Biomarkers of Sudden Unexpected Death in Epilepsy (SUDEP)

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Abstract (English)

SUDEP (Sudden Unexpected Death in Epilepsy) is the most devastating outcome in epilepsy and the commonest cause of epilepsy-related premature mortality. Studies of clinical risk factors have allowed identifying high-risk populations. However no genomic, electrophysiological or structural features have emerged as established biomarkers of an increased SUDEP risk. To elucidate the genetic architecture of SUDEP, we used an unbiased whole-exome sequencing approach to examine overall burden and over-representation of deleterious variants in people who died of SUDEP compared to living people with epilepsy and non-epilepsy disease controls. We found significantly increased genome-wide polygenic burden per individual in the SUDEP cohort when compared to epilepsy and non-epilepsy disease controls. The polygenic burden was driven both by the number of variants per individual, and over-representation of variants likely to be deleterious in the SUDEP cohort. To elucidate which brain regions may be implicated in SUDEP, we investigated whether regional abnormalities in grey matter volume appear in those who died of SUDEP, compared to subjects at high and low risk for SUDEP, and healthy controls. We identified increased grey matter volume in the right anterior hippocampus/amygdala and parahippocampus in SUDEP cases and people at high risk, when compared to those at low risk and controls. Compared to controls, posterior thalamic grey matter volume, an area mediating oxygen regulation, was reduced in SUDEP cases and subjects at high risk. It is fundamental to understand the range of SUDEP aetiological mechanisms. Our results suggest that both exome sequencing data and structural imaging features may contribute to generate SUDEP risk estimates. Translation of this knowledge into predictive algorithms of individual risk and preventive strategies would promote stratified medicine in epilepsy, with the aim of reducing an individual patient's risk of SUDEP.

Abstract (Italiano)

La SUDEP (Sudden Unexpected Death in Epilepsy) è una complicanza devastante dell'epilessia e rappresenta la più comune causa di mortalità prematura in epilessia. Studi volti alla definizione di fattori di rischio clinici hanno permesso di identificare gruppi ad alto rischio. Tuttavia al momento non esistono validati biomarkers genomici, elettrofisiologici o strutturali predittivi di aumentato rischio di SUDEP. Al fine di definire la base genetica della SUDEP, abbiamo condotto una analisi di sequenziamento esomico per esaminare la prevalenza di varianti con effetto deleterio in soggetti deceduti per SUDEP rispetto a pazienti epilettici non deceduti e controlli con altre patologie. Abbiamo riscontrato una prevalenza significativamente aumentata di varianti deleterie diffuse a livello dell'intero genoma nei soggetti deceduti per SUDEP in confronto agli altri gruppi. Un secondo studio di neuroimaging è stato dedicato alla valutazione di anomalie regionali del volume della sostanza grigia in soggetti deceduti per SUDEP, confrontati con soggetti epilettici viventi rispettivamente ad alto e basso rischio per SUDEP, e controlli sani. Abbiamo riscontrato un aumento del volume della sostanza grigia in emisfero destro a livello di amigdala, parte anteriore dell'ippocampo e paraippocampo nei soggetti deceduti per SUDEP e nei soggetti ad alto rischio, rispetto ai soggetti a basso rischio ed ai controlli. Sia il sequenziamento esomico sia il neuroimaging strutturale hanno fornito dati significativi per il profilo di rischio di SUDEP. La definizione dei meccanismi eziologici della SUDEP è fondamentale. La traslazione di tali dati in algoritmi predittivi di rischio individuale consente di promuovere la 'medicina personalizzata', allo scopo di adottare strategie preventive e ridurre il rischio individuale di SUDEP in pazienti con epilessia.

Contents

List of Tables

List of Figures

Chapter 1

SUDEP: OVERVIEW OF THE LITERATURE AND HYPOTHESES

SUDEP is defined as the sudden, unexpected, witnessed or unwitnessed, non-traumatic, and non-drowning death in patients with epilepsy with or without evidence for a seizure, and excluding documented status epilepticus, in which post-mortem examination does not reveal a structural or toxicological cause for death (Nashef, 2012). It represents the most severe degree of the spectrum of epilepsy severity and is the commonest cause of epilepsy-related premature mortality (Walczak et al, 2001). Despite an applicable definition, and clear guidance where there is uncertainty, significant variability in use has hampered efforts to integrate findings from multiple studies on epidemiological and risk factor data. Inconsistent and inaccurate methods of assessment might represent a limitation for human SUDEP studies—from physiological, EEG, genetic, neuroimaging, and pathological studies (Tellez-Zenteno et al, 2005; Monte et al, 2007).

1.1 Classification

According to the most recent proposed Unified SUDEP Definition and Classification (Nashef et al, 2012), SUDEP can be classified in the following categories:

-Definite SUDEP: a SUDEP for which post-mortem examination failed to reveal a cause of death;

-Probable SUDEP: a SUDEP for which no post-mortem examination is available and the victim died unexpectedly while in a reasonable state of health, during normal activities, and in benign circumstances, without a known structural cause of death;

-Near SUDEP: a sudden, unexpected, non-traumatic, and non-drowning cardiorespiratory arrest with no structural cause identified after investigation, occurring in benign circumstances in an individual with epilepsy, with or without evidence for a seizure excluding documented status epilepticus, where the patient survived resuscitation for more than 1 h after the cardiorespiratory arrest;

-Fatal near SUDEP: near SUDEP in which the cardiorespiratory arrest was responsible for irreversible major brain damage directly leading to death more than 1 h after the cardiorespiratory arrest;

-Non-SUDEP: sudden death in an individual with epilepsy with a clear cause of death other than SUDEP (eg, myocardial infarction, brain haemorrhage).

1.2 Epidemiology

Sudden unexpected death in the general population is extremely rare in young adults with an incidence of 5–10/100,000 person-years, while the rate climbs steeply with advancing age to approximately 300/100,000 person-years in the elderly (Elveback et al, 1981). The incidence of sudden death in patients with epilepsy is significantly higher and varies markedly with the population studied (Tomson et al, 2005). For example, in population based studies the incidence has been reported to be 0.35 and 2.7/1000 person-years depending on the methodologies employed (Leestma et al, 1989; Ficker et al, 1998; Thurman et al, 2014). This increases to between 2 and 5.9/1000 patient-years in cohorts of patients attending specialist epilepsy clinics (Nashef et al, 1995a), 3.4/1000 patient-years in pupils with epilepsy enrolled in a special residential school (Nashef et al, 1995b) up to 6.5/1000 patient-years in cohorts of people with drug-resistant epilepsy unsuitable for surgery (Bell et al., 2010). The incidence of sudden death in young adults with intractable epilepsy is therefore many times that of the general population, with a peak between the ages of 20 and 40 years (Hitiris et al, 2007). In older adults SUDEP might be underdiagnosed as sudden death might be attributed to cardiac events without careful investigation of alternative causes. The range of community or population-based estimates might reflect the methodological challenges of ascertaining the frequency of SUDEP for the following reasons: SUDEP is under-reported and under-recognised by medical examiners, coroners, and clinicians who complete death certificates (Schraeder et al, 2009); epilepsy is often not documented on death certificates; and International Classification of Disease codes used in compiled death certificate data do not describe SUDEP as a distinct entity.

1.3 Risk factors

There is significant debate regarding risk factors for SUDEP. Seizures and SUDEP are probabilistic events that cannot be accurately predicted. Relevant and independent risk factors are difficult to establish given the non-independence of patient, syndrome, seizures and treatment characteristics. Search for risk factors has been mostly done in retrospective case-control studies, which may have limitations. Multiple logistic regression analyses require large cohorts of patients to achieve statistical significance for each of the variables evaluated and this is difficult to attain (Schraeder et al, 2006). Furthermore, the high variability between studies in terms of patient cohorts, definition, choice of control group, methodology and overall study quality limits the generalisability of observation and significantly affects comparison.

1.3.1 Demographics

Descriptive studies have extensively reported that patients with SUDEP are young adults (Leestma et al, 1989; Nashef et al, 1995a; Ficker et al, 1998; Lear-Kaul et al, 2005). A number of biases exist however, including, by definition, the exclusion of patients with significant co-morbidity associated with increasing age, such as ischaemic heart disease or cerebrovascular disease, identified on postmortem examination (Lear-Kaul et al, 2005). Other examples of bias include case identification through self-referral by bereaved relatives, most commonly parents (Nashef et al, 1998), and studies with only small numbers of patients (Ficker et al, 1998). Case-control studies are less conclusive. Some studies only included defined age groups and can draw no conclusions regarding other age groups. Nevertheless, it is interesting to note that 70−80% of the studied population in a number of case-control studies were less than 45 years old (Nilsson et al, 1999; Hitiris et al, 2007). Data regarding age, however, is not available from a number of large studies due to age-matching of control subjects (Nilsson et al, 1999; Langan et al, 2005; Hitiris et al, 2007). Of the remaining studies, the use of a cohort of non-SUDEP deaths as a control group may bias the patient group towards a younger age due to exclusion of co-morbid conditions more commonly associated with advancing age (Schnabel et al, 2002; Opeskin K & Berkovic SF, 2003; Vlooswijk et al, 2007), although young age as an independent risk factor has not been universally reported (Walczak et al, 2001). The likelihood of selection bias is corroborated by the presence of significantly less co-morbidity in the SUDEP group than the non-SUDEP group (Vlooswijk et al, 2007). Although a large number of descriptive studies have suggested that male gender is a significant risk factor for

SUDEP (Tennis et al, 1995; Lear-Kaul et al, 2005), this has not been confirmed by many case-control studies (Tennis et al, 1995; Nilsson et al, 1999; Walczak et al, 2001; Schnabel et al, 2002; Langan et al, 2005; Williams et al, 2006; Vlooswijk et al, 2007). In addition, a small number of both descriptive and case-control studies have reported a significantly increased standardised mortality rate in female patients, which may be attributable to a lower background rate of death in the female non-SUDEP control group (Ficker et al, 1998; Opeskin & Berkovic, 2003). However, a pooled analysis including 289 SUDEP cases and 958 controls confirmed the role of male gender as a risk factor (Hesdorffer et al, 2011).

1.3.2 Epilepsy characteristics

A number of case-control studies have suggested that early onset of epilepsy is a significant risk factor for SUDEP (Nilsson et al, 1999; Schnabel et al, 2002; Opeskin & Berkovic, 2003; Hesdorffer et al, 2011). For example, an eight-fold higher SUDEP risk in patients with an onset of epilepsy between the ages of 0 and 15 years has been reported, when compared to patients with seizure onset after 45 years of age (Nilsson et al, 1999). However, while this may reflect a different aetiological basis for the epilepsy, it may also merely be a surrogate marker for an increased cumulative lifetime risk of having seizures for a longer period of time, as suggested by other studies (Lear-Kaul et al, 2005; Hitiris et al, 2007). Conversely, there are reports of a shorter duration of epilepsy being associated with an increased risk of SUDEP although this is most likely as a result of comparison with an older control population (Schnabel et al, 2002; Vlooswijk et al, 2007). Long duration of epilepsy (>30 years) was no longer a risk factor after adjustment for seizure frequency (Walczak et al, 2001), whilst duration of epilepsy (>15 years) emerged as a significant risk factor in the combined analysis by Hesdorffer et al. (2011).

There is some evidence of an association between the epilepsy syndrome and an increased risk of SUDEP (Nilsson et al, 1999). Nilsson et al. (1999) found that 7 out of 57 (12%) SUDEP cases had primarily generalised epilepsy compared to 12 out of 171 (7%) control subjects. Statistical comparison revealed that there was a higher risk of SUDEP in patients with primary generalised epilepsy compared to patients with focal, symptomatic epilepsy, although this was only significant in men. Nevertheless, although primary generalised epilepsy is usually less refractory to treatment, individuals with this

type of epilepsy are well represented in SUDEP cohorts. It is possible that specific epilepsy syndrome subtypes carry an increased risk of sudden death due to phenotypic expression in other cerebral and possibly cardiac structures.

High seizure frequency seems to be an independent risk factor for SUDEP. Several descriptive and large case-control studies have reported an increased risk of SUDEP in patients with poor seizure control (Tennis et al, 1995; Nilsson et al, 1999; Walczak et al, 2001; Schnabel et al, 2002; Langan et al, 2005). This increased risk is most marked for generalised tonic-clonic seizures (GTCs), with or without focal onset (Leestma et al, 1989; Ficker et al, 1998; Nashef et al, 1998; Nilsson et al, 1999; Walczak et al, 2001; Langan et al, 2005; Hesdorffer et al, 2011) rather than non-convulsive episodes, such as complex partial seizures (Schnabel et al, 2002). Moreover, on logistic regression analysis, it was noted that only the frequency of GTCs was relevant, and not the frequency of all seizures combined (Walczak et al, 2001). High seizure frequency was not an independent risk factor in a number of other reports although a number of methodological issues exist (Opeskin & Berkovic, 2003; Lear-Kaul et al, 2005). In a population-based cohort of childhood onset epilepsy drug-resistance emerged as a risk factor (Sillanpaa & Shinnar; 2010).

1.3.3 Antiepileptic medication

The number of antiepileptic drugs (AEDs) taken concomitantly has been reported to be an independent risk factor for SUDEP (Tennis et al, 1995), even after correction for seizure frequency (Nilsson et al, 1999; Walczak et al, 2001; Hitiris et al, 2007). This is not universally reported however (Opeskin & Berkovic, 2003; Langan et al, 2005; Vlooswijk et al, 2007; Hesdorffer et al, 2012). Risk of SUDEP is also increased in those whose treatment history was unclear, which may reflect the risk associated with the lack of treatment and uncontrolled seizures, although the reason for this was not objectively assessed (Langan et al, 2005). Despite several descriptive studies suggesting that sub-therapeutic levels of AEDs are a risk factor for SUDEP (Leestma et al, 1989; Ficker et al, 1998; Kloster & Engelskjon, 1999; Lear-Kaul et al, 2005; Zhuo et al, 2012), this has not been corroborated by the majority of case-control studies (Nilsson et al, 2001; Opeskin & Berkovic, 2003), most likely because this is difficult to study as an independent factor. Of note is that post-mortem levels of AEDs may not accurately reflect ante-mortem levels possibly due to, for example, redistribution and continuing

metabolism (Tomson et al, 1998a). The issue of variability of AED use was recently addressed in a study comparing hair AED concentration variability in patients with SUDEP, non-SUDEP epilepsy-related deaths, epilepsy outpatients and epilepsy inpatients. The SUDEP group showed greater hair AED concentration variability than either the outpatient or the inpatient groups, reflecting variable AED ingestion over time. However, this cannot distinguish prescribed changes from poor compliance, or identify consistent non-compliance over time. Secondly, it does not provide information on drug taking behaviour immediately before death as it takes about five days for drug sequestrated into the follicle to appear at the scalp; therefore short-term non-compliance immediately before death is not assessed by this study and may have been overlooked (Williams et al, 2006). Despite a number of descriptive and controlled studies, no specific AED has been clearly associated with an increased risk of SUDEP (Walczak et al, 2001; Walczak, 2003; Lear-Kaul et al, 2005; Hitiris et al, 2007), although a few studies have implicated treatment with carbamazepine as an independent risk factor (Hennessy et al, 2001; Langan et al, 2005). For antiepileptic medication in general, proposed mechanisms include perturbed heart rate variability, lengthening of the Q-T interval on the electrocardiogram combined with a mild pro-arrhythmic effect of epileptic seizure discharges, or excessive post-seizure brainstem inhibition producing a blunting or transient abolition of the central hypoxic and hypercarbic respiratory drive, with consequent post-ictal respiratory arrest (Tomson & Kenneback, 1997; Hennessy et al, 2001). Elevated serum levels of carbamazepine have been associated with an increased risk of SUDEP even after adjustments for seizure frequency have been made. Frequent drug changes and multiple concomitant AEDs, conventional markers of severe and unstable epilepsy, increased this risk synergistically (Nilsson et al, 2001). On this basis, it is difficult to know whether a high carbamazepine level is an independent risk factor or is merely representative of challenging epilepsy.

1.3.4 Perimortem features

There is evidence from both descriptive and controlled studies that a terminal (preceding death) GTCs (Leestma et al, 1989; Nashef et al, 1995a; Nashef et al, 1998; Opeskin & Berkovic, 2003; Thom et al, 2003; Lear-Kaul et al, 2005), being found alone in bed (Nashef et al, 1995a; Nashef et al, 1998; Opeskin & Berkovic, 2003; Zhuo et al, 2012) and in the prone position (Langan et al, 2005; Lear-Kaul et al, 2005; Zhuo et al, 2012; Shmuely et al, 2016) are independent risk factors for SUDEP. Whereas a small

number of descriptive studies have not found an association, all case-control studies that have evaluated these factors have found a positive relationship with the risk of SUDEP. In a published report of interviews with bereaved relatives, evidence for a terminal seizure was found in 24 out of 26 cases but it is of interest that only two were witnessed. The observation that, in most studies, unwitnessed cases far outnumber those witnessed suggests that enhanced surveillance of patients with epilepsy may be protective (Nashef et al, 1998). This is corroborated by a study of young patients with epilepsy at a special residential school. All sudden deaths during the period of the study occurred when the pupils were not under the close supervision of the school and most were unwitnessed (Nashef et al, 1995b). Similar findings of a protective effect of enhanced supervision at night were also found in a large controlled study, where supervision was defined as the presence in the bedroom of an individual of normal intelligence and at least 10 years old or the use of special precautions, such as checks throughout the night or the use of a listening device (Langan et al, 2005). In some cases where a prone position was not observed, other factors which might compromise breathing were identified. For example, in one study only five out of 26 people were found face down in the pillow, and a sixth with the head in carpet pile. In total however, there were 11 out of 26 cases in which an extrinsic or intrinsic positional obstruction to breathing amenable to intervention may have contributed (Nashef et al, 1998). Moreover, it is possible that this may be an underestimate as obstructive apnoea can occur in an apparently benign position (Nashef et al, 1998). A retrospective survey of patients being monitored on EEG-videotelemetry units (MORTEMUS study) found 29 cardiorespiratory arrests in 147 units. Of these, 16 were SUDEP, nine near-SUDEP and four deaths from other causes (1 et al, 2013a). They identified post-convulsive respiratory and cardiac rate and rhythm disturbances in SUDEP. Of the 16 SUDEP cases, 14 occurred at night (Ryvlin et al., 2013a).

1.3.5 Other features

There is limited evidence for an independent association between intellectual disability and an increased risk of SUDEP. Early descriptive and population-based studies, in which intellectual disability was determined by observer impressions rather than by formal IQ examination, provided only weak support for this association (Hirsch CS & Martin DL, 1971; Leestma et al, 1989). A recent study of a cohort of patients with epilepsy and intellectual disability found a high SUDEP incidence in this population but

there was no comparison with epilepsy patients without intellectual disability (Kiani et al, 2014). Other studies have found no clear correlation (Nashef et al, 1998; Opeskin & Berkovic, 2003; Langan et al, 2005; Hesdorffer et al, 2011), although others have reported an IQ of less than 70 to be a risk factor for SUDEP, even after accounting for seizure frequency (Walczak et al, 2001). It has been postulated that patients with intellectual disability are more susceptible to central apnoea and positional asphyxia that may cause SUDEP as a result of prolonged post-ictal encephalopathy (Biton et al, 1990), decreased post-ictal respiratory drive and impaired movement and righting reflexes (Walczak et al, 2001).

Despite early reports of an increased incidence of structural lesions in patients with SUDEP (Annegers et al, 1984; Leestma et al, 1989), this has not been confirmed by more recent, controlled studies (Nilsson et al, 1999; Walczak et al, 2001).

While there is evidence that psychotropic medication can influence the risk of sudden death in general, there is no convincing evidence of this being particularly relevant in SUDEP.

1.4 Pathophysiology of SUDEP

Pathophysiological mechanisms of SUDEP are likely to be heterogeneous and may be multifactorial. Theories propounded have focused on autonomic disturbance - particularly cardiac arrhythmias and central and obstructive apnoea and neurogenic pulmonary oedema. Additionally, the possibility of structural or functional cardiac pathology predisposing patients with epilepsy to cardiac events has been proposed.

1.4.1 Cerebrogenic autonomic control

The components of the central autonomic network involved in the functional relationships between cortical, subcortical and somatic regions have been elucidated from experimental and human stimulation and lesional studies. For example, it has been demonstrated that limbic structures, especially the amygdala and pyriform cortex, modulate hypothalamic function, and stimulation of these foci can elicit both sympathetic and parasympathetic visceromotor autonomic responses (Altenmuller et al, 2004).

Other than visual inspection of a standard 12-lead ECG, more sophisticated methods to interrogate the cardiac autonomic system have been developed, for example, measures of heart rate variability. In its simplest form this is measured in a time domain analysis as the standard deviation of R-R wave intervals (Persson et al, 2005). Frequency domain analysis permits the calculation of high-frequency (HF) and low-frequency (LF) components which assess the relative contribution of parasympathetic and sympathetic autonomic activity (Evrengul et al, 2005). Depressed interictal heart rate variability is seen in people with chronic epilepsy (Lotufo et al, 2012; Surges et al, 2012). AEDs, epilepsy duration, GTCs, and drug-resistant epilepsy seem to contribute to impaired interictal heart rate variability (Surges et al, 2010; Suorsa et al, 2011; Yildiz et al, 2011). There is no established association between impaired interictal heart rate variability and risk of SUDEP.

1.4.2 Cardiac mechanisms

1.4.2.1 Structural cardiac pathology. The exclusion of cardiac pathology as a contributing factor in SUDEP is challenging due to the presence of, for example, subtle abnormalities that only a detailed microscopic examination of cardiac tissue can elucidate, such as conducting system fibrosis or cardiomyopathy (Corrado et al, 2001), tissue decomposition precluding the acquisition of suitable material for evaluation, lack of an appropriate control group for comparison, and the possibility of a functional rather than a structural disorder, such as ion channelopathies or pre-excitation syndromes, with normal macroscopic and microscopic examinations being implicated (Nashef et al, 2007). Increased cardiac weight has been observed in male SUDEP cases compared to control subjects (Leestma et al, 1989) although more recent studies, using more convincing methodology, have failed to replicate this earlier finding and cardiac weight is not considered to differ between SUDEP and non-SUDEP cases (Opeskin et al, 2000; Davis GG & Mcgwin, 2004). It has been postulated that neurogenic coronary vasospasm may be implicated, and that if recurrent, this may eventually progress to perivascular and interstitial fibrosis (Cordero et al, 1995). This may, in turn, predispose the heart to arrhythmogenesis, particularly in the setting of considerable autonomic imbalance during seizures (Kawara et al, 2001). The occurrence and significance of these pathological changes in SUDEP is not universally agreed however (Opeskin et al, 2000; Codrea et al, 2005) and the full characterisation of the relationship between

myocardial pathology and acute and recurrent seizures remains unclear at the present time.

1.4.2.2 Inter-ictal. At the simplest level, inter-ictal cardiac function can be evaluated by visually assessing a standard 12-lead ECG, primarily for evidence of conduction abnormalities, although these are frequently normal (Rugg-Gunn et al, 2004) or show only minor, non-significant changes (Drake et al, 1993). Early experimental studies demonstrated that inter-ictal epileptiform activity was associated with sympathetic and parasympathetic autonomic dysfunction, in a time-locked synchronised pattern (Lathers et al, 1987). In the first clinical reports, analysis of inter-ictal heart rate variability in 19 patients with refractory temporal lobe epilepsy revealed frequent, high-amplitude fluctuations in heart rate which were most pronounced in poor surgical candidates (Frysinger et al, 1993). More recently, reduced sympathetic tone, demonstrated by decreased low-frequency power, has been seen in both focal and, albeit less markedly, primary generalised epilepsy (Toichi et al, 1998; Tomson et al, 1998b). Overall, there is some evidence for inter-ictal cardiac autonomic dysfunction in patients with both focal and generalised epilepsy, possibly modulated by antiepileptic medication, in particular carbamazepine. There are conflicting reports in the literature however, suggesting that the relationship between inter-ictal epileptiform activity, antiepileptic medication and autonomic function has not yet been fully characterised.

1.4.2.3 Ictal. Arrhythmias, conduction block and repolarisation ECG abnormalities, such as atrial fibrillation, marked sinus arrhythmia, supraventricular tachycardia, atrial and ventricular premature depolarisation, bundle-branch block, bundle-branch block, high-grade atrioventricular conduction block, ST segment depression and T wave inversion have been reported in up to 56% of seizures. Abnormalities appear to be more common in nocturnal, prolonged and GTCs than in focal seizures or those occurring during wakefulness (Tomson et al, 1998b; Zijlmans et al, 2002; Nei et al, 2004). Sinus rate change is the most common cardiac accompaniment to ictal discharge. Sinus tachycardia has been reported in 50−100% of seizures, and is dependent on the definition used and population studied (Kirchner et al, 2002; Zijlmans et al, 2002; Leutmezer et al, 2003; Mayer et al, 2004; Rugg-Gunn et al, 2004). Ictal tachycardia is most commonly seen in the early ictal phase, soon after seizure onset (Leutmezer et al, 2003; Mayer et al, 2004), or rarely before clear evidence of electroclinical onset (Zijlmans et al, 2002). This contrasts with ictal bradycardia which is seen during the late

ictal phase or in the immediate post-ictal period (Britton et al, 2006; Schuele et al, 2007). There is some evidence for right-sided lateralisation and temporal lobe localisation in patients with ictal tachycardia (Kirchner et al, 2002; Leutmezer et al, 2003; Mayer et al, 2004), corroborating the reports of early experimental and clinical stimulation studies (Oppenheimer et al, 1992; Swartz et al, 1994), although it is important to note that most temporal lobe seizures are associated with ictal tachycardia, irrespective of lateralisation. Although ictal tachycardia is almost universally observed, ictal bradycardia has received more attention due to the potential progression to cardiac asystole and intuitive but unproven association with SUDEP. The first report of ictal asystole was by Russell in 1906, who noted the disappearance of a young male patient's pulse during a seizure (Russell, 1906). The published literature since that time is, unsurprisingly, mostly case reports or small series studies, which significantly limit the number and confidence of any conclusions extracted from the data. A recent literature review revealed that ictal aystole had a mean prevalence of ictal asystole in all people admitted for a vEEG recording (including those without epilepsy) of 0.177% and a mean prevalence in all people with refractory focal epilepsy admitted for a vEEG recording of 0.318% (van der Lende et al, 2016). Ictal asystole was only reported in people with focal epilepsy. Most of the ictal asystoles occurred during the course of a focal dyscognitive seizure, on average starting 30 s after seizure onset. The mean duration of ictal asystole was 20 s (range 3–96). The seizure onset zone was reported in 78% of the cases and was temporal in 90% without consistent lateralisation. All ictal asystoles were self-limiting, except in one subject where resuscitation was started after 44 s of cardiac arrest. This event was labelled as near-SUDEP. Most of the postictal asystoles were seen after a focal seizure evolving to a bilateral convulsive seizure and had a mean duration of 30 s. They were preceded by postictal generalised EEG suppression (PGES). Seven of 13 people died of (probable) SUDEP (van der Lende et al, 2016). Twenty-five vEEG cases of ictal bradycardia without asystole were identified. Characteristics of ictal bradycardia cases were similar to those with ictal asystole. Ictal bradycardia was only reported in people with focal epilepsy during focal dyscognitive seizures. Seizure onset was predominantly temporal (van der Lende et al, 2016). Extrapolation of ictal bradyarrhythmias to a mechanistic explanation for SUDEP remains elusive. This is, at least partly, due to a lack of clinical evidence of common factors shared by patients with ictal bradyarrhythmias and SUDEP and the difficulty in ascertaining the importance of ictal bradyarrhythmias in SUDEP in relation to other

proposed mechanisms, including other intrinsic cardiac abnormalities or apnoea and hypoxia which may aggravate arrhythmias. In the MORTEMUS study (Ryvlin et al., 2013a), video-EEG recordings were used to estimate the presence of respiratory movements; all postictal asystoles were most likely preceded by apnoea. Postictal atrial fibrillation and ventricular fibrillation were detected in the context of convulsive seizures and, in contrast with ictal asystole and ictal bradycardia, atrial fibrillation was usually present for several hours. Postictal ventricular fibrillation was always classified as (near-)SUDEP. Postictal arrhythmias may be a marker of an increased SUDEP risk (van der Lende et al, 2016).

