	0.0184	2	117126557	Intergenic	*AC062016.1*	357472	A	0.285	286/1388/1770	0.267	497/2713/3723
rs11611821	$7.3x10^{-5}$	0.0042	12	119535709	Intronic	*KIAA1853*	0	G	0.071	25/432/2943	0.060	29/731/5809
rs17752721	$7.4x10^{-5}$	0.0007	6	129820505	Intronic	*LAMA2*	0	A	0.138	67/805/2524	0.157	172/1717/4678
rs1512486	$7.4x10^{-5}$	0.0051	3	78325071	Within non-coding gene	*AC108752.1*	0	G	0.150	71/890/2484	0.166	208/1888/4834
rs12273504	$7.5x10^{-5}$	$6.9x10^{-5}$	11	26007640	Intergenic	*ANO3*	-345331	A	0.017	1/116/3284	0.011	1/141/6423
rs1106753	$7.6x10^{-5}$	$3.1x10^{-5}$	6	155164271	Within non-coding gene	*RBM16*	0	A	0.468	748/1684/962	0.436	1214/3198/2034
rs745155	$7.6x10^{-5}$	0.0102	2	234386803	Intronic	*USP40*	0	C	0.328	392/1476/1577	0.308	642/2990/3301
rs6751658	$7.7x10^{-5}$	0.0107	2	117100160	Intergenic	*AC062016.1*	383869	G	0.288	294/1397/1754	0.269	505/2723/3706
rs511137	$7.7x10^{-5}$	$2.3x10^{-5}$	6	62563667	Intronic	*KHDRBS2*	0	A	0.399	548/1569/1223	0.438	1289/3124/2102
rs2600328	$8.0x10^{-5}$	0.0001	3	12998180	Intronic	*IQSEC1*	0	A	0.471	768/1707/969	0.442	1367/3399/2167
rs4843349	$8.0x10^{-5}$	0.0285	16	86131064	Intergenic	*AC092723.1*	61039	A	0.126	60/740/2601	0.110	76/1297/5194
rs11942117	$8.3x10^{-5}$	0.0003	4	38529130	Intergenic	*AC096739.1*	19374	A	0.276	256/1368/1777	0.307	624/2787/3157
rs10798069	$8.4x10^{-5}$	$6.6x10^{-5}$	1	186875459	Intronic	*PLA2G4A*	0	A	0.508	899/1705/841	0.478	1581/3470/1883
rs4074453	$8.5x10^{-5}$	0.0001	14	105998544	Downstream	*TMEM121*	2005	G	0.273	252/1375/1815	0.244	419/2348/3763
rs1948616	$8.6x10^{-5}$	0.0017	4	62487688	Intronic	*LPHN3*	0	G	0.246	194/1309/1941	0.262	465/2698/3772
rs1886049	$8.8x10^{-5}$	0.001	13	95088866	Downstream	*DCT*	2875	A	0.430	649/1665/1129	0.409	1209/3253/2468
rs1516537	$8.9x10^{-5}$	0.0002	4	183255434	Intronic	*ODZ3*	0	A	0.506	47/75/45	0.399	918/2626/2051
rs11609210	$9.0x10^{-5}$	0.0046	12	119535001	Intronic	*KIAA1853*	0	A	0.071	25/431/2944	0.060	29/731/5810
rs8103835	$9.4x10^{-5}$	0.0227	19	29599650	Intergenic	*UQCRFSL1;UQCRFS1*	98517	G	0.299	306/1419/1676	0.312	641/2811/3116
rs2924329	$9.4x10^{-5}$	0.0001	18	53135894	Intronic	*TCF4*	0	A	0.317	362/1458/1625	0.291	560/2913/3460
rs7837755	$9.7x10^{-5}$	0.0024	8	79447414	Intronic	*PKIA*	0	G	0.137	63/821/2561	0.122	114/1464/5355
rs4714634	$9.9x10^{-5}$	0.0004	6	42901120	Intronic	*CNPY3*	0	A	0.465	710/1784/951	0.442	1350/3423/2159
