Determinants of variable disease severity in *SCN1A*-related phenotypes and X-chromosomal epilepsy syndromes

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PhD dissertation

ISBN: 978-94-6380-248-2

Lay-out: RON Graphic Power, www.ron.nu
Cover design: Linda van Zijp - StudioLIN graphic design
Printed by: ProefschriftMaken || www.proefschriftmaken.nl

The research described in this thesis was financially supported by Stichting Vrienden WKZ (project 1614054) on behalf of Stichting Panta Rhei, the Dutch Epilepsy Foundation (project 2017-01), and the COFRA foundation (grant reference number 5589).

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Determinanten van variabele ernst van *SCN1A*-gerelateerde fenotypes en X-chromosomale epilepsiesyndromen

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen
op dinsdag 5 maart 2019 des middags te 2.30 uur

door

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geboren op 13 mei 1990 te Eindhoven

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Chapter 1

General introduction
and outline of this thesis

1.1 *SCN1A*-related phenotypes

Dravet syndrome is one of the most well-known genetic epilepsy syndromes. Here, we introduce three patients with Dravet syndrome. Although in each of them the disease is caused by a pathogenic variant in the *SCN1A* gene, they have very different disease stories.

Patient 1

An otherwise healthy young infant experienced a first febrile seizure at the age of 12 months. Her general practitioner was not alarmed at first, as febrile seizures occur in up to five % of young children[1,2]. However, the febrile seizures reoccurred frequently, up to seven times per week during periods with infections, and soon she started having seizures without any provocation too. Multiple anti-epileptic drugs were tried, but they were all ineffective. In addition, at the age of three years, a developmental delay was noticed. This combination of clinical symptoms was suggestive of Dravet syndrome, which was diagnosed at the age of five years, and genetic testing of *SCN1A* revealed a frameshift variant, confirming the clinical diagnosis. Her parents were counselled accordingly: they were told that their daughter likely would have severe, treatment resistant epilepsy and a moderate to severe intellectual disability (ID). Unexpectedly however, she developed a much milder phenotype: currently, this girl is 10 years old, and only has two seizures per year, while using two anti-epileptic drugs. She goes to a special needs school and her IQ was estimated to be stable around 61 (mild ID). She shows symptoms of an autism spectrum disorder.

Patient 2

A nine-year-old boy started having febrile seizures at the age of six months. Soon thereafter, he developed multiple febrile and afebrile seizures types that could occur multiple times per day. His epilepsy was unmanageable, and he has lived in an epilepsy center during weekdays for several years. A developmental delay was noticed at the age of three years. Luckily, his symptoms improved eventually, and he currently has been seizure free for two and a half years, while using three different anti-epileptic drugs. Recently, an IQ of 70 was measured and he goes to a special needs school. Symptoms of ADHD are present, which cause many behavioral problems. At 13 months old, *SCN1A* analysis was performed, revealing a missense variant that had not been described in literature previously. Both the boy's father and his paternal grandmother turned out be carriers of this variant as well, although they both had surprisingly milder phenotypes than their (grand)son. His father has epilepsy as well, but a much milder form. He attended a mainstream school, although some additional support was necessary. As an adult he is severely impaired in social functioning, struggles with drug addiction and can show aggressive behavior. He has been diagnosed with an autism spectrum disorder and shows signs of ADHD. His mother, the boy's grandmother, has only experienced three seizures in her entire life, is highly educated, and has never shown any behavioral problems. Her mild phenotype is in stark contrast to that of her more severely affected son and grandson.

Patient 3

Patient 3 is a now 24-year old male with very severe epilepsy. Febrile seizures started at the age of six months old, and before his first birthday multiple seizure types would occur regularly. At worst, he experienced up to 100 absences per day, 8 tonic-clonic seizures per day, and 10 focal seizures with impaired awareness weekly. Currently, seizures still occur every other day, despite the use of three anti-epileptic drugs. He has a severe ID, with an estimated developmental age of a one-year-old. He has been wheelchair bound since the age of 19 years, and has never been able to go to school. Dravet syndrome was only suspected at the age of six years. When *SCN1A* genetic testing became available, at the age of 18 years, it revealed a pathogenic missense variant. This patient has an *SCN1A* phenotype at the most severe end of the spectrum, even compared to other Dravet syndrome patients.

All three described patients were affected by Dravet syndrome caused by a pathogenic *SCN1A* variant. However, the patients developed very variable phenotypes, with outcomes that could not have been predicted at the time of diagnosis. Clearly, factors other than just the pathogenic *SCN1A* mutation influence the disease course, but what are these factors, can they be tested in patients, and is it maybe even possible to manipulate them? To accurately counsel parents after detecting a pathogenic *SCN1A* variant in their child, more knowledge on these modifying factors is essential. The following sections introduce Dravet syndrome, discuss the current knowledge of the effects of *SCN1A* variants, and present the specific issues we hope to resolve in this thesis.

1.2 Dravet syndrome

Dravet syndrome is one of the most well-known genetic epilepsy syndromes. First described by Charlotte Dravet in 1978,[3] the syndrome was originally named "les epilepsies myocloniques graves de l'enfant", or Severe Myoclonic Epilepsy of Infancy (SMEI) in English. The main characteristics of the disease are early onset intractable epileptic seizures, accompanied by a delayed psychomotor development in the second year of life, resulting in mild to severe ID in most patients.[3–5] No universally used guidelines for the diagnosis of Dravet syndrome exist and many studies use different criteria, although most relate to the epilepsy phenotype and cognitive development.[6–9] Dravet syndrome is a rare disease, with an estimated incidence between approximately 1 in 20.000-40.000 children.[5,10–13]

1.2.1 Clinical features

Seizures

The first sign of Dravet syndrome are seizures in an otherwise healthy child.[3,4] In nearly all patients seizure onset occurs in the first year of life, at a mean age of 6 months.[3–5,8,14–21] However, there are also rare reports of patients with onset after one year of age.[19,22] Typically,

the seizure types at first presentation are tonic-clonic or clonic, generalized or unilateral, and they are often triggered by fever.[4,6] Later on, patients soon develop more atypical febrile seizures, afebrile seizures and different seizure types, such as myoclonias, absences and focal seizures.[4–6,17,18,21] Predominant seizure types may vary during the course of the disease and differ between children and adults: younger patients are more likely to experience focal seizures while awake, whereas adolescent patients more often experience short, generalized seizures during sleep.[23–27] Hemi-convulsions and generalized clonic or tonic-clonic seizures remain the most common seizure types in Dravet syndrome. The occurrence of at least one of those types is regarded as mandatory for the diagnosis by many.[6] Status epilepticus is reported in a large majority of patients and might already occur during the first presenting seizure.[5,16,18,20,21] Status epilepticus is less common in adulthood.[4,6,25]

One of the most recognizable features of Dravet syndrome is the provocation of seizures by hyperthermia, caused by fever associated with infection or vaccination, or for example bathing.[4,6] A first seizure is typically, but not necessarily, related to one of these precipitants.[4,5,16–18] These first episodes will often be labelled as simple febrile seizures, which may impede further diagnostics.[4,6] Seizures can be triggered by hyperthermia in virtually all patients, although the effects might decrease with age.[4–6,14–16,18,21,24] Vaccination-induced seizures are reported in 21-53% of children with Dravet syndrome.[14,28–30] Visual stimuli are another important provocative factor of seizures, that occur in up to 65% of cases, and their presence might be associated with a more severe disease course.[15,16,21,25,31]

Although seizures are frequent, in most children with Dravet syndrome no abnormalities are seen during the first interictal EEG recording. This changes with increasing age, with interictal epileptiform abnormalities arising in the first three years of life and eventually occurring in up to 80% of patients later in childhood.[5,32] Similarly, background activity is generally normal in the first year of life, but may slow later in the disease course.[16,32]

Cognitive impairment

Affected children develop normally before seizure onset. However, in the second year of life psychomotor development usually lags behind which is typically first observed around the age of 18 months.[4–6,8,18] There is a strong association between older age and an increasing degree of ID:[5,16,21,33] although mean IQ/DQ scores generally remain above 70 until the age of three years, a strong decrease is observed afterwards.[16,21] One study showed that while 40% of children was shown to still have normal cognitive functioning by the age of two, all children had a learning disability by the age of 11.[5] A severe to profound developmental delay is seen in 76-80% of adult patients.[5,24] Although the majority of patients experience such a devastating disease course, some are more mildly affected: in those patients, IQ/DQ scores only drop significantly at a higher than average age, or even remain relatively favorable throughout the disease course. Several patients that live reasonably independent lives have been described.[5,6,8,21,24,25,34,35]

The deterioration of IQ/DQ scores with age reflects an increasing gap between biological age and developmental age rather than a true deterioration, as patients do continue to acquire new skills at their own pace.[16,21] Temporary regression however may be observed after prolonged seizures, and permanent regression occurs in a minority of cases.[6,8,36] Dravet patients are at risk for acute encephalopathy, associated with prolonged seizures, which can lead to an additional decline in cognitive functioning and neurological sequelae.[37–41]

Dravet syndrome is often regarded as an epileptic encephalopathy, meaning that cognitive outcomes worsen as a result of epileptic activity.[24,26,33,42–45] However, it has become clear that epileptic activity by itself cannot explain the full extent of cognitive impairment, and it is likely that the underlying pathology itself may contribute to the cognitive outcome too, independent of seizures.[4,16,21,42,46–49]

Comorbidities

Although seizures and cognitive decline are the most prominent features of Dravet syndrome, the frequent occurrence of comorbidities also severely impacts the life of patients and their families. Behavioral problems are reported in the large majority of patients.[4,16,26,33,47,50–54] Lack of attention and hyperactivity are the most frequently described features, and occur in 20-69% of patients.[4,16,21,50–55] Other common characteristics are autistic-like traits (33%->50%),[21,26,52,53,56,57] recalcitrant behavior including aggression (35-43%),[4,16,50,53–55] problems with peer relationships (75%),[50,53] a decreased sense of danger and increased impulsiveness (38-53%),[51,53,54] mood instability and affective indifference,[33] and an excessive familiarity with strangers.[53] In general, the incidence of behavioral problems seems to increase with age up until adulthood[5,56] although certain characteristics, such as hyperactivity, might become less prominent.[58] The presence of behavioral problems poses a great burden on families: they have been identified as the strongest independent predictor for lower quality of life scores[50] and are reported to often be a cause of stress and concern for parents.[51,54]

Furthermore, motor disorders are common and have been described in 36%-100% of patients.[4,5,21,56,59–61] Children typically start walking at a normal age or with a slight delay, but with age the incidence of walking disabilities increases.[4,5,33,56,60,61] Problems usually arise during the second decade of life, are present in the majority of patients before the end of puberty, and affect around 75% of adults.[5,6,8,56,61] However, one study reported that motor impairments were already present in most infants and preschool children,[56] and that some children might even be affected before the age of two years.[5,60] Most children show ataxia and hypotonia,[4–6,60] which can lead to a striking way of walking and running, sometimes referred to as "spaghetti legs".[4] Older children develop a typical crouch gait and skeletal misalignment due to joint deformities,[4,6,60–62] for which physical rehabilitation and orthopedic aids or surgical interventions may be necessary.[6,61] The presence of motor disorders is related to a worse seizure severity[56] and might reflect a higher disease burden.[5] They furthermore lead to a higher level of dependence and have been shown to contribute significantly to lower health-related quality of life-scores.[50]

1.2.2 Treatment

There is no cure for Dravet syndrome. Symptomatic treatment of seizures is difficult, as they are typically pharmacoresistant. Therapy focusses on reducing seizure frequency as much as possible and preventing status epilepticus. Although many anti-epileptic drugs (AEDs), such as valproate, clobazam, topiramate, stiripentol, bromide, clonazepam and levetiracetam, have been shown to be effective in Dravet syndrome patients,[5,63–75] complete seizure control is not possible in most cases. Most patients require polytherapy with an average of three different AEDs, although some patients have been described to use as many as 12.[6,56] The most commonly used AED is valproate, for which also the highest efficacy has been reported.[5,63] Other frequently prescribed AEDs are clobazam, topiramate and stiripentol.[5,56,59,63,76] Often, the introduction of a new drug initially shows a promising effect, which later slowly wears off (the honeymoon effect). Only nine percent of Dravet syndrome patients were reported to have been seizure free in the last 3 months in a recent study.[56] Other studies, with smaller sample sizes, report seizure freedom in 0-16% of adults.[8,24,25] Finding the optimal treatment is difficult: patients have previously tried on average three other AED's and some as many as 28.[56] Furthermore, a balance has to be struck between effectiveness and the occurrence of side-effects, which are common in AED's.[77] Research on new therapies for Dravet syndrome continues: cannabidiol and fenfluramine have been shown to successfully reduce seizures in recent trials.[78–81] In addition to reducing seizure frequency by taking maintenance AED's, all patients should have rescue medication and a seizure protocol to prevent status epilepticus.[6] Benzodiazepines are the most effective drugs for this purpose.[6,82]

Sodium channel blockers are contra-indicated in Dravet syndrome. This group comprises agents such as lamotrigine, phenytoin, carbamazepine, oxcarbazepine and vigabatrin, which are commonly used AED's in other epilepsies. Many studies report that sodium channel blockers can lead to an exacerbation of seizures in Dravet syndrome,[5,14,26,47,63,83] and their use has been suggested to lead to a worse cognitive outcome.[25,59,84,85] Despite this knowledge, many Dravet syndrome patients (21-100%) have at some point during their disease course used these drugs,[21,56,76,86] often before a diagnosis was established.

Several other therapeutic options exist in addition to treatment with AED's. The ketogenic diet, which requires the intake of high amounts of fats and low amounts of carbohydrates to shift cerebral energy supply from glucose to ketone bodies, is used by 7% of patients.[56] Although its exact mechanisms of seizure reduction are unknown, approximately 2/3 of patients respond favorably to this regimen,[75,87–89] and the diet has been shown to have a similar efficacy as the regularly used AED's in Dravet syndrome.[67] Another option is vagal nerve stimulation (VNS), which involves an implanted pulse generator that stimulates the vagus nerve, through which it can influence brain electrical activity. VNS is used by approximately 7% of Dravet syndrome patients,[56] and different studies report varying success rates (8-63% responders).[86,90,91] Furthermore, parents and patients should be advised to avoid seizure triggers, such as warm baths, exercise on hot days, and

photosensitivity triggers.[6] Regular vaccinations are recommended.[28,47] Comorbidities may require treatment as well: mobility problems may improve through physical rehabilitation, orthopedic aids or surgical interventions.[6,61] Little is known about effective treatment of behavioral problems in Dravet syndrome. Six percent of patients use antipsychotics, and a similar number of patients uses stimulants or antistimulants.[56]

1.3 GEFS+ syndrome and related syndromes

Although Dravet syndrome has a distinctive disease course, its symptoms at onset may be similar to those of related epilepsy syndromes that are also characterized by hyperthermia-induced seizures. Examples are febrile seizures (FS) or febrile seizures plus (FS+). These related syndromes often have more favorable outcomes.

FS are generalized convulsions that occur in childhood as a result of high fever, and are relatively common with an incidence of 3-5% in the general population.[1,2] Simple febrile seizures resolve spontaneously and are not associated with brain damage or any developmental delay.[2] Since most Dravet syndrome patients present with seizures provoked by fever (58%),[14–16,92–94] they may be misdiagnosed with FS when symptoms first arise.

Febrile seizures plus (FS+) is an epilepsy syndrome that is characterized by febrile seizures that may persist after the age of five years and can be accompanied by afebrile seizures and different seizure types (ILAE diagnostics https://www.epilepsydiagnosis.org/syndrome/fbp-overview.html). At a young age, this disease may also resemble Dravet syndrome. In most cases remission occurs around puberty, although in some patients seizures can persist into adulthood.[36,95]

When FS and/or FS+ occur in two or more members of the same family, genetic epilepsy with febrile seizures plus (GEFS+) syndrome can be diagnosed. GEFS+ is an autosomal dominant epilepsy disorder that describes affected families rather than individuals, although in clinical practice single patients with an FS+ phenotype may be diagnosed with GEFS+ syndrome as well. GEFS+ syndrome comprises a range of phenotypes, and affected members of the same family often present with varying disease severities.[36,96–101] Most patients from GEFS+ families have a phenotype consistent with FS; the second most common phenotype is FS+.[36,95,102] Although generalized tonic clonic seizures are predominant, also partial seizures, absences, myoclonic seizures, hemiclonic seizures and temporal lobe epilepsy are described.[36,97–100,103] Other GEFS+ symptoms may include ataxia and other neuropsychiatric deficits.[96,97] Several GEFS+ families with more severely affected members have been described, and occasionally a GEFS+ family can include a member with myoclonic-astatic epilepsy (MAE) or Dravet syndrome.[97,98,104] Although in FS and FS+ typically no ID is expected, developmental delay and cognitive impairments have also been described in occasional GEFS+ family members who could not be classified as having Dravet syndrome.[96,97,99] The findings described above led to the suggestion that FS, FS+ and Dravet syndrome are part of the same disease spectrum.[59,101,104]

1.4 The *SCN1A* gene

Dravet syndrome, FS, FS+ and GEFS+ do not only overlap clinically. They can also be caused by a similar underlying genetic defect.: mutations in the *SCN1A* gene, located on chromosome 2q24.3, are the cause of disease in the majority of Dravet syndrome patients and in some GEFS+ families. In the following sections, the *SCN1A* gene is introduced, and we discuss how *SCN1A* mutations can cause both Dravet syndrome and milder epilepsy disorders.

1.4.1 History of *SCN1A*

In 1999, several linkage studies performed in large GEFS+ families identified an associated genetic locus on chromosome 2. This locus contained four genes, which coded for isoforms of the α-subunit of different voltage-gated sodium channels: *SCN1A, SCN2A1, SCN2A2*, and *SCN3A*.[99,103,105,106] These were interesting candidate genes, since previously a pathogenic variant in the related *SCN1B* gene, coding for the β-1 subunit of voltage-gated sodium channels, had been reported to cause GEFS+ syndrome in another family.[107] Not surprisingly, pathogenic variants in *SCN1A* were indeed identified in two GEFS+ families shortly thereafter.[108] Since both GEFS+ and Dravet syndrome are characterized by fever-associated seizures, suggesting a similar underlying defect, seven Dravet syndrome patients were screened for *SCN1A* pathogenic variants as well, which were identified in all of them.[109] Subsequent studies confirmed that heterozygous pathogenic *SCN1A* variants are a major cause of Dravet syndrome, and that these can be detected in 70-100% of patients.[20,110–116] The identification of *SCN1A* pathogenic variants in both Dravet syndrome patients and GEFS+ patients strengthens the theory that both entities are part of the same disease spectrum. Over 1200 unique *SCN1A* mutations have been identified so far, including frameshift-, nonsense-, splice-site- and missense mutations, and partial or complete deletions.[117,118] The large majority of these variants is associated with Dravet syndrome (83-86%).[117,118]

1.4.2 Function of *SCN1A*

The *SCN1A* gene is composed of 26 exons and approximately 8500 base pairs. It encodes the α-subunit of Nav1.1, a neuronal voltage-gated sodium channel. Voltage-gated sodium channels are crucial for initiation and propagation of action potentials in neurons, and thus for controlling electrical excitability in the brain. The α-subunits are large transmembrane proteins that form a sodium-permeable pore and contain a voltage sensor. They consist of four identical domains (I-IV), that each consist of six α-helical transmembrane segments (S1-S6) and a pore loop that connects S5 and S6 (Figure 1.1).[119,120] When the cell-membrane of a neuron depolarizes, the channel will be activated, which causes a conformational change that allows an increased sodium flow through the pore, triggering an action potential.[121] Each sodium channel α-subunit is associated with one or several β-subunits that modulate the level of surface expression of the channels, their voltage dependence, kinetics of the α-subunit, and serve as cell adhesion molecules.[119,120] The mammalian genome contains a total of nine functional sodium channel α-subunits, that all have slightly different

biophysical properties and a different spatiotemporally expression. The *SCN1A, SCN2A*, *SCN3A* and *SCN8A* genes encode the α-subunits of channel subtypes that are primarily expressed in the central nervous system: NaV1.1, NaV1.2, NaV1.3 and NaV1.6 respectively. Other subtypes are highly expressed in the peripheral nervous system, skeletal muscle and heart muscle. Nav1.1, Nav1.2 and Nav 1.6 are expressed in adults, while Nav1.3 expression peaks at birth and then decreases again.[121]

1.4.3 Disease mechanism of *SCN1A* mutations

Each person has two copies (alleles) of the *SCN1A* gene. Genetic mutations in one of those *SCN1A* alleles may lead to impaired function of half of the Nav1.1 channels, and thus to disease. Heterozygous *SCN1A* variants that cause a complete loss of function (LoF) of the Nav1.1 channel, such as premature stop codons, frameshifts or deletions, are found in over half of Dravet syndrome patients.[117] This implicates haploinsufficiency as the main disease mechanism in these patients: a single functional copy of *SCN1A* alone does not produce enough gene product for a normal function.[110] The exacerbation of seizures that Dravet syndrome patients experience when using sodium channel blockers may also be explained by this: when disease is caused by a lack of functioning Nav1.1 channels, blocking of the remaining channels by using these drugs can be expected to lead to even worse symptoms.

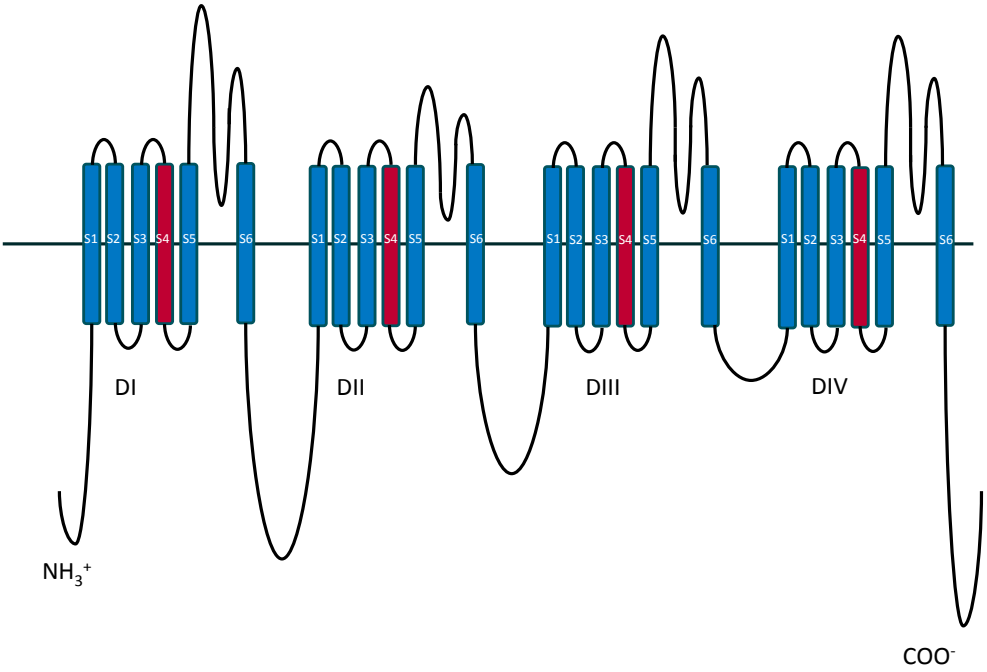

Figure 1.1. Schematic overview of the *SCN1A* protein (alpha unit of the neuronal voltage-gated sodium channel Nav1.1). *SCN1A* consists of four domains (DI-DIV), connected by intracellular loops. Each domain consists of six transmembrane segments (S1-S6). The S5 and S6 segments of all domains make up the pore of the channel, and the connecting loop between S5 and S6 is the pore loop. The S4 segment is the positively charged voltage sensor of the protein.

It may seem counterintuitive at first that a lack of sodium channels leads to epilepsy. A seizure is caused by an abnormal electric discharge of neurons, which can arise when a neuronal network is hyper-excitable. A reduction in sodium currents, caused by a lack of or defective sodium channels, would conversely be expected to lead to hypo-excitability. Nav1.1 channels are however not present in all neuron types, but mainly in neurons that have an inhibitory effect: Dravet mouse-models have shown that while a heterozygous loss of *SCN1A* significantly reduces sodium currents in GABAergic inhibitory interneurons, excitatory pyramidal neurons are unaffected.[122–125] When inhibitory neurons lose their excitability due to an *SCN1A* mutation, a decrease in GABA release, and thus a loss of inhibition will occur. A loss of inhibition will in turn lead to an overall gain of excitation in pyramidal neurons, which can cause seizures. These neuron-specific effects may also explain the occurrence of motor disorders: Purkinje cells, which are neurons located in the cerebellum that regulate and coordinate motor movements, are also GABAergic inhibitory neurons. Nav1.1 channels play a crucial role in their excitability, and loss of these channels may lead to ataxia.[126] Furthermore, Nav1.1 is highly expressed in motor neuron initial segments,[127] and neurophysiologic data suggests that a crouching gait may be (partially) caused by a motor neuropathy.[60] Impaired GABAergic neurotransmission may also cause behavioral problems and cognitive disabilities, irrespective of seizure activity.[128]

1.4.4 *SCN1A* variants in Dravet syndrome and in GEFS+ syndrome

As mentioned before, pathogenic variants in *SCN1A* can give rise to Dravet syndrome, but also to milder phenotypes, like GEFS+, FS+ and FS. This discrepancy can be partly explained by the different mutation types that are associated with the respective disorders. Over half of Dravet syndrome patients carry a pathogenic *SCN1A* variant expected to lead to a complete LoF, such as a premature stop codon, a frameshift variant or a deletion. Missense variants, that are expected to lead to a less severe protein malformation, are seen in 42% of Dravet syndrome patients.[117] In contrast, missense variants occur in much higher percentages of patients with milder phenotypes: missense variants were present in 87% of patients with mild generalized epilepsy and/or FS.[117] Furthermore, the types of missense variants differ between the syndromes: patients with severe phenotypes more often have missense variants in important functional regions of Nav1.1, such as the pore region, voltage sensor and inactivation gate, which are expected to have a more detrimental effect on channel function.[117,118,129–131]

In vitro studies have shown that GEFS+-related *SCN1A* variants can lead to both partial LoF and gain of function of Nav1.1 channels;[117,132–135] however, animal models have shown that their net *in vivo* functional effect is a reduced excitability of inhibitory neurons, as had previously been demonstrated in a more severe extent for Dravet syndrome.[122,124,136] These results support the hypothesis that *SCN1A* variants are part of a spectrum of severity and can therefore cause a spectrum of different *SCN1A* phenotypes: mild impairment of Nav1.1 channel function leads to milder phenotypes, whereas a severe or complete loss of function results in severe phenotypes.[131]

1.4.5 Other genes involved in Dravet syndrome and GEFS+

In a minority of clinically diagnosed Dravet syndrome patients (0-30%) no pathogenic variants in *SCN1A* can be demonstrated.[20,110–116] Several other genes (*SCN1B*, *GABRG2, SCN2A, GABRA1, STXBP1, HCN1* and CHD2) have been implicated to cause a phenotype similar to Dravet syndrome.[137–145] However, pathogenic variants in each of these genes only make up a very small fraction of diagnoses in Dravet syndrome patients: some have only been described in two cases at most. A slightly more common Dravet-like syndrome is *PCDH19*-related epilepsy. Variants in *PCDH19*, an X-linked gene, cause a Dravet-resembling phenotype in female patients.[146–148] However, *SCN1A* remains by far the major gene for Dravet syndrome. A recent study has shown that it is not uncommon to initially miss pathogenic *SCN1A* variants during regular diagnostics, especially due to human errors;[149] this suggests that the role of *SCN1A* pathogenic variants in Dravet syndrome is larger than previously estimated. Furthermore, Dravet syndrome patients may harbour pathogenic variants in regulatory regions of the *SCN1A* gene instead of in its coding regions, that remain undetected by regular diagnostic sequencing.[131]

In contrast, only a small percentage of GEFS+ cases (~10%) can be explained by the presence of a pathogenic *SCN1A* variant.[36,96,131,150] Variants in *SCN1B, GABRG2* and *SCN2A* have been reported to also cause GEFS+,[107,114,151–154] but often inheritance of this syndrome is complex, meaning that not one dominant gene is causal, but that multiple genes and possibly environmental factors are involved in its expression.

1.5 Variable phenotypes and prediction

The patients presented at the beginning of this introduction have shown that *SCN1A*-related phenotypes may vary widely, even in patients that are diagnosed with Dravet syndrome. In this section, we discuss the variable expression of *SCN1A* variants.

1.5.1 Predicting the effects of an *SCN1A* variant

Nowadays *SCN1A* sequencing is readily available, and children presenting with repetitive febrile seizures may be tested at a young age. Finding a pathogenic *SCN1A* variant can aid in the early diagnosis of Dravet syndrome or a milder *SCN1A* phenotype. However, the detection of an *SCN1A* variant raises several new problems. First, it has to be established whether the identified variant is in fact pathogenic or not, since some variants may represent neutral variation. Several factors plead in favor of the pathogenicity of a novel variant, such as family segregation, a likely damaging effect of the variant as assessed by *in silico* prediction tools, and the variant being very rare or absent among large cohorts of healthy individuals, such as the gnomAD database.[155,156] Then, even when a variant is deemed pathogenic, it remains difficult to predict its exact effects on Nav1.1 channel function and thereby predict phenotype. Variant types that are known to lead to a complete LoF, will virtually always

lead to Dravet syndrome.[117] However, predicting the effects of a missense variant is more difficult, since these are found in both Dravet syndrome patients and GEFS+ patients. Although strong indicators for the severity of channel disruption have been identified, such as the variant's location in the gene[59,117,129,130,157] and its physicochemical property changes,[130,158] these still cannot not predict phenotypes fully.

To complicate matters further, one and the same pathogenic *SCN1A* variant does not always result in the same phenotype. Several families have been described in which members carried the same inherited pathogenic *SCN1A* variant, but presented with different disease severities and even different clinical syndromes.[36,96–101,104,159–161] In addition, Dravet syndrome patients affected by variants that are all expected to lead to a similar LoF of Nav1.1 can show a wide phenotypic variability: while some patients are severely disabled, do not speak and need fulltime care, some lead much more independent lives and may have reasonable seizure control.[9,25,118,139,160–162]

For parents of young patients, it is understandably very important to accurately predict the clinical course after a pathogenic *SCN1A* variant has been found. It is clear that merely identifying an *SCN1A* variant is not enough to fully explain and predict phenotypes. Several factors that may modify *SCN1A*-related disease severity have already been suggested, such as mosaicism, other variants in and around *SCN1A,* clinical factors and modifier genes. Each of these is discussed in the following sections.