1.4.3 Respiratory mechanisms

It is likely that primary respiratory dysfunction is involved in an important proportion of SUDEP (Nashef et al, 1996; Langan et al, 2000; So et al, 2000; O'Regan & Brown, 2005). Alterations in respiration such as coughing, sighing, hyperventilation, irregular breathing, apnoea, increased bronchial secretions, laryngospasm, respiratory arrest, and neurogenic pulmonary oedema have all been described with seizures (Nashef et al, 1996; So et al, 2000; Blum et al, 2000O'Regan & Brown, 2005). Some form of respiratory compromise is commonly reported in witnessed cases of SUDEP (Tomson et al, 2008). Electrical stimulation of multiple brain areas, particularly in limbic and temporal regions, has been demonstrated to influence respiratory activity (Bonvallet & Bobo, 1972), supporting the potential for seizures arising from or involving these brain regions to alter respiratory function. Central apnoea can occur secondary to the ictal discharge, acting at either the cortical or medullary level or possibly as a result of secondary endogenous opioid release influencing the brainstem respiratory nuclei directly. During post-ictal impairment of consciousness, hypercapnia and hypoxia may be less potent respiratory stimuli.

Oxygen desaturations <90% have been frequently reported accompanying seizures, occurring in approximately one-third of GTCs and non-convulsive seizures (Bateman et al, 2008). Significant desaturations have also been noted in limited electrographic seizures without clear clinical accompaniments (Maglajlija et al, 2012). In a small number of cases (<5%), these desaturations may be profound, with measured oxygen saturation (SaO2) <70% (Bateman et al, 2008). Interestingly, transient bradycardia or sinus arrest has been seen in association with ictal apnoea suggesting that the reported

seizure-related arrhythmias may be consecutive to ictal apnoea (Nashef et al, 1996). In a study of 135 SUDEP cases, 15 of which were witnessed, observers described respiratory difficulties, such as apnoea and obvious respiratory obstruction, in 12 patients, although the conclusions that may be drawn are significantly limited by the quality of the retrieved information and lack of additional relevant cardiorespiratory parameters (Langan et al, 2000). Witnesses have reported a delay between the seizure and time of death which is more consistent with primary respiratory inhibition followed by respiratory arrest and the development of hypoxia and pulmonary oedema, than 'primary' ictal cardiac asystole (Lear-Kaul et al, 2005). Peri-ictal hypoxaemia has been associated with male gender, younger age in children, symptomatic generalised epilepsy, temporal onset seizures, seizure lateralisation (right in adults; left in children), seizure duration, contralateral electrographic seizure spread, AED polytherapy and MRI-negative epilepsy (Bateman et al, 2008; Moselei et al, 2011; Singh et al, 2013). Neurogenic pulmonary oedema, which may in itself be insufficient to be fatal, has been implicated in theories regarding respiratory dysfunction and SUDEP following a number of postmortem reports and case studies (Leestma et al, 1989; Swallow et al, 2002; Lear-Kaul et al, 2005). The apparent protective effect of supervision favours an important primary role for respiratory factors (Langan et al, 2005), as these can be influenced by relatively unskilled intervention, such as airway protection, repositioning, or stimulation. It is unknown what proportion of SUDEP cases may be prevented by such intervention. Suppression of cerebral activity The possibility of progressive suppression and eventually cessation of cerebral activity as a cause of SUDEP, despite normal cardiac function, was introduced with the publication of a case report of an intracranially monitored patient who died of SUDEP in which a seizure started in one hemisphere and then spread to the other after several minutes. The EEG pattern on the original side then changed to burst-suppression with spindling spike discharges, followed by complete cessation of activity. The other hemisphere continued to show spike discharges until ceasing suddenly a few seconds later. A pulse artefact on the EEG continued for a further two minutes; there was no recording of respiratory activity. It was postulated that the loss of EEG activity was not preceded by anoxia as both hemispheres were not simultaneously affected (Bird et al, 1997). Post-ictal generalised EEG suppression (PGES), is defined as the generalised absence of EEG activity greater than 10 μV in amplitude, allowing for muscle, movement, breathing, and electrode artifacts; it occurs in up to 65% or more of adult patients with convulsive seizures

(Lhatoo et al, 2010) and has been reported in monitored SUDEP or near SUDEP cases (McLean & Wimalaratna, 2007). It has been shown that >50 seconds of PGES significantly increases the adjusted odds ratios for SUDEP and for each one-second increase in the duration of PGES, the odds of SUDEP increases by a factor of 1.7% (Lhatoo et al, 2010). However, a small retrospective study of 17 SUDEP cases and matched controls found no significant differences in either presence or duration of PGES between the two groups (Surges et al, 2011) and a clear link between PGES and SUDEP continues to be elusive. Poh et al. (2012) evaluated sympathetic and parasympathetic changes in seizure patients by measuring electrodermal activity and heart rate variability. An increase in electrodermal activity response amplitude and a decrease in parasympathetic-modulated high-frequency power of heart rate variability were directly correlated to prolonged PGES. It is possible that PGES may serve as a marker of post-ictal autonomic dysregulation. The precise nature of the pathophysiological association is unclear. Excessive post-ictal brainstem inhibition due to seizure-induced release of GABA and other neuro-inhibitory peptides may contribute to death in some patients. This endogenous seizure-terminating mechanism could result in blunting of the central hypoxic and hypercarbic respiratory drive, resulting in post-ictal respiratory arrest, subsequent exacerbation of hypoxia, further cardiac destabilisation and death due to hypoxia and secondary cardiac arrhythmia. This is consistent with the observation that SUDEP occurs after a seizure, and could be a consequence of failed re-establishment of respiration in the post-ictal phase. It has been shown that patients with PGES are significantly more likely to be motionless in the post-ictal period and to have simple resuscitative interventions performed (suction, oxygen administration, placed in recovery position, vital signs checked) (Semmelroch et al, 2012). PGES in such individuals may indicate deeper post-ictal coma, more delayed arousal and, at least hypothetically, a predisposition to SUDEP. One study compared secondarily GTCs with and without PGES, and found that oxygen desaturation duration and extent, as well as peak end-tidal CO2 elevation, were more marked in patients with PGES but there was no evidence of a relationship with central apnoea (Seyal et al, 2012). Early nursing interventions that reduced peri-ictal hypoxaemia were also associated with shortening of PGES duration (Seyal et al, 2013). In the MORTEMUS study, PGES was observed in all monitored SUDEP cases once the EEG was no longer obscured by respiratory-related artifacts (Ryvlin et al, 2013a).

1.5 SUDEP and epilepsy surgery

There is compelling evidence that patients with poorly controlled, predominantly GTCs are at greatest risk of SUDEP, and a seizure is frequently seen as the terminal event. Intuitively therefore, good seizure control should translate into a reduced risk of SUDEP. A previous study evaluated the mortality rates of 393 patients who underwent epilepsy surgery; the standardised mortality ratio (SMR) for patients with recurrent seizures post-operatively was 4.69, with a SUDEP incidence of 7.5/1000 patient-years, whereas in patients who became seizure free, there was no difference in mortality rate compared with an age- and sex-matched population (Sperling et al, 1999). This compares with similar studies which, for example, found a SMR of 1.8 in those with a good post-operative outcome versus 7.4 in those who failed surgery (Salanova et al, 2002). Conversely, in a large, population-based epilepsy surgery cohort, there was no association between mortality rates and seizure outcomes, although there was a clear difference between patients who underwent surgery (SUDEP incidence 2.4/1000 patient-years) and those who failed pre-surgical assessment (SUDEP incidence 6.3/1000 patient-years) (Nilsson et al, 2003). It has been proposed that there is a common factor predisposing to surgical failure and an increased risk of SUDEP so that patients who respond poorly to surgery also carry an increased risk of SUDEP and that, overall, surgery does not alter the risk of SUDEP (Ryvlin & Kahane, 2003). Proposed common factors include temporal lobe epilepsy which extends beyond the temporal lobe into the insula, frontal orbital or frontal operculum region which may favour ictal arrhythmias, central apnoea and secondary generalisation. This, in turn, would increase the risk of SUDEP and the wide epileptogenic field would translate into a poor post-operative seizure outcome (Ryvlin & Kahane, 2003). Mortality studies performed in patients with vagal nerve stimulators have shown that excess mortality associated with refractory epilepsy reduced as a function of duration of use. The rate of SUDEP was 5.5/1000 patient-years in the first 24 months and 1.7/1000 patient-years thereafter, possibly reflecting gradual increase in efficacy over time. Stabilisation of measures of heart rate variability post-VNS implantation (Galli et al, 2003) have paralleled the improved mortality rates, although these findings are not universal (Ronkainen et al, 2006).

1.6 Neuropathology

1.6.1 Brain pathology

Post mortem examination is mandatory in SUDEP, primarily to exclude an anatomical (e.g. cardiac) or other cause of death. The examination of the brain in SUDEP cases may show mild swelling or 'fullness' of the convexities reflected in high-average brain weights but, by definition, significant swelling, shift or herniation is absent (Kloster & Engelskjon, 1999). It is perhaps a common misconception that the brain in SUDEP cases is normal in the vast majority of cases. Analysis from the larger SUDEP series report macroscopic abnormalities in half to two-thirds of cases (Shields et al, 2002). More frequently reported macroscopic abnormalities include old cerebral traumatic lesions (contusions, gliosis, previous craniotomy sites), hippocampal or cortical atrophy, cerebellar atrophy, haemangiomas, low-grade tumours and cortical malformations (Shields et al, 2002). There is no accurate data regarding the relative risk or association of any of these specific pathological lesions for SUDEP. Some lesions, including acquired old injuries and cortical neuronal damage, however may give an indirect measure of the clinical severity of the epilepsy. Histopathological examination is required in SUDEP cases for the confirmation of any type of macroscopic lesion identified but also to investigate any unsuspected pathology, e.g. meningo-encephalitis. It is not possible or necessary for a neuropathologist to perform all autopsies on patients with epilepsy. Ideally, a specialist neuropathologist should be involved in the interpretation of the histological brain findings.

1.6.2 Other organ pathology in SUDEP

There have been several studies addressing the presence of associated or significant cardiac pathology in SUDEP which may relate to the cause of death. Initial reports suggested increased heart weights and co-existing cardiac hypertrophy in some patients with SUDEP (Opeskin et al, 2000). In subsequent studies however, no difference in heart mass compared to non-SUDEP controls was noted when corrected for body mass (Davis & Mcgwin, 2004). Extensive sampling of the myocardium in SUDEP revealed frequent foci of reversible pathology (myocyte vacuolisation and interstitial oedema) in addition to irreversible pathological changes (contraction band necrosis, haemorrhage, fibrosis and hyper-eosinophilia of myocardial fibres) compared to control groups. Regions of myocardial fibrosis have been described around vessels or interdigitating

between bundles of fibres (Natelson et al, 1998). In a further study, 13 blocks of myocardium were sampled from each of 23 SUDEP cases and a significant increase in deep and sub-endocardial fibrosis was shown in 40% of the SUDEP patients compared to controls (P-Codrea Tigaran et al, 2005). Cardiac fibrosis has not however been reported in all post mortem SUDEP series (Opeskin et al, 2000). Pulmonary oedema has been reported in 50−90% of SUDEP cases (Kloster & Engelskjon, 1999; Opeskin et al, 2000; Shields et al, 2002). Lung weights in SUDEP cases did not differ from non-SUDEP cases (Davis & Mcgwin, 2004) in another study. Toxicology screening is important in the investigation of SUDEP, as in other adult sudden death cases, in order to exclude a toxic cause of sudden death and for the monitoring of AED levels to assess compliance. This should include blood, urine, and gastric contents for AEDs, drugs of abuse and alcohol level estimations. Vitreous humour should be taken for biochemistry if diabetes or other metabolic disorder is considered. Hair testing may also prove useful to test for long-term drug compliance if indicated (Williams et al, 2006).

1.7 Genetic susceptibility

Several genes have been linked to SUDEP in human and animal studies making them candidate genomic biomarkers. (Glasscock, 2013), including genes with predominantly neural expression but that might also influence cardiac function through the autonomic nervous system, genes with both cardiac and neural expression and 'neurorespiratory' genes involved in serotonin signalling including the control of respiration may also influence SUDEP risk (Richerson and Buchanan, 2011).

Dravet Syndrome is a severe epilepsy syndrome of early childhood, associated with drug resistance, developmental slowing or regression and intellectual disability, and risk of premature mortality, including SUDEP. The most frequent cause of Dravet Syndrome is mutation in the voltage-gated sodium channel alpha1 subunit gene (*SCN1A*). Mutations in *SCN1A* are also associated with milder phenotypes, such as genetic epilepsy with febrile seizures plus (GEFS+) (Ceulemans et al, 2004). GEFS+ was first reported in 1997 (Scheffer and Berkovic, 1997) and has a spectrum of phenotypes including FS+, defined as "FS extending beyond six years with or without afebrile generalized tonic–clonic seizures." Two cases of SUDEP (one definite, one probable) were reported in a family with a history compatible with GEFS+ and a novel

SCN1A mutation (Hindocha et al, 2008). Mortality is also increased in Dravet Syndrome compared with other childhood epilepsies of similar severity, ranging from 5 to 20%, ascribed largely to SUDEP (Oguni et al, 2001; Dravet et al, 2005; Genton et al, 2011; Sakauchi et al, 2011). An increased susceptibility to sudden death may be through cardiac mechanisms, reflecting underlying processes common to both neurological and cardiac functions. While *SCN1A* is primarily a neuronal gene, several studies have shown that Nav1.1 (*SCN1A* gene product) is present in various regions of the heart in rat and mouse (Rogart et al., 1989; Dhar et al., 2001; Marionneau et al., 2005), in rabbit neonate (Baruscotti et al., 1997), and in dog (Haufe et al., 2005). There is good evidence for a role for Nav1.1 in pacemaker function of the sino-atrial node. In mice, Nav1.1 was detected in the sino-atrial node, and moreover, when brain-type Na+ channels were selectively blocked, significantly reduced spontaneous heart rate and greater heart rate variability were observed (Maier et al., 2003). A role for Nav1.1 in pacemaker activity in the mouse sino-atrial node was confirmed in a similar but independent study (Lei et al., 2004). Further support comes from a study in rats, where Nav1.1 was also found in the SA node. Heart failure, induced by volume overload, resulted in SA node dysfunction and down-regulation of Nav1.1 expression (Du et al., 2007). Data from single cell and whole animal (*Scn1a*-R1407X knock-in mice) experiments suggest that altered cardiac electrical function in Dravet syndrome may contribute to the susceptibility for arrhythmogenesis and SUDEP (Auerbach et al, 2013). Other possible mechanisms for increased susceptibility to sudden death include an effect of the *SCN1A* mutation on brainstem control of respiration or autonomic function. Some individuals with Dravet Syndrome exhibit reduced heart rate variability; ECG recordings may show increased P-wave and QT dispersion (Delogu *et al.*, 2011; Ergul *et al.*, 2013). In a mouse model of Dravet Syndrome the mechanism of premature death in *Scn1a* heterozygous KO mice and conditional brain- and cardiac-specific Kos were studied. SUDEP was found to be caused by apparent parasympathetic hyperactivity immediately following tonic-clonic seizures in Dravet mice, which leads to lethal bradycardia and electrical dysfunction of the ventricle (Kalume et al, 2013).

SCN8A encodes the voltage-dependent sodium channel Nav1.6, located in both inhibitory and excitatory neurons (Wagnon & Meisler, 2015). Mutations in this gene have been found in 0.6-2.4% of cases with early infantile epileptic encephalopathy

(Larsen et al, 2015) and have been associated with increased risk of SUDEP (Veeramah et al, 2012; Wagnon & Meisler, 2015; Frasier et al, 2016)

Autosomal dominant lateral temporal lobe epilepsy (ADLTE) is a rare familial epileptic syndrome characterized by auditory and visual ictal manifestations, seizures triggered by auditory stimuli, and rare nocturnal GTCs (Gu et al., 2005). ADLTE is caused by mutations in *LGI1* (leucine-rich gene, glioma-inactivated-1), encoding a secreted neuronal protein, (Fukata et al, 2006). In a Japanese family with ADLTE two cases of sudden death were reported (Kawamata *et al.,* 2010).

Alternating hemiplegia of childhood (AHC) is a rare neurodevelopmental disorder with onset before the age of 18 months, characterized by recurrent transient plegic or paretic attacks, affecting alternate or both sides of the body, dystonic posturing, oculomotor dysfunction and seizures (Neville and Ninan, 2007; Panagiotakaki et al., 2010). Pathogenic mutations, almost always de novo, in the *ATP1A3* gene, encoding the catalytic alpha-3 subunit of the Na+/K+-ATPase transporter protein, are the cause in □80% of cases (Ishii et al., 2013). Sudden death, included SUDEP, has been reported in AHC (Panagiotakaki et al., 2010).

HCN2 is one of a family of four genes (*HCN1-4*) that encodes a hyperpolarization-activated, cyclic nucleotide-gated cation channel, which passes a mixed Na+/K+ inward current (termed If in cardiac cells and Ih in neurons) that activates with hyperpolarizing steps below about −50 mV (Robinson & Siegelbaum, 2003). *HCN2* channelopathy has been linked to both cardiac and epilepsy phenotypes in mice and to epilepsy and SUDEP in humans. In humans, screening of *HCN1* and *HCN2* genes in epilepsy patients has identified a recessive loss-of-function missense mutation in the channel gating region of *HCN2* that increases neuronal excitability and underlies idiopathic generalized epilepsy (DiFrancesco et al, 2011). In addition, a postmortem analysis of *HCN1-4* DNA variants in a group of 48 SUDEP cases found two different non-synonymous changes in *HCN2* (F738C and P802S) associated with SUDEP that were absent in controls, suggesting *HCN2* variants may underlie susceptibility to sudden death (Tu et al, 2011).

Behr et al. (2008) found features of inherited cardiac disease in 30/57 families with sudden arrhythmic death syndrome, with approximately one quarter of first-degree relatives being identified as likely to be affected. The conditions detected, likely to be

the cause of death, were long QT syndrome, Brugada syndrome, and subtle structural disease, particularly arrhythmogenic right ventricular cardiomyopathy. Also the inherited short QT syndrome is a genetic condition, associated with sudden cardiac death. In 2000, for the first time, the association of a shortened QT interval and arrhythmia was described in a sporadic case of sudden cardiac death and a family with paroxysmal atrial fibrillation. The arrhythmogenic potential of a shortened QT interval was confirmed in two unrelated families with a high number of sudden cardiac deaths in 2003 (Gaita et al, 2003). Sudden cardiac death has also been observed during the first year of life, and thus SQTS may be another potential cause of sudden infant death syndrome (SIDS) (Schimpf et al, 2008). Mutation analysis reveals a genetically heterogeneous disease with gain-of-function mutations of the cardiac I_{Kr} channel HERG (*KCNH2*, SQT-1 syndrome), the delayed rectifier potassium channel I_{Ks} (*KCNQ1*, SQT-2 syndrome), the inward rectifier potassium channel (I_{K1}) *KCNJ2*, SQT-3 syndrome) and loss-of-function mutations of cardiac L-type calcium channel with a Brugada phenotype (*CACNB2*, SQT-4 syndrome and *CACNA1C*, SQT-5 syndrome) (Schimpf et al, 2008). Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a devastating inherited disorder characterized by episodic syncope and/or sudden cardiac arrest during exercise or acute emotion in individuals without structural cardiac abnormalities (Napolitano & Priori, 2007). Although rare, CPVT has been estimated to cause up to 15% of unexplained sudden cardiac deaths in young people (Liu et al, 2008). Mutations in the ryanodine receptor 2 gene (*RYR2*), encoding the cardiac sarcoplasmic calcium channel, are known to cause dominantly inherited CPVT (Priori et al, 2001), and more than 70 different mutations are currently known. Other genes involved are the calmodulin gene (*CALM1*) (Nyegaard et al, 2012), calsequestrin-2 gene (*CASQ2*) (Lahat et al, 2001), *KCNJ2* and *ANK2* (Mohler et al, 2004; Tester et al, 2006).

1.8 Neuroimaging in SUDEP

Mueller et al (2014) compared MRI images between 7 controls, 30 cases with temporal lobe epilepsy (TLE) (of these, 16 with mesial-temporal-sclerosis (TLE-MTS) and 14 without (TLE-no)), and 2 patients with TLE who died of SUDEP. They found that TLE-MTS and to a lesser degree TLE-no is associated with volume loss in the dorsal mesencephalon that is most prominent in the region of the periaqueductal gray,

colliculi, raphe and reticular formation and extends into the diencephalon particularly the medial posterior thalamus. Graph analysis based on a measure that favored the interaction between regions with a similar degree of atrophy was used to characterize the impact of the mesencephalic volume loss on brainstem regions containing nuclei involved in the central autonomic controls. Nodal degree and local efficiency were increased in regions with volume loss in TLE-MTS compared controls. A similar pattern of graph analytical abnormalities was found in the mesencephalic nodes of TLE-no but these abnormalities did not reach significance. Mesencephalic volume losses were also seen in the two SUDEP TLE patients. In contrast to the two TLE groups though, this volume loss was not only more severe but in the case of the SUDEP TLE-MTS patient also more widespread, i.e. extended into the dorsal section of the pons and even upper medulla oblongata. In summary, there was evidence for volume loss/atrophy in brainstem regions involved in the autonomic control in TLE. These changes were not only more pronounced in the two SUDEP cases but also associated with graph analytical abnormalities that indicated an impaired interaction between those regions.

A resting-state functional connectivity study (Tang et al, 2014) compared blood oxygen level-dependent (BOLD) resting-state functional connectivity between 13 patients at high risk for SUDEP and 12 patients at low risk for SUDEP. They found that patients at high isk exhibited significant reductions in the resting-state functional connectivity between the pons and the right thalamus, the midbrain and the right thalamus, the bilateral anterior cingulate cortex and the right thalamus, and the left thalamus and the right thalamus.

Imaging studies in other conditions with high risk of sudden death have also shown structural changes in brain regions bearing autonomic regulatory or respiratory functions, i.e. the dorsal and ventral medulla, putamen, and bilateral insular cortices in recent-onset obstructive sleep apnoea (Kumar et al., 2014), and the hypothalamus, posterior thalamus, caudal raphe, locus coeruleus, insular cortex and lateral medulla in congenital central hypoventilation syndrome. People suffering from the latter condition are especially at risk for sudden death (Patwari et al., 2010).

Neuropathological studies in SIDS report brainstem abnormalities, i.e. brainstem gliosis and defects of neurotransmission in the medulla (Paine et al., 2014). Dentate gyrus abnormalities with granule cell dispersion, in the hippocampus, were reported in a large

subset of 153 SIDS cases, and may reflect defective neuronal migration and proliferation (Kinney et al., 2015). Kinney et al (2015) propose that focal granule cell bilamination, a variant of granule cell dispersion, in the dentate gyrus may be a morphological marker of an impaired forebrain/limbic network that increases the risk of sudden infant death due to instability of modulation of brainstem cardiorespiratory-related nuclei.

1.9 Need for biomarkers of SUDEP

The United States National Institutes of Health (NIH) defines a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Atkinson et al, 2001). In the highly heterogeneous and multifactorial background described above, there is a lack of reliable and validated biomarkers for SUDEP. Despite an increase in SUDEP research over the past decade, a number of substantial knowledge gaps still exist, which means prevention strategies cannot be optimised. No quantifiable SUDEP risk models have been developed for clinical use. Prediction of individual risk is fundamental to adopt preventative strategies and there is a clear and obvious urgency to test potential interventions aimed at reducing the risk of SUDEP (Tomson et al., 2016). Studies of clinical risk factors have allowed to identify high-risk populations, such as those with chronic refractory epilepsy and frequent or nocturnal GTCS. However no genomic, electrophysiological or structural features have emerged as firmly established biomarkers of an increased SUDEP.

1.10 Hypotheses

1. SUDEP represents the most severe degree of the spectrum of epilepsy severity and genomic factors are among the parameters that might indicate epilepsy severity or high-risk of drug resistance. There are no genome-wide studies exploring the genetic burden of SUDEP. This is mostly due to the fact that genetic studies of SUDEP provide a different level of challenge (SUDEP cannot be predicted, difficulties in prospective recruitment, collection after SUDEP is difficult to systematise).

To elucidate the genetic basis and architecture of SUDEP, we first proposed that there is a broad risk for SUDEP across the genome and then that risk-conferring mutations in single genes influenced the risk of SUDEP. We used an unbiased sequencing approach based on whole-exome sequencing data. We examined overall burden and over-representation of deleterious variants in people who died of SUDEP compared to living people with epilepsy and non-epilepsy disease controls.

2. To elucidate which brain regions may be implicated in SUDEP, we investigated whether regional abnormalities in grey matter volume appear in those who had SUDEP, compared to healthy controls. Due to the low incidence of SUDEP, exploring enriched risk groups has been suggested as a means to increase the yield of future studies (Ryvlin et al, 2013b). We explored whether regional imaging findings in people who died of SUDEP can be reproduced in a larger cohort of subjects at high risk for SUDEP. To assess whether imaging findings are common to SUDEP and those at high risk, independent from other epilepsy-related factors, we compared SUDEP cases and those at high risk to a population presumed to be at low risk of SUDEP. We also compared subjects at high risk and low risk of SUDEP to healthy controls.

Chapter 2

GENOME-WIDE POLYGENIC BURDEN OF RARE DELETERIOUS VARIANTS IN SUDEP

To elucidate the genetic basis of SUDEP, we analysed rare, protein-changing variants from whole-exome sequences of 18 people who died of SUDEP, 87 living people with epilepsy and 1479 non-epilepsy disease controls (Methods and Results published in Leu, Balestrini, et al, 2015).

2.1 Methods

The study was approved by the relevant institutional review boards, accredited regional/national biobanks or international cohorts with ethical frameworks.

2.1.1 Sample collection

Collection of SUDEP samples for genetic studies provides an unusual level of challenge. SUDEP cannot be predicted, so there is no 'target' population. Collection after SUDEP is difficult to systematize, as by definition death is unexpected and cannot be anticipated, leading to logistic difficulties of obtaining material after death (Smithson et al., 2014).

National Hospital for Neurology and Neurosurgery

At the National Hospital for Neurology and Neurosurgery, DNA samples have been collected from thousands of patients for an approved broad study of epilepsy genetics and pharmacogenomics. By chance, some of the individuals who gifted DNA samples sadly succumbed to SUDEP. These were the samples used in this study.

Wales Epilepsy Research Network (WERN), Swansea University

WERN has an accredited epilepsy BioBank with 3,000 samples including epilepsy families and specific cohorts and has IRAS approval for the infrastructure project. Samples submitted to this study were gifted by consent prior to the tragic SUDEP event. We thank the families for their post-SUDEP advocacy of the research and their positive bravery in the search for the cause.

Royal College of Surgeons in Ireland

At Beaumont Hospital / RCSI, Dublin, DNA samples have been collected from over 1,500 of patients with different types of epilepsy for an approved broad study of epilepsy genetics and pharmacogenomics. Using our epilepsy electronic patient record database we identified two patients from whom DNA had been collected and who had died of SUDEP. These samples were used in this study.

Epilepsy Research Centre, Melbourne

The Epilepsy Genetics Research Program at the Epilepsy Research Centre, Austin Health, University of Melbourne, has been fortunate to have many thousands of participants with epilepsy provide DNA samples over 25 years. Sadly, some participants have subsequently passed away from SUDEP. We thank our participants and their families for their ongoing support of our research, especially following such a tragic event.

Royal Hospital for Sick Children

At the Royal Hospital for Sick Children, Glasgow DNA samples have been collected from patients for clinical testing of epilepsy genes. A cohort of patients with *SCN1A*-related epilepsy was enrolled in a research project. By chance some of the individuals

who gifted DNA samples sadly succumbed to SUDEP. These were the samples used in this study.