rs4920374	0.0001	$1.1x10^{-5}$	1	17872028	Intronic	*ARHGEF10L*	0	A	0.495	849/1713/883	0.466	1536/3388/2011
rs4953133	0.0001	$1.5x10^{-5}$	2	45029525	Intergenic	*C2orf34*	29794	G	0.520	950/1686/809	0.487	1640/3469/1825
rs6723091	0.0001	$2.7x10^{-5}$	2	223584182	Intergenic	*AC016712.1*	-6994	G	0.497	849/1683/866	0.463	1402/3273/1893
rs10765118	0.0001	$3.3x10^{-5}$	10	129285183	Intergenic	*DOCK1*	34402	G	0.403	577/1586/1237	0.430	1256/3276/2202
rs10830099	0.0001	$4.2x10^{-5}$	10	129284365	Intergenic	*DOCK1*	33584	G	0.403	581/1607/1248	0.430	1258/3279/2204
rs2054263	0.0001	$4.2x10^{-5}$	6	153748051	Intergenic	*AL358134.1*	6372	A	0.246	214/1267/1964	0.221	340/2379/4211
rs12604557	0.0001	$8.7x10^{-5}$	18	71709826	Intergenic	*FBXO15*	30762	G	0.168	103/948/2393	0.188	281/2052/4601

rs732803	0.0002	$9.2x10^{-6}$	8	140969818	Intronic	*TRAPPC9*	0	A	0.395	554/1616/1275	0.428	1295/3338/2300
rs9404905	0.0002	$1.0x10^{-5}$	6	61995682	Downstream	*AL356131.1*	721	A	0.377	502/1592/1348	0.415	1236/3273/2420
rs6995955	0.0002	$1.6x10^{-5}$	8	140956133	Intronic	*TRAPPC9*	0	G	0.354	450/1542/1453	0.387	1063/3247/2625
rs13125283	0.0002	$1.8x10^{-5}$	4	28838463	Intergenic	*AC091602.1*	125406	G	0.080	30/491/2924	0.099	70/1238/5623
rs6459325	0.0002	$1.9x10^{-5}$	6	58044218	Intergenic	*AL021368.1*	112232	A	0.377	499/1560/1337	0.414	1163/3107/2291
rs1414808	0.0002	$2.9x10^{-5}$	6	62273181	Intergenic	*RP1-240B8.1*	66957	A	0.385	528/1598/1319	0.421	1287/3267/2377
rs10755544	0.0002	$3.1x10^{-5}$	6	62272667	Intergenic	*RP1-240B8.1*	67471	G	0.385	519/1578/1303	0.422	1215/3116/2235
rs4738414	0.0002	$3.4x10^{-5}$	8	74928092	Intronic	*LY96*	0	A	0.156	79/916/2450	0.177	226/2003/4702
rs646443	0.0002	$4.3x10^{-5}$	1	64994828	Intronic	*CACHD1*	0	A	0.101	35/576/2587	0.119	94/1460/5372
rs10152467	0.0002	$4.5x10^{-5}$	15	102146151	Intergenic	*TM2D3*	27729	G	0.459	706/1712/982	0.423	1217/3123/2230
rs17115302	0.0002	$5.4x10^{-5}$	21	44051855	Intergenic	*AP001626.1*	16526	G	0.150	76/868/2454	0.169	186/1844/4537
rs1743457	0.0002	$5.9x10^{-5}$	6	62520127	Intronic	*KHDRBS2*	0	G	0.400	554/1608/1234	0.436	1322/3181/2182
rs10801589	0.0002	$7.8x10^{-5}$	1	197076194	Intronic	*ASPM*	0	A	0.501	873/1708/864	0.468	1502/3492/1939
rs7930512	0.0002	$8.2x10^{-5}$	11	26057035	Intergenic	*ANO3*	-295936	G	0.410	593/1636/1216	0.438	1369/3338/2226
rs1470525	0.0002	$8.5x10^{-5}$	2	223592956	Downstream	*AC016712.1*	1403	A	0.481	802/1710/933	0.451	1397/3461/2075