1.5.2 Mosaicism

Mosaicism describes a situation in which two or more populations of cells with different genotypes exist in one individual. This happens when a pathogenic variant does not arise during fertilization, but post-zygotically. All cells descending from the one cell in which the mutation occurred will carry this variant, whereas other cells will not. When such a variant arises early in embryonic development, a high percentage of cells will be affected, whereas the variant will only be present in specific tissues when the mutation occurs in a later stadium (Figure 1.2). In the case of pathogenic variants, a higher percentage of unaffected cells may ameliorate phenotypes, as compared to patients in which all cells are affected.[163–165] Parental mosaicism for a pathogenic *SCN1A* variant has been well recognized in cases were mosaic parents of Dravet children show a mild epilepsy phenotype,[166–174] and it may explain up to two thirds of families with variable *SCN1A*-related phenotypes.[171] Furthermore, percentages of mosaicism may be so low that carriers show no epilepsy at all, although they are still able to transmit the pathogenic variant to their offspring. A number of patients with a presumed *de novo SCN1A* variant will therefore actually have a parent with low-grade mosaicism: this percentage has been estimated at 7-13%.[171,175,176] These findings implicate that postzygotic mutation of *SCN1A* occurs frequently, and that mosaicism may be an important modifier in Dravet syndrome patients with unexplained mild phenotypes as well. However, mosaicism cannot be reliably detected with techniques that are currently used in standard diagnostics.

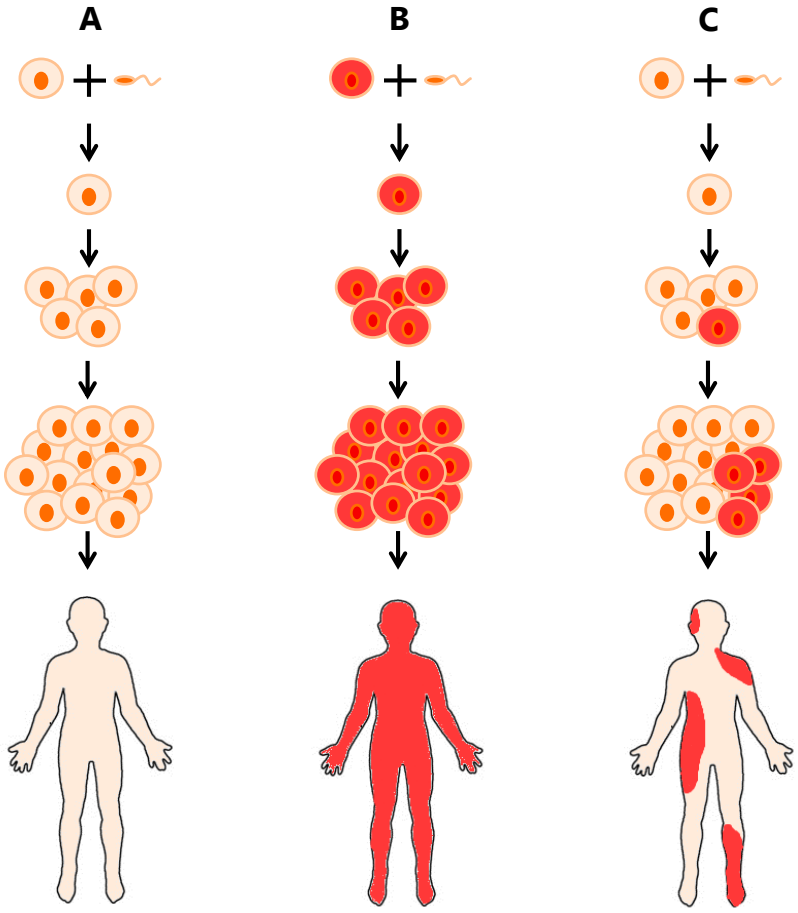

Figure 1.2. Schematic overview of the effects of a postzygotic mutation. A: no mutation; B: heterozygous mutation; C: postzygotic mutation (mosaicism).

On the contrary, in specific disorders mosaicism may actually worsen or even cause a phenotype, like in *PCDH19* related epilepsy, which may resemble Dravet syndrome.[146,147,177,178] *PCDH19* is an X-linked gene, and female patients that carry a pathogenic *PCDH19* variant can all be considered mosaics, due to random inactivation of one of their X-chromosomes in each cell.[179,180] These female patients are all clinically affected, whereas male carriers, that only have one X-chromosome and thus one homogeneous population of cells, are unaffected. In these cases, disease may occur when the two different cell populations cause disruption of cell-cell interactions, a mechanism termed cellular interference.[146]

1.5.3 Variants in and around *SCN1A*

Common variants in *SCN1A*, that may not be classified as pathogenic directly, may influence disease severity when they exist in addition to a disease-causing pathogenic variant.

Genome-wide association studies (GWAS) have implicated *SCN1A* as a susceptibility locus for epilepsy syndromes in general,[181,182] and a specific common splice site polymorphism (rs3812718) has been associated with febrile seizures in children and responses to sodium channel blockers, although not all results could be replicated.[183–187] Such variants may affect function or expression of both alleles, or of the unaffected allele, and thereby residual Nav1.1 function.

Furthermore, normal functioning of Nav1.1 requires not only an intact coding sequence of *SCN1A*; correct expression of the protein is also crucial. An undamaged promotor region and transcription factors are essential for this. Furthermore, the 5- and 3- prime untranslated regions (5'-UTR and 3'-UTR) of a gene have important roles in regulation of expression.[188] The 5'-UTR is the region of messenger RNA (mRNA) directly upstream from the first coding exon, and may enhance transcription. The 3'-UTR is the region of mRNA immediately after the terminating codon, and may regulate expression by influencing localization, stability and translation of mRNA. Untranslated exons in the 5'-UTR of *SCN1A* have been shown to be essential for full expression of the gene.[189,190] A small number of Dravet syndrome patients, and a patient with focal epilepsy and febrile seizures, have been described to be affected by pathogenic variants in the 5' or 3' region of *SCN1A*, while no coding variants could be detected.[191–193] These findings underscore the importance of the promoter and the 5'- and 3'-UTR for correct functioning of *SCN1A*, making it likely that milder variants in these regions can affect phenotypes caused by pathogenic variants in coding regions. Since these regions are often not sequenced during standard diagnostics, their exact role remains unclear.

1.5.4 Clinical factors

Non-genetic factors may also modify phenotypes,[119] as is best illustrated by the effects of clinical treatment. Dravet syndrome is often, at least partly, regarded as an epileptic encephalopathy, meaning that seizure activity may negatively influence cognitive outcomes.[24,26,33,42–45] Patients that have received optimal treatment from a young age may therefore have more favorable outcomes than patients that have been treated with sodium channel blockers for a long period of time, although this has not been established in large cohorts.[14,25,26,59,63,83–85,92] To obtain optimal seizure control in Dravet syndrome patients an early diagnosis could be essential, which is required to provide the best possible treatment and avoid contra-indicated medication. The effects of an early diagnosis have however not been investigated directly.

1.5.5 Modifier genes

Genetic variants in other genes than *SCN1A* may have a large influence on *SCN1A*-related phenotypes. An important effect of variants in such genes, so called modifier genes, has already been described for several other genetic disorders,[194–196] and there are strong indications that modifier genes also play a large role in *SCN1A*-related phenotypes.[36,95,119,131] Firstly,

mice that carry similar LoF *SCN1A* variants show very variable phenotypes, depending on their strain.[122] This implicates that genetic background strongly influences disease severity. Secondly, parents of patients with Dravet syndrome more frequently have experienced febrile seizures than observed in the general population, implying that genetic factors that cause febrile seizures may contribute to a Dravet syndrome phenotype.[129] Modifier genes for *SCN1A*-related epilepsy may be genes that function in the same pathway as *SCN1A*, that have a function in neuronal excitability, or that are associated with other causes of epilepsy, ID or for example autism. Furthermore, also genes involved in immunological responses and thereby in frequency and height of fever may indirectly modify outcomes. One or multiple additional variants in these genes, common or pathogenic, may aggravate or partially rescue the effects of aberrant Nav1.1 functioning.

Several potential modifier genes for *SCN1A*-related epilepsy have already been identified: *SCN9A, SCN8A, SCN2A, HLF, POLG, KCNQ2, CACNB4, CACNA1G* and *CACNA1A* have all been implicated to affect *SCN1A*-related phenotypes.[37,197–205] Furthermore, potential modifier loci, identified in *SCN1A* knock-out mice with variable phenotype severities, contain several potential modifier genes, including five GABA receptor subunit genes, calcium channel subunit genes (*Cacna1a* and *Cacna2d1*), the chloride channel gene (*Clcn3*), a potassium channel gene (*Kcnj11*), and several non-ion channel genes that had been previously associated with seizures or hyperexcitability (*Atp1a3, Lgi2, Mapk10, Reln* and *Slc7a10*).[206] Furthermore, an enrichment of rare variants in neuronal excitability genes in general has been identified in severely affected Dravet syndrome patients, compared to mildly affected Dravet syndrome patients.[205] However, no clinically relevant genes, that can explain unexpected phenotypes in large groups of Dravet syndrome patients, have been identified so far.

1.6 Outline of this thesis

When a pathogenic *SCN1A* variant is detected in a child with epilepsy, we are currently not always able to predict the clinical course of the disease. The variable and sometimes unexpected clinical outcomes of pathogenic *SCN1A* pathogenic variants are illustrated by the cases presented in the first section of this introduction. An accurate prediction of future perspectives is obviously of great importance to parents. In this thesis, we investigate several potential modifiers that may influence the clinical outcomes of genetic epilepsy syndromes, to ultimately improve counseling of patients and/or parents. Furthermore, if actionable modifiers are identified, these may be manipulated to improve clinical outcomes. In **Part 1** and **2**, we focus on *SCN1A*-related epilepsy; **Part 3** describes two other genetic, X-linked epilepsies in which mosaicism of a pathogenic variant can significantly affect clinical outcomes.

The overall goal of the studies described in **Part 1** and **2** is to assess whether an early screening of *SCN1A* in children with febrile seizures would be feasible. Prerequisites of such screening would be that the clinical consequences of a pathogenic variant can be predicted accurately, and that an early diagnosis improves overall outcomes. We investigate both issues in a cohort of 176 patients with pathogenic *SCN1A* variants.

Part 1 focusses on the clinical characteristics of the patients in this cohort. In **Chapter 2**, we describe in detail the clinical outcomes of Dravet syndrome patients as well as of non-Dravet syndrome patients, to investigate the potential use of comorbidities in the diagnostic process and to provide more insight in the clinical spectrum of both severe and less severe *SCN1A*-related disorders. In **Chapter 3**, we investigate which clinical features may accurately predict or influence the severity of an *SCN1A*-related disorder, with special attention for the effects of contra-indicated medication (CIM).

In **Part 2**, we investigate whether advanced genotyping can improve the prediction of *SCN1A*-related phenotypes, by analyzing the role of several genetic modifiers. In **Chapter 4**, we investigate how often high-grade mosaicism of pathogenic *SCN1A* variants occurs, and whether it has a substantial effect on disease severity. In **Chapter 5**, we investigate the frequency of low-grade mosaicism in parents of probands with *SCN1A*-related epilepsy, and whether the use of more advanced techniques may improve detection. In **Chapter 6**, we investigate the effects of rare and more common variants in potential modifier genes on *SCN1A*-related phenotypes. In **Chapter 7**, we analyze the effects of common variants in the promoter region of *SCN1A* on the expression of the unaffected allele, and therefore on clinical outcomes.

In **Part 3**, two epilepsy syndromes of a different genetic origin are discussed: *PCDH19*- and *KIAA2022*- related epilepsy. Both genes are X-linked, but show different inheritance patterns, and mosaicism of pathogenic variants may have different consequences in male and female patients. In **Chapter 8**, we describe five male patients with mosaic pathogenic variants in *PCDH19* and a phenotype resembling Dravet syndrome. In **Chapter 9**, we describe 14 female patients with pathogenic variants in *KIAA2022*, that can all be considered mosaic due to random inactivation of one of both X-chromosomes in each cell.

Chapter 10 provides a general discussion of the research described in the previous chapters.

Part 1

Clinical characteristics of patients with *SCN1A*-related phenotypes

Chapter 2

Outcomes and comorbidities of *SCN1A*-related seizure disorders

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Published: Epilepsy & Behavior. 2018 Dec 4 [Epub ahead of print]

Abstract

Purpose: Differentiating between Dravet syndrome and non-Dravet *SCN1A*-related phenotypes is important for prognosis regarding epilepsy severity, cognitive development and comorbidities. When a child is diagnosed with genetic epilepsy with febrile seizures plus (GEFS+) or febrile seizures (FS), accurate prognostic information is essential as well, but detailed information on seizure course, seizure freedom, medication use and comorbidities is lacking for this milder patient group. In this cross-sectional study we explore disease characteristics in milder *SCN1A*-related phenotypes and the nature, occurrence, and relationships of *SCN1A*-related comorbidities in both Dravet- and non-Dravet syndrome patients.

Methods: A cohort of 164 Dutch participants with *SCN1A*-related seizures was evaluated, consisting of 116 patients with Dravet syndrome, and 48 patients with either genetic epilepsy with febrile seizures plus, febrile seizures plus or febrile seizures. Clinical data were collected from medical records, semi-structured telephone interviews and three questionnaires: the Functional Mobility Scale, the PedsQL Measurement Model and the Child or Adult Behavior Checklists.

Results: Walking disabilities and severe behavioral problems affect 71% and 43% of Dravet syndrome patients respectively and are almost never present in non-Dravet patients. These comorbidities are strongly correlated to lower quality of life (QoL) scores. Less severe comorbidities occur in non-Dravet syndrome patients: learning problems and psychological/behavioral problems are reported for 27% and 38% respectively. The average QoL score of the non-Dravet group was comparable to that of the general population. The majority of non-Dravet syndrome patients becomes seizure free after 10 years of age (85%).

Conclusions: Severe behavioral problems and walking disabilities are common in Dravet syndrome patients and should receive specific attention during clinical management. Although the epilepsy course of non-Dravet syndrome patients is much more favorable, milder comorbidities frequently occur in this group as well. Our results may be of great value for clinical care and informing newly diagnosed patients and their parents about prognosis.

2.1 Introduction

Pathogenic variants in *SCN1A* can cause several different epilepsy syndromes, with varying disease severities.[108,110,111,162,207] The most common and severe associated condition is Dravet syndrome, which is characterized by intractable epileptic seizures and a slowing of the psychomotor development in the second year of life, which results in mild to severe intellectual disability. Walking difficulties and behavioral problems are common comorbidities[3,5,60,62]. Milder phenotypes include Genetic Epilepsy Febrile Seizures plus (GEFS+) syndrome and febrile seizures (FS and FS+), in which usually no intellectual disability (ID) is present and the epilepsy has a milder course.[108,208] Although the majority of school-aged, adolescent and adult patients with *SCN1A*-related disease can easily be classified as having Dravet syndrome or not, these different phenotypes may have a similar presentation at onset.[17,209]

SCN1A encodes for the α-subunit of a neuronal sodium channel, Nav1.1. Pathogenic variants cause a reduction in sodium currents in GABAergic inhibitory interneurons, which leads to hyper-excitability of neuronal networks and the occurrence of seizures.[122,123] These reduced sodium currents furthermore impair Purkinje cells, causing motor disorders,[126,208] and contribute to the development of behavioral problems and cognitive disabilities.[128] The association of *SCN1A* pathogenic variants with multiple syndromes can be partly explained by the consequences of different mutation types: pathogenic variants that lead to a complete loss of function of the channel are virtually always associated with severe phenotypes, whereas milder disturbances in channel function usually cause milder phenotypes.[117] However, in clinical practice it remains difficult to fully predict the effects of all variants on channel function.

Differentiating between Dravet syndrome and non-Dravet *SCN1A*-related phenotypes is understandably of extreme importance for families and physicians. The presence or absence of an intellectual disability, and the frequency and severity of seizures both significantly alter the level of care an affected child requires. Furthermore, severe comorbidities, such as walking disabilities or behavioral problems are frequently reported in Dravet syndrome,[4,5,52–56,60,62] and may pose a heavy burden on affected families. Behavioral problems have been identified as the strongest independent predictor for lower quality of life scores[50] and are reported to often be a cause of stress and concern for parents.[51,54] Motor disorders have been shown to contribute significantly to lower health related quality of life-scores as well.[50]

However, also when a child is diagnosed with a milder *SCN1A*-related disorder, such as GEFS+ or FS, accurate information about the disease course and prognosis is essential. Many studies have reported on the clinical spectrum of different *SCN1A*-related syndromes,[17,117,210] but detailed information on seizure course, seizure freedom, medication use and comorbidities is lacking for the milder patient group. Although ID is thought to be exclusive to Dravet syndrome patients, there are reports of non-Dravet syndrome patients that show a mild cognitive impairment or neuropsychiatric symptoms.[96,111,211] However, the

incidence of these problems in non-Dravet *SCN1A*-related epilepsy patients is unknown, as they are mostly described in case reports.

We here describe the detailed clinical data of a large cohort of Dutch patients affected by *SCN1A* pathogenic variants (n=164), consisting of patients with Dravet syndrome as well as GEFS+, FS+ or FS. We explore the nature, occurrence, and relationships of *SCN1A*-related comorbidities in both the Dravet and non-Dravet groups, to improve the counseling of patients and their parents. We furthermore give a detailed overview of the disease course of the non-Dravet syndrome patients, to provide more insight in the clinical spectrum of the less severe *SCN1A*-related disorders.

2.2 Methods

2.2.1 Participants

A previously described cohort[209,212] of 164 patients affected by *SCN1A*-related seizures was included in this study. Only symptomatic participants with heterozygous pathogenic or likely pathogenic variants (class IV and V, according to the American College of Medical Genetics and Genomics criteria[155]) in *SCN1A* were included. All eligible individuals of at least 4 years of age known to the University Medical Center Utrecht were approached. Patients below the age of 4 years were excluded since syndrome classification and estimation of disease severity is less reliable for younger children. Informed consent was obtained from participants or their legal caretakers, according to the Declaration of Helsinki. The study was approved by the Ethical Committee of the University Medical Center Utrecht.

2.2.2 Clinical data

Detailed clinical data were retrospectively collected from medical records for all participants, and a semi-structured telephone interview was conducted when possible at the time of inclusion (n=155). Interviews were conducted with participants themselves if they were adults and mentally competent; in all other cases interviews were conducted with parents of patients. Participants, or parents of participants were asked what kind of education the participants were following or had followed, whether they had any learning problems or psychological problems. If parents of patients were interviewed and not patients themselves, they were also asked whether their children showed any behavioral problems (for other patients reported as missing data). The same information was extracted from medical files. Furthermore, three questionnaires were completed by participants in specific age groups, or their parents, at the time of inclusion:

The Dutch version of the Functional Mobility Scale (FMS), to classify the general functional mobility in six categories for children aged 4-18 years[213] (Hugh Williamson Gait Laboratory, The Royal Children's Hospital Melbourne, Australia, Part of the Gait CCRE, www.rch.org.au/gait, Graham 2004);

The Dutch version of the PedsQL Measurement Model, to measure health-related quality of life (HRQOL) on a 0-100 scale for participants aged 0-25 years;[214]

The Dutch parent report version of the Child Behavior Checklist 1.5–5 years (CBCL 1.5–5) or 6–18 years (CBCL), or the Dutch version of the Adult Behavior Checklist 18-59 years (ABCL) to evaluate behavioral and emotional problems.[215,216] Behavioral problems in the clinical range on the "total problems" scale are reported (p-scores >90, according to the CBCL manual).

Cognitive functioning at the time of inclusion was classified in a consensus meeting by a child neurologist, neuropsychologist, and clinical geneticist, and rated on a five-point scale based on available data on IQ and developmental level, (1= no ID (IQ or developmental quotient (DQ) >85), 2 = borderline ID (IQ or DQ 70-85), 3 = mild ID (IQ or DQ 50-70), 4= moderate ID (IQ or DQ 30-50), 5 = severe or profound ID (IQ or DQ <30)). When no (recent) IQ or DQ was available the assessment was made based on school functioning, communication and/or adaptive behavior. All participants were categorized into two clinical subgroups: Dravet syndrome or non-Dravet syndrome. Dravet syndrome was diagnosed based on previously published criteria.[217] The diagnoses were in line with recently published recommendations.[6] The non-Dravet group consisted of patients with either GEFS+ or febrile seizures. Seizure severity was classified based on seizure frequency for both minor seizures (defined as short absences, short focal seizures or myoclonias) and major seizures (defined as all other seizure types with loss of consciousness, or prolonged seizures), at the time of inclusion (score 4 = daily seizures, score 3 = weekly seizures, score 2 = monthly seizures, score 1 = yearly seizures, score 0 = seizure free (>1 year)).

2.2.3 Descriptive analyses

Data on major disease outcomes (cognitive functioning, seizure severity, walking difficulties, behavioral problems and HRQOL) are reported as total counts, percentages or mean/median scores for all patients, and additional detailed clinical information (on seizure frequency, anti-epileptic drug (AED) use, learning problems and psychological/behavioral problems) is reported per age group for non-Dravet syndrome patients.

2.2.4 Statistical analyses

No statistical testing was performed to formally assess differences in outcomes between the Dravet and non-Dravet patients, since these outcomes were used to classify each patient and therefore differ per definition between the groups. Correlations between different outcomes were calculated with Spearman's rank-order correlation. Differences between groups were calculated with either Pearson's chi-square test or Fisher's exact test for binary and categorical variables, or a Mann–Whitney U test for continuous and ordinal variables. Statistical analyses were performed using SPSS statistics software (IBM SPSS Statistics for Windows V21, Armonk, NY: IBM Corp.). All reported tests were performed 2-tailed with an alpha-level for significance of $p < 0.05$. Correlations for the non-Dravet group only were

only investigated for the group of patients below 20 years old, because childhood learning and behavioral problems may not have been reported reliably for older patients due to recall biases.

2.3 Results

The characteristics of the study population are depicted in Table 2.1. The Dravet syndrome subgroup consisted of 116 patients belonging to 112 different families, and the non-Dravet group consisted of 48 patients belonging to 28 different families. Six families had members in both the Dravet and non-Dravet categories. The median age of the Dravet syndrome patients and the non-Dravet patients was 14 years and 22 years respectively. Important differences between both groups were seen for all five major outcomes.

2.3.1 Cognitive outcome

All patients could be assessed for cognitive outcome. Almost half of all Dravet syndrome patients had a severe cognitive disability (score 5, 45%), whereas almost all non-Dravet patients had normal cognitive capacities (score 1, 90%) except for five, who had a slight delay (score 2, 10%) (Table 2.1). Since cognitive functioning is a defining characteristic used to classify Dravet syndrome, this is an expected outcome. Cognitive disabilities worsened with age in Dravet syndrome patients; over 60% of patients of 20 years and older had a score of 5, in contrast to only 25% of patients aged 7-8 years (Figure S2.1). Interestingly, nine non-Dravet syndrome patients experienced a slowing of development after the first year of life as well; however, in two cases the delay was only temporary and none of these patients developed an intellectual disability.

2.3.2 Seizure severity

All patients could be assessed for seizure frequency. Many Dravet syndrome patients experienced major seizures weekly (44%), and minor seizures daily (41%). In the non-Dravet group, both major and minor seizures occurred much less frequently, with most patients being seizure free at the time of inclusion (73%) (Table 2.2).

If seizures did still occur in the non-Dravet group, these were mostly yearly events (23%). The youngest non-Dravet syndrome patients had most seizures: only 38% of 4-9 year olds were seizure free, whereas 82% of 10-19 years olds, and 100% of patients older than 40 years was seizure free. Although 71% of non-Dravet patients had used maintenance treatment, 60% was medication free at the time of inclusion. More older than younger patients were medication free (87% of 40+ year olds versus 38% of 4-9 year olds). Interestingly, one non-Dravet syndrome patient experienced minor seizures (myoclonias) daily. This 16-year old girl used to have generalized tonic-clonic seizures, but had been seizure and medication free between age 4 and 10 years old, after which seizures reappeared. At the time of study,

Table 2.1. Characteristics of the study population

	Complete cohort	Dravet	Non-Dravet
n	164	116	48
Age median (years, range)	15 (4-67)	14 (4-48)	22 (4-67)
Age grouped (years, n)			
4-7	29	20	9
8-11	28	22	6
12-15	31	25	6
16-19	11	8	3
20+	65	41	24
Sex: male (n)	83 (51%)	65 (56%)	18 (38%)
Mutation type (n)			
missense	87 (53%)	44 (38%)	43 (90%)
pore region/loop	58	29	29
elsewhere	29	15	14
splicing	16 (10%)	13 (11%)	3 (6%)
nonsense/frameshift/rearrangement	61 (37%)	59 (51%)	2 (4%)
Cognition score[1] (n)			
1: no ID (IQ or DQ >85)	46	3 (3%)	43 (90%)
2: borderline ID (IQ or DQ 70-85)	15	10 (9%)	5 (10%)
3: mild ID (IQ or DQ 50-69)	20	20 (17%)	0 (0%)
4:moderate ID (IQ or DQ 30-49)	31	31 (27%)	0 (0%)
5: severe or profound ID (IQ or DQ <30)	52	52 (45%)	0 (0%)
Slowing of development after first year of life (yes, n)	106 (65%)	97 (87%) (4 missing)	9 (19%)
Development of epilepsy with multiple seizure types (yes, n)	133 (83%)	113 (97%)	20 (44%)
Major seizure frequency[1] (n)			
seizure free	43 (26%)	8 (7%)	35 (73%)
yearly seizures	27 (17%)	16 (14%)	11 (23%)
monthly seizures	27 (17%)	25 (22%)	2 (4%)
weekly seizures	51 (31%)	51 (44%)	0
daily seizures	16 (10%)	16 (14%)	0
Minor seizure frequency[1] (n)			
seizure free	76 (46%)	31 (27%)	45 (94%)
yearly seizures	9 (6%)	7 (6%)	2 (4%)
monthly seizures	8 (5%)	8 (7%)	0
weekly seizures	23 (14%)	23 (20%)	0
daily seizures	48 (29%)	47 (41%)	1 (2%)
Quality of life[3]			
completed questionnaires (n)	93	71	22
average total score (range)	61.1 (13-99)	52.6 (13-86)	88.5 (63-99)
Behavioral problems[4]			
completed ABCL/CBCL questionnaires (n)	122	80	42
clinical range (n)	37 (30%)	34 (43%)	3 (7%)
behavioral problems reported by parents	72 (54%)	70 (66%)	2 (7%)
during telephone interview	(31 missing)	(10 missing)	(21 missing)
FMS score[5] (n)			
uses a wheelchair (1)	26 (31%)	26 (41%)	0
independent walking on flat surfaces (5)	19 (23%)	19 (30%)	0
independent walking on all surfaces (6)	38 (46%)	18 (29%)	20 (100%)
missing	81	53	28

[1]Based on available data on IQ and developmental level, adjusted for age at assessment. When no (recent) IQ or DQ was available, the assessment was made based on school functioning, communication and adaptive behavior. [2]Currently. 4 = daily seizures, 3 = weekly seizures, 2 = monthly seizures, 1 = yearly seizures, 0 = seizure free. Minor seizures: short absences, short focal seizures or myoclonias. Major seizures: all other seizure types with loss of consciousness, or prolonged seizures. Numbers of participants are given for dichotomized scores (score 0-1 = rarely, score 2-4 = often). [3]Quality of Life total score, based on results of PedsQL Measurement Model questionnaire. Scaled 0-100; a higher score indicates a higher health related quality of life. [4]Clinical range: patients that score >90% on the "total problems" scale on the Child Behavior Checklist 1.5–5, 6–18 years, or the Adult Behavior Checklist 18-59 years. [5]Scores measured by the Functional Mobility Scale (FMS), to classify functional mobility for children aged 4-18 years. Scores for the 500 meter range are used. No participants scored 2, 3 or 4 (uses a walker or frame, uses crutches or uses sticks, respectively).

Table 2.2. Characteristics of non-Dravet syndrome patients

	All non-Dravet patients	Non-Dravet patients 4-9 years old	Non-Dravet patients 10-19 years old	Non-Dravet patients 20-39 years old	Non-Dravet patients ≥40 years old
n	48	13 (27%)	11 (23%)	9 (19%)	15 (31%)
Major seizure frequency[1] (n)					
seizure free	35 (73%)	5 (38%)	9 (82%)	6 (67%)	15 (100%)
yearly seizures	11 (23%)	8 (62%)	1 (9%)	2 (22%)	0
monthly seizures	2 (4%)	0	1 (9%)	1 (1%)	0
weekly seizures	0	0	0	0	0
daily seizures	0	0	0	0	0
Minor seizure frequency[1] (n)					
seizure free	45 (94%)	12 (92%)	10 (91%)	8 (89%)	15 (100%)
yearly seizures	2 (4%)	1 (8%)	0	1 (11%)	0
monthly seizures	0	0	0	0	0
weekly seizures	0	0	0	0	0
daily seizures	1 (2%)	0	1 (9%)	0	0
AED[2] use (n)					
has at some point used maintenance treatment	34 (71%)	11 (85%)	9 (82%)	6 (67%)	8 (53%)
currently no maintenance treatment	29 (60%)	5 (38%)	7 (64%)	4 (44%)	13 (87%)
Learning problems[3] (n)	13 (27%)	4 (31%)	6 (55%)	3 (33%)	0
Psychological/behavioral problems[4] (n)	18 (38%)	5 (38%)	5 (45%)	5 (56%)	3 (20%)

[1]Currently. Minor seizures: short absences, short focal seizures or myoclonias. Major seizures: all other seizure types with loss of consciousness, or prolonged seizures. [2]Anti-epileptic drugs. [3]Any problem for which extra educational assistance is necessary: e.g. special education, repeating a class, extra support needed. [4]Answered "yes" to the question "are there any behavioral issues?", or behavioral issues, psychological symptoms or (signs of) psychiatric disease mentioned in interview or medical files.

she had monthly focal seizures with impaired awareness and a few myoclonias per day while being treated with topiramate and levetiracetam. She was not diagnosed with Dravet syndrome because she had a normal intellect (IQ 91) and her seizure course is unusual for Dravet syndrome.

2.3.3 Functional mobility

Sixty-three Dravet syndrome patients and 20 non-Dravet syndrome patients could be assessed for functional mobility. Dravet syndrome patients showed either no walking disabilities (independent walking on all surfaces, score 6), minor walking disabilities (independent walking on level surfaces, score 5) or used a wheelchair (score 1) on a 500 meter range. Over 40% of Dravet syndrome patients already used a wheelchair at age 4-8 years; this was 50% at 14-18 years (Figure 2.1). Non-Dravet syndrome patients did not show any walking disabilities on the FMS scale.