2.1.2 Study design

We used whole-exome sequencing (WES) data from 18 people with epilepsy who died of SUDEP and two control cohorts: a group of 87 living people with epilepsy, which we termed 'epilepsy controls', and 1,479 non-epilepsy 'disease control' samples. To ensure data homogeneity, a joint calling strategy, and stringent variant and individual-level quality control (QC) were applied for all WES datasets (Figure 2.1).

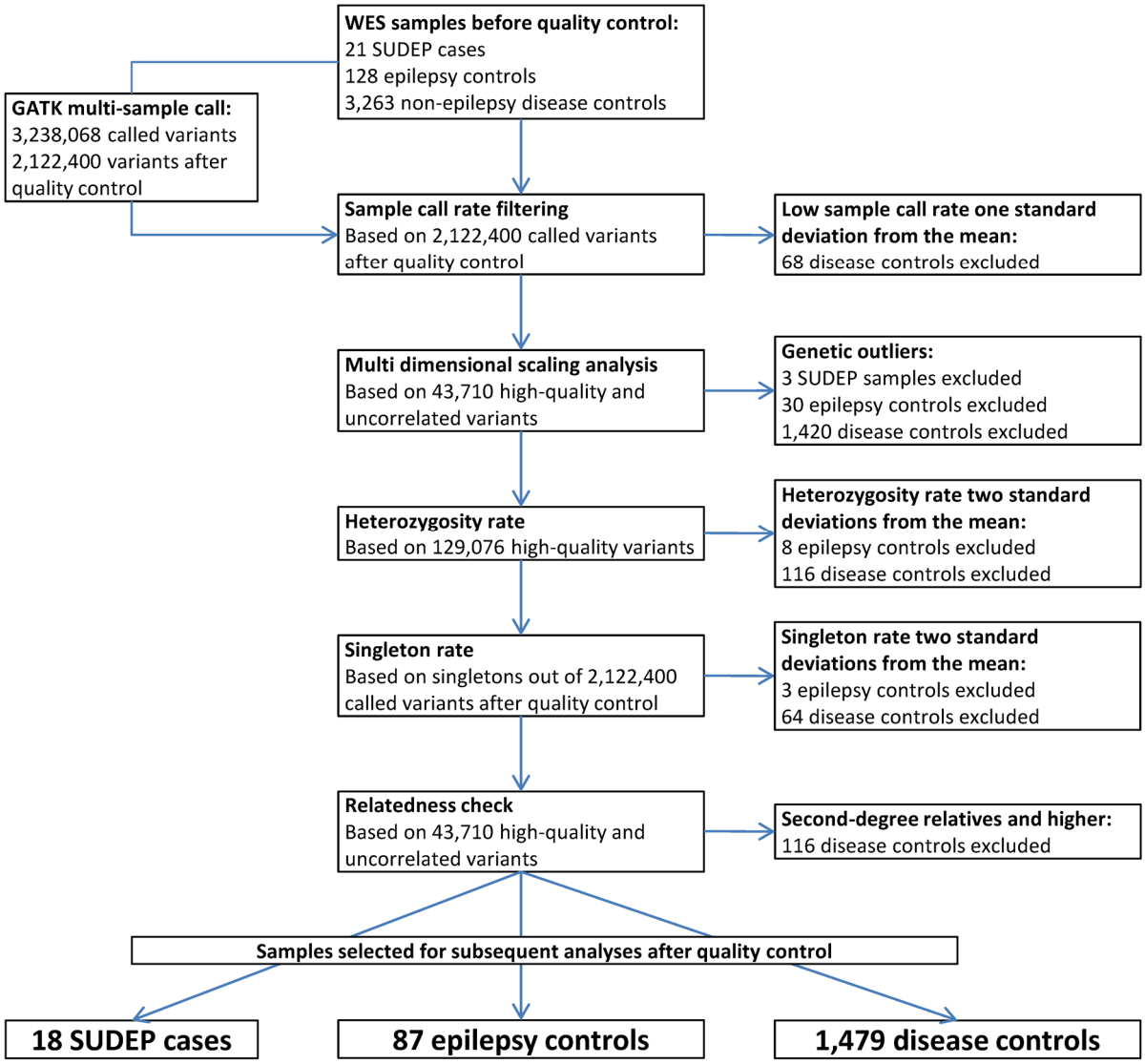

Figure 2.1. Individual-level quality control flowchart for the SUDEP, epilepsy control, and non-epilepsy disease control samples used in this study. Abbreviations: WES, whole-exome sequencing; SUDEP, sudden unexpected death in epilepsy.

2.1.3 Whole-exome sequencing

All epilepsy samples were sequenced using either Agilent's SureSelect Human All Exon V1 (38Mb, n = 42) and SureSelect Human All Exon V5 (50Mb, n = 56) or Illumina's Nextera Rapid Capture Exome kit (37Mb, n = 16). For the disease control samples, NimbleGen's SeqCap EZ and Illumina's TruSeq Exome capture technology were also used. Sequencing was performed on Illumina HiSeq2500 or GAIIx sequencing systems.

We used a multi-sample joint calling strategy across all SUDEP cases, epilepsy and disease control samples to mitigate problems caused by the heterogeneity of sequence capture kits. One major confound in case-control variant burden analyses can arise when either single-sample calling, or multi-sample calling in different batches, is used to generate the variant calls. Standard practice in single-sample calling is to call non-reference alleles only; calling of all sites is possible but impractical. In order to merge such single-sample calls into one dataset, variants not called in one sample need to be assumed as either homozygous reference, or set to missing. In contrast, multi-sample calling routinely calls homozygous reference genotypes, but only for variants with at least one non-reference allele in the entire sample. Our multi-sample joint calling strategy across all cases and controls as a set enabled us to distinguish between homozygous reference and missing genotypes (Kumar et al., 2014), and provides the basis for standardized QC across aggregated data, essential for case-control designs (Winkler et al., 2014). Details of the variant calling pipeline are given in Sergouniotis et al. (2014).

Fastq files were aligned with Novoalign (http://www.novocraft.com) against the reference human genome (GRCh37). Duplicate read removal, format conversion, and indexing were performed using Picard (http://broadinstitute.github.io/picard). The Genome Analysis Toolkit (GATK) (McKenna *et al.*, 2010) was used for variant calling, with Variant Quality Score Recalibration (VQSR) and separate models for SNPs and indels, following best practice (DePristo et al., 2011; Van der Auwera et al., 2013). Multi-sample variant calling was performed using the GATK HaplotypeCaller on 3,412 samples of the UCL-exomes consortium. We used the union of the different target regions for variant calling, +/- 100 base-pairs on each side of the target regions. Read depth was excluded from the recalibration model because of the large read depth variability generated by the heterogeneous capture kits used in the multiple studies aggregated in the UCL-exomes cohort.

2.1.4 Quality control (QC)

2.1.4.1 Variant QC

The following QC thresholds were applied for all variant calls using VCFtools (Danecek *et al.*, 2011): (i) GATK truth sensitivity 99.5% for single nucleotide variants (SNVs) and 95% for indels; (ii) genotype quality (GQ) ≥ 20 for homozygous and ≥ 40 for heterozygous calls; (iii) maximum two alleles; (iv) sample read depth (DP) of high-quality reads ≥ 10; (v) Hardy-Weinberg equilibrium (HWE) with $P > 10^{-20}$; (vi) call rate (CR) ≥ 1% in the 3,412 samples of the multi-sample call. 2,122,400 out of 3,238,068 variants called in 3,412 samples passed the QC thresholds.

2.1.4.2 Individual-level QC

To minimize the type I error rate on rare variant burden analyses (Luedtke et al., 2011), only individuals of white European ancestry were included (self-reported, and by inspecting the first 20 coordinates of a multidimensional scaling analysis (MDS), Figure 2.2). Related individuals with a proportion of alleles shared identically by descent according to second-degree relatives and higher (π-hat ≥ 25%) were excluded. In addition, extensive sample QC was applied to ensure technical (sequencing assay) homogeneity of the remaining samples. Samples were excluded for the following criteria: (i) low sample CR one standard deviation (SD) from the mean; (ii) singleton

rate two SD from the mean; (iii) heterozygosity rate two SD from the mean. Sample QCs were performed using PLINK (Purcell et al., 2007). For MDS, per-individual heterozygosity and pairwise relatedness estimation, we used a trimmed set of variants (autosomal variants only, call rate ≥ 90%, minor allele frequency (MAF) ≥ 0.1%, and linkage disequilibrium $r^2 < 0.5$ for the MDS only). Singleton rates were calculated using PLINK/SEQ (https://atgu.mgh.harvard.edu/plinkseq). Out of 21 SUDEP samples, 18 passed the individual-level QC; 87 out of 128 epilepsy controls, and 1,479 out of 3,263 UCL-exomes non-epilepsy disease controls (Figure 2.1) passed the same stringent QC.

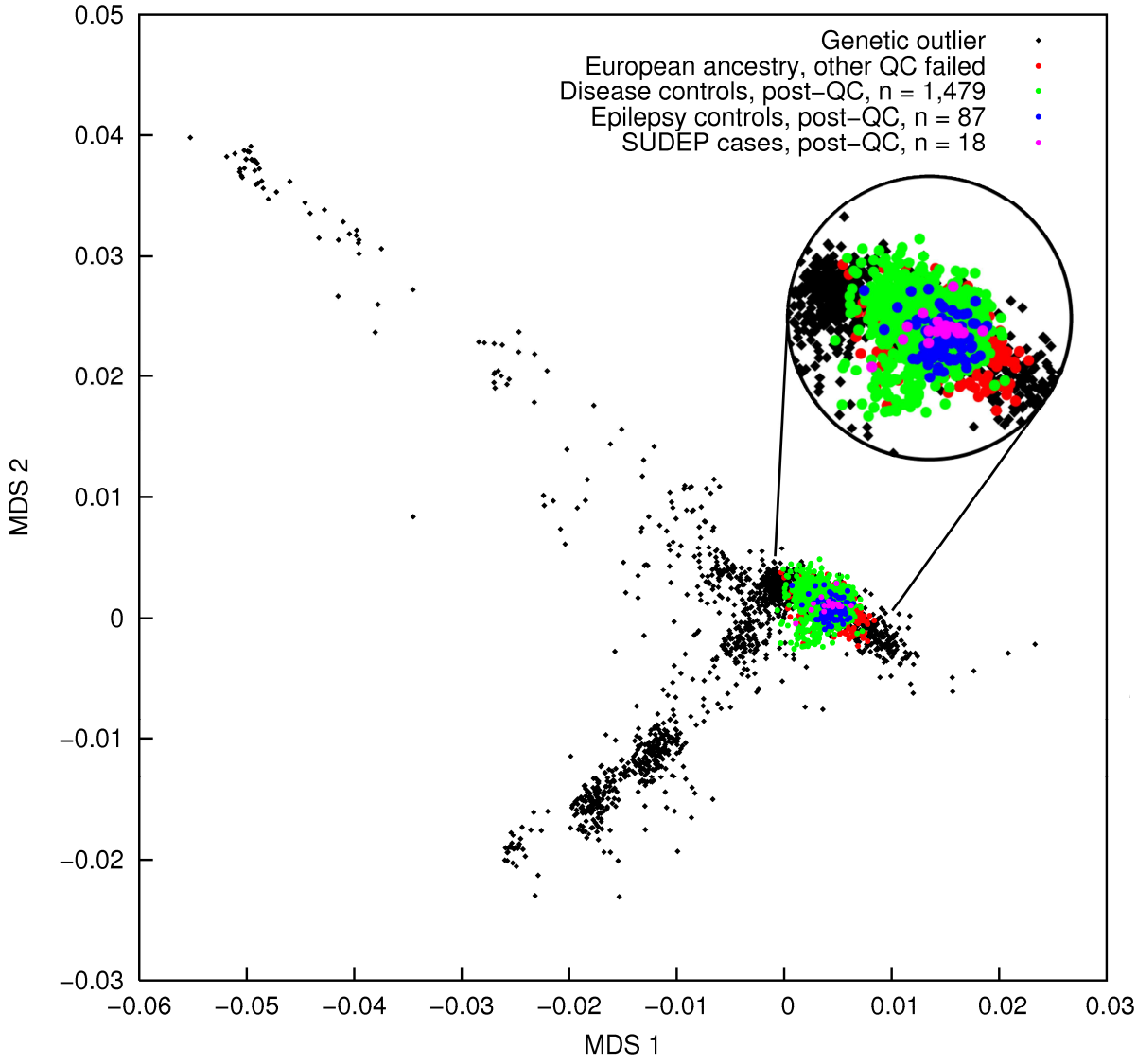

Figure 2.2. Multidimensional scaling analysis. Plotted are 3,344 UCL-exomes samples after the first individual-level QC step (68 samples with low call rate filtered out). Each point within the scatter plot represents the individual coordinates of the first two dimensions of MDS analysis using 43,710 high-quality and uncorrelated variants. Genetic outliers for 20 MDS

dimensions, indicated by black symbols, were removed as non-European samples from subsequent analyses. The zoomed area of the European cluster is indicated by black lines.

2.1.5 Prediction of variant deleteriousness

We used the recently published Combined Annotation Dependent Depletion method (CADD) (Kircher et al., 2014), to predict the deleteriousness of variants. The CADD framework integrates multiple annotations into one metric, with the advantage that it allows the ranking of every variant, based on the predicted deleteriousness, among all GRCh37/hg19 reference SNVs (~8.6 billion). We used pre-scored files provided for download (version 1.1) and the CADD web interface to generate the CADD raw and scaled scores for all sequenced and QC-filtered variants (n = 2,122,400). The CADD raw scores were used to generate the cumulative per-individual burden scores for the genome-wide burden analysis. Scaled CADD scores were used to select the most deleterious variants (scaled CADD score ≥ 15; median value for all possible canonical splice site changes and non-synonymous variants) for the gene-based association analyses.

2.1.6 Variant Annotation and filtering

We used ANNOVAR (Wang et al., 2010) to select variants based on the following criteria: (i) protein-changing variants according to the hg19 Reference Sequence (RefSeq) gene transcripts (UCSC Genome Browser, http://genome.ucsc.edu) (stop-gain/loss, splice-site variants within 2bp of an exon-intron boundary, frameshift/non-frameshift indels, and non-synonymous variants); (ii) not located within segmental duplications, to avoid artifacts due to paralogous sequence variation (Bailey et al., 2001; Wang et al., 2010). Out of 2,122,400 post-QC variants, 402,181 were classified as protein-changing. Out of 402,181 protein-changing variants, 203,089 variants, present

with at least one non-reference allele in the samples which passed individual-level QC (18 SUDEP patients, 87 epilepsy controls, 1,479 UCL-exomes non-epilepsy disease controls), were selected for subsequent analyses. ANNOVAR was also used for subsequent filtering based on the MAF.

2.1.6.1 Variant filtering for genome-wide burden analyses only

The selected 203,089 protein-changing variants were filtered to be rare, defined by a MAF $\leq$ 0.5% (arbitrary, but commonly-used, threshold to define a rare variant (Tennessen et al., 2012; Hunt et al., 2013)) according to three publicly-available datasets: Exome Aggregation Consortium (ExAC) v0.2 non-Finnish Europeans (n = 34,427), NHLBI-ESP European-Americans (n = 4,300), and 1000genomes October 2014 Europeans (n = 503). Out of 203,089 protein-changing variants, 166,603 were selected as protein-changing and rare (or novel) variants for the genome-wide burden analyses.

Additional variant QC was applied for the genome-wide analyses to mitigate batch effects. Variant missing data rates were calculated using VCFtools in the SUDEP, epilepsy, and disease control samples separately. The generated missing data rates were used as custom databases for the annotation with ANNOVAR. Subsequently, only variants sequenced in more than 80% of each test group were retained. This method was more efficient in removing sequencing batch effects than a correction method based on target interval mean coverage of the three groups, as indicated by the coefficients of variation of the cumulative per-individual burden scores (Table 2.1). A higher sequencing threshold for filtering did not lead to a lower variance of the values around their mean. Finally, 89,512 variants were included in the analysis.

Batch correction method	Observations	Mean	Standard deviation	Coefficient of variation
Per variant				
Sequencing rate ≥ 70%	1,584	324.81	74.06	22.80
Sequencing rate ≥ 80%	**1,584**	**271.44**	**61.15**	**22.53**
Sequencing rate ≥ 90%	1,584	165.98	38.81	23.38
Per target interval				
Mean average coverage ≥ 10	1,584	444.92	101.27	22.76
Mean average coverage ≥ 30	1,584	350.56	82.78	23.61
Mean average coverage ≥ 50	1,584	180.57	45.58	25.24

Table 2.1. Coefficients of variation of the cumulative per-individual burden scores after applying different methods for batch correction. The selected batch correction method with the lowest coefficient of variation in all samples (SUDEP, n = 18; epilepsy controls, n = 87; disease controls, n = 1,479) is shown in bold.

2.1.6.2 Variant filtering for gene-based association analyses only

The selected 203,089 protein-changing variants were filtered to be novel according to (i.e. not present in) the ExAC v0.2 non-Finnish Europeans, NHLBI-ESP European-Americans, and 1000genomes October 2014 Europeans. Variants present in the epilepsy control cohort were also excluded. Following our unique variant strategy, we filtered the remaining variants to be exclusive to the SUDEP or exclusive to the disease control samples. We then selected the most deleterious variants, following the recommendations of the prediction software used (scaled CADD score ≥ 15; median value for all possible canonical splice site changes and non-synonymous variants).

VCFtools was used to generate the filtered datasets for association testing.

2.1.7 Multidimensional scaling analysis

Only individuals of white European ancestry were included in subsequent analyses (Figure 2.2).

2.1.7.1 Genome-wide burden analysis

Aiming to estimate the burden of mutations at genome-wide level, we chose thresholds for variant QC metrics to maximize specificity over sensitivity, accepting loss of power to detect a significant association in favour of a reduced type I error rate (see *2.1.4.1 Variant QC*). Individual-level QC filtering generated samples with similar technical sequencing metrics, including overall call rate, singleton rate, and per-individual heterozygosity (see *2.1.4.2 Individual-level QC*). After inspection of the population

substructure by multidimensional scaling analysis, as implemented in PLINK (Purcell et al., 2007), only samples of clear European ancestry were retained (Figure 2.1).

For the genome-wide burden analysis of variants in SUDEP, we focussed on variants with the highest likelihood to be pathogenic by selecting rare (minor allele frequency (MAF) ≤ 0.5%), protein-changing variants (see *2.1.6 Variant Annotation and filtering*). We chose this strategy because variant pathogenicity is inversely correlated with the frequency of the non-reference allele in the general population (Coventry et al., 2010), with prediction of variant deleteriousness being more reliable for exonic and splice-site variants than for non-coding variants (Shihab et al., 2015). Using the selected variants, we then assigned to each individual an overall 'burden score', calculated by summing the scores for deleteriousness of every selected variant carried per individual, where the deleteriousness of each variant was determined using the Combined Annotation Dependent Depletion method (CADD v1.1) (Kircher et al., 2014), see *2.1.5 Prediction of variant deleteriousness*). The CADD method has been proven to achieve high sensitivity in identifying known pathogenic variants. To minimize batch effects between the different WES samples and cohorts, only variants sequenced in more than 80% of the SUDEP cases and the two control cohorts were retained. This strategy was enabled by our joint calling strategy across all cases and controls, and ensured that only variants sequenced in the majority of each of the testing groups were used to calculate the per-individual burden scores. This batch correction method is equivalent to a cross-sample coverage-based correction method, and is not equivalent to the filtering of poorly genotyped variants aimed at removing unreliable genotypes. The threshold of 80% was selected to obtain the lowest variability of all observed per-individual burden scores (Table 2.1).

We employed the two-tailed Wilcoxon rank-sum test, as implemented in Stata (http://www.stata.com), to compare per-individual burden scores and the number of variants per individual of the SUDEP cases against those of the two control cohorts, as well as epilepsy controls versus disease controls. The threshold for statistical significance was corrected for six tests using the Bonferroni method (two burden tests for three testing groups; $\alpha = 8.3 \times 10^{-3}$).

We tested the genome-wide burden of rare (or novel) deleterious variants in the SUDEP cohort against both control cohorts separately. Supported by the findings of the genome-

wide burden analysis, we sought to identify candidate genes for SUDEP using gene-based association analyses. The study analytic design is outlined in the Figure 2.3.

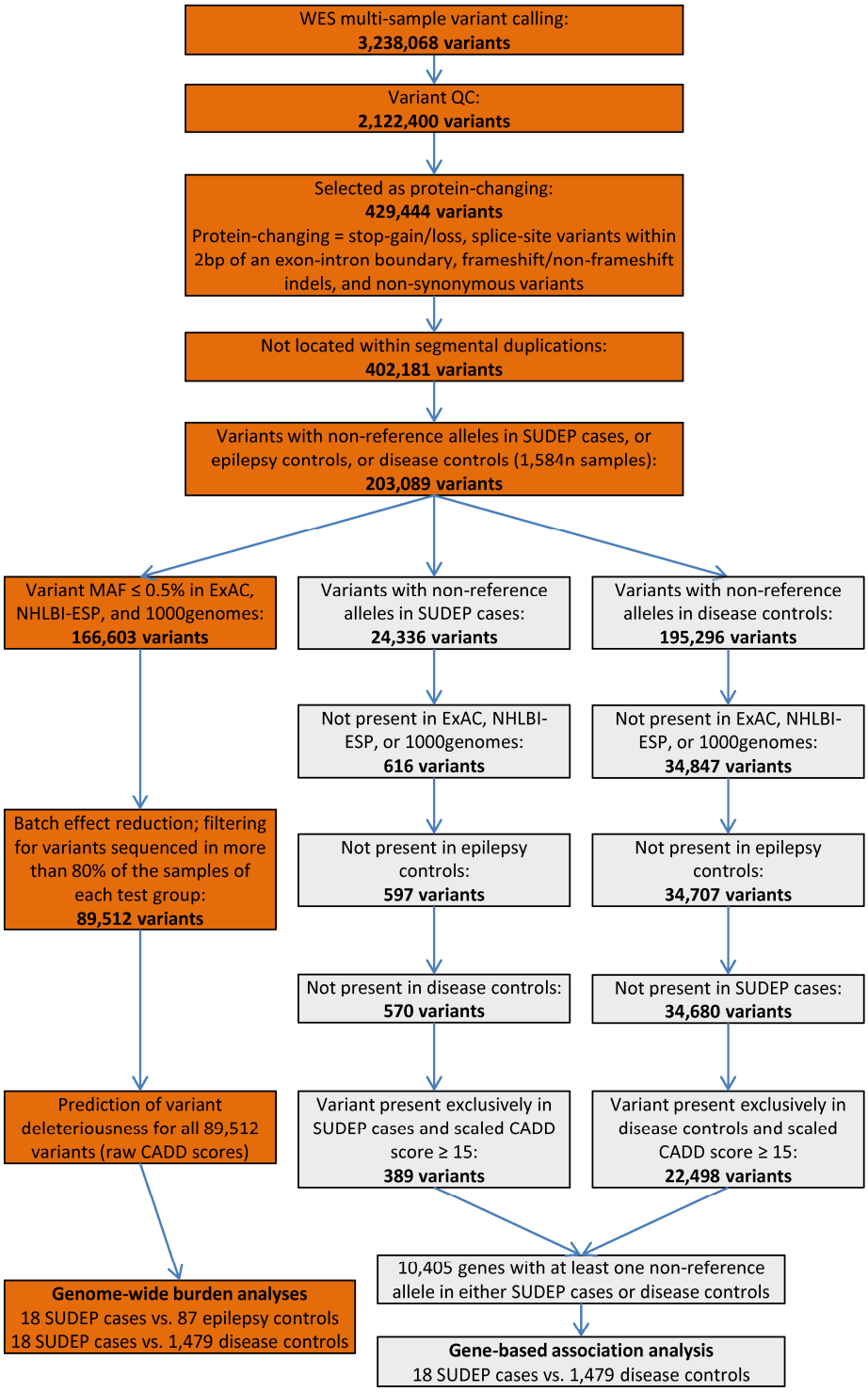

Figure 2.3. Study design and variant filtering flowchart. Main part of the study (genome-wide burden analyses) is highlighted in dark orange. Secondary part (gene-based burden analyses) is highlighted in light grey. Abbreviations: WES = whole-exome sequencing, SUDEP

= sudden unexpected death in epilepsy, ExAC = Exome Aggregation Consortium v0.2 non-Finnish Europeans (n = 34,427), NHLBI-ESP = NHLBI Grand Opportunity Exome Sequencing Project European-Americans (n = 4,300), 1000genomes = 1000 Genomes Phase 3 October 2014 Europeans (n = 503), MAF = minor allele frequency.

2.1.8 Gene-based association analysis

For the gene-based association analyses, we performed association tests based on comparison of the numbers of non-reference protein-changing variants alleles exclusive to cases versus those exclusive to controls, a well-established unique variant approach (Cohen et al., 2004; Wain et al., 2014). This approach, together with refining based on the predicted deleteriousness (scaled CADD score ≥ 15, Method 7), maximizes the power of the gene-based association tests (Ladouceur et al., 2012). Variant and individual-level QC was performed as for the genome-wide burden analysis (see *2.1.4 Quality control (QC)*). To help dissect out genes more likely conferring risk to SUDEP than to epilepsy, we excluded variants present in the epilepsy control cohort (see *2.1.6.1 Variant filtering for genome-wide burden analyses only).*

Empirical data show that the performance of rare variant association methods depends upon the underlying assumption of the relationship between rare variants and complex traits (Ladouceur et al., 2012). We employed a one-tailed burden test of an increased rare allele rate in cases (described in the supplementary information of Purcell et al. (2014)) and the two-tailed C-alpha test (Neale et al., 2011), which allows for risk and protective variants, as implemented in PLINK/SEQ (https://atgu.mgh.harvard.edu/plinkseq). An adaptive permutation procedure was used to assess *P*-values for all association tests (swapping of phenotype label across individuals; genes dropped from further permutation if clearly not associated). We used the PLINK/SEQ estimate of the smallest achievable empirical *P*-value for a gene (*I*-value) to adopt an adjusted Bonferroni correction for multiple testing, by correcting only for the number of genes for which there was power to detect association ($I < 10^{-3}$) (Kiezun et al., 2012). Based on the observed cumulative allele count in the SUDEP cohort for the tested genes, and a Bonferroni-corrected significance threshold, the epilepsy controls did not provide sufficient power to detect associations, and were not used in this component of the study. Confirmatory Sanger sequencing in the SUDEP samples was performed for variants in genes surpassing the adjusted threshold for significance.

2.1.9. Variant validation for the gene-based association analyses

Aligned sequence data for 12 variants selected from six genes significantly associated with SUDEP in the gene-based association analyses and six singletons observed in genes implicated in either cardiac death or epilepsy, were visually inspected using the IGV browser (Robinson et al., 2011).

2.1.9.1 Sanger sequencing

Confirmatory Sanger sequencing in the SUDEP samples was performed for the variants which passed the visual inspection. Primers for the regions of interest were designed using primer3 software (http://bioinfo.ut.ee/primer3/). Polymerase chain reaction (PCR) was performed according to the optimal conditions of the designed primers. PCR products were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA), and sequenced using BigDye v. 3.1 (Applied Biosystems) on an ABI3730xl automated DNA sequencer.

2.1.10 Study participants

The 18 DNA samples from people who had died of SUDEP sometime after DNA donation were selected from DNA archives at the National Hospital for Neurology and Neurosurgery, London (n = 8), the Epilepsy Research Centre, Melbourne (n = 5), the Royal College of Surgeons in Ireland, Dublin (n = 2), the Institute of Life Science, Swansea (n = 2), and the Royal Hospital for Sick Children, Glasgow (n = 1). The cause of death was classified into definite, probable, or near-SUDEP, according to the most recent proposed system: definite SUDEP required post mortem examination, without an identified toxicological or anatomical cause of death (Nashef et al., 2012). Details of SUDEP cases are given in Table 2.2.