rs11961059	0.0002	$8.9x10^{-5}$	6	128997707	Intergenic	*AL080315.1*	-22077	A	0.094	24/598/2823	0.080	51/1011/5871
rs10755808	0.0003	$3.5x10^{-5}$	6	58002475	Intergenic	*AL021368.1*	153975	G	0.375	501/1578/1365	0.409	1201/3275/2456
rs10903342	0.0003	$4.3x10^{-5}$	8	11530493	Intergenic	*GATA4*	-31220	C	0.440	663/1705/1077	0.477	1564/3296/1880
rs562294	0.0003	$7.1x10^{-5}$	6	62529924	Intronic	*KHDRBS2*	0	A	0.401	557/1607/1233	0.437	1298/3145/2127
rs9958365	0.0003	$7.7x10^{-5}$	18	1592251	Intergenic	*C18orf2*	-232621	A	0.368	465/1575/1360	0.341	803/3008/2953
rs1559930	0.0003	$8.5x10^{-5}$	2	205001563	Intergenic	*AC009965.2*	26991	A	0.378	502/1597/1346	0.352	890/3100/2945
rs11642116	0.0003	$9.6x10^{-5}$	16	23797412	Intergenic	*AC130454.1*	27140	C	0.235	186/1226/1989	0.212	315/2312/4308
rs17117335	0.0004	$2.1x10^{-5}$	15	26996126	Intronic	*GABRB3*	0	A	0.305	328/1417/1650	0.274	520/2555/3494
rs9362101	0.0004	$5.4x10^{-5}$	6	62411489	Intronic	*KHDRBS2*	0	A	0.435	655/1649/1096	0.472	1496/3199/1870
rs269166	0.0004	$8.7x10^{-5}$	8	75590311	Intergenic	*PI15*	-146461	G	0.296	307/1399/1695	0.268	466/2529/3468
rs2458625	0.0005	$2.7x10^{-5}$	4	28575606	Intergenic	*AC091602.1*	-137215	G	0.216	173/1143/2129	0.243	404/2558/3969
rs10074082	0.0005	$2.9x10^{-5}$	5	82680138	Intergenic	*XRCC4*	30561	G	0.068	17/428/2956	0.050	18/599/5782
rs1152471	0.0005	$3.0x10^{-5}$	14	56771153	Downstream	*PELI2*	2909	A	0.106	36/657/2752	0.126	94/1564/5277
rs12441037	0.0005	$5.0x10^{-5}$	15	61102657	Intronic	*RORA*	0	G	0.039	7/250/3143	0.047	18/588/5963
rs16908681	0.0005	$6.2x10^{-5}$	8	139260668	Intronic	*FAM135B*	0	A	0.303	306/1445/1647	0.267	469/2564/3532
rs1974708	0.0006	$3.4x10^{-5}$	16	55793837	Upstream	*CES4*	-623	A	0.168	106/948/2390	0.188	235/2130/4567

rs10490525	0.0006	$3.6x10^{-5}$	2	155320829	Downstream	*AC009227.2*	3497	A	0.372	465/1507/1306	0.401	1054/3070/2334
rs10484364	0.0006	$4.1x10^{-5}$	6	16553452	Intronic	*ATXN1*	0	A	0.097	34/601/2809	0.082	52/1030/5852
rs6662637	0.0006	$5.9x10^{-5}$	1	152510348	Intronic	*LCE3C*	0	A	0.259	214/1359/1872	0.281	546/2795/3587
rs16957399	0.0007	$1.7x10^{-5}$	15	33967961	Intronic	*RYR3*	0	A	0.073	27/440/2933	0.059	23/729/5814
rs10744553	0.0007	$4.7x10^{-5}$	12	1928289	Intronic	*CACNA2D4*	0	G	0.476	767/1740/933	0.447	1340/3323/2059
rs634248	0.0007	$7.8x10^{-5}$	5	103032867	Intergenic	*NUDT12*	-134373	G	0.435	654/1688/1103	0.408	1169/3319/2445