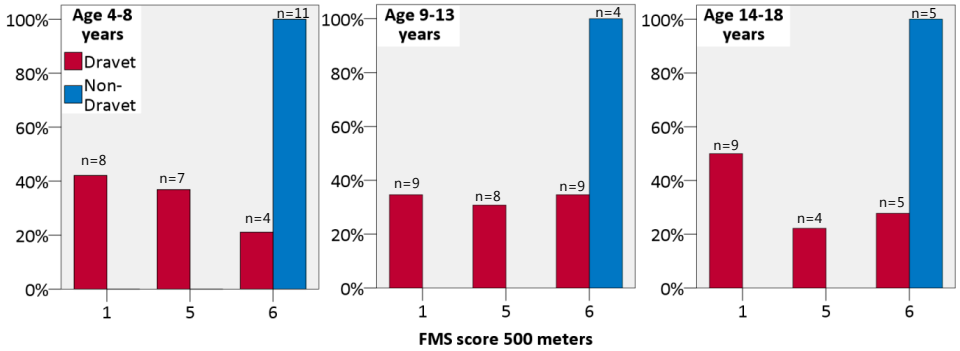

Figure 2.1. Functional Mobility Scale (FMS) scores on a 500 meter range per age group. Scores ranges from 1-6, although patients only scored 1 ("uses wheelchair"), 5 ("independent on level surfaces") or 6 ("independent on all surfaces").

2.3.3 Behavioral problems

Eighty Dravet syndrome patients and 42 non-Dravet syndrome patients could be assessed for behavioral problems by CBCL/ABCL questionnaires. Thirty-seven patients (30%) showed behavioral problems in the clinical range (Table 2.1); all but three were Dravet syndrome patients (43% versus 7%). One of the non-Dravet patients with behavioral problems in the clinical range is a 32-year old father of a son with Dravet-syndrome, who was found to be mosaic for their pathogenic *SCN1A* variant in previous research[31,39]. Since his epilepsy was well controlled and no intellectual disability was present he was not diagnosed with Dravet syndrome; he however does have psychosocial problems, among which an autism spectrum disorder, an active substance addiction and aggression. The second patient (14 years old), has an autism spectrum disorder and an IQ of 90; she has been seizure free since 2.5 years, but has previously experienced different seizure types, including multiple absences per day. The third patient is 28 years old and has a normal intellect, but was schooled at a low level and now works in a sheltered environment due to his epilepsy and memory problems. He has around 10 GTCS per year. Most behavioral problems in the clinical range were reported in younger Dravet syndrome patients: 60% of 4-11 year olds score within the clinical range, whereas less than 30% of 20+ year old patients do (Figure 2.2A). 106 Dravet syndrome patients and 27 non-Dravet syndrome patients could be assessed for behavioral problems during the telephone interview. Parents of patients responded "yes" to the question "are there any behavioral problems?" during the telephone interview in 66% of Dravet syndrome patients, and only in 7% of non-Dravet syndrome patients (Table 2.1). CBCL/ABCL data was missing for 36 patients; similar percentages of parents reported behavioral problems during the telephone interview for patients with and without CBCL/ABCL results. Most behavioral problems were reported during the telephone interview for Dravet syndrome patients between 8 and 11 years old (>80%, Figure 2.2B).

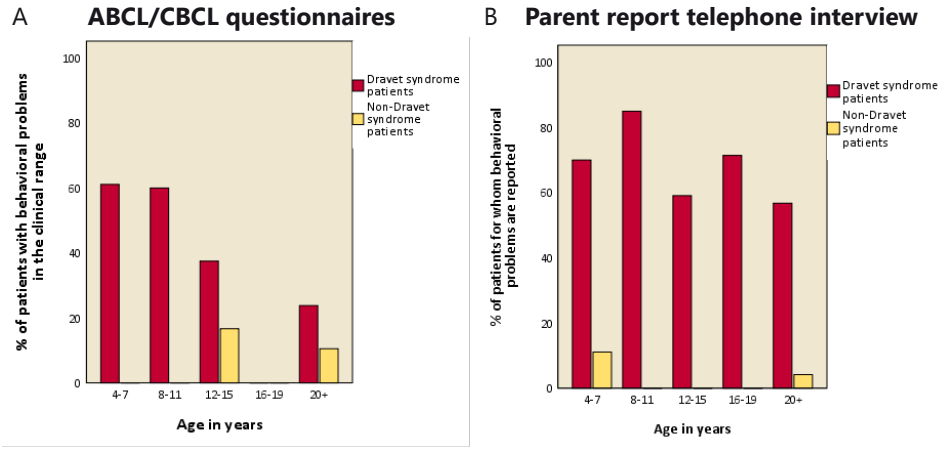

Figure 2.2. A: Percentage of Dravet syndrome patients with behavioral problems in the clinical range according to CBCL/ABCL questionnaires, per age group. B: Percentage of Dravet syndrome patients for which parents responded "yes" to the question "are there any behavioral problems?" during the telephone interview, per age group.

2.3.4 Quality of life

Seventy-one Dravet syndrome patients and 22 non-Dravet syndrome patients could be assessed for health related quality of life. Dravet syndrome patients showed lower health related quality of life scores than non-Dravet syndrome patients (average 52.6 versus 88.5, Table 2.1, Figure 2.3). Scores tended to worsen with age (Figure S2.2).

2.3.5 Learning and behavioral problems among non-Dravet syndrome patients

Although non-Dravet syndrome patients were significantly less affected than Dravet syndrome patients on all five major outcome scales, we did observe subtle problems in this group (Table 2.2). 27% had encountered some kind of learning problem for which extra educational assistance was necessary (see supplemental data S1.1 for specific problems). None of the patients over 40 years old reported to have experienced problems while following primary or secondary education. Behavioral problems, like attention deficit, autistic features or anxiety, were reported for 38% of non-Dravet syndrome patients, among all age groups, although their CBCL score was not in the clinical range except for three. Two of these 18 patients had not completed the CBCL questionnaires (see supplemental data S1.1 for specific problems).

2.3.6 Correlations between outcomes

In the complete cohort, FMS scores were strongly related to cognitive outcomes, seizure severity and quality of life scores: patients with more severe walking disabilities had a more severe cognitive disability (r_s= -0.682, p<0.0005, Figure S2.3A), a higher seizure frequency (r_s= -0.537 for major seizures and r_s= -0.492 for minor seizures, both p<0.0005, Figure

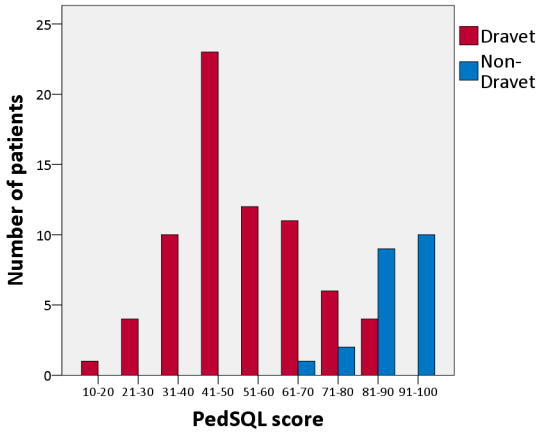

Figure 2.3. Distribution of PedsQL scores to indicate health-related quality of life (HRQOL) for Dravet syndrome and non-Dravet syndrome patients.

S2.3B) and a lower quality of life score (r_s= 0.668, p<0.0005, Figure S2.3C). Most behavioral problems were observed in patients with a mild cognitive disability (Figure 2.4A, 2.4B); less behavioral problems were seen in patients with cognitive outcomes scores on both ends of the spectrum. Behavioral problems in the clinical range of the ABCL/CBCL questionnaires were positively related to major seizure frequency (r_s=0.260, p=0.004, Figure S2.4A) in the complete cohort; however, in Dravet syndrome patients most behavioral problems were reported in patients that are seizure free or experience daily seizures (Figure S2.4B). Furthermore, patients with behavioral problems in the clinical range scored significantly lower on the quality of life questionnaire (r_s= -0.523, p<0.0005, Figure S2.4C). In the complete cohort, cognitive outcome scores were significantly related to major seizures (r_s= 0.693, p<0.0005) and minor seizure frequencies (r_s=0.511, p<0.0005) (Figure S2.5A, S2.5B).

In the non-Dravet group only, the presence of any learning problems was significantly associated with a lower quality of life score (median score of 86.45 versus 92.95, p=0.006 (Mann-Whitney U, U=19, z=-2.708), Figure S2.6A). No statistically significant differences in quality of life scores were found between patients with or without psychological/behavioral problems (p=0.188, Figure S2.6B). Patients with psychological or behavioral problems more often used AED's than patients without these problems (p=0.013, X^2-test). No differences in percentages of medication free patients were observed between patients with and without learning problems (p=0.408, X^2-test). Furthermore, no differences in percentages of seizure free patients were observed between patients with or without learning problems (p=0.421, X^2-test) or psychological or behavioral problems (0.421, Fisher's exact test).

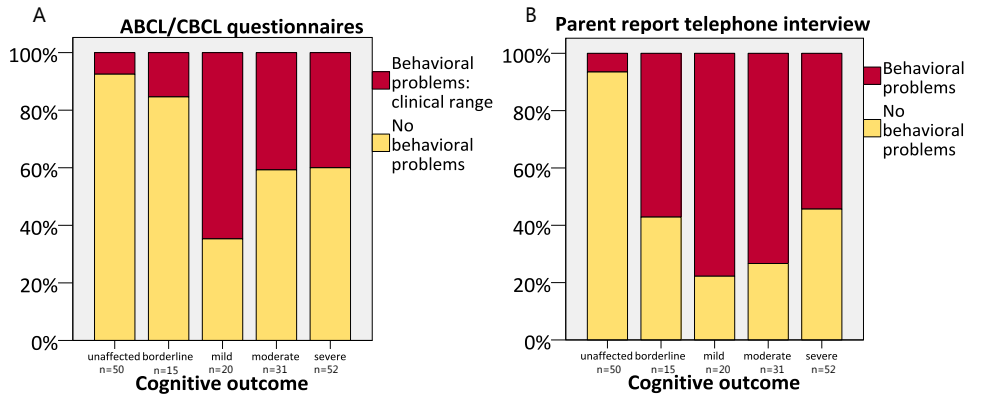

Figure 2.4. A: Percentage of patients with behavioral problems in the clinical range according to ABCL/CBCL questionnaires, per cognitive outcome score. B: Percentage of patients for which parents responded "yes" to the question "are there any behavioral problems?" during the telephone interview, per cognitive outcome score.

2.4 Discussion

No universally used consensus guidelines for the diagnosis of Dravet syndrome exist and many studies use different criteria.[6,7,9,139] The main diagnostic criteria in most studies relate to the epilepsy phenotype and cognitive development. Walking disabilities and behavioral problems are usually not included, even though many studies have already acknowledged they are common and important features of Dravet syndrome,[3,4,6,56,60–62] while they are virtually never present in non-Dravet syndrome patients with pathogenic *SCN1A* variants. Both walking disabilities and behavioral problems can emerge early in the disease course of Dravet syndrome, affecting a large percentage of patients before the age of 8 years 79% of children with Dravet syndrome between 4 and 8 years in our cohort had a walking disability, which is in line with recent research.[56] Gait disturbances have already been described in patients as young as 2 years of age.[56,60] Our overall percentage of Dravet syndrome patients with a walking disability (score 5 or lower, 54%) is between percentages previously reported: Brunklaus et al.[5] found a motor disorder in 36% of Dravet syndrome patients, whereas Lagae et al.[56] found walking disabilities in 79%. These differences are likely due to used definitions; factors such as skeletal malalignment, behavioral issues, epilepsy severity and pain can affect functional mobility as well. Furthermore, the FMS scale assesses functional mobility on a distance of maximal 500 meters, while other studies incorporated no distances.[56] Behavioral problems in the clinical range were most frequently seen between 4 and 11 years of age, when assessed by CBCL/ABCL questionnaires. This is in contrast to previous studies, that have found an increase of behavioral problems with age (although these problems had been observed in children <2 years of age already).[5,56] Our different results might be due to differences in measurement tools and data collection.

Interestingly, parents of Dravet syndrome patients reported behavioral problems more frequently in the interview (66%) than patients scored in the clinical range of the CBCL/ABCL questionnaires (43%); this might indicate that scores in the subclinical range are perceived to be very burdensome as well. This high percentage underlines the impact of these issues on families and the importance of accurate management. Behavioral problems were much more common among Dravet syndrome patients than in non-Dravet syndrome patients (43 versus 7%) and the three non-Dravet syndrome patients that scored in the clinical range of the ABCL/CBCL questionnaires had a relatively severe non-Dravet *SCN1A* phenotype. Our data suggest that walking difficulties and behavioral problems, based on official assessments, could be useful when counseling patients with *SCN1A* pathogenic variants; they are strong indicators for a more severe disorder when observed at a young age.

Both walking disabilities and behavioral problems showed a strong correlation with cognitive outcomes, seizure frequencies, and quality of life scores. Similar findings have been reported previously and behavioral problems have been identified as the most important predictors of a worse HRQoL score.[5,56] Interestingly, the relationship between cognitive disability and frequency of behavioral problems was not linear but hyperbolic: most problems were seen in patients with a mild intellectual disability. A reasons for this could be that more severely disabled patients are less capable of aberrant behavior; however, it might also be due to the fact that CBCL questionnaires have been proven to be less reliable in the assessment of children with moderate, severe or profound intellectual disability.[218] Total scores cannot be calculated when many questions are not applicable due to cognitive impairment, which occurs more frequently in patients with a worse cognitive outcome. Nevertheless, when parents were asked if they thought their child showed any behavioral problems, a similar pattern was observed. A hyperbolic relationship was also found between seizure frequency and behavioral problems in Dravet syndrome, although inversed: most behavioral problems were seen in patients that were seizure free or experienced daily seizures. This non-linear relationship indicates that behavioral problems are not completely subject to an epileptic encephalopathy disease model, as previously suggested.[42,56] Recent research has shown that *SCN1A* variants could lead to changes in the dopamine system which may contribute to behavioral problems, irrespective of seizure activity.[48] This, together with the large impact of behavioral problems on HRQoL scores and the high number of families affected by it, calls for more attention regarding recognition of behavioral problems. Handling these issues and developing coping strategies for parents require more emphasis during treatment, which should not only focus on suppressing seizures.

Non-Dravet syndrome patients seem to have a good prognosis: the majority of our patients was reported to be seizure free after 10 years of age and seizure freedom was reported for all patients over 40 years of age, in most cases without maintenance treatment. However, some of the older participants represented parents of children with *SCN1A*-related seizures, that were tested subsequently after a diagnosis was made in their children. They may have gone undiagnosed if their children had not been assessed for mutations and our results may

therefore not be fully applicable to young probands with *SCN1A*-related epilepsy. Seven of the 15 patients in the 40+ age group were parents of affected children and had never used anti-epileptic medication themselves, so it is unlikely that they would have been diagnosed individually. It is furthermore worth noting that pathogenic *SCN1A* variants do not have a 100% penetrance: although our study only describes symptomatic patients, we are aware of four mutation carrying GEFS+ family members that never experienced any seizures (not included in this study). However, although non-Dravet syndrome patients showed better outcomes on the used questionnaires, subtle comorbidities were still observed in this group. It is known that neuropsychological and cognitive problems are common in epilepsy in general,[219–223] and it is therefore not surprising that similar problems are observed in the non-Dravet group. Although the use of different methods makes comparing exact results difficult, and we investigated a small cohort (only 24 non-Dravet syndrome patients <20 years of age), we nonetheless observe relatively similar percentages of problems in our patient group and cohorts consisting of patients only diagnosed with epilepsy: 26% of patients between 7-16 years old in our cohort had repeated a grade and 60% required special educational assistance, compared to 26% and respectively 51% in an epilepsy cohort.[221] Psychological/behavioral problems were observed in 40% of patients between 5-17 years old and in 37% of patients over 16 years old in our cohort, compared to 31.4% in children with childhood seizures,[222] and versus in 30.6% in patients with epilepsy[220] in the same age groups. Furthermore, similar percentages of patients with symptoms of ADHD (10% vs 14.3%), depression (5% vs 4%) and anxiety (15% vs 12.3%) were seen in patients between 5-17 years old in our cohort and in patients with idiopathic epilepsy respectively.[219] Although according to ILAE criteria[224] usually no developmental impairments are expected in non-Dravet *SCN1A* phenotypes, we report five non-Dravet syndrome patients (10%) with a cognitive outcome score of 2 (borderline ID). Their disorder could not be classified as Dravet syndrome, since they were seizure free with minimal or no medication at the time of inclusion, and three had never developed any secondary seizure types. Furthermore, a borderline ID (IQ 70-85) represents the IQ score range between -1 and -2SD from the mean (100) and is thus observed in 13.6% of the general population. It can consequently be expected to also occur in a small part of the non-Dravet group, as described by several authors.[59,84,211] In addition, borderline ID might be a family characteristic in these patients. We therefore argue that a slight cognitive impairment does not necessarily indicate a diagnosis of Dravet syndrome.

Our study has several limitations. In the non-Dravet group, all of the more subtle problems (not captured by the standardized questionnaires) were reported by patients or their parents during telephone interviews or mentioned by clinicians in medical files, and no official assessments were used to evaluate the problems. Our results therefore reflect issues that they regarded most important, rather than structured measurements and may be subject to recall and response biases. To minimize this effect, we limited our analyses in this group therefore to patients younger than 20 years. In these patients, the occurrence of

problems at school was independent of seizure and/or medication freedom at the moment of inclusion. These problems may however already have occurred while medication was still being used, or when seizures were still active, so we cannot exclude a causal relationship. Interestingly, although psychological/behavioral problems occurred independently of seizure freedom, they occurred more often in patients that still used medication. This implies that psychological/behavioral problems might at least be partly due to medication side effects. In other studies however, no such relation was found.[219,221] It is worth noting that although significantly lower quality of life scores were found in patients with problems at school, the average QoL scores of the non-Dravet group is comparable to that of the general population.[214]

By analyzing multiple outcomes in Dravet syndrome patients together with patients showing milder *SCN1A*-related phenotypes, we are able to assess different disease burdens over a large part of the *SCN1A*-spectrum. Both groups show distributions at the different ends of the spectrum and in general a clear distinction between these syndromes can therefore be made, based on regularly used clinical criteria such as cognitive impairment and seizure severity, but also on other comorbidities: severe behavioral problems and walking disabilities can already occur at a young age in Dravet syndrome patients and are almost never seen in non-Dravet syndrome patients. These issues should receive specific attention during clinical management of the disease. However, as in patients with other epilepsies, comorbidities occur in a substantial part of non-Dravet syndrome patients as well. Although these problems are less severe than in Dravet patients, they can still have a large impact on patients and their families. Our study provides valuable information on the disease course and comorbidities in these patients, which can be of great value when counseling newly diagnosed patients and their parents.

2.5 Appendix

S2.1 Learning and behavioral problems among non-Dravet syndrome patients

Reported learning problems were: extra support is needed in one or more classes (n=7), needs extra structure (n=1), is slower/has more difficulty learning compared to peers (n=4), repeated a grade (n=3), is too sensitive to sensory stimuli (n=1), has difficulties concentrating (n=4), has dyslexia (n=3), has memory issues (n=3), works in sheltered employment (n=1). Two patients were reported to have difficulties concentrating which however did not lead to learning problems.

Reported behavioral problems were: diagnosis of ADHD (n=3), diagnosis of ADD (n=2), diagnosis of autism spectrum disorder (n=2), diagnosis of personality disorder (n=1), symptoms of (but not officially tested for) ADHD or attention problems (n=6), symptoms of (but not officially tested for) autism (n=2), stress disorder/anxiety (n=6), depressive symptoms (n=2), insecurity/low self-esteem (n=2), psychogenic seizures (n=1), problems with social functioning (n=2), addiction (n=2) and anger/aggression problems (n=2).

S2.2 Supplemental figures

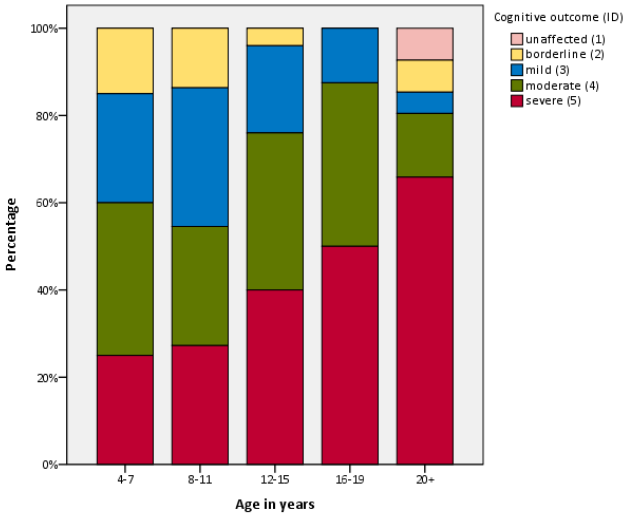

Figure S2.1. Cognitive outcome per age group in Dravet syndrome patients.

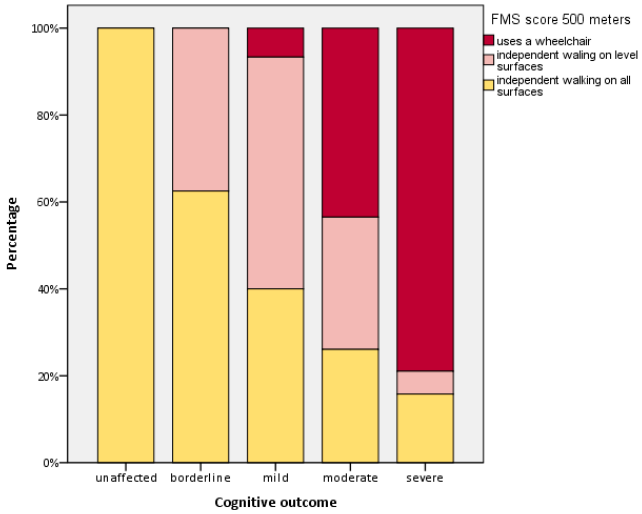

Figure S2.2. Relationship between HRQoL scores and age in the complete cohort.

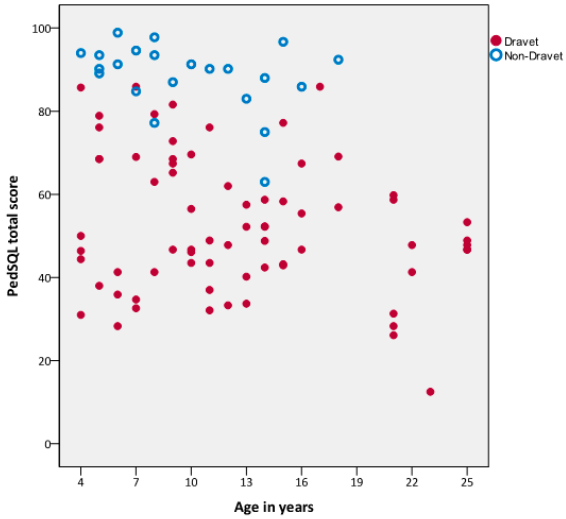

Figure S2.3A. FMS scores versus cognitive outcome scores.

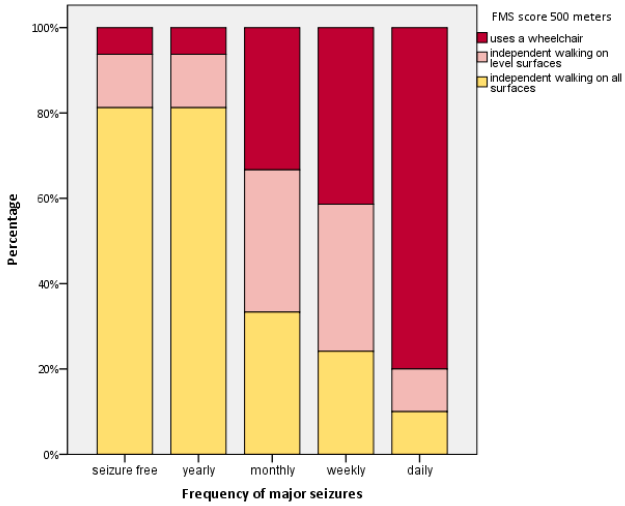

Figure S2.3B. FMS scores versus seizure severity.

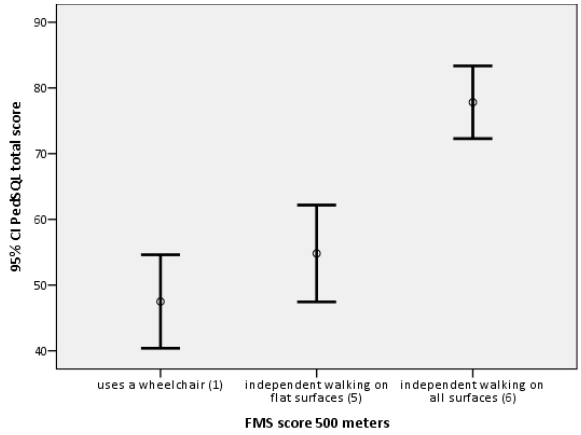

Figure S2.3C. FMS scores versus HRQoL scores.

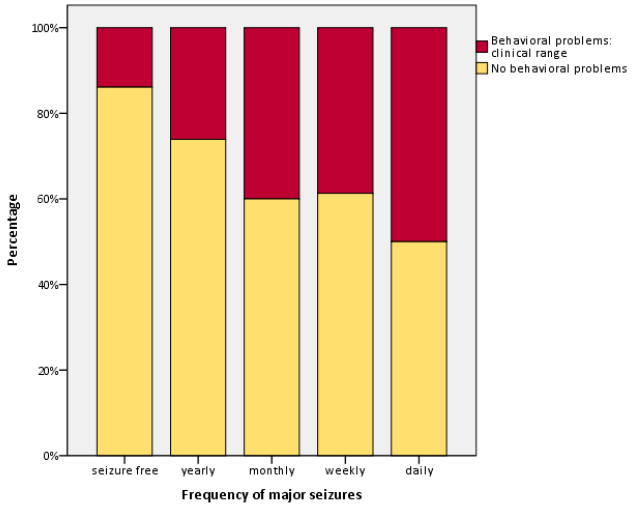

Figure S2.4A. Behavioral problems in the clinical range according to ABCL/CBCL questionnaires versus seizure frequency, complete cohort.

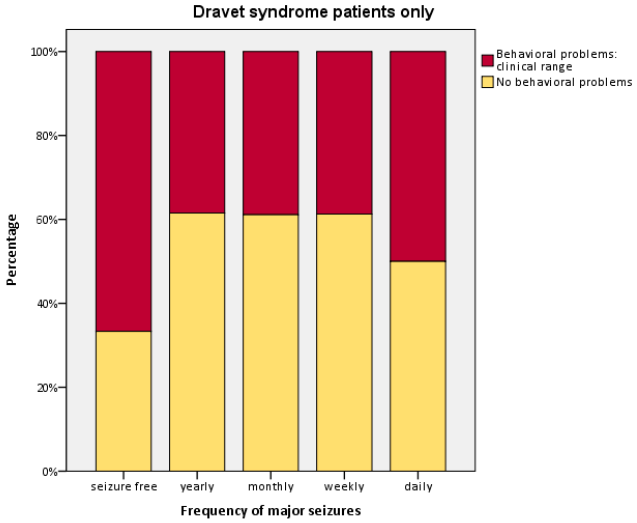

Figure S2.4B. Behavioral problems in the clinical range according to ABCL/CBCL questionnaires versus seizure frequency, in Dravet syndrome patients.

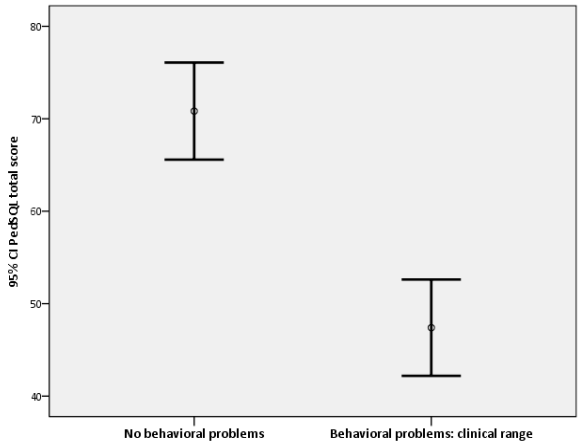

Figure S2.4C. Behavioral problems according to ABCL/CBCL questionnaires versus HRQoL scores.

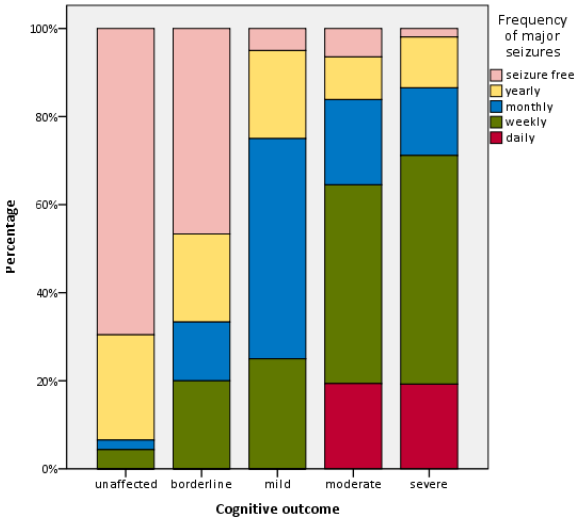

Figure S2.5A. Seizure frequency major seizures versus cognitive outcome.

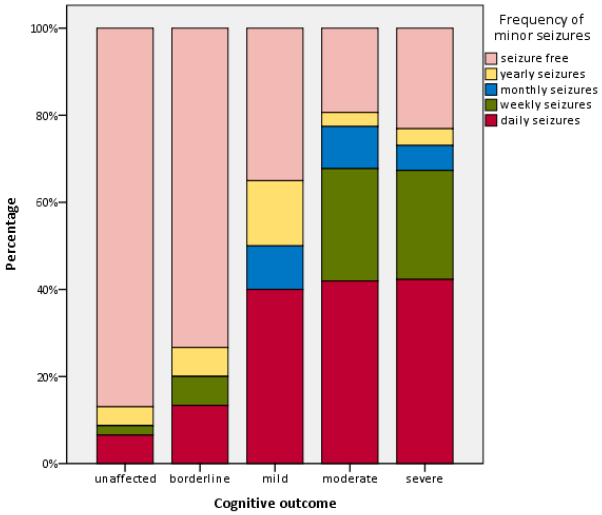

Figure S2.5B. Seizure frequency minor seizures versus cognitive outcome.