ID	Gender	Age of death	Epilepsy syndrome	SUDEP
4	F	7	DS	Definite
5	F	11	DS	Definite
6	M	6	DS	Definite
1	M	12	DS	Definite
37	F	42	Focal S.	Definite
39	M	20	Focal S.	Definite
48	M	18	Focal U.	Definite
38	F	32	GGE	Definite
3	F	3	DS	Probable
2	M	20	DS	Probable
41	M	44	Focal S.	Probable
46	M	46	Focal S.	Probable
47	M	67	Focal S.	Probable
43	M	35	Focal U.	Probable
40	M	38	Focal U.	Probable
45	M	40	Focal U.	Probable
44	M	32	UE	Probable
42	M	56	UE	Probable

Table 2.2. Details of the 18 SUDEP cases. Abbreviations: ID = identification number, SUDEP = sudden unexpected death in epilepsy, M = male, F = female, DS = Dravet Syndrome, Focal U. = Focal unknown aetiology, Focal S. = Focal symptomatic, GGE = Genetic Generalised Epilepsy, UE = Unclassified Epilepsy (Berg *et al.*, 2010).

Epilepsy controls (n = 87) were patients from the National Hospital for Neurology and Neurosurgery, London (n = 71) and the Epilepsy Research Centre, Melbourne (n = 16), who had had whole-exome sequencing for other projects and were alive at the time of selection. These controls remain at risk of SUDEP. We applied previous incidence data from a comparable group of people with chronic epilepsy, reporting a SUDEP incidence of 5.9/1000 patient-years (Nashef et al., 1995a), to the number of years that our cohort of epilepsy control subjects have already lived with epilepsy (summed minimum epilepsy duration = 2,563 years). This suggests that 15/87 would have been expected to have succumbed to SUDEP, while, in fact, none have. Thus, the epilepsy control group is enriched with those at lower risk. For all epilepsy cases, we reviewed epilepsy diagnosis (Berg et al., 2010), age at onset of first seizure, presence of intellectual disability, anti-epileptic drug (AED) treatment, and presence of convulsive or nocturnal seizures over the 12-month period prior to death or latest follow-up. Presence of intellectual disability was defined as an intelligence quotient < 70 from a previous psychometric assessment, with onset under 18 years of age, or systematic mention of "learning/intellectual disability" or "mental retardation" in the medical notes.

2.1.11 Statistical analysis for clinical phenotype

To compare the clinical features of the SUDEP cases and epilepsy controls, two-sample t-test or Wilcoxon rank-sum test were used for continuous variables showing Gaussian or non-Gaussian distributions, respectively. Pearson χ^2 test or Fisher's exact test, as appropriate according to the sample size in any of the cells of contingency tables, were used to compare categorical variables. Two-tailed *P*-values of the Fisher's exact test were calculated. Bonferroni correction of the nominal threshold for significance of 0.05 was subsequently applied. We had less than 10% missing data and we performed sensitivity analyses of deviations from the assumption of missing at random. Sensitivity analyses showed that missing data did not cause any bias to our results. We therefore present results for subjects with complete data. Data were analyzed using Stata (http://www.stata.com).

2.1.12 Disease control samples

WES data of disease control samples (pre-QC, n = 3,263; post-QC, n = 1,479; Fig. 2.3) were obtained from the University College London exomes consortium. The University College London exomes consortium (UCL-exomes) is a consortium of researchers within University College London (London, UK) designed to aggregate raw read-level data from multiple exome sequencing projects in order to facilitate case-control association studies. At the time of this study, the UCL-exomes dataset included 3,412 samples (21 SUDEP, 128 epilepsy, and 3,263 non-epilepsy disease samples). The 3,263 non-epilepsy disease samples had no diagnosis of cardiac disease.

2.2 Results

2.2.1 Clinical phenotype

Eighteen people who died of SUDEP and 87 epilepsy controls were included in subsequent analyses. Demographic and clinical data of these two groups are summarized in Table 2.3. Eight SUDEP cases fulfilled the criteria for “definite” and 10 were classified as “probable” SUDEP.

		SUDEP cases (n = 18)	Living adult epilepsy controls (n = 87)	Uncorrected P-value (test)
Mean age at last recorded follow-up/death, years (SD)		29 (18)	35 (16)	0.198 (t-test)
Gender, n = male (%)		13 (72)	36 (41)	0.021 (Fisher's exact)
Epilepsy syndrome classification, n (%)	DS	6 (33)	30 (35)	0.423 (Pearson χ^2)
	Focal S.	5 (28)	25 (29)	
	Focal U.	4 (22)	7 (8)	
	GGE	1 (6)	14 (16)	
	UE	2 (11)	11 (13)	
Median age at first seizure occurrence, years (IQR)		2.5 (0.9-13)	2 (0.5-7)	0.332 (Wilcoxon rank-sum)
Median epilepsy duration, years (IQR)		20 (10-38)	30 (19-43)	0.086 (Wilcoxon rank-sum)
Intellectual disability, n (%)*		10 (56)	38 (45)	0.402 (Pearson χ^2)
Total number of AEDs taken, median (IQR)		8 (5-11)	8 (4-10)	0.997 (Wilcoxon rank-sum)
Subject living alone in the 12-month period before last follow-up/death, n (%)*		2 (12)	6 (7)	0.617 (Fisher's exact)
Convulsive seizures in the 12-month period before last follow-up/death, n (%)*		13 (72)	35 (42)	0.021 (Fisher's exact)
History of nocturnal seizures in the 12- month period before last follow-up/death, n (%)*		5 (33)	34 (42)	0.775 (Fisher's exact)

Table 2.3. Demographic and clinical features of SUDEP cases and living epilepsy controls. The Bonferroni method was applied to correct for the following known risk factors for SUDEP: gender, epilepsy syndrome classification, age at first seizure, epilepsy duration, total number of AEDs taken, subjects living alone in the 12-month period before last appointment or death, convulsive or nocturnal

seizures in the 12-month period before last follow-up or death. The threshold for statistical significance after Bonferroni correction was set to $\alpha = 6.3 \times 10^{-3}$. Abbreviations: SUDEP, sudden unexpected death in epilepsy; DS, Dravet Syndrome; Focal U., Focal unknown aetiology; Focal S., Focal symptomatic; GGE, Genetic Generalised Epilepsy; UE, Unclassified Epilepsy (Berg et al., 2010).
* Missing data: intellectual disability ($n = 2$); subject living alone in the 12-month period before last follow-up/death ($n = 3$); convulsive seizures in the 12-month period before last follow-up/death ($n = 3$); history of nocturnal seizures in the 12- month period before last follow-up/death ($n = 8$).

The SUDEP group was compared to the living epilepsy controls for the following known clinical risk factors for SUDEP: gender, epilepsy syndrome classification, age at first seizure, epilepsy duration, total number of AEDs taken, subjects living alone in the 12-month period before last appointment or death, convulsive or nocturnal seizures in the 12-month period before last follow-up or death. Nominally significant differences were observed only for gender (72% males in SUDEP group versus 41% in living epilepsy controls, $P = 0.021$) and convulsive seizures in the 12-month period before last follow-up or death (present in 72% of SUDEP cases versus 42% of living epilepsy controls, $P = 0.021$). However, none of these differences remained significant after correction of the threshold for statistical significance using the Bonferroni method (for the eight known risk factors stated above; $\alpha = 6.3 \times 10^{-3}$).

Amongst all the epilepsy cases, there was a subset of people with Dravet Syndrome: 30 living Dravet Syndrome cases (26 with, four without, *SCN1A* mutation) and six people with Dravet Syndrome (all with *SCN1A* mutation) and SUDEP, four definite and two probable SUDEP. There was no significant difference in the distribution of the known clinical risk factors for SUDEP or in AED treatment, including exposure to sodium-channel blockers, between people with Dravet Syndrome who died of SUDEP and the living Dravet Syndrome cases, after correction for multiple testing (Table 2.4). Details of *SCN1A* mutations are presented in the Tables 2.5 and 2.6.

	Dravet Syndrome cases who died of SUDEP ***n* = 6**	**Living Dravet Syndrome cases** ***n* = 30**	**Uncorrected** ***P*-value**	**Test**
Mean age at last recorded follow-up/death, years (SD)	10 (6)	36 (11)	<0.001	t-test
Gender, n male (%)	3 (50)	12 (40)	0.677	Fisher's exact
Median age at first seizure occurrence, years (IQR)	0.7 (0.4-0.9)	0.6 (0.5-0.7)	0.732	Wilcoxon rank-sum
Mean epilepsy duration, years (SD)	9 (6)	35 (11)	<0.001	t-test
Intellectual disability	6 (100)	29 (97)	1	Fisher's exact
Total number of AEDs taken, median (IQR)	8 (5-10)	10 (9-11)	0.095	Wilcoxon rank-sum
Exposure to acetazolamide (%)	1 (17)	8 (27)	1	Fisher's exact
Exposure to carbamazepine (%)	4 (67)	29 (97)	0.066	Fisher's exact
Exposure to clobazam (%)	5 (83)	20 (67)	0.643	Fisher's exact
Exposure to ethosuximide (%)	0 (0)	8 (27)	0.302	Fisher's exact
Exposure to gabapentin (%)	1 (17)	9 (30)	0.655	Fisher's exact
Exposure to lacosamide (%)	0 (0)	4 (13)	1	Fisher's exact
Exposure to levetiracetam (%)	3 (50)	23 (77)	0.317	Fisher's exact
Exposure to lamotrigine	5 (83)	26 (87)	1	Fisher's exact

(%)				
Exposure to oxcarbazepine (%)	1 (17)	3 (10)	0.535	Fisher's exact
Exposure to phenobarbitone (%)	4 (67)	21 (70)	1	Fisher's exact
Exposure to phenytoin (%)	2 (33)	22 (73)	0.149	Fisher's exact
Exposure to pregabalin (%)	0 (0)	2 (7)	1	Fisher's exact
Exposure to primidone (%)	0 (0)	9 (30)	0.303	Fisher's exact
Exposure to stiripentol (%)	4 (67)	8 (27)	0.149	Fisher's exact
Exposure to topiramate (%)	5 (83)	20 (67)	0.643	Fisher's exact
Exposure to vigabatrin (%)	2 (33)	14 (47)	0.672	Fisher's exact
Exposure to sodium valproate (%)	6 (100)	29 (97)	1	Fisher's exact
Exposure to zonisamide (%)	0 (0)	7 (23)	0.317	Fisher's exact
Subject living alone in the 12-month period before last follow-up/death, n (%)	0 (0)	0 (0)	Not applicable	Not applicable
Convulsive seizures in the 12-month period before last follow-up/death, n (%)*	6 (100)	22 (82)	0.556	Fisher's exact
History of nocturnal seizures in the 12-month period before last follow-up/death, n (%)*	2 (33)	19 (70)	0.159	Fisher's exact

Table 2.4. Demographic and clinical features of Dravet Syndrome cases comparing those who died of SUDEP with living cases. Bonferroni method was applied to correct for exposure to each AED and for the following known risk factors for SUDEP: gender, age at first seizure, epilepsy duration, total number of AEDs taken, subjects living alone in the 12-month period before last appointment or death, convulsive or nocturnal seizures in the 12-month period before last follow-up or death. Threshold for statistical significance after Bonferroni correction was set to $\alpha = 0.002$.

ID	Variant Type	cDNA position	Predicted protein change	Inheritance	Number of mutations	SUDEP classification
1	splice site	c.4339-14T>G	unknown	de novo	1	Definite
2	nonsense	c.1738C>T	p.Arg580Ter	de novo	1	Probable
3	frameshift	c.5536_5539delAAAC	p.Lys1846SerfsTer11	de novo	1	Probable
4	nonsense	c.1837C>T	p.Arg613Ter	de novo	1	Definite
5	frameshift	c.5536_5539delAAAC	p.Lys1846SerfsX11	de novo	1	Definite
6	missense	c.4181C>T	p.Thr1394lle	de novo	1	Definite

Table 2.5. *SCN1A* mutations identified prior to WES in the Dravet Syndrome patients who died of SUDEP.

ID	Type	cDNA position	Predicted protein change	Inheritance	Number of mutations
7	frameshift	c.1714_1718delACAAG	p.Thr572ProfsTer5	de novo	1
8	in-frame deletion	c.2725_2727delATG	p.Met909del	not determined	1
9	missence	c.2729A>C	p.Glu910Pro	de novo	1
10	missense	c.3797A>C	p.Glu1266Ala	de novo	1
11	missense	c.4384T>C	p.Tyr1462His	de novo	1
12	missense	c.4568T>C	p.lle1523Thr	de novo	1
13	splice site	c.264+4_264+7delAGTG	unknown	de novo	1
14	missense	c.5639G>A	p.Gly1880Glu	One parent analysed, mother negative	1
15	nonsens	c.992delT	p.Leu331Ter	de novo	1

	e				
16	missense	c.2792G>A	p.Arg931His	not determined	1
17	premature stop codon	c.4369_4372dupCTGT	p.Tyr1458SerfsTer29	de novo	1
18	frameshift	c.111delC	p.Lys38AsnfsTer54	de novo	1
19	nonsense	c.1152G>A	p.Trp384Ter	father deceased, mother negative	1
20	missense	c.512T>A	p.Ile171Lys	de novo	1
21	frameshift	c.4062delT	p.Ile1356TyrfsTer4	de novo	1
22	frameshift	c.1209delT	p.Phe403LeufsTer12	father deceased, mother negative	1
23	nonsense	c.664C>T	p.Arg222Ter	de novo	1
24	missense	c.2792G>A	p.Arg931His	de novo	1
25	missense	c.302G>A	p.Arg101Gln	father deceased, mother negative	1
26	frameshift	c.4949dupT	p.Lys1651GlnfsTer22	de novo	1
27	missense	c.5119T>G	p.Phe1707Val	One parent analysed, mother negative	1

28	missense	c.2831T>A	p.Val944Glu	de novo	1
29	nonsense	c.4933C>T	p.Arg1645Ter	de novo	1
30	missense; nonsense	c.1811G>A; c.4573C>T	p.Arg604His; p.Arg1525Ter	father deceased, mother negative	2
31	nonsense	c.5436G>A	p.Trp1812Ter	de novo	1
32	splice site	c.2589+3A>T	unknown	de novo	1
33	Mutation not detected				
34	Mutation not detected				
35	Mutation not detected				
36	Mutation not detected				

Table 2.6. *SCN1A* mutations identified prior to WES in the living Dravet Syndrome cohort.

2.2.2 Genome-wide Burden of Rare Deleterious Variants

After individual-level QC, 18 SUDEP, 87 epilepsy, and 1,479 disease control samples were included in subsequent analyses (Figure 2.1). Variants with at least one non-reference allele in any of the SUDEP, epilepsy, and disease control samples were selected for the analyses (n = 89,512; Figure 2.3). The 89,512 variants represented 1707 genes of the human reference genome with non-reference alleles in the SUDEP samples, 5464 genes with non-reference alleles in the epilepsy controls, and 13,887 genes with non-reference alleles in the disease controls (union = 13,999 genes).

2.2.2.1 Whole exome sequencing coverage

Coverage information was generated using the DepthOfCoverage module in GATK. The union of the Agilent, NimbleGen, and Illumina target regions was used in order to obtain uniform coverage statistics across all samples corresponding to the multi-sample call.

SUDEP samples: The mean average coverage across the union of all target intervals was 55x. On average, 56% of all target bases achieved 20x or greater coverage (range 47-60%). The mean average coverage across all hg19 Reference Sequence exons was 68x.

Epilepsy samples: The mean average coverage across the union of all target intervals was 40x. On average, 48% of all target bases achieved 20x or greater coverage (range 33-81%). The mean average coverage across all hg19 Reference Sequence exons was 50x.

UCL-exomes (disease control) samples: The mean average coverage across the union of all target intervals was 45x. On average, 51% of all target bases achieved 20x or greater coverage (range 16-88%). The mean average coverage across all hg19 Reference Sequence exons was 56x.

2.2.2.2 Genome-wide burden score

We observed a significantly increased genome-wide burden score per individual in the SUDEP cohort when compared to epilepsy ($P = 5.7 \times 10^{-3}$) and non-epilepsy disease controls ($P = 1.2 \times 10^{-3}$) (Tables 2.7-8; Figure 2.4). The number of variants per individual showed suggestive over-representation against the epilepsy controls ($P = 0.022$), and significant over-representation against disease controls ($P = 4.1 \times 10^{-3}$) (Table 2.7, Figure 2.4-6). Although there was also a significant difference in the number of variants between the two control cohorts ($P = 6.1 \times 10^{-3}$), the genome-wide burden score did not differ. This genome-wide burden suggests an extensive polygenic contribution to SUDEP causation. Post hoc analysis removing all post-QC *SCN1A* variants showed that the genome-wide burden was not biassed by the enrichment of both the SUDEP and the epilepsy cohorts with Dravet Syndrome patients bearing *SCN1A* mutations (comparison against epilepsy controls: $P = 6.3 \times 10^{-3}$; disease controls: $P = 1.4 \times 10^{-3}$).

	SUDEP patients (*n* = 18)			**Epilepsy controls (*n* = 87)**			**Disease controls (*n* = 1,479)**			**Wilcoxon rank-sum test *P*-values***		
	M	**Mdn**	**IQR (Q1-Q3)**	**M**	**Mdn**	**IQR (Q1-Q3)**	**M**	**Mdn**	**IQR (Q1-Q3)**	**SUDEP vs. epilepsy controls**	**SUDEP vs. disease controls**	**Epilepsy controls vs. disease controls**
Test groups										**18 vs. 87**	**18 vs. 1,479**	**87 vs. 1,479**
Per-individual burden scores	309.2	313.3	54.3 (284-338)	282.7	276.3	47.2 (257-304)	270.3	268.4	73.5 (233-306)	5.7×10^{-3}	1.2×10^{-3}	0.023
N. of variants per individual	110.2	108.5	18 (102-120)	104.1	101	18 (96-114)	99.29	98	24 (86-110)	0.022	4.1×10^{-3}	6.1×10^{-3}
Post hoc analysis excluding SCN1A variants**:												
Per-individual burden scores	308.2	312.6	54.3 (284-338)	282.2	276.3	46.5 (256-303)	270.3	268.4	73.5 (233-306)	6.3×10^{-3}	1.4×10^{-3}	0.028

Table 2.7. Genome-wide burden analysis results based on 89,512 quality-control filtered, protein-changing, and rare variants. Threshold for statistical significance after Bonferroni correction was set to $\alpha = 8.3 \times 10^{-3}$. Abbreviations: SUDEP, sudden unexpected death in epilepsy; M, mean; Mdn, median; IQR, interquartile range; Q1, lower (first) quartile; Q3, upper (third) quartile; N., number.

* All *P*-values are two-tailed.

** *Post hoc* analysis excluding 31 *SCN1A* variants present in any of the testing groups.

ID	Group	Overall burden score	Number of variants
1	SUDEP	359.717	137
2	SUDEP	252.147	92
3	SUDEP	361.877	128
4	SUDEP	325.648	107
5	SUDEP	267.083	98
6	SUDEP	340.553	108
37	SUDEP	284.007	101
38	SUDEP	338.335	120
39	SUDEP	298.502	110
40	SUDEP	259.949	102
41	SUDEP	326.775	109
42	SUDEP	297.342	121
43	SUDEP	345.866	119
44	SUDEP	295.796	107
45	SUDEP	309.682	108
46	SUDEP	329.256	112
47	SUDEP	256.889	85
48	SUDEP	316.987	120
7	Epilepsy control	252.542	100
8	Epilepsy control	302.804	108
9	Epilepsy control	330.097	112
10	Epilepsy control	333.787	125
11	Epilepsy control	323.071	118
12	Epilepsy control	271.17	118
13	Epilepsy control	372.639	173
14	Epilepsy control	354.713	120
15	Epilepsy control	288.767	98

16	Epilepsy control	291.89	104
17	Epilepsy control	260.218	101
18	Epilepsy control	255.89	94
19	Epilepsy control	249.289	100
20	Epilepsy control	326.59	117
21	Epilepsy control	290.896	100
22	Epilepsy control	323.711	115
23	Epilepsy control	262.273	102
24	Epilepsy control	347.718	117
25	Epilepsy control	299.316	99
26	Epilepsy control	289.297	98
27	Epilepsy control	338.482	115
28	Epilepsy control	234.981	100
29	Epilepsy control	302.517	119
30	Epilepsy control	210.459	76
31	Epilepsy control	304.135	99
32	Epilepsy control	265.14	114
33	Epilepsy control	240.545	94
34	Epilepsy control	431.057	165
35	Epilepsy control	310.828	98
36	Epilepsy control	306.997	120
49	Epilepsy control	284.447	90
50	Epilepsy control	273.455	103
51	Epilepsy control	244.968	96
52	Epilepsy control	238.671	107
53	Epilepsy control	328.091	114
54	Epilepsy control	323.615	114
55	Epilepsy control	317.573	118
56	Epilepsy control	256.95	99
57	Epilepsy control	258.884	109
58	Epilepsy control	206.33	82
59	Epilepsy control	286.28	104

60	Epilepsy control	315.867	125
61	Epilepsy control	255.99	96
62	Epilepsy control	247.434	89
63	Epilepsy control	269.123	99
64	Epilepsy control	257.397	96
65	Epilepsy control	257.052	96
66	Epilepsy control	269.042	96
67	Epilepsy control	230.292	88
68	Epilepsy control	273.905	101
69	Epilepsy control	253.521	90
70	Epilepsy control	282.128	105
71	Epilepsy control	232.949	99
72	Epilepsy control	289.872	101
73	Epilepsy control	260.866	82
74	Epilepsy control	272.389	96
75	Epilepsy control	240.764	86
76	Epilepsy control	285.66	101
77	Epilepsy control	262.026	101
78	Epilepsy control	250.621	98
79	Epilepsy control	266.575	86
80	Epilepsy control	242.588	93
81	Epilepsy control	218.303	85
82	Epilepsy control	265.309	93
83	Epilepsy control	276.328	94
84	Epilepsy control	268.619	100
85	Epilepsy control	333.307	116
86	Epilepsy control	278.548	109
87	Epilepsy control	296.325	113
88	Epilepsy control	278.304	116
89	Epilepsy control	293.481	106
90	Epilepsy control	282.278	104
91	Epilepsy control	292.481	114

92	Epilepsy control	302.617	104
93	Epilepsy control	298.335	94
94	Epilepsy control	291.115	98
95	Epilepsy control	261.284	99
96	Epilepsy control	244.311	88
97	Epilepsy control	254.5	87
98	Epilepsy control	272.821	103
99	Epilepsy control	332.406	113
100	Epilepsy control	274.721	114
101	Epilepsy control	244.375	90
102	Epilepsy control	332.135	134
103	Epilepsy control	259.937	91
104	Epilepsy control	314.285	105
105	Epilepsy control	319.711	106

Table 2.8. Burden scores and variant numbers for the SUDEP and epilepsy control samples. The burden scores are calculated by summing the CADD scores for deleteriousness of every selected variant carried per individual.

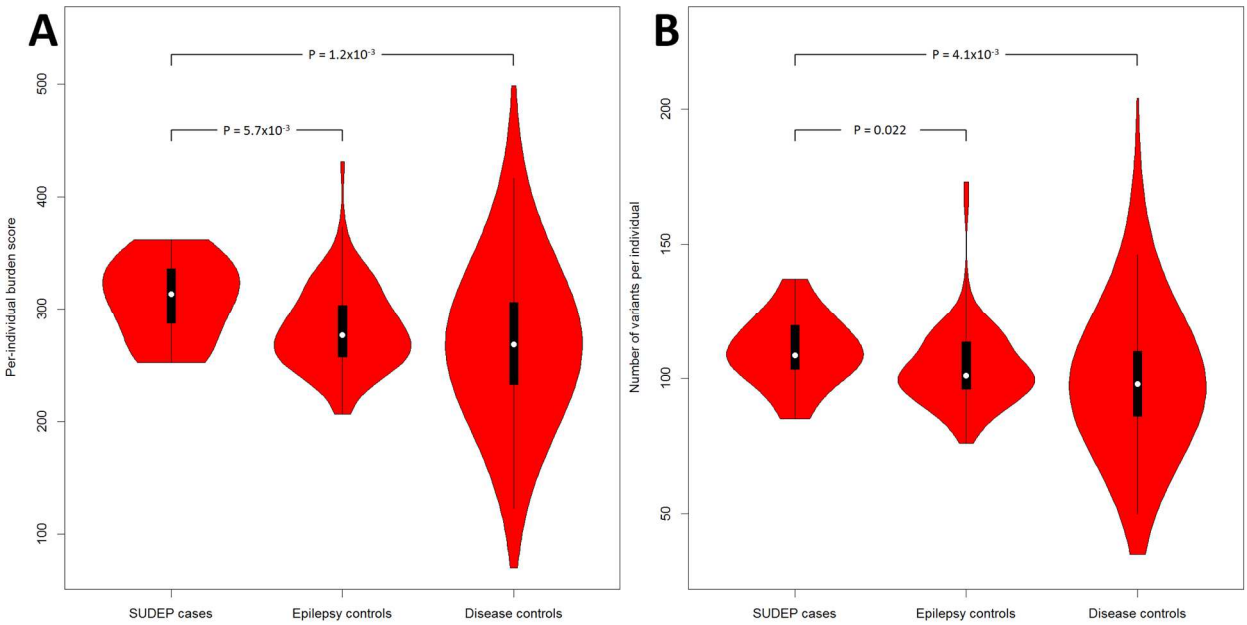

Figure 2.4. Violin plots of the burden score and variant number per individual. Plotted are the per-individual burden scores (A) and the number of variants per individual (B) of each test group. A violin plot is a box plot with the width of the box proportional to the estimated density of the observed data (proportion of cases with given ordinate value). The maximum density of the group-specific data distribution is indicated by the largest width of the violins. The density trace is plotted symmetrically to the left and the right of the box plot for better visualization. All violins have the same fixed maximum width. The white dot is the median, the thick black vertical bar represents the interquartile range (IQR), and the thin black vertical bar represents 95% confidence intervals.