rs10063779	0.0007	$9.3x10^{-5}$	5	105833108	Intergenic	*AC114940.2*	-49259	A	0.039	5/256/3139	0.030	8/376/6185
rs6494226	0.0007	$9.3x10^{-5}$	15	61105886	Intronic	*RORA*	0	A	0.039	7/251/3135	0.048	18/585/5912
rs10479370	0.0007	$9.5x10^{-5}$	5	105825670	Intergenic	*AC114940.2*	-56697	C	0.039	5/256/3140	0.030	8/376/6184
rs1591548	0.0008	$1.4x10^{-5}$	6	62036458	Intergenic	*AL356131.2*	-20312	A	0.377	499/1591/1344	0.415	1201/3159/2346
rs4458738	0.0008	$1.5\ x10^{-5}$	6	57978015	Intergenic	*AL512427.1*	-135625	A	0.468	724/1551/930	0.507	1573/2859/1494
rs12792912	0.0008	$3.4\ x10^{-5}$	11	102801303	Intergenic	*MMP13*	12424	C	0.421	615/1628/1154	0.449	1324/3248/1993
rs1338041	0.0009	$3.6x10^{-6}$	13	102058862	Intronic	*NALCN*	0	C	0.373	498/1572/1375	0.337	823/3032/3077
rs6997704	0.0009	$5.1x10^{-5}$	8	140978857	Intronic	*TRAPPC9*	0	G	0.390	538/1576/1287	0.422	1187/3160/2211
rs196002	0.0009	$9.8x10^{-5}$	16	23963237	Intronic	*PRKCB*	0	A	0.381	500/1624/1321	0.348	847/3131/2957
rs7595772	0.001	$3.4x10^{-5}$	2	126239051	Intergenic	*AC097499.2*	-229495	G	0.103	32/634/2735	0.123	105/1403/5061
rs10876993	0.001	$4.7x10^{-5}$	12	58062667	Downstream	*AC025165.3*	60	G	0.325	397/1444/1604	0.353	886/3117/2926
rs12413997	0.0011	$2.3x10^{-5}$	10	58191480	Intergenic	*ZWINT*	-70444	A	0.058	4/389/3050	0.047	9/609/6095
rs9873795	0.0011	$9.6x10^{-5}$	3	166003323	Intergenic	*AC104629.1*	-8221	A	0.089	34/540/2826	0.069	39/827/5704
rs10083154	0.0012	$7.0x10^{-5}$	12	58020933	Intronic	*B4GALNT1*	0	A	0.309	365/1400/1680	0.337	811/3049/3074
rs10080807	0.0014	$3.6x10^{-5}$	6	14551209	Intergenic	*AL359994.1*	46536	A	0.145	65/859/2477	0.167	185/1823/4561
rs2844363	0.0014	$7.8x10^{-5}$	3	37611860	Intronic	*ITGA9*	0	G	0.402	551/1665/1229	0.432	1289/3407/2238
rs1053079	0.0016	$9.0x10^{-5}$	8	74893821	Non-synonymous coding	*TMEM70*	0	G	0.129	53/786/2606	0.147	152/1736/5046
rs1372328	0.0017	$1.1x10^{-5}$	9	119484528	Intronic	*ASTN2*	0	A	0.450	713/1677/1055	0.482	1609/3461/1862
rs6899924	0.0019	$3.8x10^{-5}$	6	58071881	Intergenic	*AL021368.1*	84569	G	0.501	765/1445/757	0.462	1391/3077/1879
rs1440788	0.0023	$4.8x10^{-5}$	8	53388439	Intergenic	*FAM150A*	58161	A	0.103	62/577/2760	0.123	106/1454/5238
rs2896569	0.0024	$3.1x10^{-5}$	5	103075716	Intergenic	*NUDT12*	-177222	A	0.330	375/1524/1546	0.309	681/2918/3334