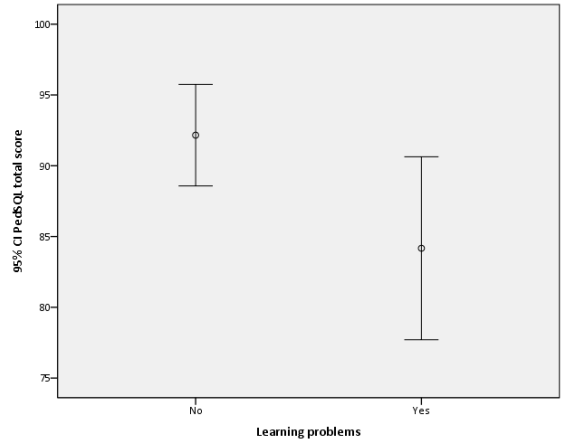

Figure S2.6A. HRQoL versus learning problems.

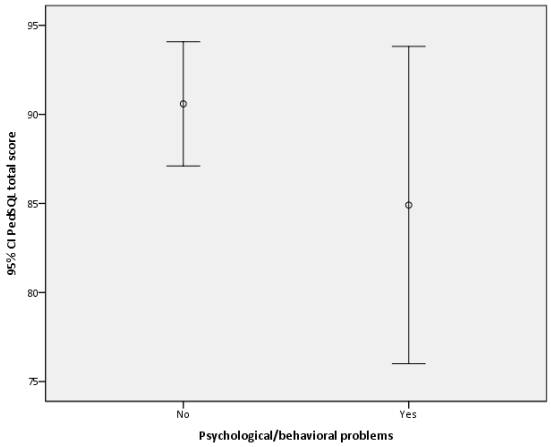

Figure S2.6B. HRQoL versus psychological/behavior problems.

Chapter 3

Influence of contra-indicated medication use on cognitive outcome in Dravet syndrome and age at first afebrile seizure as a clinical predictor in *SCN1A*-related seizure phenotypes

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Published: Epilepsia. 2018 Jun;59(6):1154-1165.

Abstract

Objective: Pathogenic variants in *SCN1A* can give rise to extremely variable disease severities that may be indistinguishable at their first presentation. We aim to find clinical features that can help predict the evolution of seizures into Dravet syndrome and clinical features that predict cognitive outcome in Dravet syndrome. We specifically investigate the role of contra-indicated medication (CIM) as a possible modifier of cognitive decline.

Methods: A cohort of 164 Dutch participants with *SCN1A*-related seizures was evaluated. Clinical data were collected from medical records and semi-structured telephone interviews. Cognitive function was classified by a child neurologist, neuropsychologist, and clinical geneticist. Several clinical variables, including duration of CIM use in the first five years of disease, were evaluated in univariable and multivariable analyses.

Results: A longer duration of CIM use in the first five years after seizure onset was significantly associated with a worse cognitive outcome at time of inclusion, and with lower interpolated IQ/DQ scores after the first five years of disease in Dravet syndrome patients. CIM use remained a significant predictor for cognitive outcome in a multivariable regression model, as did age at the first observation of developmental delay and age at first afebrile seizure. Age at first afebrile seizure was the most accurate predictor for evolution of seizures into Dravet syndrome for the complete cohort.

Significance: Our data suggest that a longer CIM use in the first five years of disease can have negative effects on cognitive outcome in Dravet syndrome. An early diagnosis is essential to avoid these drugs. Furthermore, we identified age at first afebrile seizure as an important predictor for evolution of seizures into Dravet syndrome and for the severity of Dravet syndrome, which can be used to counsel parents of young patients with *SCN1A*-related seizures.

3.1 Introduction

Pathogenic variants in *SCN1A* can give rise to extremely variable disease severities.[108,111,162,207] On the severe end of the spectrum is Dravet syndrome, characterized by intractable epileptic seizures, diminishing psychomotor development that results in mild to severe intellectual disability, and often walking difficulties and behavioral problems.[3,5,60,62] Recently, an *SCN1A* epileptic encephalopathy that is even more severe than Dravet syndrome has been described in nine patients.[207] Milder phenotypes include GEFS+ syndrome and febrile seizures, in which intellectual disability is usually absent.[108,208] Furthermore, there is a large phenotypic variability between Dravet syndrome patients: while some are severely disabled and suffer from ongoing seizures, others live much more independent lives.[9,25,139] An accurate prediction of the prognosis in affected children is understandably very important to parents.

SCN1A encodes for the α-subunit of a neuronal sodium channel, Nav1.1. Pathogenic variants that lead to a complete loss-of-function of the channel are nearly always associated with severe phenotypes.[117] Such variants include genomic rearrangements, splice-site, nonsense and frameshift variants. Certain missense variants can also lead to complete loss-of-function when located in critical regions of the gene (e.g. the pore region and voltage sensor). However, other missense variants can result in milder disturbances of channel function and thus lead to milder phenotypes, although variants causing partial loss-of-function or gain-of-function may also be associated with Dravet syndrome.[117,225] Although missense variant location is a strong indicator for the severity of channel disruption, it still cannot fully predict the effect of the variant on channel function and phenotype.[59,117,130] Physicochemical changes due to missense variants are similarly difficult to predict.[130,158] Several genetic factors have already been suggested to modify the clinical outcome of *SCN1A*-related diseases, such as variants in the *SCN1A* promoter region, the 5'- and 3' untranslated regions, and variants in other genes.[189,197,226] In clinical practice, it remains difficult to predict whether a missense variant will lead to Dravet syndrome or a milder disease. A few studies have identified clinical features that can help discriminate between Dravet syndrome and milder phenotypes, such as age at seizure onset, seizure types and number of seizures.[17,59,227]

Studies on Dravet syndrome severity have suggested that the presence of a motor disorder, certain EEG characteristics, early myoclonia and focal seizures, more severe seizures and an early onset of developmental delay are associated with a worse outcome.[5,24,25,33,52,228] Perhaps even more important are studies that focus on factors that clinicians can influence. Although it is clear that sodium channel blockers such as lamotrigine and carbamazepine can result in more frequent or severe seizures in this patient group,[5,47,83] and many studies have suggested a positive effect of an early diagnosis and appropriate treatment on long term cognitive outcomes, this has not been established in large patient groups.[25,59,84,85] Increasing knowledge about the effects of sodium channel blockers is crucial because they

are prescribed to a large proportion of patients with Dravet syndrome at some point during their disease course (21-100%)[56,86,228], often before a diagnosis is established.

Parents often experience great uncertainty about the prognosis of their children when a pathogenic *SCN1A* variant is found early in life, because accurate prediction of the consequences of an *SCN1A* variant is still not possible, and different *SCN1A*-related phenotypes may be indistinguishable at their first presentation. Consequently, there is still a critical necessity to identify more accurate predictors. Here, we study the effect of contra-indicated medication (CIM) on cognitive function in Dravet syndrome patients (n=104) and perform retrospective analyses of possible clinical predictors in a large cohort of Dutch patients with *SCN1A* pathogenic variants (n=164).

3.2 Methods

3.2.1 Participants

A cohort of 164 participants with *SCN1A* pathogenic variants was evaluated. A portion of this cohort (n=124) has previously been evaluated for mosaicism.[212] We included only symptomatic participants with pathogenic variants (class V) or likely pathogenic variants (class IV) in *SCN1A*, according to the American College of Medical Genetics and Genomics criteria.[155] *SCN1A* variants had been detected in diagnostic laboratories (University Medical Center Utrecht, the Netherlands; Laboratory for Neurogenetics, Institute Born-Bunge, University Antwerp, Belgium; Radboud University Nijmegen Medical Center, the Netherlands; Duncan Guthrie Institute of Medical Genetics, Glasgow, UK) by Sanger sequencing, NGS epilepsy gene panels, whole exome sequencing, or by MLPA (multiplex ligation-dependent probe amplification).

All eligible individuals of at least 4 years of age known to the University Medical Center Utrecht were approached for study inclusion. We excluded patients below the age of 4 years since clinical experience has shown that syndrome classification and estimation of disease severity are less reliable for younger children. Informed consent was obtained from participants or their legal caretakers, according to the Declaration of Helsinki. The study was approved by the Ethical Committee of the University Medical Center Utrecht.

3.2.2 Clinical data

Detailed clinical data were collected from medical records for all participants, and from semi-structured telephone interviews when possible (n=155). Data on several clinical variables, depicted in Table 3.1, were obtained. Variables were selected based on literature and clinical experience. Afebrile seizures were defined as seizures with a body temperature below 38°C, or seizures for which medical records reported “no fever” without mentioning the exact temperature. Our main outcome was cognitive function at the time of inclusion, which was classified in a consensus meeting by a child neurologist, neuropsychologist, and

clinical geneticist. Cognitive function at the moment of inclusion was rated on a five-point scale based on available data on IQ and developmental level, adjusted for age at assessment (1= no ID (IQ or developmental quotient (DQ) >85), 2 = borderline ID (IQ or DQ 70-85), 3 = mild ID (IQ or DQ 50-70), 4= moderate ID (IQ or DQ 30-50), 5 = severe or profound ID (IQ or DQ <30)). When no (recent) IQ or DQ was available, the assessment was made based on function in school, communication and adaptive behavior. All participants were categorized into two clinical subgroups: Dravet syndrome or non-Dravet syndrome. Dravet syndrome was diagnosed based on previously published criteria[217] and in line with recently published recommendations.[6] The non-Dravet group consisted of patients with either GEFS+ or febrile seizures.

3.2.3 Statistical analyses

We investigated predicting factors for Dravet versus non-Dravet syndrome, and for the severity of Dravet syndrome. We specifically tested the influence of contra-indicated medication (CIM) use on cognitive function. Univariable analyses were performed, and variables for which statistically significant differences between groups were observed were included in multivariable regression analyses, adjusted for age at cognitive assessment. Adjusting the models for age at cognitive assessment is necessary, since average cognition declines with older age in Dravet syndrome patients. Furthermore, sensitivity, specificity, positive- and negative predictive values and receiver operating characteristics (ROC) curves were calculated. Statistical analyses were performed using SPSS statistics software (IBM SPSS Statistics for Windows V21, Armonk, NY: IBM Corp.). All reported tests were performed 2-tailed with an alpha-level for significance of p <0.05.

Predicting factors for Dravet syndrome versus non-Dravet

Differences between the Dravet and non-Dravet group were calculated with either Pearson's chi-square test or Fisher's exact test for binary and categorical variables (type of variant, whether or not a patient had ever been admitted to an intensive care unit, secondary seizure types before the age of 12 months (comprising absences, myoclonias, focal seizures and focal seizures with impaired awareness) and the presence of mosaicism for the pathogenic variant) or Mann–Whitney U test for continuous and ordinal variables (age at seizure onset and age at first afebrile seizure). A multivariable binomial logistic regression analysis was performed with statistically significant variables.

Predicting and influencing factors for cognitive outcome in Dravet syndrome

Univariable binomial logistic regression analyses, adjusted for age at cognitive assessment, were performed in the subgroup of Dravet syndrome patients. Patients were divided into two groups: mild cognitive dysfunction (score 1-3) and severe cognitive dysfunction (score 4-5). The following variables were assessed: age at seizure onset, age at first afebrile seizure, age at first observation of developmental delay, variant type, whether or not a patient had

ever been admitted to an intensive care unit, secondary seizure types before the age of 12 months (as defined previously), the presence of mosaicism for the pathogenic *SCN1A* variant, the duration of CIM use (defined as sodium channel blockers: lamotrigine, phenytoin, carbamazepine, oxcarbazepine, vigabatrin) and age at diagnosis. The cumulative number of months CIM was used in the first five years after seizure onset was calculated for each patient with seizure onset at least five years before assessment. This timeframe was chosen since cognitive decline is generally most severe in the first years following disease onset.[5,33,228] We chose to analyze the duration of CIM use rather than whether CIM had ever been used or not, since only a small number of patients had never used CIM, thereby reducing the power of this analysis. Furthermore, we hypothesized that if CIM use is indeed damaging, a longer use will result in more severe effects. A multivariable binomial logistic regression analysis, adjusted for age at cognitive assessment was performed with all statistically significant variables.

Duration of CIM use was further investigated in relation to cognitive outcome scores 1-5 (ordinal logistic regression analysis, in contrast to the previously used dichotomy 1-3 versus 4-5) and interpolated IQ/DQ scores after five years of disease (linear regression analysis). To obtain these approximated scores, all IQ- and developmental assessment scores of each patient, conducted at different ages, were interpolated by linear regression. When the first official assessment was made later than five years after seizure onset we used the age at which a developmental delay was first observed (by either parents or clinicians) as the first moment of decline, and IQ/DQ scores up until that age were estimated to be average (=100). The duration of CIM use may depend on disease severity. To avoid confounding, clinical variables that were shown to have significant predictive value in our main analysis were added to these last two regression models.

We furthermore analyzed the effects of CIM use in year 1, 2, 3, 4 and 5 after seizure onset separately in univariate and multiple regression analyses, to investigate whether different effects are seen in different years.

3.3 Results

The characteristics of the study population are depicted in Table 3.1A (*All participants*), Table 3.1B (*Dravet syndrome patients)*, Table 3.2 and Figure 3.1A. The Dravet syndrome subgroup consisted of 116 patients, and the non-Dravet group of 48. The median ages of the participants were 14 and 22 years, respectively. Almost half of all Dravet syndrome patients had a cognition score of 5 (45%), whereas almost all non-Dravet patients had normal cognitive capacities (score 1, 90%) except for 5 who had a slight delay (score 2, 10%). In 104 Dravet syndrome patients, seizure onset was at least five years prior to cognitive assessment. After five years of disease, the average interpolated IQ/DQ score was 62 and the median duration of CIM use was 11 months. Patients older than 12 years were more likely to have

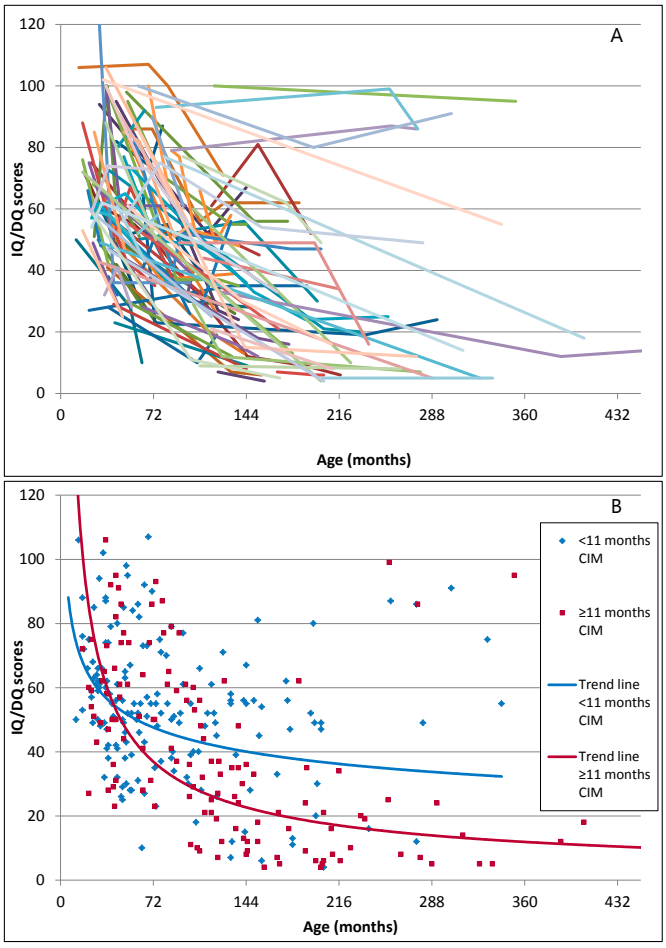

Figure 3.1. A: Decline of cognitive capacities of Dravet syndrome patients during their disease course, measured by interpolated IQ/DQ scores. B: All measured IQ/DQ scores of the Dravet syndrome patients, divided into shorter and longer duration of CIM use in the first five years of disease (blue: <11 months CIM, red: ≥11 months CIM). Trend lines were fitted for both groups.

used CIM for longer periods of time and to be diagnosed at a later age. It was not possible to accurately perform an ordinal logistic regression analysis with age at diagnosis as a variable and the cognitive score as outcome, as there was a very strong multicollinearity between age at diagnosis and age at cognitive assessment (VIF=15.375) (supplemental Figure S3.1). Due to this strong linear relation (i.e. older patients had been diagnosed at a later age), it is difficult to calculate the separate contributions of each variable to the outcome. Since a younger age at diagnosis can only positively influence outcome if clinical management is adjusted, we focused on the duration of CIM use instead.

Table 3.1. Characteristics of the study population and univariable analyses

	1a: All participants				1b: Dravet syndrome patients		
Variable	**Total**	**Dravet**	**Non-Dravet**	**p**	**Cognition score 1-3**	**Cognition score 4-5**	**p**
n	164	116	48		33	83	
Age (years, median, range)	15 (4-67)	14 (4-48)	22 (4-67)		11 (4-48)	15 (4-47)	
Sex: male (n)	83	65 (56%)	18 (38%)		16 (48%)	49 (59%)	
Variant type (n)							
missense	87	44 (38%)	43 (90%)		14 (42%)	30 (36%)	
pore/pore loop/voltage sensor	58	29	29	**<0.0005**	7	24	0.562
elsewhere	29	15	14		7	8	
splicing	16	13 (11%)	3 (6%)		4 (12%)	9 (11%)	
nonsense/frameshift/rearrangement	61	59 (51%)	2 (4%)		15 (45%)	44 (53%)	
Mosaicism: yes (n)	9	8 (7%)	1 (2%)	0.286	5 (15%)	3 (4%)	**0.03**
Cognition score[1] (n)							
1	46	3 (3%)	43 (90%)		3 (9%)	0 (0%)	
2	15	10 (9%)	5 (10%)		10 (30%)	0 (0%)	
3	20	20 (17%)	0 (0%)		20 (61%)	0 (0%)	
4	31	31 (27%)	0 (0%)		0 (0%)	31 (37%)	
5	52	52 (45%)	0 (0%)		0 (0%)	52 (63%)	
Age at seizure onset (months, median, range)	8 (1-72)	5 (1-30)	15 (4-72)	**<0.0005**	7.6 (3-30)	5.2 (1-18)	**0.007**
Secondary seizure types[2] <12 months: yes (n)	71	68 (59%)	3 (6%)	**<0.0005**	13 (39%)	55 (66%)	**0.008**
Admission to intensive care unit: yes (n)	60	54 (47%)	6 (13%)	**<0.0005**	13 (39%)	41 (49%)	0.185
Age at first observation of developmental delay (n)							
<12 months	15	15 (13%)	0 (0%)		1 (3%)	14 (17%)	
12-23 months	43	43 (38%)	0 (0%)		5 (16%)	38 (47%)	
24-35 months	24	23 (21%)	1 (2%)		9 (29%)	14 (17%)	**<0.0005**
36-47 months	24	20 (18%)	4 (8%)		9 (29%)	11 (14%)	
≥48 months	15	11 (10%)	4 (8%)		7 (23%)	4 (5%)	
no delay	39	0 (0%)	39 (81%)		0 (0%)	0 (0%)	
missing	4	4	0		2	2	
Age at first afebrile seizure[3] (n)							
<12 months	70	70 (65%)	0 (0%)		10 (34%)	60 (77%)	
12-23 months	31	27 (25%)	4 (9%)		15 (52%)	12 (15%)	
24-47 months	14	5 (5%)	9 (20%)	**<0.0005**	1 (3%)	4 (5%)	**0.001**
≥48 months	21	5 (5%)	16 (36%)		3 (10%)	2 (3%)	
never	16	0 (0%)	16 (36%)		0 (0%)	0 (0%)	
missing	12	9	3		4	5	
Age at diagnosis (months, mean)		130			104	140	
Dravet syndrome patients with disease onset >5 years ago, n=104:					29	75	
Duration of CIM[4] **use in first 5 years of disease** (mean, range)					10.5 (0-56)	22 (0-59)	
never used CIM (n)		11 (0-59)			6 (21%)	4 (6%)	
used CIM 1-6 months (n)					9 (32%)	17 (24%)	**0.015**
used CIM 7-12 months (n)					6 (21%)	10 (14%)	
used CIM 13-24 months (n)					3 (11%)	13 (18%)	
used CIM >24 months (n)					4 (14%)	27 (38%)	
missing					1 (3%)	4 (5%)	
Interpolated IQ/DQ5 score after 5 years of disease (mean, range)		62 (19-100)			81 (44-100)	55 (20-96)	

[1] Based on available data on IQ and developmental level, adjusted for age at assessment (1 = no ID (IQ or developmental quotient (DQ) >85), 2 = borderline ID (IQ or DQ 70-85), 3 = mild ID (IQ or DQ 50-70), 4= moderate ID (IQ or DQ 30-50), 5 = severe or profound ID (IQ or DQ <30). When no (recent) IQ or DQ was available, the assessment was made based on function in school, communication and adaptive behavior. [2]Absences, myoclonias, focal seizures and focal seizures with impaired awareness. [3]Defined as seizures with a body temperature below 38°C, or seizures for which medical records reported "no fever" without mentioning the exact temperature. [4]CIM = contra indicated medication (sodium channel blockers: lamotrigine, phenytoin, carbamazepine, oxcarbazepine, vigabatrin). [5]DQ = developmental quotient.

Table 3.2. CIM use in Dravet syndrome patients per age group

Dravet syndrome patients with disease onset >5 years ago, n=104:	**5-8 years**	**9-12 years**	**13-16 years**	**17-20 years**	**21+ years**
n	12	22	24	9	32
CIM[1] use in first 5 years of disease (months)					
mean	2.6	10.3	23.1	31.9	23.7
median	1.5	7.5	14	40	21.5
range	0-11	0-34	0-56	1-57	0-59
missing	0	0	0	0	5
Age at diagnosis (months)					
mean	26	39.6	74.8	117	239.6
median	19.5	31.5	66	110	239
range	12-90	10-115	39-135	83-148	110-516
Used CIM (prescribed at some point during the disease course to % of patients)					
lamotrigine	25%	64%	92%	100%	63%
phenytoin	33%	36%	67%	56%	47%
carbamazepine	33%	45%	71%	89%	88%
oxcarbazepine	0%	23%	29%	33%	22%
vigabatrin	0%	9%	21%	44%	50%

[1]CIM = contra indicated medication (sodium channel blockers)

3.3.1 Predicting factors for Dravet syndrome versus non-Dravet

Five tested variables showed a significant difference between the Dravet syndrome and non-Dravet subgroups in univariable analyses (all $p < 0.0005$, Table 3.1A). Missense variants were the predominant variant type in non-Dravet patients (90%) and nonsense/frameshift/rearrangements were present in half of Dravet syndrome patients (51%). Dravet syndrome patients had a significantly earlier age of seizure onset (median 5 versus 12 months), more often secondary seizure types before the age of 12 months (59% versus 6%) and were more likely to have been admitted to an ICU (47% versus 12%). Additionally, Dravet syndrome patients had their first afebrile seizure at an earlier age: 65% before 12 months, whereas 36% of non-Dravet patients experienced their first afebrile seizure only after 48 months and 36% never had an afebrile seizure. Specificity, sensitivity, positive- and negative predictive values and ROC areas of all five significant variables are shown in supplemental Table S3.1A. Occurrence of a first afebrile seizure before the age of 24 months was shown to be the best predictor for Dravet syndrome, with a positive predictive value of 96% and a negative predictive value of 81%.

The multivariable binomial logistic regression model, including all five significant variables, showed that the variables most significantly associated with Dravet syndrome were age at first afebrile seizure ($p<0.0005$), a truncating or splice-site variant ($p=0.006$) and admittance to an ICU ($p=0.039$) (Table 3.3A).

3.3.2 Predicting and influencing factors for cognitive outcome in Dravet syndrome

Six variables analyzed in univariable binomial regression analyses differed significantly between the mild and severe groups of Dravet syndrome patients: mosaicism of the pathogenic variant (p=0.03), age at seizure onset (p=0.007), secondary seizure types before the age of 12 months (p=0.008), age at first observation of developmental delay (p<0.005)), age at first afebrile seizure (p=0.001) and the duration of CIM use in the first 5 years of disease (p=0.015) (Table 3.1B). Specificity, sensitivity, positive- and negative predictive values and ROC areas of all six significant models are shown in supplemental Table S3.1B. Age at first observation of delay and age at first afebrile seizure without fever were the best clinical predictors (ROC area under curve: 0.797 and 0.792 respectively).

The multivariable binomial logistic regression model, including all six statistically significant variables, showed that the best predictors for a worse cognitive outcome were age at first observation of developmental delay (p=0.004), age at first afebrile seizure (p=0.019) and duration of CIM use (p=0.022) (Table 3.3B). Ordinal logistic regression analysis, corrected for age at inclusion, age at first observation of developmental delay and age at first afebrile seizure showed a significantly higher chance of a worse cognitive outcome category score with a longer duration of CIM use (p=0.001, Table 3.3C, Figure 3.2). Linear regression analysis, corrected for age at first observation of developmental delay and age at first afebrile seizure, showed that a longer duration of CIM use was also a significant predictor for a lower interpolated IQ/DQ score five years after disease onset (B=-0.267, p=0.010, Table 3.3D, Figure 3.1B and Figure 3.3).

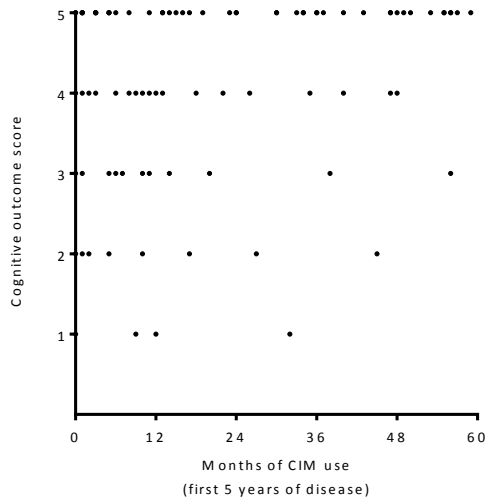

Figure 3.2. Duration of contra-indicated medication use by Dravet syndrome patients during the first five years of disease, divided into cognitive outcome categories.

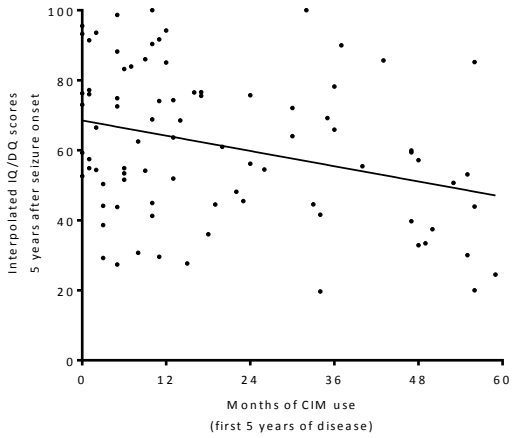

Figure 3.3. Relationship of interpolated IQ/DQ scores and duration of contra-indicated medication use by Dravet syndrome patients after five years of disease.

Repeated analyses, to investigate whether the use of estimated data for the interpolated IQ/DQ scores may have influenced our results, yielded very similar results: B=-0.237, p=0.05 without the 29 patients for which estimated IQ/DQ scores were used, and B=-0.244, p=0.011 when the IQ/DQ scores before the first notice of delay were estimated to be 85 instead of 100. We furthermore found the strongest effect for the second year of disease when analyzing the first five years separately (B=1.961, p=0.002, supplemental Table S3.2).

3.4 Discussion

We studied several clinical variables in patients with pathogenic *SCN1A* variants to determine those that can be used as predictors for the development of Dravet syndrome and its severity. The most novel finding of our study is negative influence of longer CIM use on cognition in Dravet syndrome patients. Previous studies have suggested that appropriate treatment may have a positive effect on long term cognitive outcomes in Dravet syndrome.[25,59,84,85] To our knowledge, only one study tried to correlate CIM to cognitive outcomes and found no effect;[228] however, only 17 participants (25%) had used CIM and none of them had taken it for more than 3 months. In our current study, 86% of Dravet syndrome patients had used CIM and the median duration of use in the first five years of disease was 11 months. We identify a significantly higher probability of a worse cognitive outcome, as well as a more severe cognitive decline in the first five years of the disease, when CIM was used for longer periods of time. Although reliable data on seizure frequency during CIM use are not available for our cohort, increases in seizure frequency due to sodium channel blocker use have been reported previously.[5,47,83] High seizure frequency has been associated with a worse cognitive outcome,[26,33] and similar findings have recently

been shown in *SCN1A*+ mice.[45] Furthermore, if CIM is used, indicated medication is less likely to be prescribed, and therefore the negative effects of CIM can also partly be due to the deprivation of optimal treatment. It is therefore likely that an increased seizure severity, at least to some extent, explains the worse cognitive outcome associated with longer CIM use, in accordance with the epileptic encephalopathy disease model. However, since we did not observe an association between longer CIM use and ICU admittances (data not shown), the worse cognitive outcome is probably not due to an increase in status epilepticus. Furthermore, multiple authors have suggested that cognition in Dravet syndrome patients declines independent of seizures due to the direct impact of sodium channel dysfunction on cognitive processes,[42,228] which may also explain the negative effects of CIM use on cognition.

We specifically observed the largest effect of CIM use in the second year of disease, indicating that the developing brain might be particularly sensitive to CIM during this timeframe. This finding emphasizes the importance of an early diagnosis and correct clinical management as soon as possible in the disease course. We suggest that *SCN1A* analysis should be considered in every child with atypical febrile seizures at the moment that maintenance treatment is indicated. Not surprisingly, older patients were more likely to be diagnosed at a later age and to have used CIM for a longer period of time, likely due to the upcoming availability of *SCN1A* genetic testing and evolving insights regarding best treatments. Better cognitive outcomes and a shorter duration of CIM use are to be expected if a younger and more recent cohort would be studied. Future research could therefore also focus on the effects of indicated treatment in this patient group.