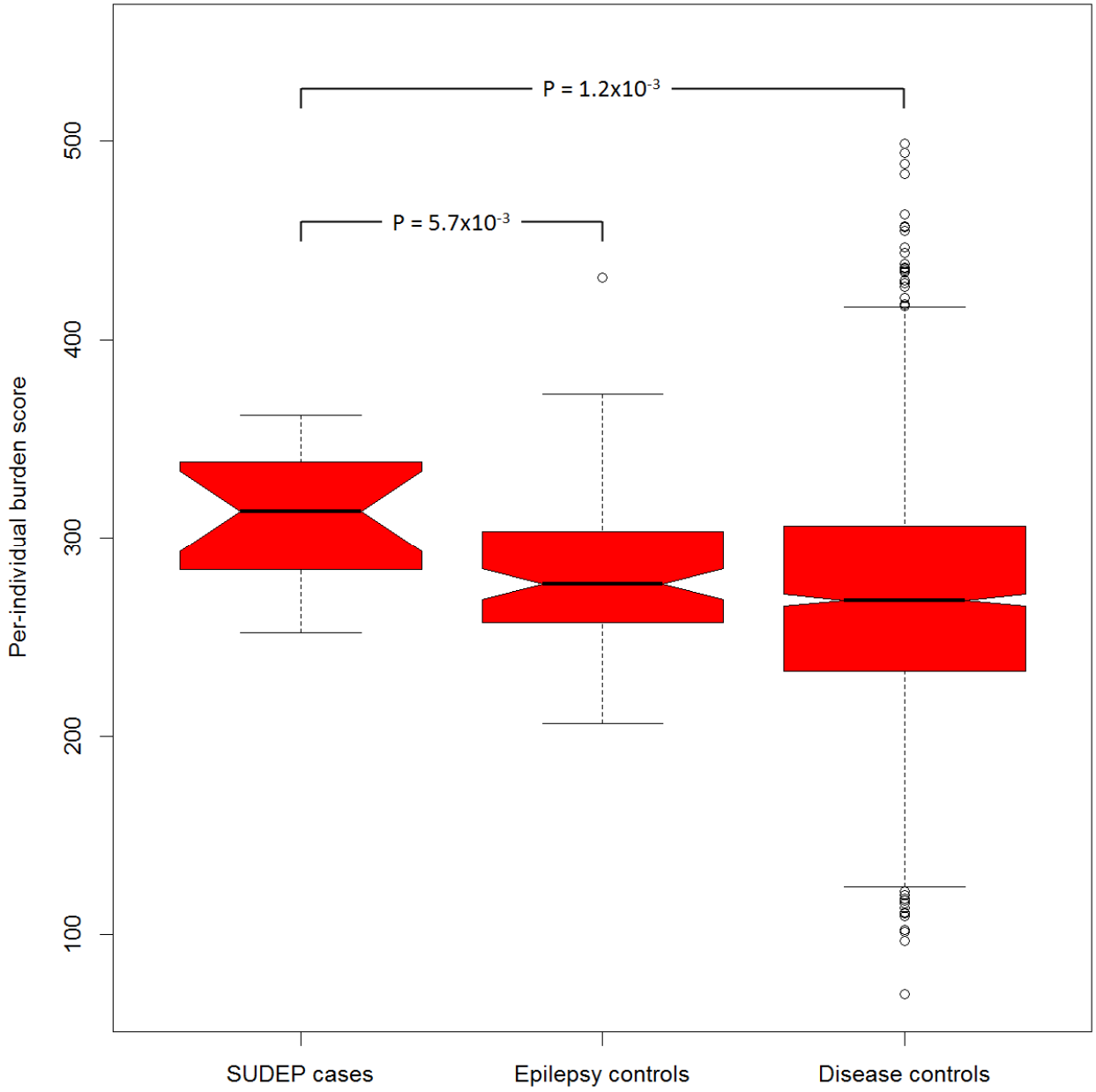

Figure 2.5. Notched boxplot of the per-individual burden scores. Plotted are the per-individual burden scores for each test group. The thick black horizontal line is the median. The notched section represents the confidence interval around the median (median +/- 1.57 x IQR/$n^{0.5}$). According to Chambers *et al.* (1983) (Graphical Methods for Data Analysis, p. 62), there is "strong evidence" (95% confidence) that their medians differ when the notches of two boxes do not overlap. The box represents the IQR, while the whiskers extend to the furthest observations within ± 1.5 IQR of the lower (first) quartile and the upper (third) quartile. Empty dots represent outliers beyond 1.5 IQRs.

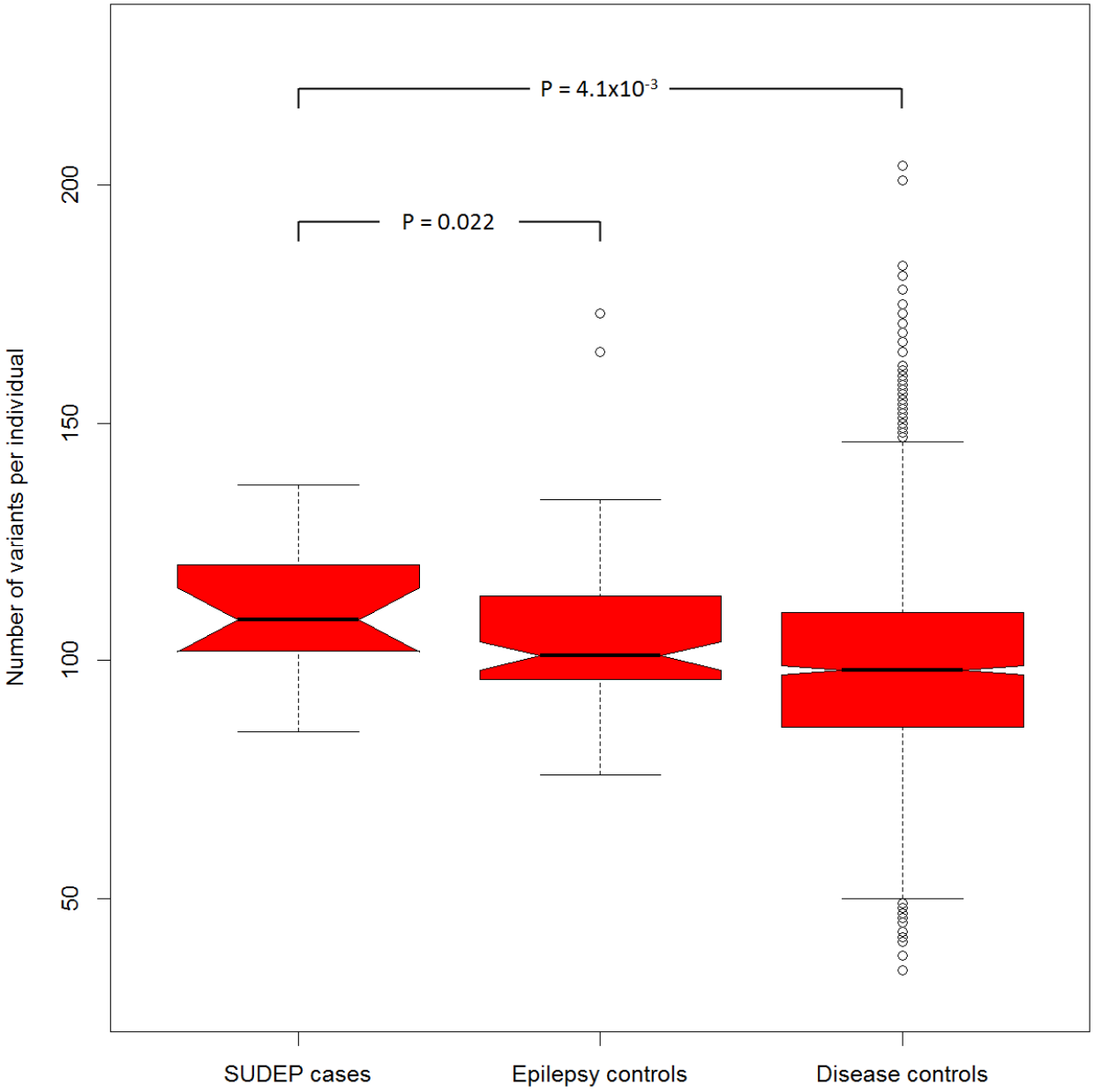

Figure 2.6. Notched boxplot of the number of variants per individual. Plotted are the numbers of variants per individual of each test group. The thick black horizontal line is the median. The notched section represents the confidence interval around the median (median +/- 1.57 x IQR/$n^{0.5}$). According to Chambers *et al.* (1983) (Graphical Methods for Data Analysis, p. 62), there is "strong evidence" (95% confidence) that their medians differ when the notches of two boxes do not overlap. The box represents the IQR, while the whiskers extend to the furthest observations within ± 1.5 IQR of the lower (first) quartile and the upper (third) quartile. Empty dots represent outliers beyond 1.5 IQRs.

2.2.3 Gene-based association of unique deleterious variants

Gene-based association tests were performed for all genes with at least one non-reference allele in either SUDEP cases or disease controls (373 genes in the SUDEP cases; 10,319 genes in the disease controls; union = 10,405). The threshold for statistical significance was corrected for 32 tests using the adjusted Bonferroni method (two association tests for 16 genes with $I < 10^{-3}$; $\alpha = 1.56 \times 10^{-3}$). Five genes harbouring Sanger-confirmed variants were significantly associated with SUDEP when compared to the 1479 disease controls (Table 2.9). The most strongly associated gene was *SCN1A* (C-alpha $P = 1.61 \times 10^{-4}$), followed by *LGI1* (lowest $P = 3.12 \times 10^{-4}$), *SMC4* (lowest $P = 5.39 \times 10^{-4}$), *COL6A3* (lowest $P = 7.27 \times 10^{-4}$), and *TIE1* (lowest $P = 1.48 \times 10^{-3}$). Sanger sequencing failed to confirm one of two variants of the *PIK3C2A* gene. We note that we considered only *SCN1A* variants that passed the same QC filtering applied to every other WES-derived variant in any other gene. Coverage statistics for the WES target intervals within the genes are given in Table 2.9.

		Cumulative non-reference allele count (cumulative minor allele frequency, %)*			Mean average coverage		
Gene	**Cytoband GRCh37**	**SUDEP cases (*n* = 18)**	**Epilepsy controls (*n* = 87)****	**Disease controls (*n* = 1,479)**	**SUDEP cases**	**Epilepsy controls**	**Disease controls**
SCN1A	2q24.3	2 (5.56)	18 (11.19)	4 (0.16)	51x	44x	75x
LGI1	10q23.33	2 (5.56)	0 (0)	2 (0.08)	90x	67x	45x
PIK3C2A	11p15.1	2 (5.56)	1 (0.61)	1 (0.04)	81x	58x	69x
SMC4	3q25.33	2 (5.56)	0 (0)	1 (0.05)	92x	57x	36x
COL6A3	2q37.3	2 (5.56)	0 (0)	5 (0.19)	77x	63x	51x
TIE1	1p34.2	2 (5.56)	0 (0)	4 (0.14)	85x	58x	37x

		Percent of target bases with 10x or greater coverage			18 SUDEP cases vs. 1,479 disease controls	
Gene	**Cytoband GRCh37**	**SUDEP cases**	**Epilepsy controls**	**Disease controls**	**Burden *P*-value*****	**C-alpha *P*-value*****
SCN1A	2q24.3	87%	81%	89%	1.21×10^{-4}	1.61×10^{-4}
LGI1	10q23.33	80%	78%	68%	3.12×10^{-4}	3.12×10^{-4}
PIK3C2A	11p15.1	93%	90%	87%	$3.12 \times 10^{-}$	$3.34 \times 10^{-}$

					4	4
SMC4	3q25.33	79%	73%	63%	5.39×10^{-4}	5.39×10^{-4}
COL6A3	2q37.3	83%	84%	76%	7.27×10^{-4}	7.27×10^{-4}
TIE1	1p34.2	71%	70%	59%	1.48×10^{-3}	2.01×10^{-3}

Table 2.9. Gene-based association analysis results. Shown are six genes significantly associated with SUDEP when compared to the 1,479 disease controls. *P*-values surpassing the Bonferroni-corrected threshold for significance ($\alpha = 1.56 \times 10^{-3}$) are highlighted in grey. Sanger sequencing failed to confirm one variant for the *PIK3C2A* gene shown in red; the gene is not considered as associated with SUDEP. Abbreviations: SUDEP, sudden unexpected death in epilepsy; GRCh37, Genome Reference Consortium Human genome build 37.

* Cumulative counts and frequencies are the summed counts and frequencies of the non-reference alleles.

** Cumulative counts and frequencies for the epilepsy controls are given for comparison only. Association tests were not performed, as explained in the text.

*** All *P*-values are based on adaptive permutations. Burden *P*-values are one-tailed; C-alpha *P*-values are two-tailed. Genes are ranked by the C-alpha *P*-value.

2.2.4 Deleterious singleton variants in genes implicated in cardiac death or epilepsy causation

Of 373 genes with at least one non-reference variant present in the SUDEP cohort only (Table 2.10), we also found deleterious variants in one gene implicated in sudden cardiac death (*CACNB2* (Antzelevitch et al., 2007)) and five genes implicated in different epilepsy syndromes (*CNTN2* (Stogmann et al., 2013), *GABRG2* (Baulac et al., 2001), *MAGI2* (Marshall et al., 2008), *POLG* (Uusimaa et al., 2013), and *SYNGAP1* (Carvill et al., 2013)), each present as a singleton in the SUDEP cohort.

	Carrier of deleterious alleles				
Gene	Exclusive to SUDEP cases (*n*)	Exclusive to disease controls (*n*)	Burden *P*-value	C-alpha *P*-value	Comment
SCN1A	2	4	1.21E-03	1.61E-04	variants in SUDEP cases confirmed by Sanger sequencing
LGI1	2	2	3.12E-04	3.12E-04	variants in SUDEP cases confirmed by Sanger sequencing
PIK3C2A	2	1	3.12E-04	3.34E-04	one variant not confirmed by Sanger sequencing
SMC4	2	1	5.39E-04	5.39E-04	variants in SUDEP cases confirmed by Sanger sequencing
COL6A3	2	5	7.27E-04	7.27E-04	variants in SUDEP cases confirmed by Sanger sequencing
TIE1	2	4	1.48E-03	2.01E-03	variants in SUDEP cases confirmed by Sanger sequencing
MAGI2	1	3	8.93E-03	1.21E-02	epilepsy gene; variant in SUDEP case confirmed by Sanger sequencing
GABRG2	1	0	9.80E-03	1.40E-02	epilepsy gene; variant in SUDEP case confirmed by Sanger sequencing
CACNB2	1	1	2.14E-02	2.14E-02	sudden cardiac death gene;

					variant in SUDEP case confirmed by Sanger sequencing
CNTN2	1	6	2.67E-02	2.49E-02	epilepsy gene; variant in SUDEP case confirmed by Sanger sequencing
POLG	1	3	4.01E-02	3.12E-02	epilepsy gene; variant in SUDEP case confirmed by Sanger sequencing
SYNGAP1	1	2	4.71E-01	5.88E-01	epilepsy gene; variant in SUDEP case confirmed by Sanger sequencing
DNAH8	2	11	4.92E-03	3.62E-03	
VPS13D	2	7	3.61E-03	5.15E-03	
SYNE1	2	23	9.95E-03	6.64E-03	
MRPS6	2	1	1.02E-02	8.96E-03	
TENM3	2	9	7.33E-03	9.42E-03	
DNAH17	2	12	4.78E-01	3.91E-01	
ANO7	1	0	2.47E-03	2.03E-03	
HSPB7	1	0	2.34E-03	2.34E-03	
SLC9A3	1	0	3.12E-03	3.34E-03	
FLT3LG	1	0	4.04E-03	3.54E-03	
CENPP	1	1	3.17E-03	3.62E-03	
N4BP3	1	2	3.69E-03	3.69E-03	
CPE	1	0	3.95E-03	3.95E-03	
OSBPL6	1	0	7.39E-03	4.31E-03	

FCN1	1	2	1.22E-02	4.38E-03	
TOP3A	1	3	6.32E-03	4.42E-03	
POMGNT2	1	0	4.96E-03	4.63E-03	
TTC17	1	0	5.66E-03	4.67E-03	
PRAF2	1	0	5.45E-03	4.77E-03	
SLITRK2	1	1	1.48E-02	5.19E-03	
MPP7	1	0	7.69E-03	5.38E-03	
CD96	1	0	6.94E-03	5.40E-03	
RPL13A	1	0	7.23E-03	5.42E-03	
CYYR1	1	0	5.93E-03	5.53E-03	
FOPNL	1	0	4.86E-03	5.55E-03	
PITX2	1	0	4.25E-03	5.77E-03	
XPNPEP1	1	0	5.06E-03	5.78E-03	
R3HCC1L	1	0	7.87E-03	5.80E-03	
SFTPB	1	0	4.34E-03	5.88E-03	
PM20D2	1	0	6.31E-03	5.89E-03	
DHX32	1	0	5.57E-03	5.94E-03	
KLK7	1	0	6.38E-03	5.96E-03	
KIAA1549	1	0	8.13E-03	5.99E-03	
CRISP3	1	0	6.10E-03	6.10E-03	
CHMP2A	1	0	8.73E-03	6.11E-03	
TMED5	1	0	7.28E-03	6.37E-03	
ACTR10	1	0	8.70E-03	6.41E-03	

MAP10	1	0	6.42E-03	6.42E-03	
ZNF264	1	0	6.93E-03	6.47E-03	
ERCC1	1	0	8.49E-03	6.61E-03	
PKLR	1	0	8.51E-03	6.62E-03	
ST6GAL1	1	0	6.22E-03	6.66E-03	
PTGES2	1	0	8.63E-03	6.71E-03	
OTOA	1	0	5.59E-03	6.79E-03	
PSENEN	1	0	6.40E-03	6.86E-03	
THBS4	1	0	5.65E-03	6.87E-03	
SLC25A3	1	0	9.87E-03	6.91E-03	
TIMM9	1	0	7.90E-03	6.91E-03	
ZNF513	1	0	6.46E-03	6.92E-03	
OR2AP1	1	0	7.96E-03	6.96E-03	
C12orf10	1	0	6.53E-03	7.00E-03	
DNAJA3	1	0	8.01E-03	7.01E-03	
TERT	1	0	6.58E-03	7.05E-03	
ZNF451	1	0	6.18E-03	7.06E-03	
PDZD3	1	0	6.24E-03	7.13E-03	
HIST1H2BB	1	0	8.16E-03	7.14E-03	
TACC1	1	0	5.90E-03	7.16E-03	
PRSS21	1	0	8.29E-03	7.25E-03	
NSMAF	1	0	6.79E-03	7.27E-03	
SYAP1	1	0	8.44E-03	7.39E-03	

MON1A	1	0	5.77E-03	7.41E-03	
DMXL2	1	1	7.02E-03	7.53E-03	
BOLL	1	0	9.19E-03	7.57E-03	
MEX3A	1	1	1.03E-02	7.59E-03	
ACTA2	1	0	8.16E-03	7.61E-03	
WHSC1L1	1	1	7.71E-03	7.71E-03	
CCDC9	1	0	8.83E-03	7.73E-03	
ALOX12B	1	0	4.93E-03	7.75E-03	
NLK	1	1	8.86E-03	7.76E-03	
SPNS1	1	0	6.80E-03	7.77E-03	
PIGR	1	1	1.01E-02	7.88E-03	
SPTLC3	1	1	1.14E-02	8.01E-03	
ZCCHC9	1	0	8.07E-03	8.07E-03	
CPT1A	1	1	7.55E-03	8.09E-03	
MTAP	1	0	5.43E-03	8.15E-03	
PTPN5	1	0	1.05E-02	8.18E-03	
OR4B1	1	0	8.27E-03	8.27E-03	
SCML4	1	0	9.12E-03	8.51E-03	
TROVE2	1	0	9.74E-03	8.52E-03	
DYRK4	1	0	7.15E-03	8.68E-03	
SUCO	1	0	8.68E-03	8.68E-03	
GRHL3	1	0	6.43E-03	8.73E-03	
RBM12	1	0	7.30E-03	8.86E-03	

DLK2	1	1	1.46E-02	8.90E-03	
RPL32	1	0	7.79E-03	8.90E-03	
DSCAML1	1	0	1.02E-02	8.97E-03	
TBCEL	1	0	7.89E-03	9.01E-03	
WNT2B	1	1	1.10E-02	9.03E-03	
MFAP1	1	0	7.17E-03	9.22E-03	
TECPR2	1	1	1.08E-02	9.43E-03	
TMEM95	1	1	8.35E-03	9.54E-03	
CCDC60	1	0	7.47E-03	9.60E-03	
DACH2	1	0	1.03E-02	9.62E-03	
CORO1C	1	0	7.11E-03	9.65E-03	
NUP88	1	3	1.38E-02	1.01E-02	
NYNRIN	1	2	8.91E-03	1.02E-02	
ADAMTS12	1	5	1.32E-02	1.03E-02	
SLC24A4	1	1	1.03E-02	1.03E-02	
TANGO2	1	0	8.03E-03	1.03E-02	
NUMA1	1	2	1.48E-02	1.09E-02	
TCF7L2	1	0	8.62E-03	1.11E-02	
NR1H3	1	1	1.27E-02	1.11E-02	
TTC7A	1	1	9.77E-03	1.12E-02	
TSHZ3	1	0	1.05E-02	1.12E-02	
DENND6A	1	0	1.20E-02	1.12E-02	
INCA1	1	1	1.05E-02	1.13E-02	

SLC7A1	1	0	1.30E-02	1.13E-02	
OLFML2B	1	1	1.06E-02	1.14E-02	
SIPA1L2	1	1	1.73E-02	1.15E-02	
IPO5	1	1	1.57E-02	1.15E-02	
CTH	1	1	1.51E-02	1.18E-02	
NCKAP5	1	0	1.10E-02	1.18E-02	
NARF	1	2	1.65E-02	1.21E-02	
OR6C76	1	1	1.13E-02	1.21E-02	
GLB1L2	1	3	1.31E-02	1.22E-02	
PHKA2	1	1	1.22E-02	1.22E-02	
PRPH2	1	1	1.57E-02	1.22E-02	
CNBD2	1	1	8.63E-03	1.23E-02	
STRN	1	1	1.59E-02	1.24E-02	
MRPL50	1	0	1.25E-02	1.25E-02	
WDR12	1	1	1.04E-02	1.27E-02	
CWH43	1	1	1.12E-02	1.28E-02	
UNC13A	1	1	1.74E-02	1.28E-02	
VSIG8	1	0	8.97E-03	1.28E-02	
PRPF39	1	1	1.75E-02	1.29E-02	
NUDT9	1	1	1.29E-02	1.29E-02	
MASP2	1	3	1.21E-02	1.30E-02	
CSRNP3	1	0	1.08E-02	1.31E-02	
CHI3L2	1	0	1.34E-02	1.34E-02	

PPP3R1	1	1	1.64E-02	1.35E-02	
CEP97	1	1	1.05E-02	1.35E-02	
MFN1	1	1	1.11E-02	1.35E-02	
ZNF48	1	2	1.74E-02	1.35E-02	
STRIP2	1	2	1.55E-02	1.36E-02	
MTBP	1	1	1.45E-02	1.36E-02	
ACOXL	1	1	1.20E-02	1.37E-02	
KIF5C	1	1	1.29E-02	1.38E-02	
FN3KRP	1	1	1.08E-02	1.39E-02	
OR3A1	1	1	1.22E-02	1.39E-02	
KCNH1	1	1	1.09E-02	1.40E-02	
CTRL	1	1	1.83E-02	1.43E-02	
TNRC6C	1	1	1.14E-02	1.46E-02	
POLR3B	1	2	1.77E-02	1.46E-02	
ASXL1	1	1	1.67E-02	1.46E-02	
COG2	1	1	1.31E-02	1.50E-02	
SLC30A6	1	1	1.61E-02	1.50E-02	
CCDC33	1	1	1.11E-02	1.51E-02	
PPP1R12A	1	0	1.51E-02	1.51E-02	
PAPPA	1	1	1.62E-02	1.51E-02	
CHODL	1	2	1.53E-02	1.53E-02	
ERAP1	1	1	1.34E-02	1.53E-02	
TXNDC16	1	1	1.08E-02	1.55E-02	

HHAT	1	2	1.44E-02	1.55E-02	
LGALS13	1	1	1.67E-02	1.56E-02	
THAP1	1	1	1.56E-02	1.56E-02	
SCN11A	1	2	1.83E-02	1.60E-02	
NDST4	1	1	1.26E-02	1.62E-02	
ENOX1	1	1	1.86E-02	1.63E-02	
EBNA1BP2	1	1	1.87E-02	1.63E-02	
KCNB2	1	3	1.53E-02	1.64E-02	
FAM115A	1	1	1.76E-02	1.65E-02	
OLFML3	1	3	3.65E-02	1.65E-02	
CAD	1	7	1.49E-02	1.70E-02	
BAI3	1	1	1.19E-02	1.70E-02	
MRPS28	1	0	1.95E-02	1.71E-02	
RBM28	1	1	1.72E-02	1.72E-02	
EBF2	1	1	1.21E-02	1.72E-02	
RBCK1	1	1	1.42E-02	1.73E-02	
PZP	1	1	1.61E-02	1.73E-02	
CCNL2	1	1	1.85E-02	1.73E-02	
MDGA1	1	2	1.35E-02	1.73E-02	
BUB1B	1	3	2.12E-02	1.75E-02	
IL1RN	1	1	2.14E-02	1.76E-02	
TAC1	1	1	2.14E-02	1.77E-02	
TMED8	1	1	2.44E-02	1.79E-02	

TACR1	1	1	2.07E-02	1.81E-02	
BECN1	1	3	1.50E-02	1.82E-02	
KLHDC7A	1	2	1.61E-02	1.84E-02	
LRSAM1	1	2	1.85E-02	1.85E-02	
FAM171A1	1	2	2.12E-02	1.86E-02	
ZNF365	1	2	1.87E-02	1.87E-02	
LCMT2	1	3	1.92E-02	1.92E-02	
MAGEC2	1	1	2.89E-02	1.93E-02	
GOLGA4	1	4	2.50E-02	1.94E-02	
ART3	1	1	2.09E-02	1.96E-02	
GPALPP1	1	1	1.96E-02	1.96E-02	
DLC1	1	2	2.24E-02	1.96E-02	
RAB35	1	1	1.97E-02	1.97E-02	
CDK15	1	2	1.63E-02	1.98E-02	
NOC3L	1	1	2.12E-02	1.98E-02	
GLI2	1	3	2.83E-02	1.98E-02	
SIL1	1	3	2.13E-02	1.99E-02	
FAM168A	1	3	2.43E-02	2.00E-02	
DGKB	1	2	2.00E-02	2.00E-02	
NEMF	1	2	2.47E-02	2.03E-02	
SLC17A5	1	2	2.34E-02	2.04E-02	
CD97	1	1	1.69E-02	2.05E-02	
GAS2L3	1	1	1.92E-02	2.05E-02	

POLI	1	2	1.38E-02	2.07E-02	
CTNS	1	2	1.41E-02	2.11E-02	
MED12L	1	3	3.34E-02	2.12E-02	
ILF3	1	3	1.99E-02	2.14E-02	
HK2	1	2	2.60E-02	2.14E-02	
TPBG	1	1	2.01E-02	2.15E-02	
MAB21L1	1	1	2.62E-02	2.15E-02	
ETFA	1	2	2.47E-02	2.16E-02	
ZC3H7A	1	2	1.53E-02	2.19E-02	
PID1	1	2	2.19E-02	2.19E-02	
ZNF223	1	3	2.19E-02	2.19E-02	
IMPG2	1	1	2.36E-02	2.20E-02	
IPO9	1	1	2.06E-02	2.21E-02	
ATXN2	1	4	1.56E-02	2.22E-02	
MTMR3	1	1	2.54E-02	2.23E-02	
CDH7	1	1	2.24E-02	2.24E-02	
FRMPD3	1	4	1.31E-02	2.25E-02	
PHGDH	1	2	2.11E-02	2.26E-02	
NBEA	1	3	3.40E-02	2.27E-02	
NUB1	1	1	1.49E-02	2.34E-02	
LRRC17	1	2	2.51E-02	2.35E-02	
GPATCH3	1	2	1.96E-02	2.38E-02	
CCNB2	1	2	2.39E-02	2.39E-02	

ATM	1	6	3.42E-02	2.40E-02	
PRDM16	1	1	2.40E-02	2.40E-02	
CNTNAP5	1	3	2.59E-02	2.41E-02	
IL16	1	3	2.99E-02	2.46E-02	
NCOA2	1	2	2.07E-02	2.51E-02	
ATP9A	1	3	2.53E-02	2.53E-02	
ZNF470	1	2	2.72E-02	2.54E-02	
GNPDA1	1	2	2.76E-02	2.58E-02	
NDE1	1	2	2.96E-02	2.59E-02	
ERCC3	1	3	2.28E-02	2.61E-02	
PKD1L1	1	3	2.46E-02	2.64E-02	
RABGAP1L	1	4	3.21E-02	2.65E-02	
FNIP1	1	3	1.96E-02	2.66E-02	
CWF19L1	1	2	2.48E-02	2.66E-02	
NCF2	1	3	2.86E-02	2.67E-02	
TRAPPC9	1	2	1.97E-02	2.67E-02	
AKT1	1	3	2.69E-02	2.69E-02	
BRCA2	1	6	2.51E-02	2.69E-02	
NFAT5	1	3	2.36E-02	2.69E-02	
MLLT4	1	0	3.09E-02	2.71E-02	
RBM23	1	3	2.74E-02	2.74E-02	
NR1H4	1	2	3.23E-02	2.82E-02	
CAPN11	1	1	2.00E-02	2.85E-02	