rs9839172	0.0024	$6.3x10^{-5}$	3	78469764	Intergenic	*AC112508.1*	-104487	G	0.305	321/1435/1645	0.337	815/3039/3078
rs7571928	0.0025	$7.3x10^{-5}$	2	126327505	Intergenic	*AC097499.2*	-141041	A	0.103	34/639/2771	0.121	108/1457/5368
rs4434970	0.003	$7.5x10^{-5}$	11	102772798	Downstream	*AP000789.1*	1824	G	0.337	404/1481/1515	0.365	881/3037/2652

rs7581261	0.0035	$7.3x10^{-5}$	2	126229399	Intergenic	*AC097499.2*	-239147	C	0.103	34/641/2768	0.121	108/1463/5362
rs8008756	0.0038	$8.2x10^{-6}$	14	33546266	Intronic	*NPAS3*	0	A	0.145	114/753/2520	0.170	220/1745/4457
rs12445022	0.0044	$5.4x10^{-5}$	16	87575332	Intergenic	*ZCCHC14*	-49681	A	0.352	417/1557/1427	0.325	701/2870/2997
rs2676790	0.0045	$5.2x10^{-5}$	17	47662683	Downstream	*NXPH3*	1511	A	0.357	437/1587/1421	0.330	773/3024/3137
rs10511688	0.0048	$2.6x10^{-5}$	9	20744371	Intronic	*KIAA1797*	0	A	0.372	480/1602/1363	0.399	1154/3221/2554
rs9646952	0.0053	$5.6x10^{-5}$	2	110102339	Intronic	*SH3RF3*	0	A	0.263	252/1306/1886	0.237	408/2469/4057
rs10865566	0.0057	$6.6x10^{-5}$	3	78455571	Intergenic	*AC108752.1*	111690	G	0.193	129/1070/2246	0.220	345/2361/4228
rs2898295	0.0062	$10.0x10^{-5}$	8	11595969	Intronic	*GATA4*	0	A	0.454	710/1666/1023	0.490	1590/3253/1720
rs9827826	0.0069	$8.3x10^{-5}$	3	192024014	Intronic	*FGF12*	0	G	0.075	21/477/2947	0.089	54/1121/5756
rs3789090	0.0078	$5.4x10^{-5}$	2	111789092	Intronic	*ACOXL*	0	A	0.372	474/1616/1354	0.399	1084/3363/2484
rs17776169	0.0097	$4.4x10^{-5}$	2	142931146	Intergenic	*AC078882.1*	-35747	A	0.115	38/703/2656	0.096	54/1156/5350
rs17310162	0.0114	$2.3x10^{-5}$	14	97171691	Intergenic	*AL137786.1*	-66840	G	0.021	1/143/3257	0.033	11/418/6140
rs7638304	0.0133	$9.7x10^{-5}$	3	18911259	Within non-coding gene	*AC099053.2*	0	A	0.369	481/1580/1384	0.347	831/3150/2951
rs7583748	0.0146	$9.5x10^{-5}$	2	142855291	Intronic	*LRP1B*	0	G	0.112	42/689/2711	0.095	55/1211/5662
rs6972422	0.0186	$4.0x10^{-5}$	7	18834375	Intronic	*HDAC9*	0	G	0.232	193/1188/2018	0.251	450/2393/3723
rs2123478	0.0221	$9.7x10^{-5}$	4	41290052	Intergenic	*AC095043.2*	15202	G	0.396	506/1544/1179	0.372	890/2862/2493
rs195770	0.0277	$6.8x10^{-5}$	17	8710465	Intronic	*PIK3R6*	0	G	0.312	333/1485/1627	0.288	561/2864/3507

Table A3.1 SNPs with *p*-values below 1.0×10^{-4}, either in logistic regression (LR) or Cochran-Mantel-Haenszel (CMH) tests, for the genome-wide association study of partial epilepsy.

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