There are a few limitations to our strategy of IQ/DQ interpolation. Different testing methods have been used, and IQ scores are not necessarily completely comparable to DQ scores. Furthermore, interpolated values become less reliable as the difference increases between the ages at which the tests are performed, as deterioration might not have been linear but fluctuated or logarithmic instead. This can especially be a problem when a first assessment is performed a long time after a period of steep decline. Most importantly, it is likely that development had already began to decrease before it was first observed by parents or clinicians, and while the assumption of an IQ/DQ of 100 before this moment might be correct for the group as a whole, in reality individual scores will differ. However, the same estimation was made for patients with longer and shorter CIM use and it therefore has probably not affected our results: repeated analyses without the 29 patients for which estimated IQ/DQ scores were used and analyses in which the IQ/DQ scores before the first observation of delay were estimated to be 85 instead of 100 yielded very similar results. Ideally, CIM use should be tested in a prospective cohort with regular IQ/DQ assessments at similar ages for all participants, including an assessment before developmental delay is present. However, because of the known negative effects of CIM use this is not ethically responsible. Our method, while probably not completely accurate, seems to lead to a close

Table 3.3. Regression analyses

	3a. Outcome: Dravet syndrome vs. non-Dravet. Binomial regression, all patients (Nagelkerke R^2=0.831)			3b. Outcome: "Mild" vs. "severe" cognitive dysfunctioning. Binomial regression, patients with disease onset >5 years ago (Nagelkerke R^2=0.647)			3c. Outcome: Cognition scores at inclusion (1-5)[1]. Ordinal regression, patients with disease onset >5 years ago (X^2=51.84)			3d. Outcome: interpolated IQ/DQ[2] scores after 5 years of disease. Linear regression, patients with disease onset >5 years ago (R^2=0.386)		
Variable	**Odds ratio**	**95% CI**	**p**	**Odds ratio**	**95% CI**	**p**	**Odds ratio**	**95% CI**	**p**	**B**	**95% CI**	**p**
Age at inclusion	1.074	0.992 - 1.162	0.077	1.250	1.050 - 1.488	**0.012**	1.105	1.036 -1.179	**0.002**			
Age at first seizure	0.956	0.824 - 1.110	0.558	0.919	0.657 - 1.284	0.620						
Age at first afebrile seizure[3]	0.101	0.035 - 0.287	**<0.0005**	0.341	0.139 - 0.835	**0.019**	0.644	0.380 - 1.093	0.103	3.658	-0.920 - 8.236	0.116
ICU[4] admittance	6.044	1.097 - 33.294	**0.039**									
Secondary seizure types[5] <12 months	5.450	0.655 - 45.354	0.117	0.275	0.056 - 1.358	0.113						
Variant type (truncating/splice-site versus missense)	16.591	2.253 - 122.178	**0.006**									
Age at first observation of developmental delay				0.238	0.089 - 0.636	**0.004**	0.394	0.255 - 0.607	**<0.0005**	9.510	6.104 - 12.917	**<0.0005**
Mosaicism				1.981	0.110 - 35.552	**0.643**						
Duration of CIM[6] use in first 5 years of disease				1.091	1.012 - 1.175	**0.022**	1.057	1.022 - 1.092	**0.001**	-0.267	-0.469 - -0.065	**0.010**

[1]Based on available data on IQ and developmental level, adjusted for age at assessment (1 = no ID (IQ or developmental quotient (DQ) >85), 2 = borderline ID (IQ or DQ 70-85), 3 = mild ID (IQ or DQ 50-70), 4= moderate ID (IQ or DQ 30-50), 5 = severe or profound ID (IQ or DQ <30). When no (recent) IQ or DQ was available, the assessment was made based on function in school, communication and adaptive behavior. [2]DQ = developmental quotient. [3]Defined as seizures with a body temperature below 38°C, or seizures for which medical records reported "no fever" without mentioning the exact temperature. [4]ICU = intensive care unit. [5]Absences, myoclonias, focal seizures and focal seizures with impaired awareness.[6] CIM = contra indicated medication (sodium channel blockers: lamotrigine, phenytoin, carbamazepine, oxcarbazepine, vigabatrin.

approximation of the real situation, as all different approaches, including our main analysis, show the same effect of CIM use on cognition.

A previous study has suggested that older patients respond differently to CIM use than younger patients.[229] While there is anecdotal evidence for improved cognition after withdrawal of CIM following long term use in adults,[26] there are also adult patients in which CIM discontinuation cannot be established because of increased seizure severity.[229] It is hypothesized that this could be due to secondary lesions or compensatory mechanisms after a long disease course, leading to seizures that respond to sodium channel blockers.[229] Eighty-one percent of adults in our cohort have a severe or profound intellectual disability (score 4-5), and only one patient had never used CIM. The small group of mildly affected participants (score 1-3, n=8) gives us little power to show an effect of CIM use in adulthood. We can therefore not state whether continued CIM use at higher ages results in further cognitive deterioration. Ideally, a cohort of adults would be analyzed prospectively, with functional assessment before and after withdrawal. If discontinuing CIM in adult patients is shown to have positive effect on function or reduces further decline, this would advocate for changing medication even in patients that have acceptable seizure control at the moment of diagnosis.

Although CIM seems to be able to influence cognitive outcomes, it only explains a small part of the differences in cognitive capabilities: adding duration of CIM use to the multivariable binomial regression model increased the explained variance by 14% (Nagelkerke R^2: from 0.498 to 0.647). There are many patients that have used CIM for only short periods of time and still have a severe cognitive outcome, and IQ/DQ scores after 5 years of disease ranged over 60 IQ/DQ points for participants that had used CIM for almost the complete duration of that period. It is very likely that other genetic factors have a substantial influence on cognitive outcomes and also on responses to CIM, as multiple genetic modifiers have already been implicated to influence *SCN1A* phenotypes[189,197,212,226] and responses to anti-epileptic medication in general.[230] Further research investigating the role of other genetic modifiers and pharmacogenetics is needed. However, even if CIM use is responsible for only a small part of cognitive decline, it should still be avoided to optimize disease courses in individual patients. An early diagnosis is crucial in achieving this result.

We have also studied several other clinical variables in patients with pathogenic *SCN1A* variants. We identified that having a first afebrile seizure at a young age is the most significant predictor for seizures to evolve into Dravet syndrome: 96% of patients experiencing afebrile seizures before age 24 months developed Dravet syndrome, in contrast to 20% of patients that did not. Age at the first afebrile seizure was shown to be a better predictor than age at seizure onset, a variable that was previously mentioned as the most relevant parameter associated with Dravet syndrome.[17] Having a truncating or splice-site variant, a well-known predictor,[117] added significant predictive value to the regression model, as was expected. ICU admittances were also significantly associated with the outcome. Interestingly, six non-Dravet syndrome patients (13%) also experienced ICU admittances. These patients

were not classified as having Dravet syndrome because of their readily achieved seizure control and absence of intellectual disability at the time of inclusion. Having secondary seizure types at a young age, previously described as having predictive value,[59,227] was in our cohort only significant in univariable analysis.

A younger age at first afebrile seizure was also shown to be a significant predictor in analyses for the cognitive outcome in Dravet syndrome, together with a longer duration of CIM use and an earlier onset of developmental delay. While age at the start of developmental delay was already identified as a significant prognostic factor,[5] age at first afebrile seizure is not mentioned in previous studies. The fact that age at the first afebrile seizure is a significant predictor for distinguishing between Dravet syndrome and non-Dravet, and for the severity of Dravet syndrome itself, provides support for the theory that FS, GEFS+ and Dravet syndrome are all part of the same disease spectrum, as previously proposed.[59,104] Having secondary seizure types at an early age, another variable that was found to be significant in other studies,[5,52,228] was again only significant in a univariable analysis.

3.4.1 Conclusion

Our data suggest that longer use of CIM in the first five years of disease may have negative effects on cognitive outcomes in Dravet syndrome patients and should therefore be avoided. An early diagnosis is essential to achieve this result. The negative effect of CIM might be mediated through a direct impact on cognitive processes and/or an increased seizure frequency, caused by the medication itself or by deprivation of indicated treatment. We furthermore identified age at first afebrile seizure as an important predictor for evolution of seizures into Dravet syndrome, as well as for the severity of Dravet syndrome, which can be used to counsel parents of young patients with *SCN1A*-related seizures.

Table S3.1. Diagnostic accuracy of analyzed variables

Variable	1a: all participants prediction of Dravet syndrome					1b: Dravet syndrome patients prediction of a severe outcome (score 4-5)				
	Sensitivity (%)	Specificity (%)	PPV[1] (%)	NPV[2] (%)	ROC AUC[3]	Sensitivity (%)	Specificity (%)	PPV[1] (%)	NPV[2] (%)	ROC AUC[3]
Age at first seizure (months)						95	21	75	64	0.705
0-6	74.8	90.0	95.5	57.9	0.828					
0-12	97.4	27.3	77.7	80	0.623					
Age at first seizure without fever (months)						92	24	77	54	0.792
0-12	65.4	100	100	54.9	0.827					
0-24	90.7	91.1	96.0	80.4	0.909					
ICU[4] admittance (yes)	47.4	87.5	90	41.2	0.674					
Secondary seizure types[5] <12 months (yes)	58.6	93.8	95.8	48.4	0.762	95	6.1	72	33	0.675
Mutation type										
F/N/R[6]	50.9	95.8	96.7	44.7	0.733					
F/N/R/S	62.1	89.8	93.5	49.4	0.758					
F/N/R/S/pore region	95.5	29.2	74.8	48.3	0.581					
Age at first observation of developmental delay						89	38.7	79	57	0.797
Mosaicism (yes)	6.9	97.9	88.9	30.1	0.524	97.6	12.1	73.6	66.7	0.647

[1]Positive predictive value. [2]Negative predictive value. [3]Receiver operating characteristic area under curve. [4]Intensive care unit. [5]Absences, myoclonias, complex partial and focal seizures. [6]F = frameshift, N = nonsense, R = rearrangement, S = splice site.

Table S3.2. Regression analyses for CIM use in separate years and combined years of disease on IQ/DQ scores after 5 years of disease (all Dravet syndrome patients)

	2a. Univariable linear regressions				2b. Multivariable regression[1]			
Year of disease	**Adjusted R^2**	**B**	**95% CI**	**p**	**Adjusted R^2**	**B**	**95% CI**	**p**
1	0.020	-1.188	-2.618 - 0.243	0.102		0.008	-1.471 - 1.486	0.992
2	0.187	-2.082	-3.002 - -1.163	**<0.0005**		-1.961	-3.106 - -0.727	**0.002**
3	0.079	-1.222	-2.072 - -0.372	**0.005**	0.198	-0.746	-1.852 - -0.359	0.183
4	0.032	-0.762	-1.672 - 0.149	0.100				
5	0.012	-0.427	-1.286 - 0.431	0.325		0.473	-0.489 - -1.435	0.33
1-2	0.155	-1.300	-1.942 - -0.659	**<0.0005**				
1-3	0.184	-0.939	-1.359 - -0.519	**<0.0005**				
2-3	0.167	-1.057	-1.554 - -0.560	**<0.0005**				
3-5	0.034	-0.319	-0.638 - -0.001	0.050				
4-5	0.010	-0.308	-0.763 - 0.146	0.181				

[1]Year 4 was removed from the multivariable regression model because high correlation coefficients were seen with year 3 and year 5 (>0.7).

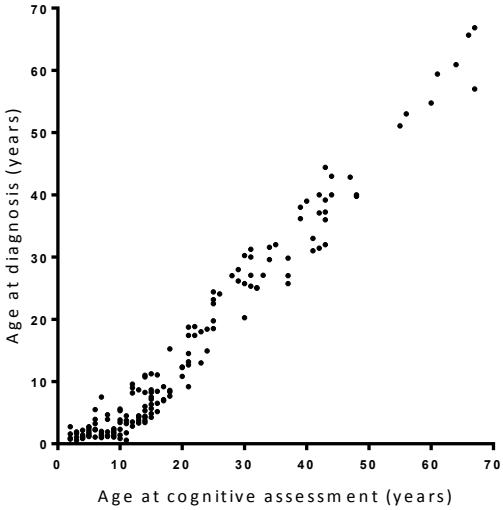

Figure S3.1. Age at cognitive assessment plotted for age at diagnosis.

Part 2

Advanced genotyping in *SCN1A*-related epilepsy

Chapter 4

Mosaicism of *de novo* pathogenic *SCN1A* variants in epilepsy is a frequent phenomenon that correlates with variable phenotypes

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Published: Epilepsia. 2018 Mar;59(3):690-703.

Abstract

Objective: Phenotypes caused by *de novo SCN1A* pathogenic variants are very variable, ranging from severely affected patients with Dravet syndrome to much milder GEFS+ cases. The most important determinant of disease severity is the type of variant, with variants that cause a complete loss of function of the *SCN1A* protein (α-subunit of the neuronal sodium channel Nav1.1) being detected almost exclusively in Dravet syndrome patients. However, even within Dravet syndrome disease severity ranges greatly, and consequently other disease modifiers must exist. A better prediction of disease severity is very much needed in daily practice to improve counseling, stressing the importance of identifying modifying factors in this patient group. We evaluated 128 participants with *de novo*, pathogenic *SCN1A* variants to investigate whether mosaicism, caused by postzygotic mutation, is a major modifier in *SCN1A*-related epilepsy.

Methods: Mosaicism was investigated by re-analysis of the pathogenic *SCN1A* variants using single molecule molecular inversion probes and Next Generation Sequencing with high coverage. Allelic ratios of pathogenic variants were used to determine whether mosaicism was likely. Selected mosaic variants were confirmed by ddPCR and sequencing of different tissues. Developmental outcome was classified, based on available data on IQ and school functioning/education.

Results: Mosaicism was present for 7.5% of *de novo* pathogenic *SCN1A* variants in symptomatic patients. Mosaic participants were less severely affected than non-mosaic participants if only participants with truncating variants are considered (distribution of developmental outcome scores, Mann-Whitney U p=0.023)).

Significance: Postzygotic mutation is a common phenomenon in *SCN1A*-related epilepsies. Participants with mosaicism have on average milder phenotypes, suggesting that mosaicism can be a major modifier of *SCN1A*-related diseases. Detection of mosaicism has important implications for genetic counseling and can be achieved by deep sequencing of unique reads.

4.1 Introduction

De novo pathogenic *SCN1A* variants are found in the majority of Dravet syndrome patients.[108,110,111,162] Dravet syndrome is characterized by onset in the first year of life, with generalized or unilateral clonic seizures triggered by fever, illness or vaccination as first symptoms. Other seizure types often develop at a later stage and prolonged status epilepticus can occur. Psychomotor development slows, usually in the second year of life, resulting in mild to severe intellectual disability (ID) in most patients. In addition, many patients experience walking difficulties and behavioral problems.[3,5,61] Pathogenic variants in *SCN1A* are also found in patients with the much milder Genetic Epilepsy Febrile Seizures Plus (GEFS+) syndrome or febrile seizures only.[108]

SCN1A encodes for the α-subunit of a neuronal sodium channel, Nav1.1 (Figure 1.1). The main disease mechanism of *SCN1A*-related phenotypes is haploinsufficiency, caused by complete or partial loss of function. Truncating variants are expected to lead to a complete absence of expression of the mutant allele and thus to complete haploinsufficiency of Nav1.1. These variants are virtually always associated with severe phenotypes:[59,117,130] 97.7% of genomic rearrangements, splice site-, nonsense- and frameshift *SCN1A* variants are mainly associated with Dravet syndrome.[117] The effect of pathogenic missense variants is more difficult to predict. Functional studies have shown varying degrees of loss of function through a lack of sodium current when pathogenic variants are located in critical regions of the gene (voltage sensor and/or ion-pore regions).[59,117,129,130,157] Most pathogenic missense variants associated with Dravet syndrome lead to a complete loss of sodium current, whereas missense pathogenic variants associated with milder phenotypes, such as GEFS+, lead to milder disturbances of channel function.[117] The location of missense variants is a strong indicator for the severity of channel disruption. However, it still cannot predict the effect of the variant on channel function and disease severity fully.[59,117,129,130] The same is true for physicochemical property changes due to missense variants.[130,158]

Although type and location of pathogenic *SCN1A* variants are a major determinant of disease severity, a significant disease variability remains unexplained; a wide phenotypic variability between patients with Dravet syndrome exists[9,25,139] and variable phenotypes have even been associated with the same pathogenic variant.[129,162,231] These different *SCN1A*-related phenotypes may be indistinguishable at their first presentation, often leaving parents in great uncertainty about the future of their children when a pathogenic *SCN1A* variant is found early in life. A better prediction of disease severity is very much needed in daily practice to improve counseling, stressing the importance of identifying modifying factors in this patient group. Several modifying factors have already been suggested, such as variants in the *SCN1A* promoter region and in 5'- and 3' untranslated regions, and variants in other genes.[25,189,198,226,232] Moreover, parental mosaicism for the pathogenic *SCN1A* variant has been well recognized in cases were mosaic parents of Dravet children show a mild epilepsy phenotype. This observation suggests that postzygotic pathogenic variants may be present

in a significant percentage of carriers of *de novo* pathogenic *SCN1A* variants and that the percentage of mosaicism can affect the severity of the disease.[167–169,233] However, these are results from single case reports or studies aimed at the detection of low-grade parental mosaicism. A study to investigate the occurrence and effect of high-grade mosaicism in patients with *de novo SCN1A* pathogenic variants themselves has never been reported to our knowledge. Because mosaicism has been known to influence expression in other diseases,[164] we here investigate whether mosaicism is a common phenomenon in *de novo* pathogenic *SCN1A* variants, and whether it has a substantial effect on the severity of the disease. If both are true, then mosaicism might be a major modifier for *SCN1A*-related phenotypes, which can help predict the disease course. We here describe testing for mosaicism in 128 participants with *de novo* pathogenic *SCN1A* variants, using single molecule molecular inversion probes (smMIPs) and Next Generation Sequencing (NGS), to determine the incidence and clinical effects of mosaicism.

4.2 Materials and Methods

4.2.1 Participants and clinical data

Participants

A cohort of 128 participants with *de novo SCN1A* pathogenic variants was evaluated. Only participants with (likely) pathogenic *SCN1A* variants (class 4 and 5) were included (see supplemental data S4.1. *Severity classification of pathogenic variants* for more details). Multiplex Ligation-dependent Probe Amplification (MLPA) was performed in all patients to exclude deletions or duplications. Since mosaicism is not expected in inherited pathogenic variants, we included only participants with *de novo* pathogenic variants (confirmed *de novo* by Sanger sequencing or NGS: n=107; presumed *de novo* (negative family history) in the absence of parental DNA: n=21). Since we aim to improve counseling in patients with *SCN1A* pathogenic variants detected in clinical care, all individuals with pathogenic variants detectable by standard diagnostic procedures were considered for inclusion. 124 participants exhibited epilepsy or febrile seizures (Dravet syndrome: n=106, according to criteria published previously)[217]. The other four participants were fathers of children with Dravet syndrome (n=3) and the oldest member of a GEFS+ family (n=1), who carried the same causal *SCN1A* pathogenic variant as their children but did not have any seizures themselves. In two of these four asymptomatic participants and in two others, mosaicism was already suspected based on their diagnostic results (Sanger sequencing or MLPA), one of whom has been described earlier.[170] Diagnostic testing in the four asymptomatic participants was only performed after their children were diagnosed, so one could argue that they should not have been included. Our main analyses are therefore performed without these participants, in order to achieve results applicable to symptomatic patients, which

is clinically most meaningful. We however repeated the analyses including them, since asymptomatic carriers of pathogenic *SCN1A* variants have a higher a priori probability of being mosaic. By including them in our analyses we achieve a more complete understanding of the effects of mosaicism as a modifier, as it gives the opportunity to investigate at what grade of mosaicism symptoms arise.

All eligible individuals known to the University Medical Center Utrecht were approached, to avoid any selection bias for milder phenotypes or known mosaic cases. Informed consent was obtained from participants or their legal caretakers according to the Declaration of Helsinki. The study was approved by the Ethical Committee of the University Medical Center Utrecht.

Clinical data and statistical analyses

Detailed clinical data were collected from medical records for all participants, and a semi-structured telephone interview was conducted when possible (n=118). Furthermore, for a subset of participants, based on age, the PedsQL Measurement Model questionnaire was completed, to measure health-related quality of life for participants aged 0-25 years.[234] A classification of the developmental outcome was made, rated in a consensus meeting by a child neurologist, neuropsychologist, and clinical geneticist who were blinded for the outcome of the mosaicism assessment. Developmental outcome was rated on a five-point scale based on available data on IQ and developmental level, adjusted for age at assessment (1= no ID (IQ or developmental quotient (DQ) >85), 2 = borderline ID (IQ or DQ 70-85), 3 = mild ID (IQ or DQ 50-70), 4= moderate ID (IQ or DQ 30-50), 5 = severe or profound ID (IQ or DQ <30)). When no (recent) IQ or DQ was available, the assessment was made based on school functioning, communication and adaptive behavior. Differences in outcomes between mosaic participants and non-mosaic participants were analyzed (Mann-Whitney U test for cognitive development and age at seizure onset; Fishers' exact test for seizure severity and age at first notice of developmental delay; independent samples T-test for PedsQL results;), for symptomatic participants as well as for the complete group of participants. We furthermore performed a second analysis for both groups in which only participants with truncating variants were assessed, to control for variation in outcomes due to the variant types themselves. This group comprises patients with frameshift and nonsense variants, large rearrangements and splice site variants leading to frameshifts, that can all be expected to lead to a similarly severe channel dysfunction of *SCN1A*. This in contrast to missense variants, for which the precise effects on channel function cannot be accurately predicted.

4.2.2 Molecular analyses

Mosaicism screening by smMIPs and NGS

All *SCN1A* exons were captured by smMIPs, as described earlier,[235] and sequenced (see

supplemental data S4.2. *smMIP capture and sequencing* for more details). The resulting data were analyzed using commercial software (SeqNext module of Sequence Pilot; JSI medical systems, Ettenheim, Germany) (see supplemental data S4.3. *JSI medical systems Sequence Pilot/SeqNext settings* for more details). Reads with the same single-molecule tag were assembled into one consensus read, to correct for PCR and sequencing artefacts. In addition, the molecular tag discriminates unique reads from PCR duplicates, allowing the determination of quantitative sequence coverage of reads originating from unique DNA molecules. *SCN1A* pseudogene reads were removed from alignment and analysis. The earlier identified pathogenic variants were located and the percentages of mutated reads were used to determine whether mosaicism for these pathogenic variants was likely based on a binomial distribution. Only pathogenic variants with a coverage of at least 20X were initially analyses. Two types of technical artefacts were identified, and pathogenic variants with a deviating alternative allele frequency (AAF) influenced by these were discarded as possible mosaics (see supplemental data S4.4. *Technical artefacts smMIP data* for more details).

Statistical analysis of smMIP data

We expect true heterozygous variants to follow a binomial distribution, in which variants with a higher coverage (= number of observations) will have an AAF closer to 0.5. For each pathogenic variants *p*-values were calculated (X^2 –test), to test whether the AAF deviated significantly from 0.5 (see supplemental data S4.5. *Determining the p-value threshold for mosaic pathogenic variants* for more details).

Confirmation by droplet digital PCR

Part of the samples with screening results compatible with mosaicism were re-evaluated by ddPCR (see supplemental data S4.6. *ddPCR* for more details).

Confirmation in other tissues

DNA from buccal cells, saliva, urine and/or brain tissue could be obtained for five participants that were suspected of mosaicism based on smMIP screening, and was analyzed with smMIPs and NGS or ddPCR as described above. No tissue samples were available for the other mosaic participants.

4.3 Results

4.3.1 Statistical analysis of mosaicism screening by smMIPs

NGS results were obtained for 122 out of 128 participants (Figure 4.1). The pathogenic variants of six participants could not be detected by smMIPs due to the nature of the variants (deletions or duplications spanning more than one smMIP, with no heterozygous

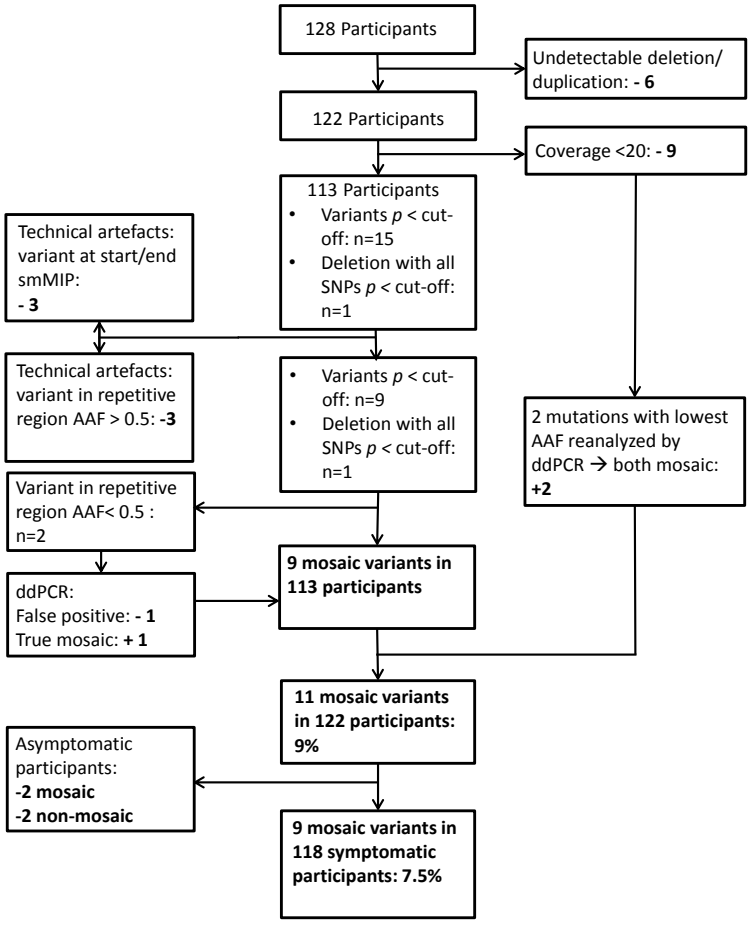

Figure 4.1. Flowchart of detected mosaic pathogenic variants.

SNPs present in that region to deduct possible mosaicism). Nine other participants did not meet the threshold of 20X coverage at the site of their pathogenic variant. The average unique read depth on the location of the known pathogenic variant of the 113 remaining participants was 1599X (ranging from 20X-7320X, median: 1281X).

Fifteen pathogenic variants had *p*-values below the mosaicism cut-off. Six were discarded as possible mosaic variants due to the technical artefacts described earlier. Among the remaining nine were two pathogenic variants in a repetitive region with an AAF<0.5 that were selected for further testing by ddPCR. This confirmed true mosaicism in one and disproved it in the other, leaving eight true mosaic pathogenic variants. All mosaic participants carried at least one neutral SNP with an AAF close to 0.5, excluding additional deletions or duplications of *SCN1A* as the cause for their deviating AAF.

The pathogenic variant in one of the participants of which the SNP data were regarded as outliers was an *SCN1A* deletion of exon 2-23. Results of diagnostic genetic testing by

MLPA had already suggested mosaicism for this deletion in 50% of cells. The significantly deviating AAFs of the SNPs present in this region confirmed mosaicism (alternative allele frequency 0.72-0.75, p = $1.75x10^{-34}$ – $3.63x10^{-55}$, Figure 4.2), thereby raising the number of mosaic participants detectable by smMIPs to nine out of 113 (7.9%). AAFs of the mutated alleles ranged between 0.10 and 0.39 (Table 4.2).

As mentioned above, for nine participants insufficient coverage was reached (<20X), and 14 others had a coverage <100X. With these read depths high-grade mosaicism is virtually impossible to detect at our level of significance: at 100X an AAF of <0.25 is necessary to drop below the *p*-value threshold for significance, and at 20X even zero alternative allele reads only give a *p*-value of $7.7x10^{-6}$, still above the threshold. We therefore reanalyzed two low-coverage pathogenic variants with the lowest AAFs (<0.25) with ddPCR.

4.3.2 Droplet Digital PCR

DdPCR was used to reanalyze seven likely mosaic pathogenic variants detected by NGS, including the two poorly covered pathogenic variants described above. It was not feasible to design functional ddPCR essays for each likely mosaic pathogenic variant, so a selection was made based on the expected specificity of the probes. As mentioned earlier, one pathogenic variant in a repetitive region was disproved (Figure 4.2). The other six, including the two low-coverage variants, were confirmed as mosaics (Table 4.1). This raises the final number of mosaic participants in our complete cohort to 11 out of 122 (9%). For symptomatic patients only, the incidence is 9 out of 118 (7.5%).

4.3.3 Sequencing of other tissues

DNA from buccal cells, saliva, urine and/or brain tissue was analyzed with smMIPs in five mosaic participants. In all five mosaicism could be confirmed, in most with similar AAFs as in blood (Table 4.1).

4.3.4 Read-frame restoring mosaic pathogenic variant

In one participant (6), not only her known frameshift pathogenic variant (insG) was seen at the variant site, but also a missense variant three basepairs upstream, that had not been identified in diagnostic testing. The known frameshift pathogenic variant was present in ~32% of the reads, and the second variant in ~13% of the reads (supplemental Figure S4.1A), together accounting for ~50%. No reads were present with both variants, proving independent haplotypes. We hypothesize that at first a heterozygous insG pathogenic variant was present, and that in a subpopulation of cells this allele acquired another variant, a delT three basepairs upstream. This leads to restoring of the original reading frame along with a T>G missense variant, resulting in mosaicism for both the frameshift and the new missense variant in different cell populations (supplemental Figure S4.1B). The missense variant is estimated to be less severe than the frameshift pathogenic variant, as it is located in pore loop 1. DdPCR confirmed that both variants were present in less than 50% of

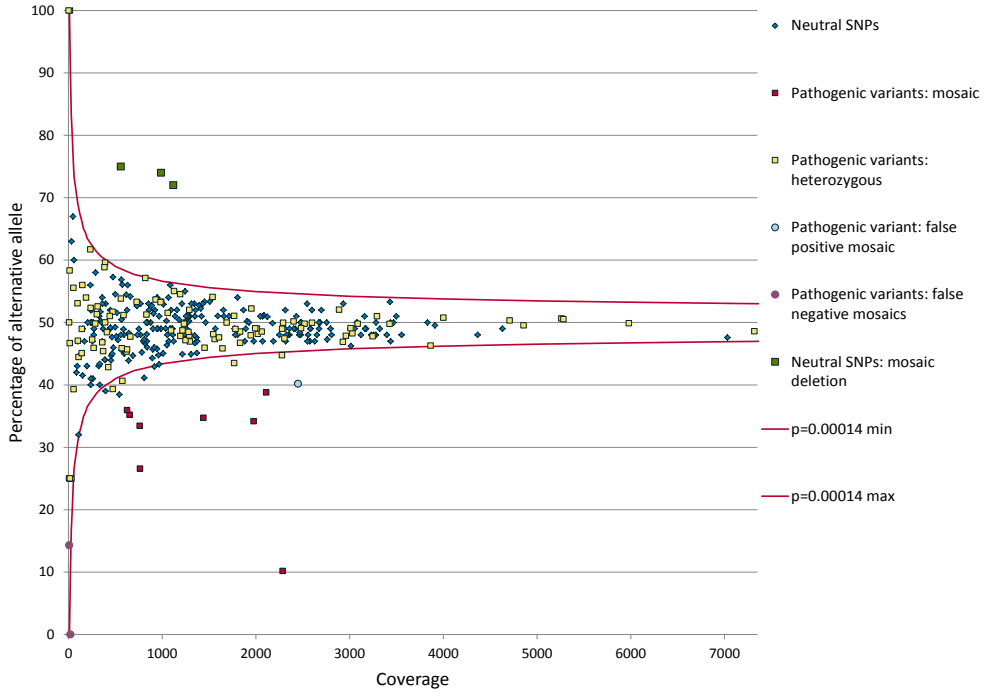

Figure 4.2. Overview of neutral SNPs and pathogenic variants. For each analyzed neutral SNP or pathogenic variant the percentage of alternative allele and achieved the unique coverage at its location is depicted.

alleles, with more insG than delT alleles, although no exact percentages can be given since the Poisson statistics implemented in the software are not appropriate to calculate this in a three-allelic situation.