FERMT1	1	4	2.51E-02	2.87E-02	
TBC1D15	1	1	2.37E-02	2.88E-02	
PNMAL1	1	3	2.03E-02	2.90E-02	
CCKAR	1	4	3.11E-02	2.90E-02	
CEP76	1	7	3.37E-02	2.95E-02	
ZDHHC5	1	1	3.24E-02	3.02E-02	
COL6A6	1	2	1.96E-02	3.08E-02	
RFX2	1	3	2.54E-02	3.09E-02	
ATXN7	1	3	4.95E-02	3.15E-02	
LDHAL6A	1	1	2.95E-02	3.16E-02	
PRCP	1	3	3.66E-02	3.20E-02	
CD207	1	3	3.90E-02	3.21E-02	
PARD3	1	3	3.45E-02	3.22E-02	
ITGAE	1	3	2.38E-02	3.23E-02	
PSD4	1	3	2.83E-02	3.23E-02	
GPR37	1	3	4.10E-02	3.33E-02	
SOX6	1	2	4.56E-02	3.36E-02	
PRKCH	1	3	3.38E-02	3.38E-02	
CFAP43	1	3	5.33E-02	3.39E-02	
MTRF1	1	3	2.81E-02	3.41E-02	
PSTK	1	2	3.95E-02	3.42E-02	
PRKD3	1	4	2.31E-02	3.47E-02	
SEC24C	1	5	3.48E-02	3.48E-02	

DLD	1	2	3.49E-02	3.49E-02	
VPS41	1	5	6.23E-02	3.52E-02	
SALL4	1	3	3.33E-02	3.57E-02	
CEP128	1	4	3.60E-02	3.60E-02	
TFDP2	1	1	3.01E-02	3.66E-02	
MSL2	1	3	3.26E-02	3.72E-02	
TTC3	1	4	4.87E-02	3.72E-02	
GUCY1A2	1	1	3.09E-02	3.75E-02	
NUP98	1	3	3.54E-02	3.80E-02	
ABHD8	1	1	3.53E-02	3.80E-02	
ANKRD50	1	4	3.82E-02	3.82E-02	
NBAS	1	4	4.19E-02	3.89E-02	
MKI67	1	6	5.42E-02	3.92E-02	
TRPM7	1	3	2.50E-02	3.92E-02	
PSMD13	1	3	4.02E-02	4.02E-02	
ARHGAP30	1	6	5.70E-02	4.11E-02	
MARCH8	1	4	5.43E-02	4.15E-02	
PIKFYVE	1	3	4.22E-02	4.22E-02	
PCNXL4	1	1	4.05E-02	4.36E-02	
PLCG2	1	4	2.83E-02	4.45E-02	
TDRD9	1	6	9.31E-02	4.48E-02	
NUP205	1	8	4.18E-02	4.50E-02	
NFATC3	1	5	3.14E-02	4.71E-02	

ATF7IP	1	4	4.17E-02	4.81E-02	
CLUH	1	3	3.06E-02	4.81E-02	
SLC25A32	1	2	4.92E-02	4.92E-02	
ITPR1	1	7	5.08E-02	5.08E-02	
GBA2	1	4	2.55E-02	5.10E-02	
OR5M10	1	2	6.00E-02	5.20E-02	
PIGN	1	3	4.00E-02	5.23E-02	
NCAPD3	1	3	4.87E-02	5.24E-02	
PHLDB2	1	4	6.15E-02	5.33E-02	
KIAA2018	1	6	4.68E-02	5.40E-02	
TTLL6	1	3	6.96E-02	5.65E-02	
EML5	1	4	6.70E-02	5.80E-02	
CYP2R1	1	5	8.52E-02	5.83E-02	
CCDC141	1	7	5.88E-02	5.88E-02	
PTPRT	1	3	3.83E-02	5.90E-02	
USP24	1	8	1.03E-01	6.07E-02	
MATN2	1	4	4.83E-02	6.32E-02	
GPR125	1	5	6.47E-02	6.47E-02	
LRP1B	1	14	9.60E-02	6.57E-02	
THADA	1	8	1.12E-01	6.95E-02	
KCNQ5	1	4	5.65E-02	6.96E-02	
ERICH2	1	2	6.07E-02	7.01E-02	
PCDH15	1	6	5.73E-02	7.05E-02	

DNAH3	1	11	8.20E-02	7.10E-02	
PTPRD	1	5	6.44E-02	7.43E-02	
MYO10	1	6	5.49E-02	7.59E-02	
PPL	1	38	7.30E-02	7.87E-02	
LAMA2	1	8	8.54E-02	7.93E-02	
FER1L6	1	7	9.76E-02	7.93E-02	
FRYL	1	6	6.63E-02	8.67E-02	
CDH1	1	6	7.18E-02	8.84E-02	
LAMC1	1	8	7.56E-02	1.05E-01	
PKHD1L1	1	14	2.37E-01	2.03E-01	
PITPNM1	1	2	5.33E-01	3.33E-01	
LOXHD1	1	14	5.00E-01	3.75E-01	
BEAN1	1	2	5.22E-01	3.91E-01	
TRIM56	1	1	6.09E-01	3.91E-01	
MARK2	1	2	2.86E-01	4.29E-01	
MKNK1	1	2	3.57E-01	4.29E-01	
PI4KA	1	2	4.44E-01	4.44E-01	
USP25	1	2	6.67E-01	4.44E-01	
MPP3	1	1	6.11E-01	4.44E-01	
ARHGEF1	1	2	4.29E-01	4.76E-01	
CACNA1B	1	7	6.25E-01	5.00E-01	
MICAL3	1	6	6.88E-01	5.00E-01	
RARG	1	2	5.63E-01	5.00E-01	

COL11A2	1	6	5.33E-01	5.33E-01	
ASB6	1	4	5.33E-01	5.33E-01	
EPHB2	1	3	5.33E-01	5.33E-01	
GLTPD2	1	3	6.92E-01	5.38E-01	
PPAPDC2	1	1	5.38E-01	5.38E-01	
MYO7A	1	9	4.44E-01	5.56E-01	
ZNF831	1	3	3.91E-01	5.65E-01	
JUN	1	2	8.33E-01	5.83E-01	
PHF2	1	3	8.00E-01	6.00E-01	
SYNPO2L	1	3	7.00E-01	6.00E-01	
GAREM	1	1	3.91E-01	6.09E-01	
NCOR2	1	9	5.00E-01	6.25E-01	
ZNF335	1	3	4.09E-01	6.36E-01	
TLE2	1	6	5.33E-01	6.67E-01	
LAMA5	1	4	6.67E-01	6.67E-01	
EMILIN1	1	2	4.44E-01	6.67E-01	
COL6A2	1	7	5.38E-01	6.92E-01	
TTN	1	85	7.14E-01	7.14E-01	
RNH1	1	2	7.14E-01	7.14E-01	
BLOC1S5	1	1	8.57E-01	7.14E-01	
FBRSL1	1	3	4.44E-01	7.78E-01	
OTOG	1	24	8.33E-01	8.33E-01	
MAST2	1	12	8.33E-01	8.33E-01	

STRA6	1	1	8.33E-01	8.33E-01	
ITGA4	1	7	7.14E-01	8.57E-01	

Table 2.10. List of all 373 genes with at least one non-reference variant in the SUDEP cases. Genes with *P*-values surpassing the Bonferroni-corrected threshold for significance ($\alpha = 1.56 \times 10^{-3}$) are highlighted in grey. One gene with significant *P*-values but without Sanger confirmation is shown in red.

2.2.5 Summary of results

We found significantly increased genome-wide polygenic burden per individual in the SUDEP cohort when compared to epilepsy and non-epilepsy disease controls. The polygenic burden was driven both by the number of variants per individual, and over-representation of variants likely to be deleterious in the SUDEP cohort. More than a thousand genes contribute to the observed polygenic burden within the framework of this study. Subsequent gene-based association analysis revealed five possible candidate genes significantly associated with SUDEP or epilepsy, but no one single gene emerges as common to the SUDEP cases.

Chapter 3

STRUCTURAL IMAGING BIOMARKERS OF SUDEP

To assess whether structural changes potentially attributable to sudden death pathogenesis were present on MRI in people who subsequently died of SUDEP, we conducted a retrospective, voxel-based analysis of T_1 volume scans, and compared grey matter volumes in 12 cases of SUDEP, with 34 people at high risk, 19 at low risk of SUDEP, and 15 healthy controls (Methods and Results published in Wandschneider et al, 2015).

3.1 Methods

The study was conducted at the National Hospital for Neurology and Neurosurgery as part of database research on the 'Prevention and Risk Identification of SUDEP', approved by the National Research Ethics Committee.

3.1.1 Subjects

The scans for SUDEP cases, those at low and high risk of SUDEP, and healthy controls were obtained from an overlapping period of case ascertainment, ensuring same imaging protocols were used for acquisition. Subjects with epilepsy were identified from a general clinical database at the National Hospital for Neurology and Neurosurgery. We identified 12 people who died with definite or probable SUDEP, and matched those with 53 living subjects with epilepsy identified from the same database according to the criteria below. All subjects had to have undergone a high-resolution T1 volume scan using the identical 3 Tesla MRI scanner as part of their clinical care. Individuals with major brain lesions, such as those after partial temporal lobe resection, were not included to avoid problems with imaging normalization. Sufficient clinical data had to be available to subsequently identify subjects at low or high risk of SUDEP, as described below. All three groups were matched for gender, age, epilepsy syndrome, and epilepsy duration to control for duration-related structural changes. Groups were also matched for lesion pathology where possible. Healthy controls were comparable to the epilepsy populations for gender and age.

3.1.1.1 Characteristics of SUDEP cases

Those deceased were classified as probable (n = 10) or definite (n = 2) SUDEP, according to Nashef et al.'s classification (2012). The median age at death was 35.5 [interquartile range (IQR) 2.8] years. Scans were acquired at a median of 2 (IQR 2.8) years antemortem. Videotelemetry data of seizures were available in five SUDEP cases.

Further clinical information on the SUDEP cases are shown in Table 3.1. SUDEP, subjects at high or low risk, as well as control subjects, were comparable for gender and age at scan (Table 3.2).

Case	Epilepsy syndrome	SUDEP Category	Lesion on MRI?	Duration tonic phase (sec)	PGES?	Duration PGES (sec)
1	Juvenile myoclonic epilepsy	probable	no	N/A	N/A	N/A
2	Focal, left temporal Primary generalized	probable	Bulky left amygdala with mild FLAIR signal increase	N/A	no	N/A
3	Focal, bitemporal	probable	no	11	yes	30 – 43
4	Focal, probably bitemporal	probable	no	N/A	N/A	N/A
5	Multifocal, left mesial temporal and frontal	probable	Left hippocampal sclerosis	10	yes	33
6	Focal, frontal	probable	no	N/A	N/A	N/A
7	Focal, unclassified	definite	Bilateral periventricular leucomalacia	N/A	N/A	N/A
8	Focal, frontal	probable	Mild left hippocampal sclerosis	N/A	yes	5
9	Focal, left hemisphere neocortical	definite	Cavernoma left superior frontal gyrus	6 - 23	no	N/A
10	Unclassified ?primary generalized	probable	Cavernoma right inferior frontal, in white	N/A	N/A	N/A

			matter			
11	Focal, probably bitemporal	probable	Enlarged left amygdala > hippocampus	N/A	N/A	N/A
12	Focal, left hemisphere	probable	Right superior temporal DNET	N/A	N/A	N/A

Table 3.1. Additional clinical characteristics of the SUDEP cohort

	SUDEP cases	At high risk	At low risk	Controls (n=15)	df	X^2	p-

	(n=12)	(n=34)	(n=19)				value
Age at scan (yrs) Median (IQR)	33·5 (21·5)	30·5 (12)	30·0 (7·5)	37 (16)	3	2·85	0·241
Age at onset (yrs) Median (IQR))	16·5 (10)	13·5 (7)	14 (6)	N/A	2	6·21	0·045
Epilepsy duration (yrs) Median (IQR)	11·5 (24·3)	17 (11·25)	15 (15)	N/A	2	5·74	0·057
Gender, male	8	19	12	7	3	1·42	0·722
>3 GTCs/year	8	24	0	N/A	2	26·09	0·000
Nocturnal seizures	8	27	0	N/A	2	31·9	0·000
Polytherapy	4	14	4	N/A	2	2·21	0·347

Table 3.2. Demographic and clinical parameters. Chi-Square test was employed for dichotomous variables and Kruskal-Wallis test for all other variables.

3.1.1.2 Characteristics of people at high or low risk for SUDEP

A risk score was created for each subject according to the most robust epilepsy-specific risk factors for SUDEP identified in recent combined-risk factor analyses (Hesdorffer et al., 2011) that were also implemented in a recent SUDEP imaging study (Tang et al., 2014). Odds ratios for individual SUDEP risk factors were therefore adjusted for different study groups. Those with either nocturnal seizures [odds ratio (OR) = 3.9], or frequent (≥3/year) GTCs (OR = 15.46), were considered 'high risk.' Increased SUDEP risk is also associated with young age at disease onset (onset age<16 years: OR = 1.72), and long disease duration (duration>15 years: OR = 1.95) (Hesdorffer et al., 2011). For each subject, odds ratios for risk factors were added to define an individual overall risk score. In the SUDEP cohort, 11 of 12 SUDEP cases (91.7%) were correctly identified as high risk subjects if the summed risk score was at least 3.9 (median risk score 19.1, IQR 16.7). One subject with juvenile myoclonic epilepsy had died, probably from SUDEP, but was not known to have suffered from nocturnal seizures or frequent convulsive seizures. A cut-off of 3.9 was therefore used to stratify others into those with high (≥3.9) and low risk (<3.9) of SUDEP.

Individual risk scores and pathology identified on MRI of people at low and high risk for SUDEP are listed in Table 3.3.

SUDEP cases, and those at low and high risk, were matched for epilepsy syndrome (SUDEP: 1/12 generalized genetic epilepsy; high risk: 0/34; low risk: 3/19), and as far as possible for type of pathology (Table 3.4). We were primarily interested in identifying common structures and pathophysiological mechanisms underlying SUDEP and high risk for SUDEP, and the majority of those at low risk (10/19), and high risk (22/34), had no identifiable lesions on a high-resolution 3 T epilepsy protocol clinical MRI brain scan. Videotelemetry data of seizures were available in 30/34 of those at high risk, and 7/19 at low risk. Further information regarding epilepsy classification (as per videotelemetry and/or history) is shown in Table 3.5.

Subject	Group	Total risk score	Early onset	Long duration	GTCs >3/year	Nocturnal seizures	Lesion on MRI
1	Low risk	0	0	0	0	0	right temporal occipital FCD
2	Low risk	1.95	0	1.95	0	0	no lesion
3	Low risk	1.95	0	1.95	0	0	no lesion
4	Low risk	3.67	1.72	1.95	0	0	left superior temporal gyrus, non specific focus
5	Low risk	3.67	1.72	1.95	0	0	no lesion
6	Low risk	0	0	0	0	0	no lesion
7	Low risk	3.67	1.72	1.95	0	0	right inferior pareital cystic lesion (likely DNET)
8	Low risk	1.95	0	1.95	0	0	no lesion
9	Low risk	1.72	1.72	0	0	0	no lesion
10	Low risk	1.72	1.72	0	0	0	no lesion
11	Low risk	0	0	0	0	0	no lesion
12	Low risk	0	0	0	0	0	no lesion
13	Low risk	0	0	0	0	0	right anterior temporal/ amygdala cavernous haemangioma
14	Low risk	3.67	1.72	1.95	0	0	no lesion
15	Low risk	3.67	1.72	1.95	0	0	hypoxic injury
16	Low risk	0	0	0	0	0	exophytic cavernoma or lipoma left inferior colliculus

17	Low risk	1.72	1.72	0	0	0	left hippocampal cavernoma or DNET/left hippocampal sclerosis
18	Low risk	3.67	1.72	1.95	0	0	right fusiform cavernoma
19	Low risk	1.72	1.72	0	0	0	left hippocampal sclerosis
20	High risk	21.08	1.72	0	15.46	3.9	right hippocampal sclerosis
21	High risk	23.03	1.72	1.95	15.46	3.9	no lesion
22	High risk	5.62	1.72	0	0	3.9	left hippocampal sclerosis
23	High risk	7.57	1.72	1.95	0	0	left precentral DNET
24	High risk	15.46	0	0	15.46	0	no lesion
25	High risk	19.13	1.72	1.95	15.46	0	subtle left insular malformation
26	High risk	3.9	0	0	0	3.9	no lesion
27	High risk	19.13	1.72	1.95	15.46	0	right parietal damage or dysplasia
28	High risk	19.36	0	0	15.46	3.9	no lesion
29	High risk	21.08	1.72	0	15.46	3.9	left temporal dysplasia, small left hippocampus

30	High risk	7.57	1.72	1.95	0	3.9	no lesion
31	High risk	5.62	1.72	0	0	3.9	no lesion
32	High risk	19.36	0	0	15.46	3.9	left hippocampal sclerosis
33	High risk	7.57	1.72	1.95	0	3.9	right inferior parietal cortical dysplasia
34	High risk	23.03	1.72	1.95	15.46	3.9	no lesion
35	High risk	3.9	0	0	0	3.9	no lesion
36	High risk	21.31	0	1.95	15.46	3.9	left frontal non specific white matter focus
37	High risk	7.57	1.72	1.95	0	3.9	lesion
38	High risk	19.36	0	0	15.46	3.9	right superior temporal gyrus/ polar haematoma (old)
39	High risk	23.03	1.72	1.95	15.46	3.9	cerebellar atrophy
40	High risk	5.62	1.72	0	0	3.9	no lesion
41	High risk	19.13	1.72	1.95	15.46	0	no lesion
42	High risk	23.03	1.72	1.95	15.46	3.9	mature damage, left>right gyrus rectus
43	High risk	21.31	0	1.95	15.46	3.9	no lesion
44	High risk	15.46	0	0	15.46	0	no lesion
45	High risk	7.57	1.72	1.95	0	3.9	hypothalamic hamartoma
46	High risk	23.03	1.72	1.95	15.46	3.9	no lesion

47	High risk	19.13	1.72	1.95	15.46	0	no lesion
48	High risk	19.13	1.72	1.95	15.46	0	no lesion
49	High risk	21.08	1.72	0	15.46	3.9	no lesion
50	High risk	23.03	1.72	1.95	15.46	3.9	no lesion
51	High risk	23.03	1.72	1.95	15.46	3.9	no lesion
52	High risk	21.08	1.72	0	15.46	3.9	left supramarginal gyrus dysplasia
53	High risk	23.03	1.72	1.95	15.46	3.9	no lesion
54	SUDEP	0	0	0	0	0	no lesion
55	SUDEP	23.03	1.72	1.95	15.46	3.9	bulky left amygdala with mild FLAIE signal increase
56	SUDEP	19.36	0	0	15.46	3.9	no lesion
57	SUDEP	15.45	0	0	15.46	0	no lesion
58	SUDEP	19.13	1.72	1.95	15.46	0	left hippocampal sclerosis
59	SUDEP	5.85	0	1.95	0	3.9	no lesion
60	SUDEP	21.08	1.72	0	15.46	3.9	bilateral periventricular leucomalacia
61	SUDEP	23.03	1.72	1.95	15.46	3.9	left hippocampal sclerosis
62	SUDEP	3.9	0	0	0	3.9	cavernoma left superior frontal gyrus
63	SUDEP	21.08	1.72	0	15.46	3.9	cavernoma right inferior frontal, in

							white matter
64	SUDEP	19.13	1.72	1.95	15.46	0	enlarged left amygdala >hippocampus
65	SUDEP	3.9	0	0	0	3.9	right superior temporal DNET

Table 3.3. Additive odds ratios and individual pathology demonstrated on MRI

	Low risk **N = 19**	**High risk** **N = 34**
No lesion	10	22
Hippocampal sclerosis **Left/Right**	1/0	2/0
Focal cortical dysplasia **Left/Right**	3 1/2 1 left/ 1 right temporo-occipital 1 right parieto-occipital	6 4/2 1 left insular 1 left medial temporal 1 right supramarginal gyrus 1 entire left temporal lobe 1 left inferior parietal 1 right anterior temporal
Cavernoma **Left/Right**	2 0/2 - right temporal pole - right fusiform gyrus	1 0/1 - right temporal pole
DNET **Left/Right**	1 0/1 - left amygdala	0
Hamartoma	0	1 - hypothalamic
Ischaemic lesions **Left/Right**	1 - perinatal, leading to ventriculomegaly	1 0/1 - right parieto-occipital

Unclassified lesions **Left/Right**	1 1/0 - left frontal	1 1/0 - left superior frontal
Lateralization **Left/Right**	3/5	7/3

Table 3.4. Structural abnormalities.

	Low Risk N = 19	High risk N = 34
Videotelemetry data available	7	30
Epilepsy syndrome		
temporal (left/right/bitemporal)	4 (1/1/2)	6 (3/2/1)
temporo-occipital (L/R/non-lateralizing)	2 (0/2/0)	1 (0/0/1)
fronto-temporal (L/R/bilateral)	0	6 (4/1/1)
frontal (L/R/non-lateralizing)	0	6 (1/2/3)
parieto-occipital (L/R)	3 (1/2)	3 (1/2)
hemisphere (L/R)	0	1 (1/0)
Lateralisation	2 left / 5 right / 11 non-lateralized	10 left / 7 right / 13 non-lateralized
Focal, non-localisable	6	6
Idiopathic generalised	3	0

Table 3.5. Epilepsy classification in the at risk populations.

3.1.1.3 Controls

Scans of 15 age- and gender-matched healthy control subjects were included from a previous study (Stretton et al., 2013). All controls had normal MRI scans.

3.1.2 MRI data

3.1.2.1 MRI data acquisition

All participants had been previously scanned on the same 3 T GE Signa HDx scanner (General Electric), and were scanned with identical acquisition parameters. We used standard imaging gradients, with a maximum strength of 40 mT/m and slew rate of 150 T/m/s. As part of the clinical sequences, a coronal T_1-weighted volumetric (3D) scan was acquired with 170 contiguous 1.1-mm thick slices (matrix 256 × 256, in-plane resolution 0.9375 × 0.9375 mm).

3.1.2.2 MRI data analysis

We used the Voxel Based Morphometry 8 toolbox (http://dbm.neuro.uni-jena.de/vbm), implemented in Statistical Parametric Mapping (SPM) 8 software (http://www.fil.ion.ucl.ac.uk/spm) for data analysis. Preprocessing included spatial normalization to the Montreal Neurological Institute (MNI) template, segmentation into the different tissue classes (grey matter, white matter, CSF), and modulation to correct for volume changes due to normalization. Intersubject registration was optimized by using the DARTEL (Diffeomorphic Anatomical Registration Through Exponentiated Lie Algebra) algorithm. A quality check implemented in the VBM8 Toolbox did not identify any outliers, and grey matter images were then smoothed with a 10-mm full-width at half-maximum Gaussian kernel (Yasuda et al., 2010).

The smoothed grey matter images were entered into a full-factorial design with group as factor to test for local differences in grey matter volume between groups. Voxels with grey matter values <0.2 (absolute threshold masking) were excluded to avoid edge effects between different tissue types. Age at scan was entered as a nuisance variable into the model.

The statistical threshold was set at $P < 0.001$, with a minimum cluster size of 30 contiguous voxels.

3.1.3 Statistical analysis of demographical and clinical data

Statistical analysis of demographical and clinical data was performed with SPSS Version 20.0 (SPSS Inc.). Pearson's chi-square test with an exact significance test for cells with a count of less than five was used for dichotomous data. Kruskal-Wallis test was used for all other data (Table 3.2).

3.2 Results

3.2.1 Demographic and clinical data

All groups were comparable for gender and age at scan. Epilepsy groups were generally comparable for clinical parameters, except for factors included in the risk scoring, i.e. frequent convulsive seizures, nocturnal seizures, and onset of disease (Table 3.2). Of the epilepsy groups, 66.7% of SUDEP cases, 35.3% of high risk and 47.3% of low risk had a lesion on the scan (Table 3.4).

3.2.2 Voxel-based morphometry

SUDEP cases showed increased grey matter volume within the right anterior hippocampus, and parahippocampal gyrus (Figure 3.1A), and decreased grey matter volume in the pulvinar of the thalamus bilaterally (Figure 3.1C), compared to controls. In those at high risk, we found similar changes within these regions, i.e. grey matter volume increase in the right hippocampus and parahippocampal gyrus (Figure 3.1B), and decreased grey matter volume in the left pulvinar (Figure 3.1C), when compared to controls.

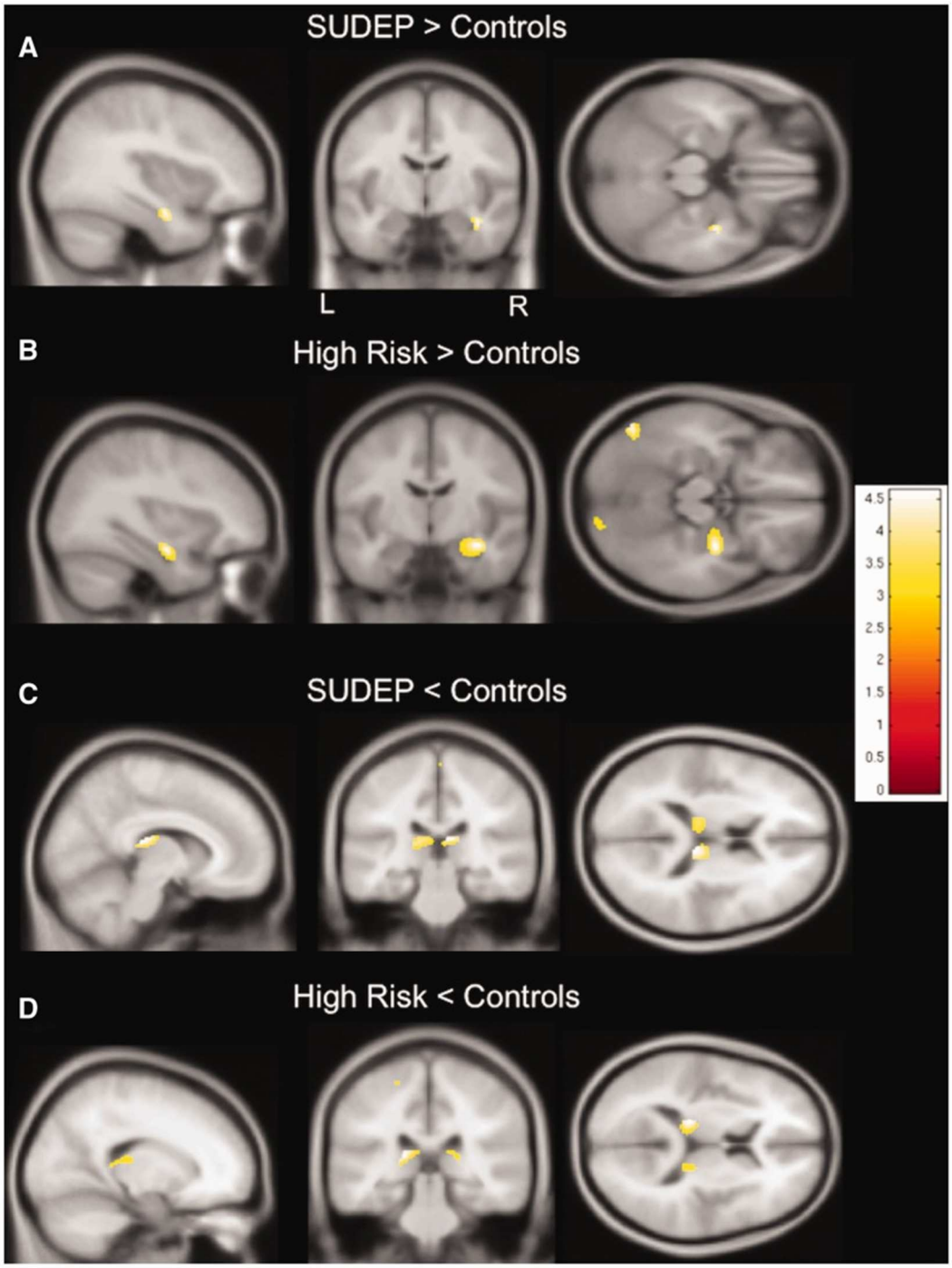

Figure 3.1. Regional grey matter volume differences between SUDEP and people at high risk and controls. (**A**) SUDEP cases show increased grey matter volume in the right hippocampus and parahippocampal gyrus compared to healthy subjects. (**B**) Similarly to SUDEP cases, subjects at high risk show increased grey matter volume in the right hippocampus and parahippocampal gyrus compared to healthy controls. (**C**) Compared to controls, grey matter volume is decreased in SUDEP cases in the pulvinar bilaterally. (**D**) Likewise, grey matter volume is decreased in those at high risk in the left pulvinar, compared to healthy controls. T-values are represented in the coloured bars. $P < 0.001$, 30 voxel threshold extent; L = left; R = right.