4.3.5 Phenotypic features and statistical analysis of clinical outcomes

Clinical features and molecular details of the 11 mosaic participants are summarized in Table 4.1. Two participants were clinically unaffected. Overall, 54 participants carried truncating variants, of which ten belonged to the mosaic participants. No statistical significantly differences were seen in disease severity when comparing all symptomatic participants (Table 4.2). However, the mosaic group contained a much higher percentage of truncating variants (89% versus only 40% in the non-mosaic group). Therefore, we also compared the outcomes of participants with mosaic and non-mosaic truncating variants only (Table 4.2), so our analysis is not biased by differences in severity based on the variant type itself. In this group, statistically significant differences regarding developmental outcome were seen in favour of mosaic patients (median score 3 (mild ID) in mosaic participants versus median score 4.5 (moderate to severe ID) in non-mosaic participants, p=0.023 (Mann-Whitney U test)). Mosaic participants furthermore had a later onset of developmental delay (86% after

Table 4.1. Clinical features of mosaic participants

Participant	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Age (years)	10	60	10	6.5	25	15	44	39	7	41	43
Gender	Male	Male	Female	Female	Female	Female	Male	Male	Male	Male	Male
Pathogenic variant	c.622_657del insT, p.Asp208fs	c.C3430-3G, splicing	c.5193delA, p.Ile173fs	c.C5348T, p.Ala1783Val	Deletion exon 2-23	c.982insG, p.Glu328fs and c.T980G, p.Leu327Arg	c.1537delG, p.Glu513fs	c.992delT, p. Leu 331*	c.4262_4275del14, p.Gly1421fs	c.G3880-1A, splicing	c.G603-1T, splicing
Truncating variant	Yes	Yes	Yes	No	Yes	Yes and no	Yes	Yes	Yes	Yes	Yes
smMIPs: % of alternative allele in blood (total read depth at variant location)	35% (653X)	35% (1440X)	33% (760X)	36% (626X)	n.a.: ~50% of cells (MLPA), 72-75% (SNPs) (559-1119X)	32% and 13% (1968X)	27% (764X)	39% (2210X)	14% (7X)	0% (20X)	10% (2285X)
ddPCR: % of mosaicism in blood			38%			Both mosaic	27%	38%	28%	14%	
% of alternative allele other tissues			28% in buccal cells (smMIPs: 354X)	38% in buccal cells (smMIPs: 32X)		34% and 12% in buccal cells (smMIPs: 176X)	8% in saliva (26X) and 0% in urine (10X) (smMIPs)		16% in brain tissue (ddPCR)		
Syndrome diagnosis	Dravet	GEFS+	Dravet	Dravet	Dravet	Dravet	Dravet	Dravet	Dravet	Unaffected	Unaffected
ID	+	-	+	+	+	+	+	+	+	-	-
Developmental outcome[a]	3	1	3	5	5	3	2	5	2	1	1
Age at first notice of developmental delay (months)	30	n.a.	38	18	12	36	72	36	60	n.a.	n.a.
Seizure severity[b]	3	0	1	4	2	Major sz: 1; Minor sz: 4	0	Major sz: 3; Minor sz: 4	Major sz: 2; Minor sz: 4	0	0

Participant	1	2	3	4	5	6	7	8	9	10	11
Status epilepticus (number of experienced episodes)	1	0	1	2	12	1	0	2	2	0	0
Seizure types[c]	GTCS, hemiconvulsions, absences, tonic, myoclonias	GTC	GTCS, atonic, CP, absences	GTCS, CP, focal, hemiconvulsions, absences, tonic, myoclonias,	GTCS, focal, CP, tonic	GTCS, hemiconvulsions, focal, CP, absences, myoclonias, tonic	GTCS, absences (possibly other types)	GTC, CP, absences, myoclonias	GTC, CP, hemiconvulsions, atonic, absemnces, myoclonias, tonic	n.a.	n.a.
Current treatment[d]	VPA, TPM	PB	VPA, LEV	TPM, LEV, STP	LEV, OXC, TPM	TPM, VPA	-			n.a.	n.a.
Age at seizure onset (months)	5	24	12	3	5	6	8	3	11	n.a.	n.a.
Behavior difficulties[e]	+	-	+ (ASS)	+	+ (ASS)	+ (aggressive, impulsive)	+	+ (ADHD, autism, aggressive, often angry)	ADHD	-	-
Walking difficulties	Ataxia, endorotation feet, pes plano valgus (surgery planned)	-	Pes plano valgus, orthopedic shoes, hypotonia, cannot run well	Ataxia, wears walking frame outside	Ataxia, mostly wheelchair dependent	Genua valga, arch support necessary but can play sports	-	Ataxia, orthopedic shoes	-	-	-
Comorbities[f]					Acute metabolic encephalopathy (Reye syndrome) at age 7 caused regression			SUDEP at age 39	SCAD, SUDEP at age 7 years		

n.a. = not applicable, MLPA = Multiplex ligation-dependent probe amplification. [a]1 = no learning disability, 2 = borderline learning disability, 3 = mild learning disability, 4 = moderate learning disability, 5 = severe or profound learning disability. [b]Currently. 4 = daily seizures, 3 = weekly seizures, 2 = montly seizures, 1 = yearly seizures, 0 = seizure free. Minor sz: short absences, short focal seizures or myoclonias. Major sz: all other seizure types with loss of consciousness, or prolonged seizures (specified only if big and small seizures do not have the same score). [c]Currently and/or in the past. GTC = generalized tonic-clonic, CP = complex partial. [d]VPA = valproic acid, TPM = topiramate, LEV = levetiracetam, OXC = oxcarbazepine, STP = stiripentol, VNS = vagus nerve stimulation, PB = phenobarbital. [e]ADHD = attention deficit hyperactivity disorder, ASS: autism spectrum disorder. [f]SCAD = short-chain acyl-CoA dehydrogenase deficiency, SUDEP = sudden unexpected death in epilepsy.

24 months versus 40% of non-mosaic participants, p=0.022 (Fisher's exact test)). A trend for later seizure onset (median 7 months versus 5 months, p=0.054 (Mann-Whitney U test)) and for lower seizure frequency of major seizures (50% "rarely" versus 16% "rarely", p=0.051 (Fishers' exact test)) was observed in the mosaic participants compared to non-mosaic participants. Repeated analyses, including the four asymptomatic participants, showed similar results (Table 4.3).

4.4 Discussion

4.4.1 Prevalence of mosaicism in *SCN1A* pathogenic variants

Although mosaicism has been detected in many other genetic diseases caused by *de novo* heterozygous pathogenic variants, only a few studies have tried to estimate its prevalence, which ranges from 3.3 to 30%.[236–240] However, these studies all had different designs and inclusion criteria, which makes it difficult to compare results. Two studies are most similar to ours. The first[239] found mosaicism in 6.5% of *de novo* pathogenic variants in severe ID patients. SmMIPs were used in this study as well, but only to confirm possible mosaic pathogenic variants with a deviating AAF, that were detected by other methods. The other study[240] found mosaicism in 0.6-12.5% of pathogenic variants in genes related to epilepsy-related neurodevelopmental disorders, and in 1.3% of *SCN1A* variants specifically. Conclusions were based on the results of multigene epilepsy panels or whole-exome sequencing. Differences in sequencing methods could explain the lower prevalences compared to the current study (mosaicism in 7.5% of symptomatic patients): by not screening with smMIPs, it is likely that some (high-grade) mosaic pathogenic variants were missed. The added value of the single molecule tag to deduplicate PCR copies is highlighted by our results: without deduplication, 4 mosaic participants show AAFs that differ significantly from frequencies after deduplication (5, 8, 10 and 15 percentage point differences). At least two mosaic participants would not have been identified if not for the single molecule tag.

By using MIPs with single molecule tags and high coverage we were able to make very accurate estimates of the percentages of mosaicism, which was confirmed by ddPCR. This makes it possible to distinguish high-grade mosaicism from heterozygous pathogenic variants. Three of four participants in which mosaicism was already suspected or shown, based on earlier clinical results, were indeed detected as a mosaics. The fourth participant did not meet our criteria for mosaicism because of low coverage at his variant site, although sequencing results were still very suggestive of mosaicism, and further testing by ddPCR confirmed this. It is possible that more mosaic pathogenic variants were missed for this reason, since high coverage could not be reached for all regions of *SCN1A*. We recommend reanalysing variants that have a strongly deviating AAF but a coverage that is too low to reach statistical significance, as we did for our two variants with an AAF <0.25. It is also possible to miss mosaics with relatively high coverage, as they require higher coverage to

Table 4.2. Comparison of outcomes between mosaic and non-mosaic participants in clinically affected patients

Group		All symptomatic patients			Symptomatic patients with truncating variants		
		Mosaic patients	Non-mosaic patients	Statistics[a]	Mosaic patients	Non-mosaic patients	Statistics[a]
Number of participants		9	109	-	8	44	-
Mean age (years)		25	19	-	27	15	-
Developmental outcome[b]	1 (n)	1	17		1	1	
	2 (n)	2	7		2	2	
	3 (n)	3	16	p=0.393	3	5	**p=0.023**
	4 (n)	0	27	(Mann-Whitney U, U=409, z=-0.854)	0	14	(Mann-Whitney U, U=87, z=-2.408)
	5 (n)	3	42		2	22	
	Median score	3	4		3	4.5	
Age at first notice of developmental delay (months)[c]	Young (<24)	2 (25%)	52 (58%)	p=0.170 (Fisher's exact)	1 (14%)	26 (60%)	**p=0. 022** (Fisher's exact)
	Older (>24)	6 (75%) (no delay n=1)	38 (42%) (no delay n=16)		6 (85%) (no delay n=1)	17 (40%) (no delay n=1)	
Average age at seizures onset (months)	Mean	8.67 (3-24)	6.98 (1-48)	p=0.294 (Mann-Whitney U, U=571, z=1.050)	9.25 (3-24)	5.20 (1-11)	p=0.054 (Mann-Whitney U, U=251, z=1.941)
	Median	6	5		7	5	
Seizure severity[d]	Major seizures often (2-4)	5 (56%)	77 (71%)	p=0.452 (Fisher's exact)	4 (50%)	37 (84%)	p=0.051 (Fisher's exact)
	Major seizures rarely (0-1)	4 (44%)	32 (29%)		4 (50%)	7 (16%)	
	Minor seizures often (2-4)	5 (56%)	63 (58%)	p=1.000 (Fisher's exact)	4 (50%)	32 (73%)	p=0.231 (Fisher's exact)
	Minor seizures rarely (0-1)	4 (44%)	46 (42%)		4 (50%)	12 (27%)	
Quality of Life[e]	Completed questionnaires	n=4	n=66	p=0.260 (independent samples T-test, t_{68}=1.136)	n=4	n=46	p=0.087 (independent samples T-test, t_{32}=1.765)
	Average (total) score	67.68 (57-86)	56.02 (13-99)		67.68 (57-86)	52.04 (26-98)	

Table 4.3. Comparison of outcomes between mosaic and non-mosaic participants (complete cohort including unaffected participants)

Group		All patients			Patients with truncating variants		
		Mosaic participants	Non-mosaic participants	Statistics[a]	Mosaic patients	Non-mosaic patients	Statistics[a]
Number of participants		11	111	-	10	44	-
Mean age (years)		28	20	-	30	15	-
Developmental outcome[b]	1 (n)	3	19		3	1	
	2 (n)	2	7		2	2	
	3 (n)	3	16	p=0.126	3	5	**p=0.002**
	4 (n)	0	27	(Mann-Whitney U,	0	14	(Mann-Whitney U,
	5 (n)	3	42	U=445. z=-1.530)	2	22	U=88, z=-3.114)
	Median score	3	4		2.5	4.5	
Age at first notice of developmental delay (months)[c]	Young (<24)	2 (25%)	52 (58%)	p=0.135	1 (14%)	26 (60%)	**p=0.039** (Fisher's exact)
	Older (>24)	6 (75%)	38 (42%)	(Fisher's exact)	6 (86%)	17 (40%)	
		(no delay n=3)	(no delay n=18)		(no delay n=3)	(no delay n=1)	
Average age at seizures onset (months)	Mean	8.67 (3-24)	6.98 (1-48)	p=0.294	9.25	5.20	p=0.054
	Median	6	5	(Mann-Whitney U,	7	5	(Mann-Whitney U,
		(no seizures n=2)	(no seizures n=2)	U=571, z=1.050)	(no seizures n=2)	(no seizures n=0)	U=251, z=1.941)
Seizure severity[d]	Major seizures often (2-4)	5 (45%)	77 (69%)		4 (40%)	37 (84%)	
	Major seizures rarely (0-1)	6 (55%)	34 (31%)	p=0.174 (Fisher's exact)	6 (60%)	7 (16%)	**p=0.008** (Fisher's exact)
	Minor seizures often (2-4)	5 (45%)	63 (57%)		4 (40%)	32 (73%)	
	Minor seizures rarely (0-1)	6 (55%)	48 (43%)	p=0.535 (Fisher's exact)	6 (60%)	12 (27%)	p=0.067 (Fisher's exact)
Quality of Life[e]	Completed questionnaires	n=4	n=66		n=4	n=30	
	Average (total) score	67.68 (57-86)	56.02 (13-99)	p=0.260 (independent samples T-test, t_{68}=1.136)	67.68 (57-86)	52.04 (26-98)	p=0.087 (independent samples T-test, t_{32}1.765)

[a]All reported test were performed 2-tailed with an alpha-level for significance of p <0.05. Significant p-values are bolded. [b]Based on available data on IQ and developmental level, adjusted for age at assessment (1 = no ID (IQ or developmental quotient (DQ) >85), 2 = borderline ID (IQ or DQ 70-85), 3 = mild ID (IQ or DQ 50-70), 4= moderate ID (IQ or DQ 30-50), 5 = severe or profound ID (IQ or DQ <30). When no (recent) IQ or DQ was available, the assessment was made based on school functioning, communication and adaptive behavior. Numbers of participants and statistical test results are given for dichotomized scores (score 1-3 = mild, score 4-5= severe). [c]Numbers of participants and statistical test results are given for dichotomized scores (young versus older). [d]Currently. 4 = daily seizures, 3 = weekly seizures, 2 = monthly seizures, 1 = yearly seizures, 0 = seizure free. Small sz: short absences, short focal seizures or myoclonias. Big sz: all other seizure types with loss of consciousness, or prolonged seizures. Numbers of participants are given for dichotomized scores (score 0-1 = rarely, score 2-4 = often). [e] Quality of Life total score, based on results of PedsQL Measurement Model questionnaire.

be reliably detected. For example, a pathogenic variant with an AAF of 0.4 can only fall below the confidence interval (Figure 4.2) at ~1000X or higher. By assuming that these variants are heterozygous while there is still a small possibility that they are in fact mosaic, our result of mosaicism in 7.5% of symptomatic participants might be an underestimation. Furthermore, it is estimated that 7-10% of parents of Dravet syndrome patients are low-grade mosaics for the *SCN1A* pathogenic variants of their offspring.[175] Therefore, it is likely that not all tested pathogenic variants were truly *de novo*. By excluding cases with parental mosaicism the percentage of mosaic participants may increase even more.

Conversely, smMIPs may generate false positive mosaics as well. Skewed AAFs at the ends of each smMIP can be due to the use of a NextSeq, which has lower quality base calls in the first few cycles. Future studies should take this in consideration during their experimental design, since smMIPs always start and end at the same coordinate. Skewed AAFs in repetitive regions should also be interpreted with caution, since this can be due to technical artefacts. By reanalysing two pathogenic variants in repetitive regions by ddPCR, we could confirm mosaicism in one (participant 8), while disproving it in the other.

4.4.2 Mosaicism as a modifier in *SCN1A*-related epilepsy

Overall, participants with mosaic truncating variants were less severely affected than participants with heterozygous truncating variants, suggesting that mosaicism is an important modifier for *SCN1A*-related phenotypes. This is in line with previously published results.[117,171] Remarkably, in two of the 11 mosaic participants (18%) sudden unexpected death in epilepsy (SUDEP) occurred, while this occurred in none of the other 111 patients. This may be pure coincidence, but further studies on a possible association between SUDEP and mosaic *SCN1A* pathogenic variants are warranted. Two of the mosaic participants were clinically unaffected, leading to the estimation that symptoms arise between 14 and 27 percent of mutated allele in blood. This is in line with earlier studies.[117,241] The effects of mosaicism would be underestimated if we did not take into account participants with such low AAFs that they do not show any symptoms. However, by including them in our clinical analysis, our results might not be applicable to the group of symptomatic patients for which we want to improve clinical counseling. Therefore, these participants were excluded from our main analyses (Table 4.2), but included in repeated analyses (Table 4.3). Cognitive abilities usually decline as patients with Dravet syndrome age, which could create a bias in the analyses. However, the mosaic participants are older on average in all analyzed (sub) groups, so the effect of mosaicism on cognitive outcome might even be underestimated.

Interestingly, we also found mosaicism in three severely affected participants (4, 5 and 8). To our knowledge, this is the first report of mosaic *SCN1A* pathogenic variants that do not lead to a relatively mild phenotype. This suggests other modifiers, such as variants in other genes, have a large influence as well. Furthermore, it is likely that the degree of mosaicism is of more importance than just its presence or absence. In addition, levels of mosaicism in lymphocytes do not necessarily correspond to the levels in brain. Clinical

factors such as medication use and comorbidities should also be taken into account. For example, participant 5 got affected by the unrelated Reye syndrome, which caused major developmental regression. Participant 4, 5 and 8 were all treated with sodium channel blockers, which are contraindicated drugs in Dravet syndrome. This emphasizes the importance of accurate clinical management for cognitive development; even patients with a favourable genotype can ultimately have a poor outcome.

4.4.3 Implications for genetic counseling

Besides partially explaining and predicting differences in phenotype severity, our results also reveal other important consequences for genetic counseling. Patients that are only mildly affected because they are mosaic for a pathogenic variant, should be made aware that their children are at risk to be more severely affected, when they inherit the variant in a heterozygous state. Furthermore, mosaicism in a proband virtually rules out germline mosaicism in the parents, which lowers their recurrence risk to zero. Nevertheless, participant 7 shows that simply assessing mosaicism is not enough for accurate counseling. At first sight, she seemed mosaic for her known insG pathogenic variant. However, because we also discovered her mosaic T>G variant three base pairs upstream, we could reason that this was most likely a mosaic read frame-restoring variant of an originally heterozygous insG pathogenic variant. Similar read-frame restoring mosaic variants and human reverse mutations in general have been previously described.[242–245] This is the first report of a reverse mutation in Dravet syndrome. Several hypotheses about the occurrence of reverse mutations have been proposed.[245,246] In Dravet syndrome however, these do not seem applicable, so this might be pure coincidence. Nonetheless, by deducing that the insG pathogenic variant only seemed mosaic but was probably originally heterozygous, we now cannot exclude that one of the patient's parents might be germline mosaic for this variant, with an inherent increased recurrence risk.

Overall, mosaicism is present in 7.5% of *de novo* pathogenic *SCN1A* variants in clinically affected patients, which implicates that postzygotic mutation is a common phenomenon in *SCN1A*-related epilepsies. Patients with mosaicism of truncating variants have on average milder phenotypes which makes mosaicism an important modifier in *SCN1A*-related phenotypes. However, mosaicism is also seen in severely affected patients, implicating an important role of other modifiers, including accuracy of clinical management. Detection of mosaicism has important implications for genetic counseling regarding recurrence risk and phenotype prediction, and can be achieved by deep sequencing of unique reads. Our results stress the importance of implementing high coverage NGS with attention for possible mosaic pathogenic variants in standard diagnostics.

4.5 Appendix

S4.1 Severity classification of pathogenic variants

Only participants with pathogenic *SCN1A* variants were included in this study; these comprised variants classified as class V and VUS (variants of unknown clinical significance) class IV (according to American College of Medical Genetics and Genomics criteria[155]). Decisions on causality were made based on medical literature and databases of *SCN1A*-variants, the participants' phenotype and segregation of the *SCN1A*-variant with the phenotype in families. Pathogenic variants had been detected in diagnostic laboratories (University Medical Center Utrecht, Utrecht, the Netherlands; Laboratory for Neurogenetics, Institute Born-Bunge, University Antwerp, Antwerp, Belgium; Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands; Duncan Guthrie Institute of Medical Genetics, Glasgow, UK) by Sanger sequencing, an NGS epilepsy gene panel, whole exome sequencing, or by MLPA (multiplex ligation-dependent probe amplification).

S4.2 SmMIP capture and sequencing

SmMIPs were designed using the MIPgen software package[247] to cover all *SCN1A* exons and flanking regions single-stranded. A unique stretch of 6 random nucleotides was inserted between the extension probe and the backbone of each smMIP. SmMIPs were pooled equimolarly and used to capture their target in 100ng of DNA (derived from peripheral blood mononuclear cells) of each sample. This was done as described earlier,[235] although a molecular ratio between gDNA and smMIPs of 1:800 was used, instead of 1:3200. Libraries were sequenced on a NextSeq500 (Illumina, San Diego, CA) using custom sequencing primers according to the manufacturer's protocol (mid output configuration), resulting in 150 bp paired-end reads.

To optimize results for participants with an initial low coverage on the site of their pathogenic variant, due to a suboptimal performing smMIP, results of multiple experiments (at most four) were combined to reach sufficient coverage. After three experiments a small number of smMIPs was re-designed to improve performance in the subsequent experiment, and instead of 100 ng DNA, 200 ng was used.

S4.3 JSI medical systems Sequence Pilot/SeqNext settings

The following software settings were used for single molecule tag processing, consensus building and variant calling:

- Tags enabled: yes
- R1 tag length: 0
- R2 tag length: 6
- Ignore tags with "N" bases: yes
- Ignore tags with low Qs: yes

- Min abs. coverage (per base pos): 1
- Min %. coverage (per base pos): 50%
- Ignore consensus read threshold: 50%
- Required Coverage - Min abs. coverage: 10 combined
- Mutations - Min abs. coverage: 5 combined
- Min % coverage: 5% per direction

S4.4 Technical artefacts smMIP data

Two types of technical artefacts were identified, and pathogenic variants with a deviating alternative allele frequency (AAF) influenced by these were discarded as possible mosaics. In type 1, pathogenic variants were covered by two overlapping smMIPs, of which one smMIP showed a convincing heterozygous variant, while the other gave a deviating AAF. In all cases this was due to the variant being present in the first three nucleotides of the second smMIP, for which less reliable results were seen. In type 2, the variant was in a repetitive region, also leading to false positive deviating AAFs. Pathogenic variants with an AAF>0.5 were discarded as possible mosaics immediately for this reason, while pathogenic variants with an AAF<0.5 were tested further by droplet digital PCR (ddPCR) before mosaicism was considered.

S4.5 Determining the p-value threshold for mosaic pathogenic variants

We expect true heterozygous variants to follow a binomial distribution, in which variants with a higher coverage (= number of observations) will have an AAF closer to 0.5. To assess whether this is true for smMIP-data, we evaluated 8 different neutral *SCN1A* single-nucleotide polymorphisms (SNPs) in 82 participants and parents of participants. We calculated the average AAF of heterozygous variants to see whether a bias for either the reference or alternative allele exists. For each heterozygous SNP and pathogenic variants *p*-values were calculated (X^2 –test), to test whether the AAF deviated significantly from 0.5. Data on heterozygous SNPs and pathogenic variants were analyzed together, to assess which of the pathogenic variants were outliers compared to the group of heterozygous SNPs and to determine the cut-off *p*-value for mosaicism.

Analysis of neutral SNPs confirmed a binomial distribution (Figure 4.3). Two participants were removed from the SNP analysis since all of their SNPs (three per participant) were clear outliers (AAF 0.62-0.75, p-values 3.94×10^{-6} – 3.63×10^{-55}). The average AAF of the remaining 240 SNP data points was 0.491 (SD 0.038), meaning no important bias for either the reference or alternative allele is seen with this technique. This average was nevertheless used as the expected AAF to calculate *p*-values for the pathogenic variants (X^2-test), to correct for technical bias.

In total, 368 data points were taken into account for a binomial distribution, leading to a *p*-value threshold for significance, corrected for multiple testing, of 0.05/368=0.00014 (depicted by red lines in Figure 4.3). Although by far most neutral SNPs fell between

these lines, a few did not. Therefore, we adjusted the *p*-value threshold to the lowest (non-outlier) SNP *p*-value ($6.86x10^{-7}$). Pathogenic variants that would classify as mosaic variants under the unadjusted threshold, but not after adjusting it (n=11) were reanalyzed with ddPCR when specific probes could be designed (n=8). All eight variants were proven to be heterozygous upon analysis. We can therefore conclude that variants in the range between the two different p-value thresholds are very likely to be noise and that the adjustment of our threshold is appropriate.

S4.6 ddPCR

Customized probes were designed for each specific pathogenic variant (Integrated DNA Technologies, Coralville, Iowa). Droplets were generated in a QX100TM and thermal cycling was done using a Bio-rad C1000 Touch Thermal Cycler. A QX200 Droplet Reader and Quantasoft™ software were used for data acquisition and analysis. Percentages of positive versus negative droplets were used to calculate the level of mosaicism of the pathogenic variants, using the Poisson statistics as implemented in the Quantasoft™ software.

S4.7 Read-frame restoring mosaic pathogenic variant

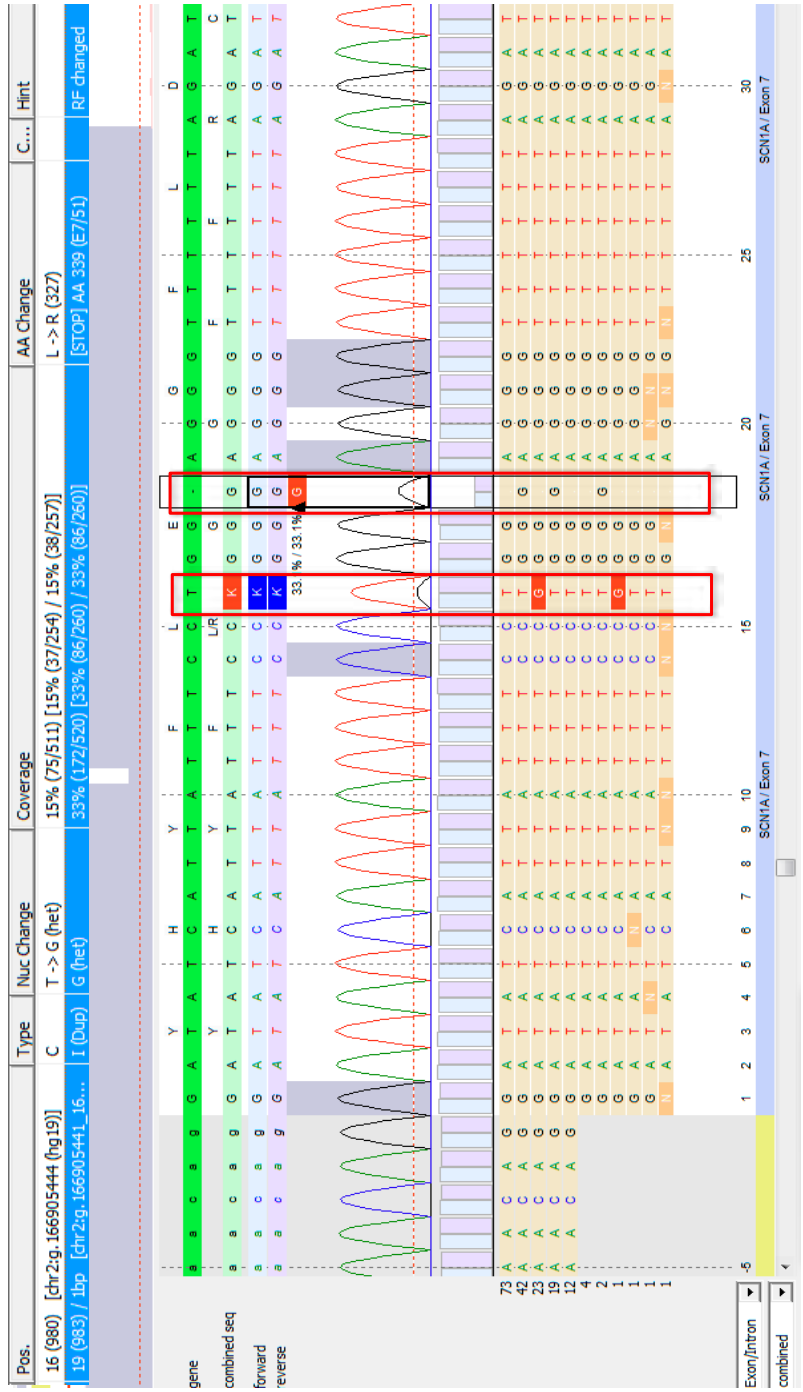

Figure S4.1A. NGS reads of the two mosaic variants in patient 6.

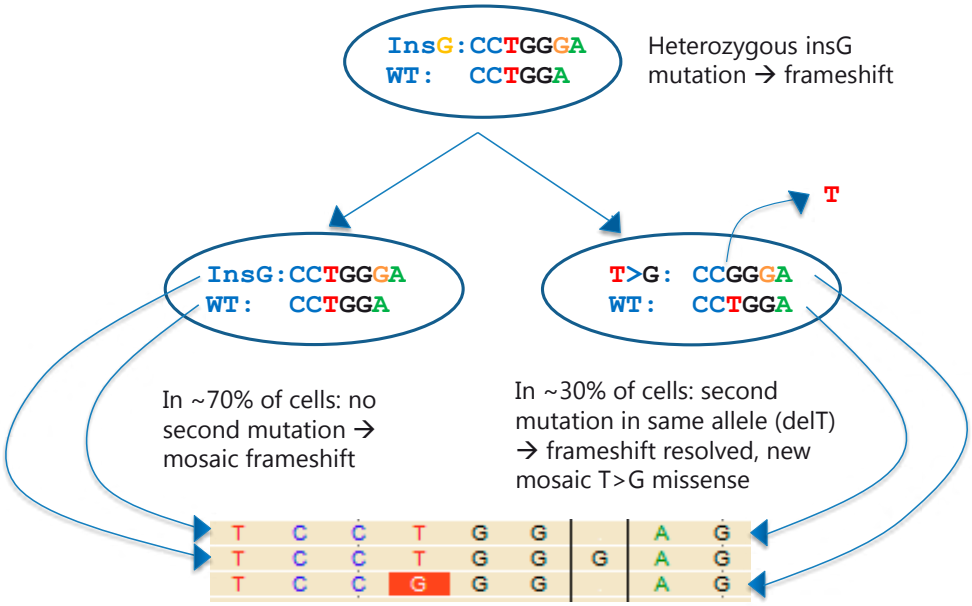

Figure S4.1B. Schematic overview of the proposed frameshift reversing second mosaic variant in patient 6. A heterozygous insG pathogenic variant, originated during gametogenesis, is present in all cells. One of the cells then acquired a second delT variant, 3 basepairs upstream of the insG, on the same allele, which results in restoring of the original read frame and introduction of a seemingly T>G variant in a subpopulation of cells.

Chapter 5

Assessment of parental mosaicism in *SCN1A*-related epilepsy by single molecule molecular inversion probes and next generation sequencing

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Published: J Med Genet. 2018 Oct 27. [Epub ahead of print]

Abstract

Background: Dravet syndrome is a severe genetic encephalopathy, caused by pathogenic variants in *SCN1A*. Low-grade parental mosaicism occurs in a substantial proportion of families (7-13%) and has important implications for recurrence risks. However, parental mosaicism can remain undetected by methods regularly used in diagnostics. In this study, we use single molecule molecular inversion probes (smMIP), a technique with high sensitivity for detecting low-grade mosaic variants and high cost-effectiveness, to investigate the incidence of parental mosaicism of *SCN1A* variants in a cohort of 90 families and assess the feasibility of this technique.

Methods: Deep sequencing of *SCN1A* was performed using smMIPs. False positive rates for each of the proband's pathogenic variants were determined in 145 unrelated samples. If parents showed corresponding variant alleles at a significantly higher rate than the established noise ratio, mosaicism was confirmed by droplet digital PCR (ddPCR).

Results: Sequence coverage of at least 100X at the location of the corresponding pathogenic variant was reached for 80 parent couples. The variant ratio was significantly higher than the established noise ratio in eight parent couples, of which 4 (5%) were regarded as true mosaics, based on ddPCR results. The false-positive rate of smMIP analysis without ddPCR was therefore 50%. Three of these variants had previously been considered *de novo* in the proband by Sanger sequencing.

Conclusion: SmMIP technology combined with NGS performs better than Sanger sequencing in the detection of parental mosaicism. Because parental mosaicism has important implications for genetic counseling and recurrence risks, we stress the importance of implementing high sensitivity NGS based assays in standard diagnostics.

5.1 Introduction

Dravet syndrome (MIM: 607208) is a severe genetic encephalopathy, characterized by intractable epileptic seizures and a delayed psychomotor development, resulting in mild to severe intellectual disability (ID) in most patients. Walking difficulties and behavioral problems are common comorbidities.[3,5,56,62] Pathogenic variants in *SCN1A*, which codes for the α-subunit of the neuronal sodium channel Nav1.1, are found in 70-100% of Dravet syndrome patients. *SCN1A* variants can however also cause milder phenotypes, such as Genetic Epilepsy Febrile Seizures Plus (GEFS+) syndrome or febrile seizures only.[108,111,162]

The varying disease severity of phenotypes caused by *SCN1A* pathogenic variants are partly due to differences in mutation types and amino acid changes, which can cause different grades of channel dysfunction.[117] However, an important part of the disease variability is still unexplained, and multiple modifying factors have been suggested.[189,197,209,212,226] We have recently shown that mosaicism in patients is an important modifier of *SCN1A*-related disease severity.[212] Mosaicism arises when a variant occurs post-zygotically, leading to genetically distinct cell populations. Patients that carry a mosaic pathogenic variant might be less severely affected, since unaffected cells are also present.[117,164,165,170,171,212,246] Variant carriers can even be free of symptoms when very low percentages of mosaicism are present, caused by mutations occurring relatively late in embryonic development. They can however still transmit the variant to their children when it is present in gonadal tissue. Unfortunately, low-grade parental mosaicism can be missed in molecular diagnostics, as regularly used Sanger sequencing fails to detect low percentages of a variant allele,[248] which may also be the case when NGS is used with limited coverage. Moreover, variants are not always present in DNA isolated from blood. A number of patients with a presumed *de novo SCN1A* variant, will therefore actually have a parent with low-grade mosaicism: this percentage has been estimated to be 7-13%[171,175,176] and is illustrated by multiple case reports of families with healthy or mildly affected parents and multiple severely affected children.[167–169,172,173,233,249]

Detecting parental mosaicism has important implications for genetic counseling: recurrence risks rise when father or mother carries the pathogenic variant allele too, which might affect decisions regarding family planning and prenatal testing when they are aware of this. Other techniques are needed to reliably test for parental mosaicism. Droplet digital PCR, although proven to be an extremely sensitive method for detecting low-grade mosaicism,[176,250] has the disadvantage of needing specifically designed probes for each assessed variant, which is not feasible in regular diagnostics. Another assay that can be used is smMIP (single molecule molecular inversion probes) capture and sequencing, a deep NGS technique with high sensitivity for detecting low-frequency variants and high cost-effectiveness.[235,251,252] In this study, we use smMIPs to investigate the incidence of low-grade parental mosaicism of *SCN1A* variants in a cohort of 90 families and assess the feasibility of this technique.

5.2 Methods

5.2.1 Participants

A portion of the parents of a previously described cohort of 176 patients clinically affected by *SCN1A*-related seizures[209,212] were tested for low-grade mosaicism. In this cohort, only participants with pathogenic variants (class 5) and likely pathogenic variants (class 4), according to the American College of Medical Genetics and Genomics criteria,[155] in *SCN1A* were included. *SCN1A* variants in index patients had been detected in diagnostic laboratories (University Medical Center Utrecht, Utrecht, the Netherlands; Laboratory for Neurogenetics, Institute Born-Bunge, University Antwerp, Antwerp, Belgium; Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands; Duncan Guthrie Institute of Medical Genetics, Glasgow, UK) by Sanger sequencing, NGS epilepsy gene panels, whole exome sequencing, or by MLPA (multiplex ligation-dependent probe amplification). Only parents for whom DNA was available were considered for inclusion. In most families, parents had been assessed for carriership of the pathogenic *SCN1A* variant by Sanger sequencing in a regular diagnostic setting; families in which parents were shown to be heterozygous or mosaic carriers of their children's pathogenic variants were regarded as participants themselves and excluded from additional DNA testing. Families in which mosaicism was demonstrated in the proband in previous research[212] were also excluded , since this rules out parental mosaicism. Informed consent was obtained from participants, according to the Declaration of Helsinki. The study was approved by the Ethical Committee of the University Medical Center Utrecht.

5.2.2 Molecular analyses

Mosaicism screening by smMIPs and NGS

All *SCN1A* exons were captured by smMIPs, as described earlier,212,235 and sequenced (see supplemental data S4.2. smMIP capture and sequencing for more details) in parental DNA extracted from lymphocytes. The resulting data were analyzed using commercial software (SeqNext module of Sequence Pilot; JSI medical systems, Ettenheim, Germany) (see supplemental data S4.3. JSI medical systems Sequence Pilot/SeqNext settings for more details). Reads with the same single-molecule tag were assembled into one consensus read, to correct for PCR and sequencing artefacts. In addition, the molecular tag discriminates unique reads from PCR duplicates, allowing the determination of quantitative sequence coverage of reads originating from unique DNA molecules. *SCN1A* pseudogene reads were removed from alignment and analysis.

Parents of patients with deletions and duplications spanning more than one smMIP were excluded from analyses, since those variants cannot be detected by smMIPs.

Statistical analysis of smMIP data

We determined whether the patients' pathogenic variants were present in the sequencing data of their respective parents. Only variants that were present in both forward and reverse reads were counted, to filter out likely false positive reads. High coverage is needed to detect low-grade mosaicism with a high confidentiality: a unique coverage of 300X is needed to detect 1% of alternative allele reads with a 95% probability level (calculated based on a binomial distribution). Therefore, parents with a coverage <100X (needed to detect 3% of alternative allele reads with a 95% probability level, calculated based on a binomial distribution) and no alternative allele reads at the location of their child's pathogenic variant, were excluded from statistical analyses.

We established the false positive rate of the variants detected in the parents, to determine whether they were true variants and not sequencing errors leading to false positives. The overall percentage of variant reads of each possible low-grade mosaic variant was determined in 145 unrelated samples. P-values were calculated for each parent in whom a possible mosaic variant was present, based on a binomial distribution and the average percentage of variant reads in unrelated controls, to determine whether the percentage of variant reads deviated significantly from the established noise ratio. Variants in parents with significant p-values, corrected for multiple testing (below 0.05 divided by the number of tested parents), were considered to be likely true low-grade mosaic variants.

Confirmation by droplet digital PCR

All likely true mosaic variants were validated by ddPCR if probes could be designed (see supplemental data S4.6. *ddPCR* for more details).

5.3 Results

5.3.1 Participants

DNA was available for both parents in 101 families. The probands of six families carried a duplication or deletion of *SCN1A* that could not be detected by smMIPs and were therefore excluded. High-grade mosaicism was previously established in probands of seven of these families, which were excluded. Ninety complete families remained and were analyzed as described. The 26 families for which DNA of both parents was not available contained 5 mosaic family members, of which 4 were parents of probands that were found to carry the same mutation as their children in regular diagnostics (Sanger sequencing).

5.3.2 Molecular analyses

Mosaicism screening by smMIPs and NGS

A coverage of at least 100X unique coverage at the location of the known pathogenic variant of their children was reached for 80 complete parent couples. 70 of these 80 families had been previously assessed for parental carriership by Sanger sequencing in standard diagnostic procedures, and their children's variants had been deemed *de novo* (Figure 5.1). Parents of the other 10 families had previously declined Sanger sequencing of their own DNA. The average unique read depth in these parents was 1663X (ranging from 112X-8990X, median: 1203X). No corresponding pathogenic variant alleles were detected in parents with a unique coverage <100X.

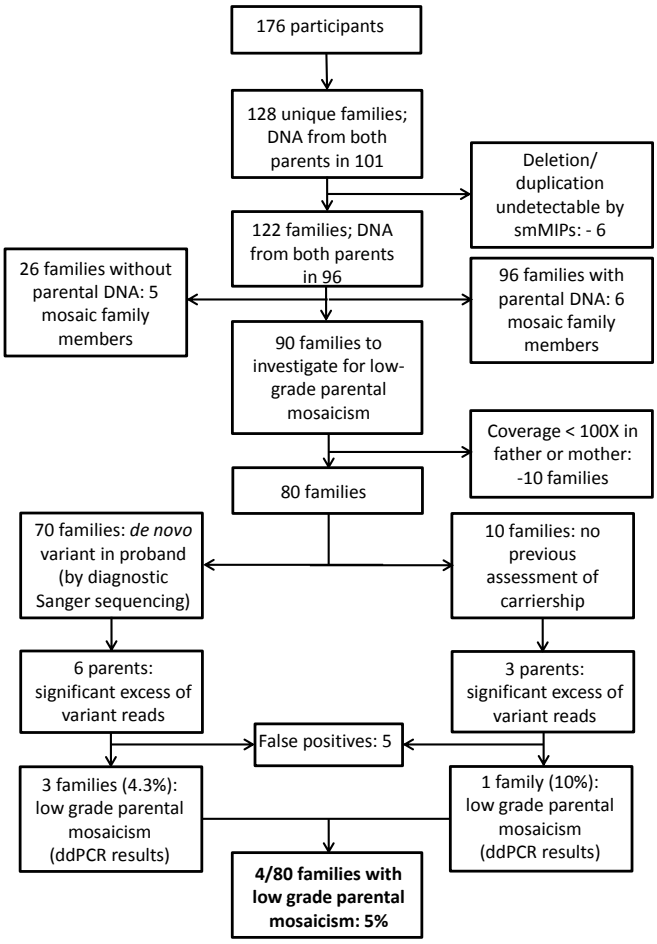

Figure 5.1. Flowchart of detected mosaic pathogenic variants in the complete cohort described here and in previous work.

Corresponding variant alleles were detected in 29 parents, belonging to 22 different families; in 7 families both parents carried variant alleles. Variants in six parents, of which two belonged to families in which both parents carried variant reads, were regarded as false positives, since their variant reads were never present in both a forward and reverse read and had low quality scores (14). The remaining 23 parents belonged to 18 different families and carried 16 different variants (two variants were present twice in the cohort of probands).

Statistical analysis of smMIP data

The percentages of variant reads in unrelated parents (noise ratios) are shown in Table 5.1 for all 16 variants. A percentage of variant reads significantly higher than the established noise ratio was found in nine parents (Table 5.1, bolded p-values). Unexpectedly, a significantly high ratio of variant allele was found in both father and mother of one family (variant 1). The father of this parent couple, in which a much lower ratio of variant allele was found than in the mother (0.63% versus 6.47%), was regarded as a false positive, since it is extremely unlikely that both parents are true mosaics for the same variant. The low p-value in this father might be explained by the location of the variant in a poly-T sequence, for which less reliable NGS results are seen.'

Confirmation by droplet digital PCR

Mutation specific ddPCR probes could be designed for six of the eight likely mosaic parents. Low-grade mosaicism could be confirmed in three: a significantly higher percentage of droplets positive for the variant allele was observed than in negative controls (Table 5.1.). The confirmed mosaic variants belonged to the parents with the lowest p-values that we established for the percentages of variant compared to noise ratios. One of the parents (family 1) for whom ddPCR probes could not be designed (due to their variant being in a poly-T sequence) had an even lower p-value ($7.5x10^{-88}$), the highest percentage of variant alleles (6.47%), and two affected sons; we therefore regard her as a true mosaic variant carrier as well. The other parent for whom no ddPCR probes could be designed however (variant 7), had a p-value in the same range as the variants that could not be confirmed by ddPCR (0.0006) and the lowest percentage of variant alleles (0.17%). This father was therefore regarded as a false positive. In summary, we have detected low-grade parental mosaicism in four out of 80 parent couples (5%) (Figure 5.1). In three of these four families, parents had been assessed for carriership of their child's variant, which had been deemed *de novo* by Sanger sequencing.

5.3.3 Clinical symptoms

Two of the four mosaic parents (50%) had experienced seizures in the past, although limited details were available: the father of family 4 (0.5% variant alleles) had experienced epilepsy as a child for which phenobarbital was prescribed; the father of family 2 (2.43% variant alleles) had epilepsy as a child and was prescribed antiepileptic drugs until 10 years

of age. Seizures were reported in 5% of the remaining non-mosaic parents, who experienced mostly typical febrile seizures (6/8), one single seizure (1/8), and one parent had had two seizures as a child and one as an adult, provoked by alcohol and fatigue. Antiepileptic drugs were prescribed in none.

5.4 Discussion

The presence of low-grade parental mosaicism for pathogenic variants has important implications for genetic counseling. A recurrence risk of 1% is often counselled to parents in the case of a presumed *de novo* genetic disorder;[253] recurrence risks however rise when father or mother are shown to carry variant alleles as well. Parents may make different choices regarding prenatal diagnostics when this knowledge is available. In standard clinical practice, Sanger sequencing in parents is often the method of choice to determine whether the pathogenic variant in a proband is *de novo* or not. However, Sanger sequencing and regular NGS fail to detect low percentages of variant allele,[248] and a normal result might therefore give parents a false sense of reassurance. By using smMIPs and deep sequencing, we here detected low-grade parental mosaicism in 3 out of seventy families (4.3%) with a previously presumed *de novo SCN1A* variant (based on Sanger sequencing in regular diagnostics), and in a total of 4 out of 80 families (5%).

The use of smMIPs to detect mosaicism has several advantages. Firstly, the assembly of smMIP-reads into one consensus read based on the molecular barcode in each smMIP probe, corrects for PCR or sequencing artefacts and therefore reduces sequencing errors. Furthermore, the removal of PCR duplicates from the analyses leads to very accurate percentages of variant alleles, since only one read per unique DNA molecule is taken into account. The smMIP-technique with deep sequencing, allows us to detect parental mosaicism even when only very small fractions of variant alleles are present. However, this method also has several limitations. No definitive conclusions about mosaicism can be made without confirmation by a second technique, especially when percentages of variant reads are very low. This is illustrated by the three variants for which significant percentages of variant alleles were seen based on smMIP sequencing, of which none could not be validated by ddPCR. Another example of the need for confirmatory tests is the parent couple that both showed a significant percentage of variant reads, of which only one is likely a true mosaic. A solution for this could be to lower the p-value threshold for significance, since the choice of threshold influences the false positive rate. Since all false positive variants had p-values in the same range (much higher than the true mosaic variants), this may be a feasible resolution.

Besides generating false positive results, mosaic variants may also be missed when using smMIPs, which causes false negative results. While we were able to detect percentages of mosaicism as low as 0.5%, theoretically even lower percentages of mosaicism are possible.

Table 5.1. Possible low-grade parental mosaic variants

Variant		% variant reads in **father** (coverage)	% variant reads in **mother** (coverage)	% variant reads in **unrelated parents** (coverage)	***p*-value**[1] (father; mother)	**ddPCR results**[2]**: % of variant alleles**
1	c.1209del p.(Phe403fs)	0.63 (630X)	6.47 (804X)	0.06 (325,481X)	**0.0004**; **$7.5x10^{-88}$**	Probes could not be designed – mosaicism likely
2	c.5348C>T p.(Ala1783Val)	2.34 (770X)		0.07 (123,200X)	**$3.4x10^{-21}$**	0.58% (NC: 0.0001%)
3	c.5674C>T p.(Arg1892*)	6.35 (126X)		0.06 (114,869X)	**$1.8x10^{-14}$**	8% (NC: 0.15%)
4	c.5656C>T p.(Arg1886*)	0.50 (2,403X)		0.03 (317,306X)	**$3.0x10^{-11}$**	0.83% (NC: 0.05%)
5	c.5164A>G p.(Thr1722Ala)		0.24 (841X)	0.001 (172,609X)	**$4.7x10^{-5}$**	No confirmation
6	c.4757G>A p.(Gly1586Glu)		0.31 (651X)	0.002 (248,849X)	**0.00012**	No confirmation
7	c.3706-1G>A p.(1236splice)	0.17 (3,580X)	0.10 (3,913X)	0.03 (435,939X)	**0.0006**; 0.021	Probes could not be designed – mosaicism unlikely
8	c.4219C>T p.(Arg1407*)	0.56 (718X)		0.07 (117,985X)	**0.0013**	No confirmation
9	c.602+1G>A p.(201splice)	0.16 (4,324X)	0.08 (4,842X)	0.049 (685,966X)	0.0043; 0.121	
10	c.2836C>T p.(Arg946Cys)	0.09 (4,350X)	0.07 (2,921X) 0.34 (1,170X)	0.07 (420,914X)	0.165; 0.271 0.007	
11	c.602C>T p.(Ala201Val)	0.04 (4,508X)		0.004 (696,006X)	0.0164	
12	c.1738C>T p.(Arg 580*)	0.22 (912X) 0.09 (2,113X)		0.06 (285,028X)	0.079 0.216	
13	c.1178G>A p.(Arg393His)	0.12 (1,669X)		0.05 (226,584X)	0.155	
14	c.580G>A p.(Asp194Asn)	0.04 (5,271X)	0.04 (5,466X)	0.05 (736,234X)	0.246; 0.239	
15	c.3637C>T p.(Arg1213*)		0.03 (5,947x)	0.02 (527,215X)	0.249	
16	c.5269G>A p.(Gly1757Arg)	0.04 (5,371X)		0.033 (465,391X)	0.266	

[1]Threshold values for significance: <0.05/23 = 0,00217. Significant *p*-values are bolded.
[2] NC = negative control

Fractions of variants in the noise range (0.001%-0.07%, dependent on variant location) will however not be detected. Even variants with percentages above this noise ratio can be missed when coverage is insufficient at their location; for mosaic variants of 0.5% a coverage of at least 600X is needed to detect it with a 95% probability level (calculated based on a binomial distribution). In 44 of the non-excluded parents this coverage level was not reached, and therefore some very low-grade mosaics have possibly remained undetected. Finally, variant alleles will not be detected by sequencing of DNA from blood in the case of purely gonadal mosaicism. Our percentage of families affected by parental mosaicism (5%) is therefore likely an underestimation, which is demonstrated by one family in our cohort with two affected brothers and absence of variant alleles in both parents (905X and 1564X). A previous study[176] has demonstrated paternal mosaicism for *SCN1A* pathogenic variants in sperm that could not be detected in DNA from blood in three fathers. This highlights the limitations of mosaicism detection if only DNA from lymphocytes is investigated.

Studies to investigate the incidence of parental mosaicism of *SCN1A* pathogenic variants have been performed previously in Dravet syndrome families. A recent study detected parental mosaicism in 3 out of 40 families with apparent *de novo* pathogenic *SCN1A* variants by using smMIPs.[254] No validation studies were performed, which may have led to false positive results; however, as the reported percentages of mosaicism were relatively high (16.7-30.6%), this risk may be low. Xu et al.[175] found parental mosaicism in 10% of Dravet syndrome families as well. Yang et al.[176] reported an even higher incidence: parental mosaicism was detected in 25% of Dravet syndrome families. However, part of these parental mosaics could also be detected by Sanger sequencing, which was an exclusion criterion in our study based on which 4 families were excluded. The incidences of parental mosaicism in the mentioned studies were 8.6% and 13.3% respectively if only mosaics undetectable by conventional methods in clinical practice are taken into account. In our study, the relatedness of families was not confirmed, which makes it possible that additional mosaic parents were missed. However, the much higher incidence of Yang et al.[176] is most likely due to the use of ddPCR as screening method, which can reach a higher sensitivity than NGS based methods; variant allele percentages as low as 0.03% were reported, which is in the noise range of our technique. However, the disadvantage of ddPCR as a screening tool is that specifically designed probes are needed for each mutation, which makes it infeasible to implement in standard diagnostics. SmMIPs that cover *SCN1A* only have to be designed and ordered once, and can then be used for the assessment of all pathogenic variants detectable by NGS, making them very inexpensive: a full *SCN1A* smMIP NGS run for 96 patients could be performed in our lab for approximately €22 per sample (including the use of plastics, reagents, oligo's and sequencing, excluding labour costs for laboratory procedures and analyses). In contrast, ddPCR costs €600-800 per sample for merely ordering custom probes for each patient, and Sanger sequencing could be performed at out diagnostics lab for ~€25-30 per sample. SmMIPs might therefore be an attractive cost-effective method to assess parental mosaicism: although it will detect less instances of

parental mosaicism than more sensitive methods like ddPCR, it is clear that it outperforms Sanger sequencing: in three families mosaicism, detected by smMIPs, was missed in regular diagnostics.

In this study, we identified low-grade mosaicism of variants that had remained undetected by Sanger sequencing. Alternatively, mosaicism can be present at higher grades in variants that can be detected by Sanger sequencing. We have recently used smMIPs and NGS to identify high-grade mosaicism in 9% of pathogenic *SCN1A* variants detected in regular diagnostics, of which 9 out of 11 were previously considered heterozygous based on Sanger sequencing results.[212] Similar percentages of high-grade mosaic variants in other genes have been reported previously.[237,239,240] The grade of mosaicism reflects the timing of mutagenesis: variants occurring during some of the first cell divisions after fertilization will be present in many cells in multiple tissues, whereas variants that occur later in embryonic development might only be present in a single tissue type.[239] Although the methods of studies differ, which makes comparing exact percentages difficult, relatively similar incidences of high and low-grade mosaic variants are reported, implicating that mutations occur at roughly the same rate during early and slightly later embryonic development. Detecting high-grade mosaicism in a proband is currently the only way to virtually rule out low-grade mosaicism in a parent, since only one member of a family can be mosaic for a specific variant and all techniques can miss very small percentages of (gonadal) mosaicism. Testing probands for high-grade mosaicism therefore adds value to testing parents for low-grade mosaicism in the assessment of recurrence risks, especially because this occurs in a substantial amount of patients (9%).[212] We suggest that analyzing both parents and the proband with smMIPs and NGS is currently the most cost-effective way of assessing parental mosaicism in *SCN1A*-related epilepsy after a pathogenic variant is found in regular diagnostics. Since either low- or high-grade mosaicism could be detected in a total of 12% of families carrying pathogenic *SCN1A* variants in this cohort (11 probands[212] and 4 parents in 122 families), a substantial amount of families will likely benefit from improved counseling this way.

The percentages of pathogenic variant allele reads detected in parents ranged between 0.5 and 6.5. Two of the four mosaic parents had experienced seizures or epilepsy in the past, in contrast to 5% of non-mosaic parents. Furthermore, more severe epilepsy phenotypes were observed. Unlike in a previous report,[176] the percentage of mosaicism was not necessarily related to seizure symptoms: the two parents with the highest percentages of mosaicism (6.47% and 6.35%) reported no seizures. A reason for this may be that levels of mosaicism in blood do not necessarily correspond to those in brain. Furthermore, different genetic backgrounds are likely to modify the effect of the mosaic pathogenic *SCN1A* variant on the disease outcomes. Symptoms have previously been reported to arise between 12.5-25% of pathogenic *SCN1A* variant alleles.[117,212,241] Our results and earlier studies[175,176] suggest that symptoms might already arise below 1%, although this risk of seizures is much lower and we cannot be sure that the seizures have been caused by the *SCN1A* variant.

In conclusion, this study confirms that parental mosaicism of *SCN1A* pathogenic variants is a common phenomenon. Using molecular barcoding to obtain unique sequence reads per DNA fragment analysed, by combining smMIP technology and deep sequencing, can detect parental mosaicism in 4.3% of families with mutations that were previously considered *de novo* by standard diagnostics. Our reported total incidence of 5% is likely an underestimation, since ultra-low percentages of variant allele and gonadal mosaicism cannot be detected by the used methods. However, our methods perform better than Sanger sequencing used in regular diagnostics and implementing smMIPs could be a cost-effective way to improve the accuracy of counseling recurrence risks, which has important implications for families.

Chapter 6

Modifier genes in *SCN1A*-related epilepsy syndromes

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Submitted for publication

Abstract

Objective: *SCN1A* is one of the most important epilepsy-related genes, with pathogenic variants leading to a range of phenotypes with varying disease severity. Different modifying factors have been hypothesized to influence *SCN1A*-related phenotypes. We investigate the presence of rare and more common variants in epilepsy-related genes as potential modifiers of *SCN1A*-related disease severity.

Methods: 87 patients with *SCN1A*-related epilepsy were investigated. Whole exome sequencing was performed by the Beijing Genomics Institute (BGI). Functional variants in 422 genes associated with epilepsy and/or neuronal excitability were investigated. Differences in proportions of variants between the epilepsy genes and four control gene sets were calculated, and compared to the proportions of variants in the same genes in the ExAC database

Results: Statistically significant excesses of variants in epilepsy genes were observed in the complete cohort and in the combined group of mildly and severely affected patients, particularly for variants with minor allele frequencies of <0.05. Patients with extreme phenotypes showed much greater excesses of epilepsy gene variants than patients with intermediate phenotypes.

Significance: Our results indicate that relatively common variants in epilepsy genes may play a large role in modulating *SCN1A* phenotypes. They may modify the phenotypes of both severely and mildly affected patients.

6.1 Introduction

SCN1A is one of the most important epilepsy-related genes, with pathogenic variants leading to a wide range of phenotypes with varying disease severity.[111,121,162,207] One of the most severe associated diseases is Dravet syndrome, which is characterized by intractable epileptic seizures, a diminishing psychomotor development that results in mild to severe intellectual disability (ID), and often walking difficulties and behavioral problems.[62,92,255] Milder phenotypes include GEFS+ syndrome and febrile seizures, in which seizures show a milder course and a normal intellect is expected.[121,208]

SCN1A encodes for the α-subunit of a neuronal sodium channel, Nav1.1. Different pathogenic variants in *SCN1A* can have different effects on channel function, which partly explains why the gene is associated with multiple phenotypes. Variants leading to a complete loss of function (LoF) of the channel are virtually always associated with severe phenotypes, whereas milder disturbances in channel function usually cause milder clinical pictures.[117] However, a large part of the phenotypic variability of patients remains unexplained: there are several reports of families in which multiple members carry the exact same pathogenic *SCN1A* variant, but nevertheless show an intra-familial variability in phenotype severity.[96,97,159–161,256] Furthermore, Dravet syndrome patients with similar loss of function variants may show important phenotypic differences, ranging from severely disabled individuals to patients that live much more independent lives.[9,25,139] This variability makes it difficult to accurately predict clinical outcomes in newly diagnosed young patients, which is very important to parents.

Several factors have been suggested to modify the clinical outcome of *SCN1A*-related epilepsy and to explain these phenotypic differences. Mosaicism for a pathogenic *SCN1A* variant can have a major ameliorating impact on disease severity.[167,169,212,256] Furthermore, variants in regulatory regions of *SCN1A* may modulate disease severity.[189,257] Additionally, clinical management and especially the use of contra-indicated medication can affect clinical outcomes.[47,83,209]

Moreover, variants in modifier genes may influence *SCN1A*-related phenotypes. An important effect of modifier genes has already been described for several other genetic disorders,[195,196,258] and there are strong indications that genetic background can modulate the clinical effects of pathogenic *SCN1A*-related phenotypes too, in human patients as well as in *SCN1A* knock-out mice.[36,95,122,159–161,199,206,208,256] Several potential modifier genes have already been identified: variants in *SCN9A, SCN8A, SCN2A, HLF, POLG, KCNQ2, CACNB4, CACNA1G* and *CACNA1A* might aggravate or partially rescue clinical outcomes.[37,197–205] Potential modifier loci, identified in *SCN1A* knock-out mice with different disease severities, also contain several candidate modifier genes, including GABA receptor subunit genes, ion channel genes and genes associated with seizures or neuronal hyperexcitability.[206] Furthermore, an enrichment of rare variants in neuronal excitability genes in general has been identified in severely affected Dravet syndrome patients, compared

to mildly affected Dravet syndrome patients.[205] However, these potential modifiers each account for only a small portion of the clinical variability of *SCN1A*-related phenotypes. Many only show an effect when studied in large groups of patients and different patients might be affected by different modifiers or by multiple modifiers simultaneously. Currently, no clinically relevant modifier genes have been identified for which diagnostic testing can be offered, and thus more research is needed to understand clinical variability and to improve the counseling of patients.