A *post hoc* analysis across all cases suggested a negative correlation of grey matter volume within the pulvinar bilaterally with disease duration (Figure 3.2; $P < 0.005$, 30 voxel threshold extent).

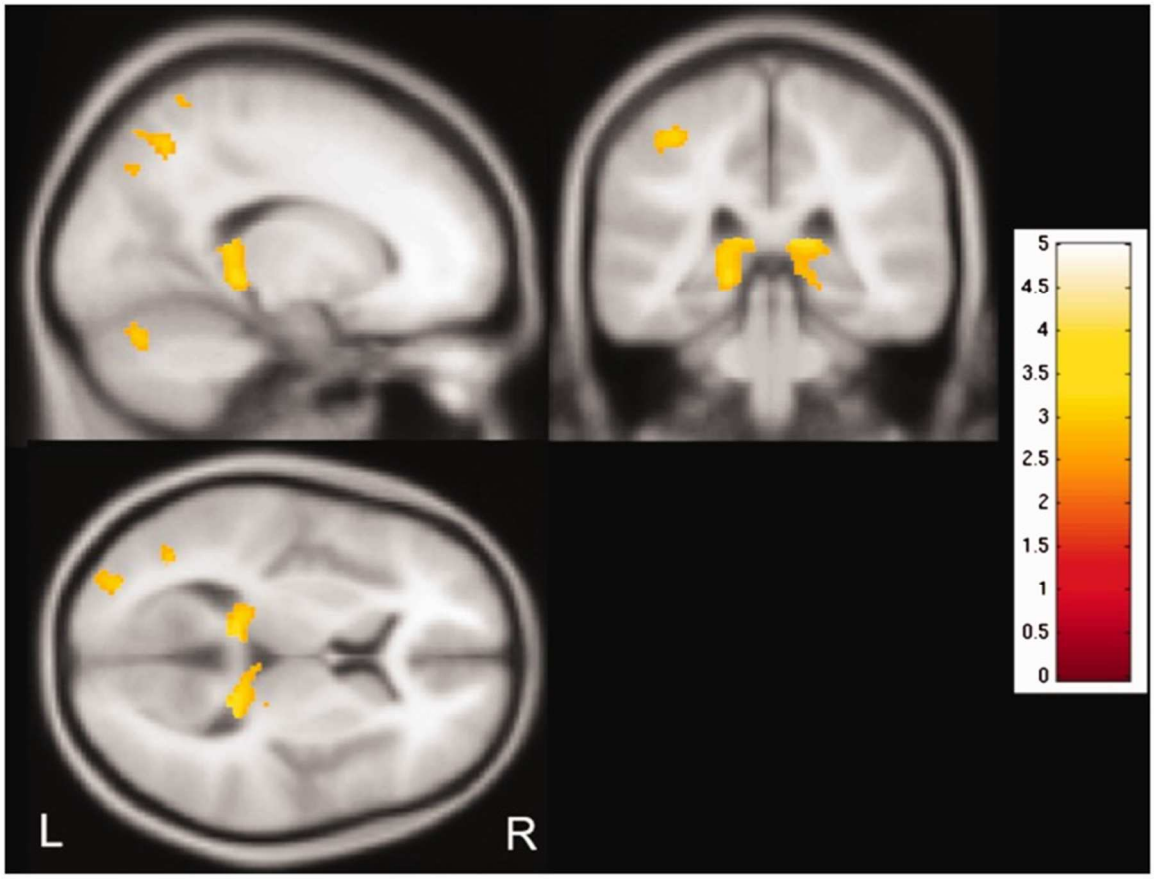

Figure 3.2. Correlation of grey matter volume with disease duration. Regional grey matter volume in bilateral thalamic pulvinar shows a negative correlation with epilepsy duration, i.e. grey matter volume decreases with longer duration ($P < 0.005$, 30 voxel threshold extent). T-values are represented in the coloured bar. L = left; R = right.

Both SUDEP cases and those at high risk showed areas of increased grey matter volume in the right hippocampus and parahippocampal gyrus, compared to those at low risk (threshold of significance $P < 0.05$, 30 voxels threshold extent; Figure 3.3).

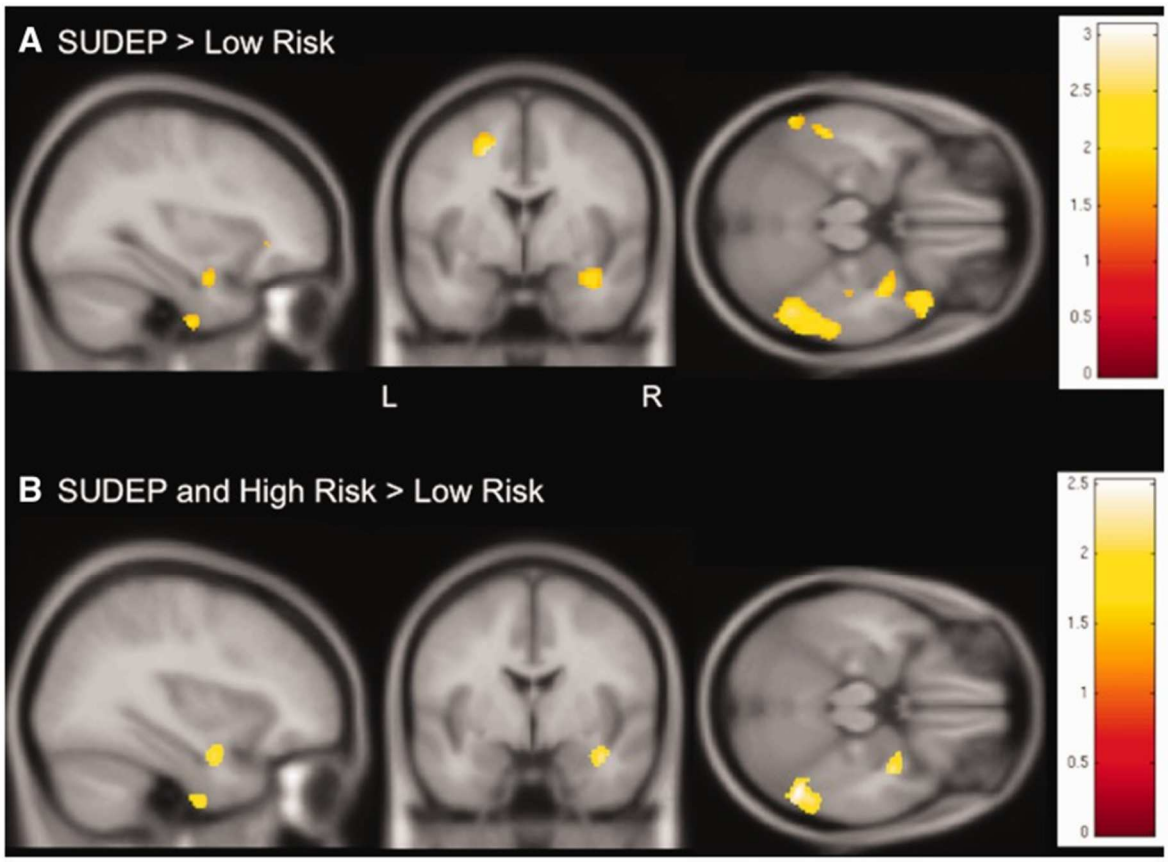

Figure 3.3. Regional grey matter volume differences between SUDEP cases and those at high risk in comparison to people at low risk. (**A**) Similar to findings in comparison to controls (Fig. 1A), but at a lower threshold level ($P < 0.05$, 30 voxels threshold extent), SUDEP cases show increased grey matter volume in the right hippocampus and parahippocampal gyrus in comparison to those at low risk. (**B**) People at high risk and SUDEP cases share common areas of increased grey matter volume within the right hippocampus and parahippocampal gyrus when compared to those at low risk (conjunction, $P < 0.05$, 30 voxels threshold extent). T-values are represented in the coloured bars. L = left; R = right.

3.2.3 Subgroup analyses

To ensure that the findings were not driven by gross brain pathologies, we conducted a subgroup analysis in those at risk who had non-lesional MRI scans (low risk $n = 10$; high risk $n = 22$). In the majority of SUDEP cases (66.7%), lesions were evident on clinical scans; hence, due to small sample size, the same subgroup analysis could not be conducted. Age at scan was entered as a nuisance variable. Compared to controls, those at high risk and without lesions still showed increases in anterior hippocampal grey matter volume, as well as in the amygdala, albeit this time bilaterally (Figure 3.4A). Similarly, grey matter volume in both hippocampi and amygdalae was increased in people at high risk compared to those at low risk, but more prominent in the right than left amygdala and hippocampus (Figure 3.4B).

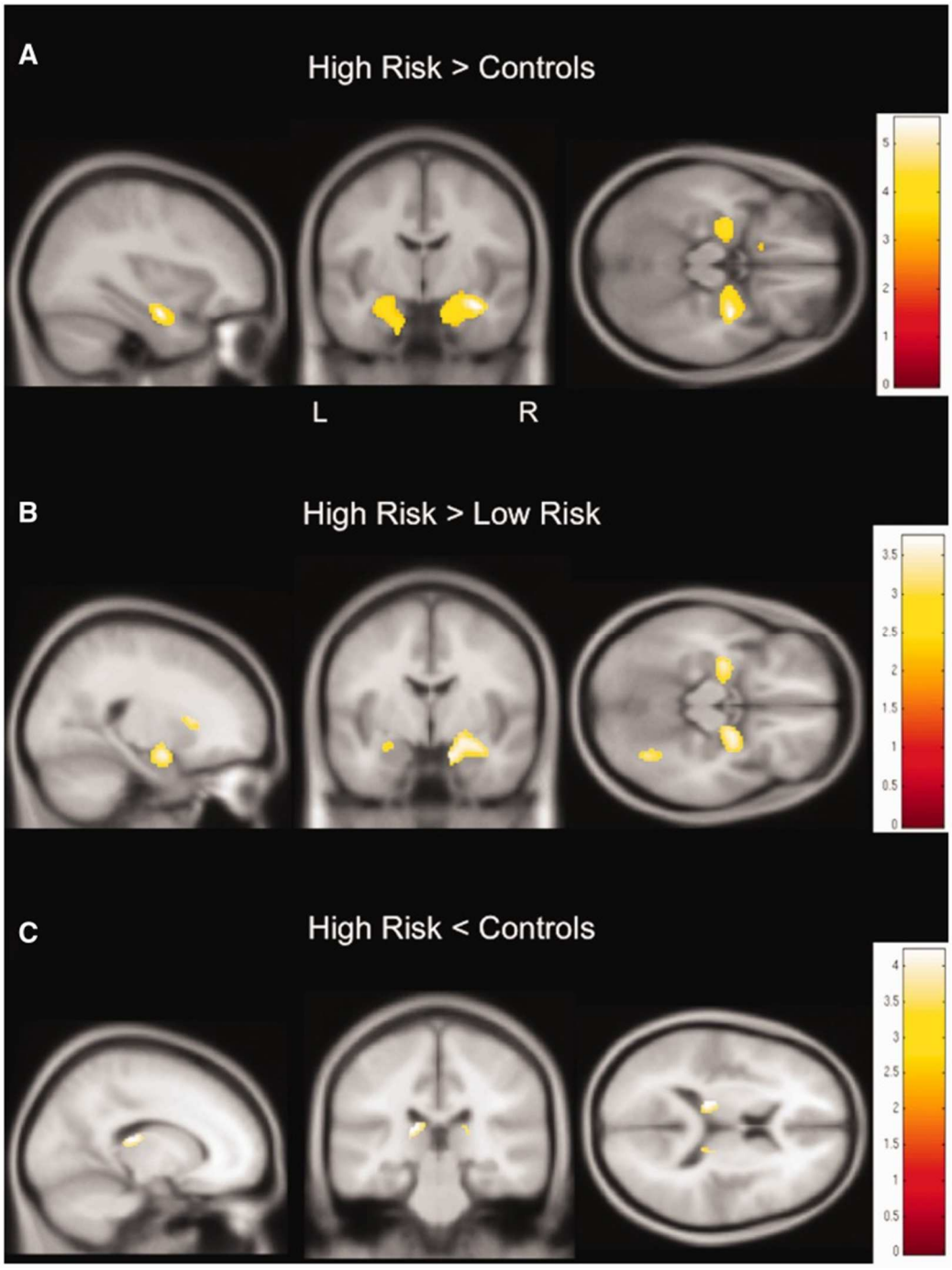

Figure 3.4. Regional grey matter volume differences between those at low and high risk with non-lesional epilepsy and controls. Findings appear similar to previous findings in the whole sample (Figure 3.1), but are more bilateral: subjects at high risk without identifiable pathology on clinical structural scans show an increase of grey matter volume in both anterior hippocampi and amygdalae when compared to controls (**A**; $P < 0.001$, 30 voxels threshold extent) and when compared to people at low risk (**B**; $P < 0.005$, 30 voxels threshold extent). Grey matter volume is decreased in the bilateral posterior thalamus in those at high risk when compared to controls (**C**; $P < 0.005$, 30 voxels threshold extent). T-values are represented in the coloured bars.

To explore whether findings in the right medial temporal lobe are only related to frequent convulsive seizures, we compared those with more than three convulsive seizures per year to those with fewer convulsive seizures per year in the high risk and SUDEP groups. Fourteen subjects had fewer convulsive seizures (four SUDEP, 10 high risk) and 32 had frequent convulsive seizures (eight SUDEP, 24 high risk). Age at scan and gender were entered as nuisance variables. There were no differences within the medial temporal region between both groups. Compared to controls, both groups showed common areas of increased grey matter volume in the right hippocampus (conjunction, $P < 0.005$; Figure 3.5).

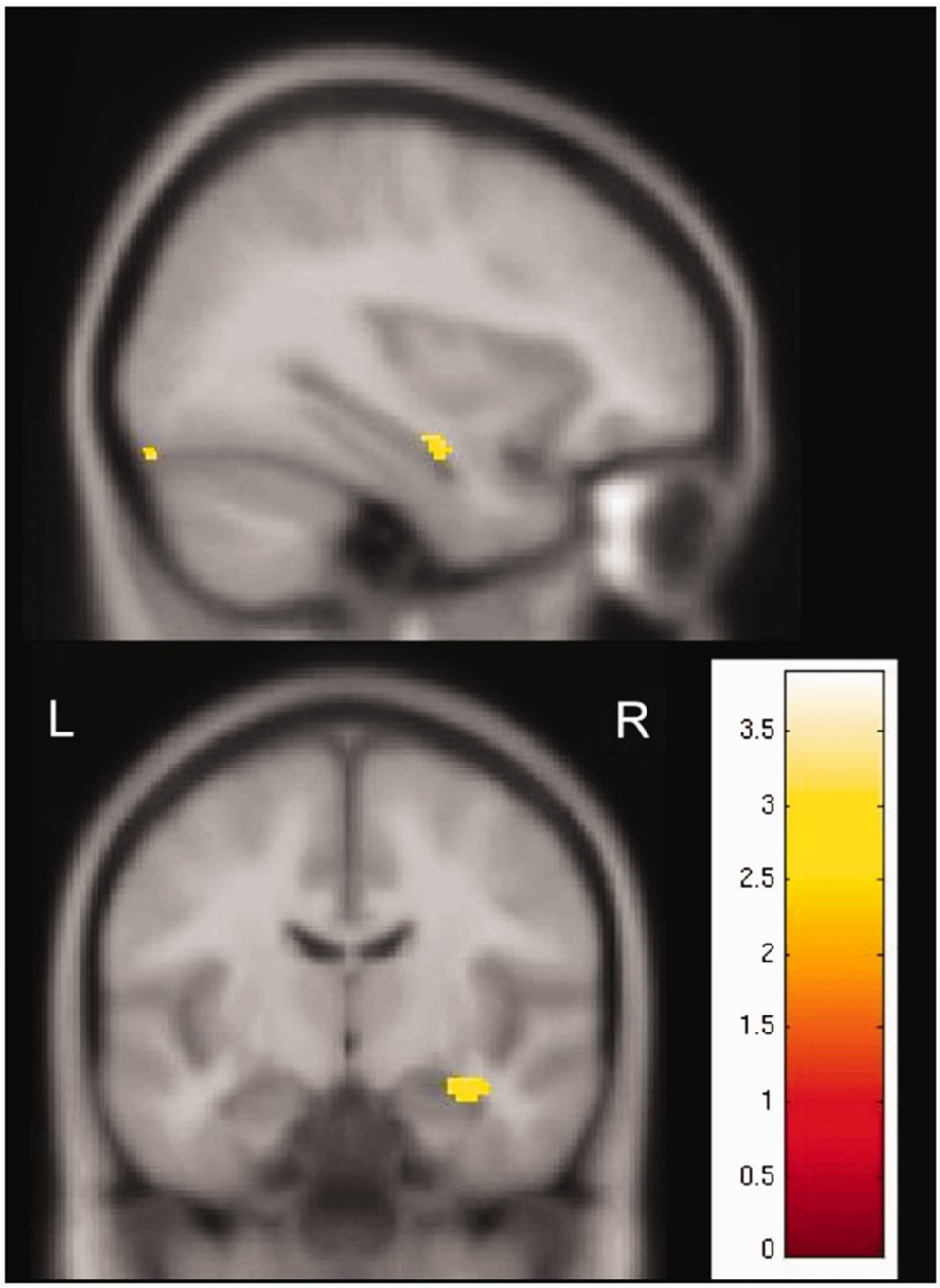

Figure 3.5. Common areas of increased grey matter volume in subjects with frequent and less frequent convulsive seizures compared to controls. Amongst SUDEP cases and people at high risk of SUDEP, subjects with frequent convulsive seizures (i.e. ≥3/year) and less frequent convulsive seizures (<3/year) share common areas of increased grey matter volume in the right hippocampus when compared to healthy controls. Conjunction, $P < 0.005$. T-values are represented in the coloured bars. L = left; R = right.

We also compared total right hippocampal volumes in both groups using an automated segmentation tool (Winston et al., 2013). There were no significant differences in right hippocampal volumes between subjects with frequent and less frequent convulsive seizures (right hippocampal volume in cm^3 in subjects with less than three convulsive seizures per year: median 3.036, IQR 0.65; in subjects with three or more convulsive seizures per year: median 2.90, IQR 0.52 cm^3; Mann-Whitney U = 130.000, $P = 0.646$).

To relate seizure onset site to right medial temporal findings, subjects with right temporal seizure onset were compared to those with a different, right extratemporal or left hemisphere onset. Ictal EEG data were available in nine SUDEP, 30 high risk and four low risk individuals. In six high risk and one SUDEP case, seizure onset could not be localized and these cases were therefore excluded. There were no differences between these two groups in volumetric findings. In comparison to controls, both groups showed an increase in grey matter volume in the right hippocampus ($P < 0.005$, 30 voxels threshold extent; Figure 3.6).

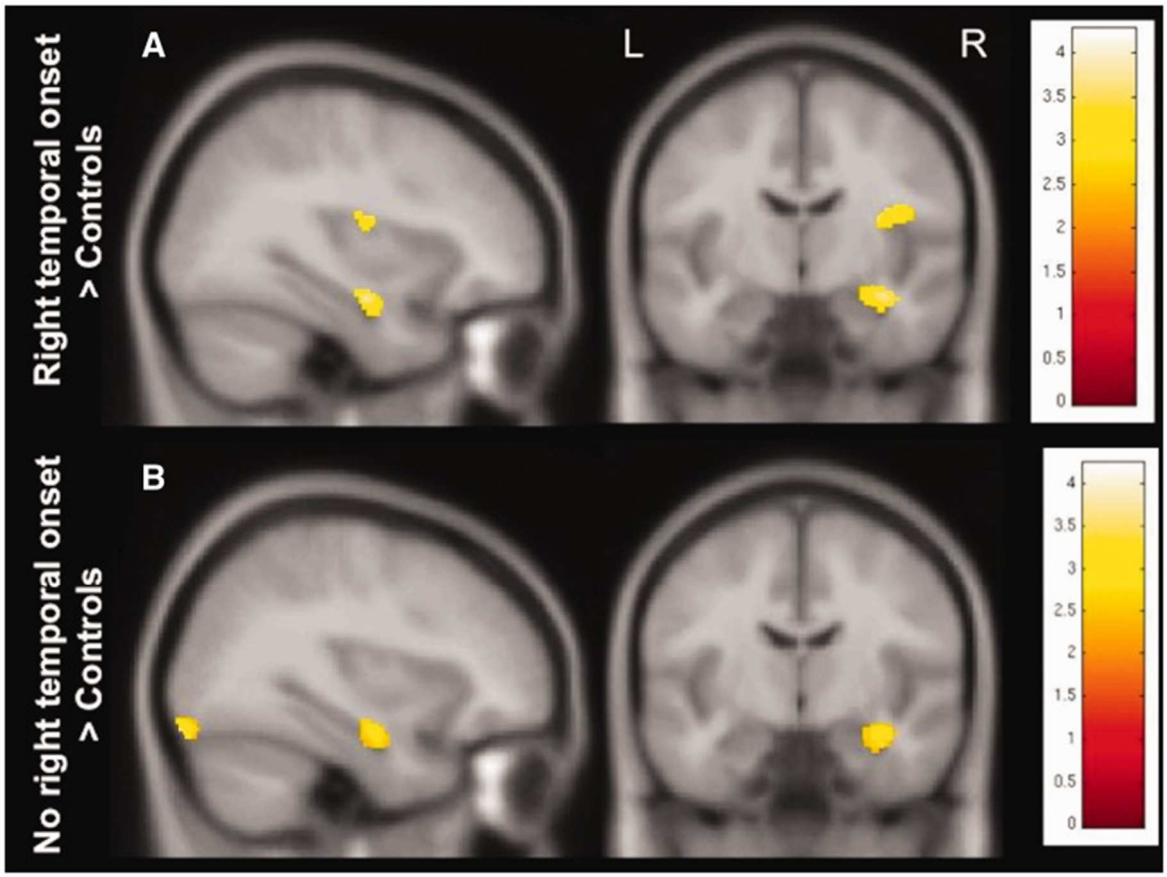

Figure 3.6. Grey matter volume changes in subjects with and without right temporal seizure onset. In comparison to healthy controls, subjects with right temporal seizure onset (A), as well as those without (B) showed an increase of grey matter volume in the right hippocampus ($P < 0.005$, 30 voxels threshold extent). T-values are represented in the coloured bars. L = left; R = right.

3.2.4 Summary of results

We explored whether regional imaging findings in people who died of SUDEP can be reproduced in a larger cohort of subjects at high risk for SUDEP. At-risk subjects were defined based on risk factors of sudden unexpected death in epilepsy identified in a recent combined risk factor analysis. To assess whether imaging findings are common to SUDEP and those at high risk, independent from other epilepsy-related factors, we compared SUDEP cases and those at high risk to a population presumed to be at low risk of SUDEP. We also compared subjects at high risk and low risk of SUDEP to healthy controls.

We identified increased grey matter volume in the right anterior hippocampus/amygdala and parahippocampus in sudden death cases and people at high risk, when compared to those at low risk and controls. Compared to controls, posterior thalamic grey matter volume, an area mediating oxygen regulation, was reduced in cases of sudden unexpected death in epilepsy and subjects at high risk. The extent of reduction correlated with disease duration in all subjects with epilepsy.

Chapter 4

INTERPRETATION OF THE RESULTS AND FUTURE PERSPECTIVES

SUDEP is the most devastating outcome in epilepsy. Whilst a number of risk factors and terminal pathophysiological phenomena have been determined, the cause of SUDEP remains unknown. Our results suggest that both exome sequencing data and structural imaging features may contribute to generate SUDEP risk estimates, promoting stratified medicine in epilepsy, with the eventual aim of reducing an individual patient's risk of SUDEP.

4.1 Genome-wide burden of rare deleterious genetic variants

Given evidence for heterogeneity of genetic risk, we proposed that genetic risk is spread across the genome. We show that, in people who have succumbed to SUDEP, there is a higher burden of deleterious genetic variants, with a higher cumulative deleteriousness score, compared to the burden in people with epilepsy who had not succumbed to SUDEP, and compared to the burden in people without epilepsy. Gene-based analysis in this group of SUDEP cases identifies some possible candidate genes that may carry some of the excess burden in this small sample. Our results provide further evidence for genetic susceptibility to SUDEP.

The identified genetic susceptibility is spread across the genome. Deleterious variants exclusively present in the exomes of this SUDEP group were found in 373 genes in the human genome. One of these genes, *CACNB2*, is associated with cardiac arrhythmia (see 2.2.4 and Table 2.10). Antzelevitch et al. (2007) reported that a loss of function in calcium channel activity secondary to mutations in *CACNB2b* can contribute to a sudden death syndrome that consists of a shorter-than-normal QT interval and ST-segment elevation (Brugada syndrome phenotype).

No other genes previously implicated in sudden cardiac death emerged. There are some genes that in our small SUDEP group appear overburdened ($n = 5$), but no one single gene, nor one single pathway, emerges as common to all SUDEP cases. Our findings require confirmation in an independent cohort. Taking the known genetic heterogeneity of syndromes associated with a higher risk of SUDEP together with our findings, we suspect that there is indeed not one culpable pathway or gene set for SUDEP. Studies of other SUDEP case groups might identify additional sets of risk variants. Even though

observational studies report mutations in SUDEP in candidate genes, we note that single candidate gene studies have not revealed a robust association with SUDEP in humans. For instance, congenital central hypoventilation syndrome (CCHS) is a potentially lethal autonomic nervous system disorder characterized by hypoventilation and impaired ventilatory response to hypercapnia and hypoxemia during sleep. An increased frequency of bradyarrhythmias has been reported in children with CCHS, sometimes requiring cardiac pacemaker therapy (Silvestri et al, 2000). CCHS is predominantly caused by expansion of an alanine repeat in the homeobox gene *PHOX2B*, with frameshift, nonsense, and missense mutations in *PHOX2B* accounting for a small proportion of cases (Amiel et al, 2003). Patients with smaller *PHOX2B* expansions can present in later life with nocturnal hypoventilation and some have coexistent epilepsy (TRochet et al, 2008). Bagnall et al. (2014) investigated *PHOX2B* sequence variations in 68 unrelated SUDEP cases; no *PHOX2B* polyalanine repeat expansion alleles or point mutations were found.

We propose that an overall increased burden of deleterious variants in a highly polygenic background is important in rendering a given individual more susceptible to SUDEP.

4.2 SUDEP in Dravet Syndrome

Some deleterious variants we have identified may per se contribute to, or be the cause of, the epilepsy, as well as increasing SUDEP risk. This may be the case, for example, for some *SCN1A* mutations that were already known in the Dravet Syndrome cases and held responsible for the condition. It is unlikely that these single mutations were solely responsible for SUDEP in these cases, as SUDEP is not universal in Dravet Syndrome, although a higher frequency of SUDEP is well recognised to occur (Sakauchi et al., 2011). Notably, *SCN1A* emerged as a burdened gene even when considering only WES-derived variants that passed variant selection. The exclusion of many *SCN1A* variants considered causal before QC is due to our strict and conservative QC, emphasising specificity above sensitivity. Nevertheless, *SCN1A* still emerged as a burdened gene. A possible dual role in both disease and SUDEP causation may apply to variants in other genes as well.

4.3 Limitation and future perspectives of genetic studies of SUDEP

The number of individuals who succumbed to SUDEP is small. Whilst there are new efforts to address this problem, to date case recognition and ascertainment (Smithson et al., 2014), collection of suitable samples and difficulties in obtaining WES data from certain types of material, have hampered progress and limited numbers. WES data from certain types of material, have hampered progress and limited numbers. Dravet Syndrome is over-represented in both SUDEP and epilepsy control groups compared to the general population of people with epilepsy, though we note that SUDEP is also more common in people with Dravet Syndrome than in the overall population of people with epilepsy. Whilst we cannot exclude the possibility that any individual in our epilepsy control might succumb to SUDEP in the future, none has yet despite an expectation that a proportion might have been expected to do so, such that our epilepsy control group is enriched with those at lower risk of SUDEP. Although a significantly higher prevalence of male gender and convulsive seizures in the 12-month period before last follow-up or death was observed in the SUDEP cases compared to the epilepsy controls, these differences do not survive correction for multiple comparisons. Nevertheless, the differences merit some discussion. Male gender has been associated with a 1.4-fold increased risk for SUDEP in a combined analysis of case–control studies (Hesdorffer et al., 2011). Other previous studies did not confirm this association (Walczak et al., 2001; P-Codrea Tigaran et al., 2005; Vlooswijk et al., 2007) and more recently a mouse model of SUDEP did not show significantly different susceptibility to seizure-induced respiratory arrest between males and females (Faingold and Randall, 2013). Overall, the difference in the proportion of males in the SUDEP and epilepsy control groups may therefore not be biologically relevant, and is not in any case statistically significant after correction for multiple comparisons. The difference in convulsive seizure frequency between the SUDEP and epilepsy control groups is also not significant after correction for multiple comparisons, but it is interesting to speculate whether genome-wide burden of deleterious variants is an explanation that might underlie this epidemiologically-derived risk factor, tying epilepsy severity into genomic burden.

The burden test used in our genome-wide burden analysis is sensitive to linkage disequilibrium (increased type I error rate). The comparatively small epilepsy control dataset may mean that we have not adequately filtered out deleterious variants related to

epilepsy causation rather than to SUDEP in our gene-based association analyses only. The associated genes may contribute to both epilepsy and SUDEP causation. We used different tests for the gene-based association analyses: replication of the results in an independent sample using the same statistical tests is needed. Our strategy focuses on deleterious rare variants: other types of genetic variant may also influence SUDEP risk. We did not undertake functional studies, but such studies are likely to prove extremely challenging, requiring not only construct complexity or multiple knock-ins, but also a whole animal model, as agonal changes in SUDEP typically occur outside the brain.

The finding of genome-wide increased burden of deleterious variants, rather than the individual genetic results, needs replication. If substantiated, these results provide scope for individualised risk estimates of SUDEP in people with epilepsy, with direct consequences for use of current strategies to reduce risk through improved seizure control or environmental measures, and may also assist with recurrence risk estimation in affected family members. The results highlight the value of exome sequencing in people with epilepsy: one test can provide insights into possible genetic causation, pharmacogenomic variants and outcome risk estimation. Overall, the findings provide new perspectives into SUDEP.

4.4 Anatomical differences between subjects with SUDEP and high risk versus those at low risk

We identified increased grey matter volume within the right hippocampus and parahippocampal gyrus in SUDEP cases and in subjects at high risk for SUDEP, compared to those at low risk and controls. There was increased grey matter volume in both hippocampi, extending to the amygdala when comparing non-lesional high and low risk subjects. The posterior thalamus (pulvinar) showed disease duration-dependent grey matter volume reduction in all patient groups.

MRIs of all cases and controls were subsequently reviewed again by an experienced neuroradiologist, specifically looking for the presence or absence of hippocampal pathology (Table 1 and 3). No new lesions within these regions were identified on visual inspection of individual cases, suggesting that the findings are at a group level.

Neuropathological studies in sudden unexplained death in childhood and in sudden infant death syndrome have found abnormalities in the same region. Kinney et al (2015)

found granule cell dispersion in the dentate gyrus of the hippocampus characterized by focal granule cell bilamination in 41.2 % of infants with sudden unexplained death (compared to 7.7 % of explained deaths). This finding suggests that focal granule cell bilamination may be a morphological marker of an impaired forebrain/limbic network that increases the risk of sudden infant death. They hypothesize that this might be due to instability of modulation of brainstem cardiorespiratory-related nuclei, or to a subclinical seizure in an infant with a predisposition to epilepsy, which had not yet manifested as a clinical seizure. They propose that this morphological marker "identifies" a vulnerable infant at risk for sudden death during a critical developmental period when the infant meets an exogenous stressor, i.e., the vulnerable infant of the triple-risk model for SIDS (Filiano & Kinney, 1994).

Hippocampal and temporal lobe anomalies were also described in 62% of sudden unexplained death in childhood cases (Kinney et al., 2009). Microdysgenetic features of the hippocampal formation included dentate gyrus and subicular anomalies, granular nodular heterotopia, subventricular neuroblasts and hamartia, all indicative of aberrant neurodevelopment. Similar to SUDEP cases, those sudden unexplained death in childhood individuals with structural anomalies were found dead during sleep and in the prone position, and more commonly had an individual or family history of febrile seizures, creating a potential link between hippocampal/temporal lobe maldevelopment, susceptibility to seizures, and sudden death.

Increase in grey matter volume, which has appeared in several epilepsy syndromes in previous voxel-based morphometry studies (Yasuda et al., 2010), has been suggested as indicative of dystopic neurons and diminished grey-white matter demarcation (Yasuda et al., 2010), and findings in the current study may therefore reflect abnormal neurodevelopmental processes. Neuropathological studies in SUDEP show that pathology can be present in the hippocampus: a retrospective study of forensic autopsy SUDEP cases reported hippocampal gliosis, atrophy or acute hypoxic-ischemia change (Zhuo et al., 2012). There are so far, however, no quantitative neuropathological studies of the hippocampus in SUDEP, which would be needed to confirm any subtle abnormalities such as microdysgenesis.

Increased grey matter volumes in the hippocampus may also represent gliosis. Gliosis has been defined as a spectrum of changes in astrocytes that occur in response to all forms and severities of CNS injury and disease including subtle perturbations; the changes undergone by reactive astrocytes vary with the nature and severity of the insult

along a gradated continuum of progressive alterations in molecular expression, progressive cellular hypertrophy and, in severe cases, proliferation and scar formation; the changes of astrogliosis are regulated in a context-specific manner by specific signaling events that have the potential to modify both the nature and degree of those changes; the changes undergone during reactive astrogliosis have the potential to alter astrocyte activities both through gain and loss of functions that can impact both beneficially and detrimentally on surrounding neural and non-neural cells (Sofroniew, 2009). One striking hallmark of the hippocampal sclerosis in temporal lobe epilepsy is that while there is a specific pattern of neuronal loss, there is also "reactive gliosis" with hypertrophic glial cells exhibiting prominent GFAP staining and long, thick processes (Binder and Steinhäuser, 2006). In addition to changes in preexisting glial cell populations, newly-generated glial cells with distinct properties may migrate into the hippocampus and contribute to enhanced seizure susceptibility (Hüttmann et al., 2003; Parent et al., 2006). Gliosis within the hippocampus may therefore alter neuronal activity facilitating the risk of SUDEP, e.g. through hyperexcitability and/or limbic network dysfunction implicating also autonomic function.

A recent study evaluating structural imaging prediction patterns for seizure freedom after surgery in temporal lobe epilepsy found unilateral or bilateral atrophy of the hippocampus, amygdala and entorhinal cortex in most subjects, although one subgroup showed bilaterally increased hippocampal and amygdala volumes (Bernhardt et al., 2015). Subjects in this group were more likely to have unsuccessful epilepsy surgery, supporting the concept that gliosis may facilitate processes of treatment-resistant disease. Histopathology confirmed hippocampal gliosis in almost all subjects of this subgroup. Astrogliosis and cellular hypertrophy have been described in neuropathological studies in hippocampal tissue of subjects with refractory temporal lobe epilepsy and considered likely to be a major contributor to both disease development and severity in temporal lobe epilepsy (Das et al., 2012). Of 11 drug-resistant patients with mesial temporal lobe epilepsy and hippocampal sclerosis, diagnosed with amygdala enlargement based on presurgical MRI, nine were found with mild gliosis on histopathology (Minami et al., 2015).

Longitudinal voxel-based morphometry studies report a decrease of grey matter volume in mesio-temporal structures and the thalamus with longer disease duration and more active disease, i.e. frequent seizures (Bernhardt et al., 2009, 2013; Coan et al., 2009). Similarly, changes in the posterior thalamus correlate with disease duration in our

cohort and this suggests a dynamic origin of grey matter volume alterations in our study. A potential mechanism for gliosis in epilepsy could be repeated hypoxic insults, particularly through convulsive seizures (Macey et al., 2009).

That increased grey matter volume may represent gliosis and plasticity following neural injury (Yasuda et al., 2010) is corroborated by data in other sudden death entities: increased grey matter volume in the putamen appears in people with newly-diagnosed obstructive sleep apnoea, who are subjected to repeated hypoxic episodes, with the increased volumes usually attributed to transitional processes in glial death accompanying the neural injury in the syndrome (Kumar et al., 2014).

4.5 Association with autonomic dysfunction and significance of laterality of findings

Several functional imaging studies in humans (Shoemaker et al., 2012, 2015), and stimulation studies in animals (Terreberry and Neafsey, 1987) have identified the hippocampus as an essential component of limbic circuitry modulating autonomic function, with substantial influences on blood pressure regulation (Harper et al., 2000). Major hippocampal influence on autonomic activity through efferent projections can also be assumed from intracerebral stimulation studies in subjects with refractory epilepsy (Catenoix et al., 2011). These influences are corroborated by reports of people with mesial temporal lobe epilepsy who show decreased heart rate variability in relation to seizures and interictal epileptic discharges, which were more pronounced during sleep, when most cases of SUDEP occur (Moseley et al., 2011). Of interest, changes of cardiovascular autonomic modulation after temporal lobe surgery have been demonstrated by Hilz et al (2002). In this study they monitored heart rate, systolic blood pressure and respiration in 18 temporal lobe epilepsy patients before and after temporal lobe surgery. While the standard measures of cardiovascular function, heart rate, blood pressure and respiration, remain stable, the autonomic modulation in the low frequency range shows an average reduction of 43% and the baroreflex sensitivity is lowered by almost 40% after surgery. The reduction of sympathetic cardiovascular modulation and baroreflex sensitivity after surgery might result from decreased influences of interictal epileptogenic discharges on brain areas involved in cardiovascular autonomic control. Temporal lobe epilepsy surgery seems to stabilize the cardiovascular control in epilepsy

patients by reducing the risk of sympathetically mediated tachyarrhythmias and excessive bradycardiac counter-regulation.

Hippocampal grey matter volume increases in our cohort may partially underlie seizure generation and ictal and peri-ictal autonomic dysfunction. However, increased right hippocampal grey matter volume was even present in those individuals with known right medial temporal epilepsy when compared to healthy controls (no cases of hippocampal sclerosis in either group). The increased grey matter volume was surprising, as longitudinal voxel-based morphometry data describe progressive atrophy of the ipsilateral hippocampus in the medial temporal lobe, especially in those subjects with higher seizure frequency and longer epilepsy duration, i.e. higher SUDEP risk (Bernhardt et al., 2009, 2013; Coan et al., 2009). This may suggest that our findings are not associated with a primarily seizure-related autonomic dysfunction; but they may be associated with an interictal autonomic dysregulation. At this time this is a speculative suggestion, and needs investigation of autonomic function in similar cohorts.

4.6 Asymmetry of grey matter volume increases

A significant aspect of the grey matter volume hippocampal increase in SUDEP cases and subjects at high risk was the asymmetry, with the volume changes on the right side. The lateralization of tissue change in an autonomic regulatory area poses a serious concern for sympathetic and parasympathetic outflow. If laterality on sympathetic influences is preserved to medullary output nuclei, the consequences to cardiac arrhythmia generation are severe, as asymmetric sympathetic outflow leads to such phenomena as potentially fatal long Q-T syndrome (Schwartz, 1998). A series of stimulation, lesion, stroke, and imaging studies, including human epilepsy surgical studies (Oppenheimer et al., 1992; Oppenheimer, 2006) investigated the cortical lateralization of cardiovascular regulation. Lesions confined mainly to the right posterior insula of the rat increase blood pressure and heart rate without altering baroreceptor sensitivity (Butcher et al, 1995; Zhang & Oppenheimer, 1998). Conversely, left posterior insular lesions do not alter cardiovascular variables, but increase baroreceptor sensitivity (Oppenheimer, 2006). Right posterior insular stimulation was shown to increase cardiac sympathetic tone in the absence of heart rate, blood pressure or respiration changes (Oppenheimer et al, 1998). Interestingly, baroreceptor sensitivity decreased, a finding also linked to increased mortality after

stroke (Robinson et al, 2003). Left caudal anterior insular stimulation during surgery for intractable epilepsy increases the frequency of bradycardia and depressor responses, whereas stimulation of a similar region of the right anterior insula is associated with heart rate and diastolic blood pressure elevation (Oppenheimer et al., 1992). Although both types of response were elicitable from either insula, the proportion varied, and the degree of bradycardia was greater on left insular stimulation. These data indicate that in the human at least, some lateralization of cardiovascular representation may exist with sympathetic predominance of cardiovascular regulation being a right insular function, and parasympathetic cardiac neural regulation relating to the left insula (Oppenheimer, 2006). Tokgözoglu et al (1992) showed that stroke in the right insula leads to decreased heart rate variability and to increased incidence of sudden death. Sudden death after acute right-sided insular strokes and increased complex arrhythmias appears more often than in any other lesion localization (Soros and Hachinski, 2012). Right insular injury in obstructive sleep apnoea shows distorted blood pressure recovery patterns to a challenge (Harper et al., 2003; Henderson et al., 2003) and right hemisphere strokes, particularly when involving the insula, are accompanied by increased nocturnal blood pressure, higher noradrenaline levels and QTc prolongations (Oppenheimer, 2006). The insular effects appear to be mediated by projections to the ventral medial frontal cortex, hypothalamus, and hippocampus through integrated circuitry (Shoemaker et al., 2015). The lateralized (right) increased mesiotemporal grey matter volume in our cohort may contribute to chronic, asymmetric hyper-sympathetic activation, or a sympathetic system lacking in appropriate responsiveness, which would contribute to mechanisms that pose a risk for sudden death.

Similar scenarios develop for obstructive sleep apnoea and for heart failure, which induce severe injury preferentially in the right insula, and consequential very high resting, and unresponsive, sympathetic tone (Macey et al., 2002; Woo et al., 2005). An imbalance between parasympathetic and sympathetic drive places an individual at risk, resulting in a tendency to postictal bradycardia/asystole as noted in the MORTEMUS study (Ryvlin et al., 2013a).

4.7 Decreased grey matter volume in the posterior thalamus

A second major finding was that grey matter volume was reduced in the posterior thalamus, and correlated with disease duration. The finding was not unique to SUDEP. A decrease of grey matter volume in the posterior thalamus correlated with disease

duration in all subjects with epilepsy (Figure 3.2), and one may speculate that those changes may develop in low risk subjects, given sufficient duration of seizures. However, the finding of posterior thalamic grey matter volume should be taken in the context of roles for that structure in respiratory regulation. Substantial evidence, ranging from lesion and stimulation studies in the foetal lamb (Koos et al., 1998, 2004), to functional MRI studies in adolescents and children with congenital central hypoventilation syndrome (Macey et al., 2005), show the significant role of the posterior thalamus in mediating breathing responses following manipulation of oxygen levels, with special participation in the inhibition of breathing following hypoxic exposure (Koos et al., 1998, 2004). We speculate that injury to the posterior thalamus is common in people with epilepsy, that the evidence suggests that disease duration potentiates that injury, and that such injury poses particular risk to the hypoxia normally accompanying ictal episodes, causing thalamic structures to fail to adequately recover from low oxygen. A thalamic role must, however, be viewed in the context that in people who succumbed to SUDEP or who were at high risk also were burdened with right-sided grey matter volume increases in the hippocampal region, which would compromise appropriate blood pressure responses that accompany apnoea. Thus, the combination of injury, diminished posterior thalamic and altered right-sided hippocampal grey matter volume may impose a set of circumstances leading to vital failure.

The mechanisms underlying decreased thalamic grey matter volume should be considered; the decline emerges in several epilepsy syndromes (Yasuda et al., 2010), and appears to be, in part, independent of epilepsy severity, presence of MRI lesions, and duration (Keller et al., 2002). Strong relationships of disease duration and declines in grey matter volume and changes in white matter tract microstructure, i.e. mean fractional anisotropy declines, have been described, and may underlie progressive brain changes in response to active disease, i.e. recurrent seizures (Keller et al., 2012).

4.8 Limitation and future perspectives of structural imaging studies of SUDEP

The criteria used to define our risk groups, and the cut-off between high and low risk, were arbitrary. The finding that SUDEP and those at high risk show similar patterns is consistent with our definition of risk groups. Eleven of 12 SUDEP cases were classified as high risk with our criteria.

A major limitation of our study is to disentangle whether our finding of right hippocampal grey matter volume increase is a specific SUDEP risk factor or rather a marker of severe epilepsy.

As there was only one low risk case in our SUDEP group, we could not establish whether increased right hippocampal grey matter volume is present in SUDEP cases despite being labelled low risk. This would have marked our finding as more SUDEP-specific. In vivo imaging biomarkers of SUDEP risk should be present in both subjects who later on died from, and those at high risk of, SUDEP. We argue that the smaller the difference we observe between those two groups, the better our classification and definition of high risk criteria. Similarly, main risk factors for SUDEP—like frequent, uncontrolled convulsive seizures—will have to be present in both SUDEP and high risk groups (Hesdorffer et al., 2011), and hence, are also the major distinguishing factor of high risk versus low risk subjects in our study. By the nature of SUDEP and our study, it is therefore impossible to fully disentangle the effect of severe epilepsy from a specific SUDEP biomarker itself.

Due to methodological challenges (Ashburner and Ridgeway, 2013), there are only few longitudinal voxel-based morphometry studies in people with mesial temporal lobe epilepsy. All of them show grey matter atrophy within mesial temporal structures and beyond (e.g. thalamus) over time, which are more progressive with longer disease duration and higher seizure frequency (Bernhardt et al., 2009, 2013; Coan et al., 2009). Evaluation of subregional mesiotemporal disease progression revealed that progressive atrophy particularly involves the anterior part of the hippocampus (CA1 subfields) (Bernhardt et al., 2013). These reports are in clear contrast with our findings of increased grey matter volume particularly in the anterior hippocampus, and suggest that these are not only caused by frequent seizures. There are poor data on exact seizure counts in our groups, but when subjects in the high risk and SUDEP groups where dichotomized into those with frequent (i.e. more than three convulsive seizures per year) and those with less frequent convulsive seizures, there were no significant group differences within the right hippocampus, but both groups showed common areas of increased right hippocampal grey matter volume when compared to healthy controls (Fig. 3.5). In addition, total right hippocampal volume measures did not differ between groups. This underscores our argument that the findings represent more specific SUDEP markers than just markers of severe epilepsy.

In keeping with the longitudinal data, posterior thalamic grey matter atrophy correlates with disease duration in our cohort and we can therefore confirm that this finding is not a specific SUDEP biomarker.

We appreciate that epilepsy groups in this study combine various different epilepsy subtypes, and include subjects with lesional and non-lesional MRI scans (Table 3.4). Right hippocampal sclerosis was, however, not present in either epilepsy group, and therefore does not explain differences in right hippocampal grey matter volume. Structural abnormalities were common among our SUDEP population (66.7% of cases), and we acknowledge that our SUDEP group may therefore not be representative of all SUDEP cases.

A previous study (Mueller et al., 2014) described mesencephalic volume losses using graph analysis methodology in two SUDEP cases compared to controls. We did not aim to examine brainstem volumes, although our whole-brain analysis included the brainstem; we found no abnormal changes in the brainstem within any group. Voxel-based morphometry has substantial limitations in evaluating brainstem segmentation, due to the difficulty in resolving internal brainstem architecture reliably and consistently (Lambert et al., 2013). Disturbances in brainstem attributes may be better evaluated with newer procedures for examining tissue changes, such as diffusion MRI.

Increased right hippocampal and parahippocampal grey matter volume and grey matter volume decline in the posterior thalamus appear to be related to SUDEP risk. In the case of grey matter volume increases, the relationship is independent of markers of severe epilepsy, such as frequent convulsive seizures. The volume increases are potentially of dynamic origin, representing gliosis in response to repetitive injury from severe epilepsy, while the thalamic volume declines may result from excitotoxic or other injury sources. The thalamic injury may lead to an inability to recover breathing to a hypoxic challenge from apnoea, while the hippocampal/parahippocampal pathology may contribute to asymmetric influences on autonomic outflow, establishing circumstances for cardiac arrhythmia and hypotension. The structural changes may be useful biomarkers to assist determination of pathophysiology of SUDEP.

4.9 Biomarkers and implication for management

Despite a wealth of studies reporting on proposed risk factors or mechanisms of SUDEP this has not yet been translated into targeted therapeutic interventions and a reduced incidence of SUDEP. Given the disturbance in cardiac autonomic control, there has

been speculation as to whether cardiotropic medication, such as beta-antagonists, may have a protective effect, although no studies have been performed in this regard (Opherk et al, 2002). Experimental studies in rats with audiogenic seizures and ictal apnoea have shown that selective serotonin reuptake inhibitors have a protective effect (Tupal & Faingold, 2006), although relevant confirmatory clinical studies are lacking. There is neuropathological evidence of involvement of the medullary serotonergic network in SIDS cases with a significantly lower density of serotonin receptor binding sites, particularly in male SIDS cases compared to controls (Paterson et al, 2006). Whether pharmacological modulation of the brainstem serotonergic network or cardiac autonomic function results in a protective effect remains to be seen. The implications of the observed ictal asystole in a small cohort of patients to a larger, more representative, group of epilepsy patients is unknown. If this finding is confirmed, the potential role of pacemaker insertion in preventing a proportion of SUDEP cases needs to be assessed. Supervision of patients with epilepsy has emerged as the only clinically important protective factor, independent of seizure control. The basis for this remains unclear but may relate to body positioning and alleviation of obstructive apnoea or possibly brainstem arousal mechanism (Nashef et al, 1998; Langan et al, 2000, 2005). Existing techniques for monitoring apnoea in other clinical contexts suffer from limitations which make them unsuitable for SUDEP prevention. These include the size and weight of monitors, duration of real-time monitoring, difficulty of use in unsupervised conditions and, most importantly, very poor sensitivity and specificity, mostly due to signal artefacts. Oximetry, for example, suffers from artefacts and false alarms, and the delay between beginning of apnoea and detection of oxygen saturation drop causes warnings to come late.

The first clinical study of a novel wearable apnoea detection device (WADD) has been undertaken, which proved that the device works even in the presence of artefacts in healthy subjects and individuals with sleep apnoea; it can provide over 90% sensitivity and specificity for detection of potentially dangerous apnoeas (Rodriguez-Villegas et al, 2014). The ideal system for monitoring a patient's movements should have a high sensitivity and specificity, be easy to operate and be unobtrusive. Several attempts have been made to develop devices in order to alert patients and carers to an ongoing seizure, but unfortunately these attempts have universally had a very low sensitivity and specificity (Carlson et al, 2009; Narechania et al, 2013). Current approaches for SUDEP

prevention are primarily based on detecting rhythmic movement caused by tonic-clonic seizures, with devices that are either worn by the person or installed in the bed. The ideal device should be validated with simultaneous ictal EEG recordings. The stationary bed seizure monitors function by either detecting noises originating from the rhythmic banging on the bed during the clonic phase of GTCs or from the bed springs (Carlson et al, 2009), or changes in mattress pressure during abnormal movements (Narechania et al, 2013). Several products are commercially available, but none of them have been tested in a clinical setting, and sensitivity and specificity are disappointingly low. Surface electromyography (EMG) during convulsive seizures is another way to detect ongoing epileptic seizures. The tonic phase of GTCs is characterised by a marked increase in amplitude-derived parameters and tonic seizures have a marked increase in frequency. The devices are worn on the biceps and multicenter studies are currently ongoing. In the UK the NICE Guidelines state that tailored information and discussion between the individual, family and/or carers and healthcare professional should take account of the small but definite risk of SUDEP (NICE, Clinical Guideline 20, 2004).

For many years, there was reluctance by some health professionals to talk openly about SUDEP. However, the weight of opinion is shifting to full disclosure. A report from the joint working party of the American Epilepsy Society and Epilepsy Foundation summarises risks and preventative strategies (So et al, 2009). In particular, information about SUDEP is important for patients at risk of AED non-adherence and for those who are candidates for surgery and can be reassuring for patients with well-controlled epilepsy who are at low risk.

4.10 Conclusions

The cause of SUDEP is not known, and its occurrence unpredictable. It is fundamental to understand the range of SUDEP mechanisms and translation of this knowledge into predictive algorithms of individual risk and preventive strategies.

Based on evidence from a few familial studies in humans, evidence from specific genetic epilepsy syndromes, and animal models, we proposed that there might be a polygenic contribution to SUDEP risk. We used whole exome sequencing of DNA samples from 18 people who had SUDEP, compared to sequence data from a range of controls, to show that there is indeed an over-representation of rare deleterious variants

in people who had SUDEP. This is the first such study in SUDEP. Whole exome sequencing studies require careful execution. We adopted strict quality control and a joint calling strategy across cohorts to overcome problems inherent to such studies, especially when different batches of samples are examined. Our sample size is modest, but this is the inevitable nature of studies in this catastrophic phenotype. We were also careful in selecting our disease controls. We used a set (UCL-exomes) for which we knew the participants had no history of epilepsy or cardiac disease. We used another set, of people with epilepsy, in which the occurrence of SUDEP was less than expected, representing 'super-controls'. Overall, our meticulous and reproducible methods render our findings robust. The findings are novel: no such studies have yet been published and no such approach has yet been used in SUDEP studies. Previous work has focussed on a number of single candidate genes per manuscript. The findings will also make the community think more on the spectrum of severity in epilepsy and how severity might be generated and measured. SUDEP is a resultant co-morbidity of epilepsy. Several co-morbidities, neurological, psychiatric and somatic, are over-represented in epilepsy, and our findings demonstrate a new way in which the spectrum of severity might be understood, with seizure-freedom (e.g. due to the first anti-epileptic drug) at one of the spectrum, and SUDEP at the other. Our findings therefore represent a first insight into genomic risk burden for SUDEP in epilepsy.

Peri-ictal cardiac arrhythmias are common occurrences in SUDEP. The role of changes in heart rate variability in seizure survivability is uncertain. Impaired baroreflex sensitivity, with subsequent compromised cerebral blood flow and an inability to recover from extreme postictal hypotension is a potential SUDEP mechanism. Peri-ictal respiratory apnea has been reported in near-SUDEP and recorded SUDEP cases. Previously unrecognized patterns of tachypnea and profound cardiorespiratory dysfunction were followed by terminal apnea and cardiac arrest in reported monitored cases of SUDEP. Brainstem dysfunction may contribute to fatal cardiorespiratory dysfunction, although this is a challenging region for human neurophysiologic study. We identified increased grey matter volume in the right anterior hippocampus/amygdala and parahippocampus in 12 SUDEP cases and people with epilepsy at high risk of SUDEP, when compared to those at low risk and controls. Compared to controls, posterior thalamic grey matter volume, an area mediating oxygen regulation, was reduced in cases of SUDEP and subjects at high risk. The extent of reduction correlated

with disease duration in all subjects with epilepsy. Increased amygdalo-hippocampal grey matter volume with right-sided changes is consistent with histo-pathological findings reported in SIDS. We speculate that the right-sided predominance reflects asymmetric central influences on autonomic outflow, contributing to cardiac arrhythmia. Pulvinar damage may impair hypoxia regulation. Evidence of structural abnormalities in cardiac and respiratory control structures in the forebrain suggest a role for premortem imaging in individuals with epilepsy.

Genomic risk burden and structural abnormalities in autonomic and respiratory regulatory sites may significantly contribute to develop and validate a quantifiable SUDEP risk model for clinical use.

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