Here, we investigate the presence of rare and more common variants in epilepsy-related genes that could potentially modify disease severity, in a cohort of 87 patients with *SCN1A*-related epilepsy. We provide a descriptive overview of variants present in patients with phenotypes on the most extreme ends of the spectrum, and furthermore investigate variants in six families with multiple affected members that show varying disease severities.

6.2 Materials and Methods

6.2.1 Editorial Policies and Ethical Considerations

The study was approved by the Ethical Committee of the University Medical Center Utrecht. Informed consent was obtained from participants or their legal caretakers, according to the Declaration of Helsinki.

6.2.2 Cohort and clinical data

Participants

A cohort of 87 participants with pathogenic *SCN1A* variants was evaluated, most of whom have been described previously.[209,212] Only participants with pathogenic variants (class V) or likely pathogenic variants (class IV) in *SCN1A* were included, according to the American College of Medical Genetics and Genomics criteria.[259] All variants had been detected and classified in diagnostic laboratories. Patients that had previously been shown to be mosaic for their pathogenic *SCN1A* variant were excluded.[212]

Disease severity classification

For all participants detailed clinical data were collected from medical records and semi-structured telephone interviews. Patients were either part of families with multiple *SCN1A* variants carriers, or the only affected member in their family. In all patients absolute disease severity was defined as cognitive functioning at the age of 6 years. We assessed cognitive functioning retrospectively at the age of 6 years old as previously described.[209] This was done to limit the influence of an older age on cognitive outcomes, since average cognition declines with age in Dravet syndrome patients. IQ- and developmental assessment scores, established at different ages, were interpolated by linear regression, to obtain approximate

scores at the age of 6 years. This age was chosen since cognitive decline is generally most severe in the first years following disease onset.[5,228] Patients with an IQ or developmental quotient (DQ) of >70 (no or borderline ID) at age 6 were classified as "mild", while patients with an IQ or DQ of <50 (moderate or severe to profound ID) were classified as "severe". Patients with an IQ/DQ score of 50-70 were classified as "intermediate". Participants under the age of 6 years old were not classified, unless they already showed an IQ/DQ of <50. Participants for whom a classification at age 6 could not be reliably obtained were also not classified. Furthermore, if clearly varying phenotypes were present in families with multiple variant carrying family members (different syndromes, or large differences in seizure frequencies or cognitive outcomes), disease severity was defined as "mild" or "severe" relative to other affected family members (e.g. a participant with Dravet syndrome and an unaffected father, both carrying the same pathogenic *SCN1A* variant, would be classified as "relatively severe" and "relatively mild" respectively).

To compare subgroups, we then excluded "mild" patients that did not carry an *SCN1A* variant that was predicted to cause a loss of function (LoF), or a variant that has been described before in Dravet syndrome patients, to limit the influence of the different pathogenic *SCN1A* variants on the phenotypes. Without this restriction it would be impossible to distinguish between patients with a severe *SCN1A* variant and a modifier that ameliorates the phenotype, and patients with mild *SCN1A* variants, that are relatively severely affected due to the presence of a negative modifier. The "severe" and "intermediate" groups included patients with all mutation types.

6.2.3 Molecular analyses

Exome sequencing

Whole exome sequencing was performed on DNA from lymphocytes in all patients by the Beijing Genomics Institute (BGI), using the Agilent V5 50M exome kit enrichment, followed by paired-end sequencing on an Illumina Hiseq. The resulting data was processed using an in-house developed pipeline,[260] according to the best practices guidelines.[261] Briefly, sequencing reads were mapped using BWA-MEM v0.7.5a,[262] duplicates were marked and lanes were merged. Next, using GATK IndelRealigner (v3.4-46)[263] indels were realigned and the GATK HaplotypeCaller tool was used to create a GVCF per patient containing SNPs and indels. These GVCFs were jointly genotyped using GATK GenotypeGVCFs for the described cohort. Variants were flagged using GATK VariantFiltration if they did not meet the certain criteria. For SNPs the criteria were: QD < 2.0, MQ < 40.0, FS > 60.0, HaplotypeScore > 13.0, MQRankSum < -12.5, ReadPosRankSum < -8.0, snpclusters >=3 in 35bp. The criteria for indels were: QD < 2.0, FS >200.0, ReadPosRankSum < -20.0. Finally, variants were annotated using SnpSift (v4.3t) and dbNSFP (v3.5).

Filtering of variants

We investigated variants in 422 genes that are all either associated with epilepsy, are implicated to modify epilepsy phenotypes, are associated with neuronal excitability, or function in the same pathway as *SCN1A* , based on epilepsy gene panels used in the University Medical Center Utrecht (EPI00v18.1), previous literature and the KEGG pathway database (https://www.kegg.jp/kegg-bin/show_pathway?ko04728, accessed June 2016) (further referred to as "epilepsy genes"; see supplemental data S6.1 for the complete list (available online), and supplemental table S6.2 for characteristics). A distinction was made between established monogenic epilepsy genes (when present in the diagnostic epilepsy gene panel of the University Medical Center Utrecht) (EPI00v18.1) and candidate genes (all other genes). We filtered for PASS-variants that were predicted to alter protein function (frameshift, stop-gain, stop-loss, start-loss, in-frame deletion, in-frame insertion, splice donor, splice acceptor and non-synonymous missense variants). Five categories of variants were established (type A-E), based on different minor allele frequencies (MAF) of the variants (in both exomes and genomes in the gnomAD database, r2.0,[156] all populations) and on their deleteriousness as predicted by CADD scores (Combined Annotation Dependent Depletion, v1.3):[264] Type A variants have a MAF of <0.01 and a (PHRED-scaled) CADD-score of >20 (representing the top 1% deleterious substitutions in the human genome); type B variants have a MAF of <0.01 and a CADD-score of >10 (representing the top 10% deleterious substitutions in the human genome), type C variants have a MAF of <0.01 and any CADD score; type D variants have a MAF of <0.05; and type E variants have a MAF of <0.1. The known pathogenic *SCN1A* variant of each patient was excluded.

The same categories of variants were established for variants in four sets of control genes (control 1: immunodeficiency related genes, n=360; control 2: genes related to cardiovascular disease (excluding genes related to conduction abnormalities), n=109; control 3: genes related to kidney disease, n=223; control 4: genes related to either hemostasis, erythroid cell membrane defects, congenital diarrhea, neonatal erythroderma, or angioedema, n=297)), and in genes associated with ID (excluding genes also present in the epilepsy gene-list),n=659), based on genes included in diagnostic gene panels of the University Medical Center Utrecht (version 9, http://www.umcutrecht.nl/NGS) (see supplemental data S6.1 for the complete lists, and supplemental table S6.2 for characteristics).

6.2.4 Data analyses

Proportions of variants in epilepsy genes and control genes compared to the ExAC database

We investigated whether groups of patients carry an excess of variants in our selection of epilepsy genes, as compared to the number of variants in the different sets of control genes (1-4). Since these control sets contain different numbers of genes than the set of epilepsy genes, with different lengths and mutation rates, we first investigated a healthy control

population to establish the normal ratios of variants between the epilepsy genes and the different control sets. For this, we extracted variants in the same genes from the ExAC database [156], using the same filters as applied in our cohort (frameshift, stop-gain, stop-loss, start-loss, in-frame deletion, in-frame insertion, splice donor, splice acceptor and non-synonymous missense variants, with MAFs of either <0.01, <0,05 or <0,1). Only variants present in non-Finnish Europeans were analyzed, as this population best resembles our own cohort.

Directly comparing numbers of variants found in the ExAC database to other data can lead to incorrect results, as differences in sequencing methods, coverage and variant calling may lead to biases. However, we expect the ratios of variants in epilepsy genes and control sets of genes to be roughly similar in both ExAC data and in our own sequencing data, as within each cohort the same protocols are used to analyze the various gene sets. We therefore compare these ratios, rather than absolute numbers of variants, in both cohorts. The ratio of variants in epilepsy genes and in the different sets of control genes in the ExAC database (=numbers of epilepsy gene variants divided by the number of control gene variants) was used to calculate the expected number of variants in epilepsy genes in our cohort (the established ExAC ratio times the number of variants in control genes in our cohort). We compared this expected number of variants in epilepsy genes to the actual number of variants found in our cohort, to obtain the percentage of over- or underrepresentation.

Fishers' exact test was used to determine whether this over- or underrepresentation of epilepsy gene variants was statistically significant (p-value threshold for significance: <0.05 divided by the number of tests to corrected for multiple testing). These analyses were performed for the ratio between epilepsy gene variants and variants in all four sets of control genes, for all three frequency thresholds (<0.01, <0,05 or <0,1; type C, D and E variants) and for different groups of patients (the complete cohort, only patients on the extreme ends of the disease spectrum, and only intermediate patients). We hypothesized that patients with a phenotype on both the severe and mild ends of the disease spectrum would carry more variants in epilepsy genes than intermediate patients, as both groups are likely to have a modified phenotype.

Differences between mild and severe patients

We then assessed the distribution of epilepsy gene variants present in our cohort in the different categories of patients (mild, severe and intermediate). The total number of alleles per group was calculated (=the number of genes in which at least one variant was found in at least one of the patient groups, multiplied by two alleles, multiplied by the number of patients in the group, minus one for each X-linked gene for each male in the group). The number of found variants per group was then divided by the total number of alleles per group, to obtain a percentage of variants corrected for group size. Differences between groups were calculated using Fishers' exact test. Analyses were performed 5 times, once for each category of variants (type A-E). For each of these categories, we furthermore identified

in which genes severe patients carried most variants compared to mild patients, and vice versa (Fishers' exact test, based on the numbers of variants and total alleles per gene in each group).

Variants found in families with variable phenotypes and in patients with the most extreme phenotypes

In families with multiple affected family members with different disease severities we report variants that were present in only severe patients and not in their milder family member(s) (=possible negative modifiers that could aggravate the phenotype) and variants that were present in mild patients but not in their severe family member(s) (=possible positive modifiers that could ameliorate the phenotype). Only the most pathogenic variants are described (type A), as it is difficult to prove the influence of more common and milder variants.

We furthermore report the most pathogenic variants in the patients with the most extreme phenotypes from the mild and severe groups (IQ at the age of six <30 and all the mild patients (IQ >70)). For each patient, the predicted most deleterious variant in an established epilepsy gene and the predicted most deleterious variant in a candidate gene is reported, based on the highest CADD score. When the variant with the highest CADD score was present in a recessive gene, the highest CADD score in a dominant gene was reported, if these were present.

6.3 Results

For 87 participants whole exome data was obtained. Coverage values of the analyzed gene sets differed between cohorts (ExAC and the described cohort), but was similar for sets within the same cohort (supplemental table S6.3 and S6.4). In all patients their known *SCN1A* pathogenic variants could be identified, except for large structural variants (e.g. deletions of the complete *SCN1A* gene), meaning no samples swaps had occurred.

Varying phenotypes were observed in six families (supplemental table S6.5). For 69 participants an estimation of cognitive functioning at the age of 6 years old could be made: 22 participants were severely affected, 29 were mildly affected, and 18 patients were categorized as intermediate. For 18 patients no estimation could be made, because they were either under the age of 6 and still mildly affected, or they were severely affected but no official IQ/DQ assessments were available close to the age of 6, meaning we cannot be sure when the exact decline happened. Ten of the mildly affected patients carried a LoF variant or a variant that was previously associated with a severe phenotype, and were included in the "mild" group. The mild group included both brothers from family 3; although one brother is significantly more severely affected than the other, both brothers were still categorized as mild at the age of 6 years old. The "mild" and "severe" patients combined are referred to as "extreme" patients.

6.3.1 Proportion of variants in epilepsy genes and control genes as compared to the ExAC database

Table 6.1 depicts the numbers of variants found in epilepsy genes, ID genes and different sets of control genes, for each groups of patients, in this cohort and in the ExAC database. We observed a significant excess of variants in epilepsy genes in the complete cohort, most strongly for type D variants but also for type E variants, when compared to ratios of variants in the ExAC database (111%-126%, p=0.000). A statistically significant overrepresentation of type D epilepsy gene variants was furthermore observed for extreme patients in relation to the control gene sets combined (118%, p=0.000). Overall, extreme patients showed a two- to fourfold (type E) and five- to sevenfold (type D) greater excess of epilepsy gene variants than intermediate patients (Table 6.2, Figure 6.1A). This pattern was observed in relation to all sets of control genes except for set 2. No significant excess of variants in intellectual disability genes was observed (Table 6.2, Figure 6.1B). There was no significant excess of variants in control set 1 in relation to variants in control set 3 and 4, conform expectation and suggesting validity of these analyses (Table 6.2, Figure 6.1C).

6.3.2 Differences between mild and severe patients

When assessing the distribution of variants present in epilepsy genes in our cohort between the different groups of patients (mild, severe and intermediate), we again observed a similar trend: both the severe and mild groups of patients tended to carry more variants than intermediate patients. This was true for all categories of variants except for the most restricted one (type A) (Table 6.3, Figure 6.2). These results were however not statistically significant, perhaps due to smaller sample sizes. The largest differences between the groups were again observed for type D variants. For each category of variants, we report the genes in which severe patients carried most variants compared to mild patients, and vice versa in Table 6.4.

6.3.3 Variants detected in families with variable phenotypes

Detailed clinical data on the phenotypes of participants from the six families with varying phenotypes are described in supplemental data S6.5. **Family 1** consists of a severely affected 10 year old proband with Dravet syndrome, and a father with mild epilepsy and normal cognitive functioning. **Family 2** is a large GEFS+ family in which exome sequencing was performed in two mildly affected family members (only febrile seizures/no seizures at all) and in one severely affected family member (severe epilepsy and a severe social-emotional delay, classified as Dravet syndrome). **Family 3** consists of two brothers with Dravet syndrome, one of whom is more severely affected than the other. **Family 4** consists of a proband with a phenotype on the border of Dravet syndrome and GEFS+, with regression over the years. His father has never had any seizures. **Family 5** consists of two brothers of whom the oldest has severe Dravet syndrome and the youngest has a much milder phenotype. **Family 6** consists of a proband with mild Dravet syndrome, a father with a milder epilepsy-

Table 6.1. Number of variants found in the ExAC database and in the current cohort, for different groups of patients and different categories of variants

	Type C variants (MAF[a] <0.01)				Type D variants (MAF <0.05)				Type E variants (MAF <0.1)			
Gene set	**ExAC**	**Complete cohort**	**Extreme patients**	**Intermediate patients**	**ExAC**	**Complete cohort**	**Extreme patients**	**Intermediate patients**	**ExAC**	**Complete cohort**	**Extreme patients**	**Intermediate patients**
Epilepsy genes	300155	851	337	161	556293	1876	662	335	805686	2455	888	481
Control 1	299750	858	305	176	641440	1778	626	371	950857	2608	893	546
Control 2	90285	278	130	49	185549	497	218	83	260406	765	327	140
Control 3	260659	707	270	147	513655	1378	501	300	812452	2165	798	467
Control 4	261763	702	247	131	565998	1603	579	331	853276	2291	824	473
Control 1-4 (total)	912457	2545	952	503	1906642	5256	1924	1085	2876991	7829	2842	1626
ID genes	557769	1572	582	355	1099721	3172	1132	708	1707615	4919	1739	1071

[a]Minor allele frequency; only variants with a frequency below this threshold in both the exomes and genomes in the gnomAD database are included.

Table 6.2. Overrepresentation of variants in epilepsy genes in the cohort, calculated based on different sets of control genes

		Over- or underrepresentation of epilepsy gene variants in cohort (% more or less than expected[a] (p-values[b]))								
		Type C variants (MAF[c] <0.01)			Type D variants (MAF <0.05)			Type E variants (MAF <0.1)		
Assessed group of genes	**Based on control set**	**Complete cohort**	**Extreme patients**	**Intermediate patients**	**Complete cohort**	**Extreme patients**	**Intermediate patients**	**Complete cohort**	**Extreme patients**	**Intermediate patients**
Epilepsy genes	**1**	-1 (0.846)	10 (0.221)	9 (0.414)	**22 (0.000)**	22 (0.000)	4 (0.598)	**11 (0.000)**	17 (0.001)	4 (0.552)
	2	-8 (0.229)	-22 (0.017)	-1 (0.941)	**26 (0.000)**	1 (0.904)	35 (0.016)	4 (0.388)	-12 (0.044)	11 (0.283)
	3	5 (0.387)	8 (0.329)	-5 (0.690)	**26 (0.000)**	22 (0.001)	3 (0.721)	**14 (0.000)**	12 (0.019)	4 (0.581)
	4	6 (0.285)	19 (0.038)	7 (0.597)	**19 (0.000)**	16 (0.008)	3 (0.727)	**13 (0.000)**	14 (0.007)	8 (0.257)
	1-4 (total)	2 (0.677)	8 (0.245)	-3 (0.785)	**22 (0.000)**	**18 (0.000)**	6 (0.374)	**12 (0.000)**	12 (0.005)	6 (0.292)
ID genes	**1**	-2 (0.718)	3 (0.750)	8 (0.042)	4 (0.184)	5 (0.288)	11 (0.101)	5 (0.045)	8 (0.051)	9 (0.097)
	2	-8 (0.178)	-28 (0.001)	17 (0.315)	8 (0.127)	-12 (0.073)	44 (0.002)	-2 (0.612)	-19 (0.001)	17 (0.092)
	3	4 (0.405)	1 (0.940)	13 (0.231)	8 (0.025)	6 (0.326)	10 (0.166)	8 (0.003)	4 (0.407)	9 (0.120)
	4	5 (0.280)	11 (0.193)	27 (0.020)	2 (0.561)	1 (0.918)	10 (0.159)	7 (0.006)	5 (0.216)	13 (0.025)
	1-4 (total)	1 (0.784)	0 (1.000)	15 (0.041)	5 (0.045)	2 (0.599)	13 (0.011)	6 (0.002)	3 (0.320)	11 (0.008)
Control set genes	**3**	6 (0.298)	-2 (0.835)	4 (0.738)	3 (0.370)	0 (1.000)	-1 (0.908)	3 (0.323)	-4 (0.367)	0 (1.000)
	4	7 (0.204)	8 (0.393)	17 (0.170)	-2 (0.535)	-5 (0.419)	-1 (0.910)	2 (0.465)	-3 (0.578)	4 (0.594)

[a]Numbers represent the percentage of over- or underrepresentation of variants in epilepsy genes, based on a comparison of ratios of variants found in epilepsy genes and in different control groups, in the ExAC database and the current cohort (e.g. the first "7" means that 107% of the expected number of epilepsy genes was identified in our cohort, based on the ratio of variants present in epilepsy genes and control 1-genes in the ExAC database, and the number of variants in control-1 genes present in our cohort). [b]*P*-values are based on Fishers' exact tests on the ratios of variants found in the ExAC database and the current cohort. Significant values are bolded. [c]Minor allele frequency; only variants with a frequency below this threshold in both the exomes and genomes in the gnomAD database are included.

Figure 6.1. Overrepresentation of variants in epilepsy genes in the cohort. Bars represent the percentage of over- or underrepresentation of variants in the different patient groups, based on the ratio of variants found in epilepsy genes and different control groups (ctrl 1, 2, 3, 4 and 1-4), compared to ratios in the ExAC database. A = variants in epilepsy genes compared to different control groups; B = variants in intellectual disability genes compared to different control groups; C = variants in control group 1 genes compared to control group 3 and 4 (negative control). Results are presented for categories of variants with different allele frequency cut-offs (<0.01, <0.05, <0.1). Significant values are depicted by asterisks.

Table 6.3. Distribution of variants in the epilepsy genes between groups of patients, for different categories of genes

	Group of patients	Type A variants (CADD[a]>20/ MAFb <0.01)	Type B variants (CADD>10/ MAF<0.01)	Type C variants (all CADD/ MAF<0.01)	Type D variants (all CADD/ MAF<0.05)	Type E variants (all CADD/ MAF<0.10)
% of variant alleles (based on total number of alleles per group)	Mild (n=10)	1.79	2.33	2.76	4.38	5.70
	Severe (n=22)	2.04	2.33	2.76	4.52	5.81
	Mild+severe (n=32)	1.96	2.33	2.76	4.47	5.78
	Intermediate (n=18)	1.95	2.06	2.35	4.03	5.57
P-values Fishers' exact test	Mild versus severe	0.456	1	0.997	0.73	0.812
	Mild versus intermediate	0.663	0.379	0.195	0.334	0.765
	Severe versus intermediate	0.783	0.294	0.112	0.109	0.491
	Mild+severe versus intermediate	0.998	0.259	0.089	0.113	0.727

[a] PHRED-scaled CADD (Combined Annotation Dependent Depletion). A score of >20 represents the top 1% deleterious substitutions in the human genome. [b]Minor allele frequency; only variants with a frequency below this threshold in both the exomes and genomes in the gnomAD database are included.

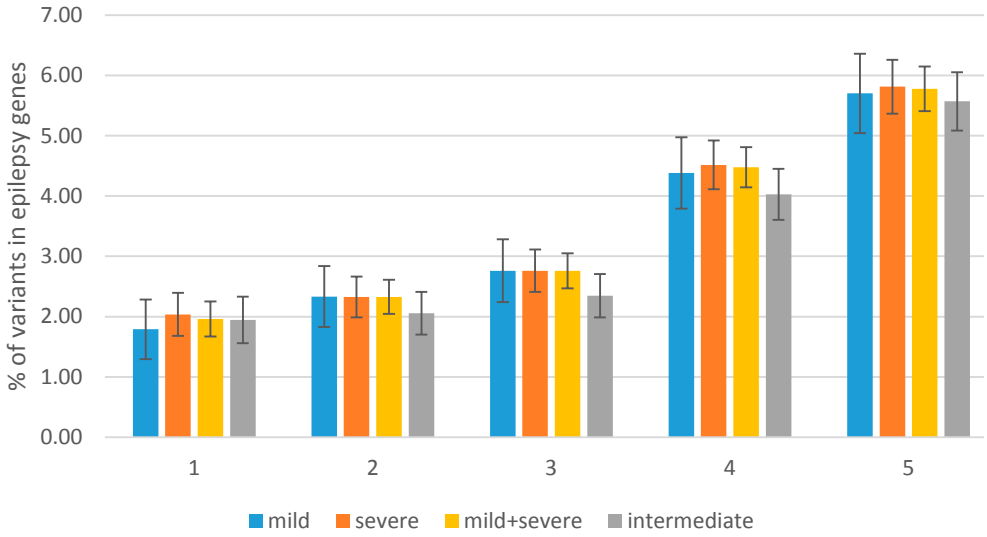

Figure 6.2. Distribution of variants in epilepsy genes among different groups of patients, depicted for different types of variants (A-E). A: CADD>20/MAF<0.01; B: CADD>10/MAF<0.01; C: all CADD/MAF<0.01; D: all CADD/MAF<0.05; E: all CADD/MAF<0.10. Percentages of variants relative to the total number of alleles per group are shown.

data from the ExAC database. Comparing absolute numbers of variants found in cases versus the ExAC database controls can lead to incorrect results, as differences in sequencing methods, coverage, variant calling, and population differences may lead to biases,[265] We therefore used the ratio of variants in epilepsy genes versus variants in different groups of control genes, and tested for differences between this ratio found in our patient cohort and this ratio observed in the ExAC database. Although exact frequencies of variants may differ between the ExAC cohort and our own, we expected the ratios of variants in epilepsy genes versus unrelated genes to remain similar, which was confirmed when assessing the ratios observed in the different control genes (Figure 6.1C). We observed the largest overrepresentation of epilepsy gene variants in the MAF <0.05 category (type D variants, Figure 6.1). This indicates that relatively common variants in epilepsy genes, which would not necessarily be classified as pathogenic, may have a large influence on phenotypes. Although we defined phenotype severity by cognitive capacities, no significant overrepresentation of variants in ID genes was observed, indicating that their role as modifier genes is limited. Similar outcomes were observed for comparisons to each control set, except for set 2; this may be due to the much smaller number of genes in this control set. A surplus of variants in epilepsy genes was observed in patients with extreme phenotypes, but not for intermediate patients. Furthermore, both mild and severe patients tended to carry more variants than intermediate patients in our cohort (the largest differences again being in the MAF <0.05 category (type D)), indicating that indeed both the phenotypes of severe as well as mild

and better cognitive functioning but with many social- and behavioral problems, and a grandmother who only experienced three seizures in her life. Type A variants that were only present in either the mild or the severe family members of each family are depicted in Table 6.5.

6.3.4 Variants detected in patients with the most extreme phenotypes

We further investigated type A variants in patients with the most extreme phenotypes in this cohort (IQ at the age of six <30 or >70): this comprised all 10 "mild" patients, and seven of the "severe" patients. For each patient, their most pathogenic variant in both an established epilepsy gene and in an epilepsy candidate gene is depicted in Table 6.6.

6.3.5 Comparison of current data and previous literature

A list of all previously implicated modifier genes for *SCN1A*-related epilepsy is shown in Table 6.7. The number of variants found in these genes in the current cohort is depicted for two different categories of variants: type A, representing the most deleterious variants with large effect size, and type D, as this is the category in which the largest overrepresentation of variants in epilepsy genes was found in patients with extreme phenotypes.

6.4 Discussion

Despite many efforts, we are still not able to fully explain variable phenotypes caused by similar pathogenic *SCN1A* mutations. More insight in modifying factors is essential for understanding genotype-phenotype relations and for accurate counseling of patients. We hypothesized that phenotypes of both severely and mildly affected patients are influenced by modifier genes, as both are on the most extreme ends of the disease spectrum. However, different hypotheses are possible as to which kinds of variants can modify these phenotypes: rare variants with large effects, or multiple more common variants with smaller effects. Previously, rare and/or pathogenic variants in genes involved in neuronal excitability and other known epilepsy genes were suggested to be modifiers.[37,197,206,198–205] Although such variants may have large effects on phenotypes (a second hit in severely affected patients, or a compensating variant in mildly affected patients), they are unlikely to be present in all patients with extreme phenotypes: none of these single modifier genes has been shown to be clinically relevant in a large patient group [205], Another possibility is the presence of (multiple) more common variants in modifier genes, that each have a smaller effect, but may simultaneously tip the balance over to a milder or more severe phenotype. Especially protective variants in mildly affected patients may not be rare, as they are not necessarily subject to negative selection.

To investigate both of the above described mechanisms, we explored in which categories of variants the largest excess was present in patients with extreme phenotypes, using variant

patients may be under the influence of modifier genes. The only exception to this occurred in the category with the most deleterious variants (type A); mild patients tended to have the lowest amount of variants in this category, suggesting that pathogenic variants in modifier genes are more likely to lead to more severe than to milder phenotypes.

The findings above suggest that it is difficult to draw conclusions from testing individual patients for variants in modifier genes: it is hard to prove whether a relatively common variant will have a substantial effect, and if so, what this effect will be, since variants are found in both mild and severely affected patients. For rare, pathogenic variants this may be easier. Although no significant excess of these variants was observed in mild or severe patients, they may still be present in several patients: only one extra variant with a large effect size may be necessary to drastically change outcomes, which is difficult to statistically detect. We therefore also report the type A variants (CADD>20, MAF<0.01) that were detected in the most extremely mild and severe patients, and those that were only present in either the mild or the severe members of affected families, in a descriptive way. Studying families in which the same *SCN1A* variant leads to variable phenotypes has several advantages: not only is the primary influence of different *SCN1A* variants themselves removed from analyses, it also means that variants that are shared between both severe and mild family members can be excluded to have significant effects. Statistically proving the modifying effects of single genes or even specific variants remains difficult; there are only small numbers of patients with extreme phenotypes and *SCN1A* mutations of which the effect can reliably be predicted, which consequently leads to low detection power. Furthermore, the variety of different possible modifier genes that may act simultaneously makes it difficult to attribute effects to specific variants. In addition, since some genes can carry both LoF and gain of function (GoF) variants, and also variants that cause no relevant effect at all, they may be incorrectly dismissed as modifier genes when variants are present in both severe, mild and intermediate patients. Functional testing is required to conclusively prove or disprove any modifying effects of single variants, which is not feasible for all variants detected in this study. However, by presenting the most significant genes in each category of variants (Table 6.4) and variants that are likely to have the largest effects (Table 6.5 and 6), we provide data for future reference. Combined with data from future studies similar to ours, trends in the cumulative data may be detected and groups of patients with similar genotype-phenotype correlations may be assembled for further research.

Despite a lack of statistical significance, some interesting results were observed in our study in relation to previous literature: a likely pathogenic *SCN8A* variant was detected in an extremely mild patient. *SCN8A* has previously been implicated to ameliorate *SCN1A* phenotypes by restoring normal seizure thresholds.[198,201] Furthermore, several severe patients carried variants in genes that were previously described to worsen phenotypes *(POLG*, *SCN2A*, *CACNA1A*and *CACNA1G),* strengthening those associations.[37,201,202,204] *GPR98*, a gene implicated in myoclonic epilepsy,[267] showed the highest overrepresentation of variants in severe patients in three categories of variants, and *SCN10A*, another sodium channel

Table 6.4. Top 5 genes with an overrepresentation of variants in mild or severe patients, per category of variants

	Type A variants (CADD[a]>20/ MAF[b] <0.01)	**Type B variants** (CADD>10/MAF<0.01)	**Type C variants** (all CADD/MAF<0.01)	**Type D variants** (all CADD/MAF<0.05)	**Type E variants** (all CADD/MAF<0.10)
Genes with an excess of variants in mild patients (gene name (p-value))[c]	*SCN10A* (0.027)	*SLC6A8* (0.013)	*EFHC1* (0.002)	*MOCS2* (0.003)	*MOCS2* (0.003)
	ACTL6B (0.094)	*SCN10A* (0.03)	*SCN10A* (0.01)	*KCNH1* (0.008)	*KCNH1* (0.008)
	COL3A1 (0.094)	*RAI1* (0.087)	*SLC6A8* (0.013)	*EFHC1* (0.01)	*DRD4* (0.009)
	DEPDC5 (0.094)	*ACTL6B* (0.094)	*DSC2* (0.027)	SLC6A8 (0.013)	*SLC6A8* (0.013)
	KPNA7 (0.094)	*COL3A1* (0.094)	*RAI1* (0.087)	*MYT1* (0.027)	*CTSD* (0.026)
Genes with an excess of variants in severe patients (gene name (p-value))	*GPR98* (0.049)	*GPR98* (0.201)	*GPR98* (0.193)	*RYR2* (0.025)	*RYR2* (0.013)
	RYR2 (0.419)	*CUX1* (0.314)	*ANKRD11* (0.3)	*CUX1* (0.049)	*CUX1* (0.049)
	ITPR1 (0.564)	*RYR2* (0.417)	*ANK2* (0.314)	*KCNB1* (0.088)	*KCNB1* (0.088)
		SIK1 (0.564)	*CUX1* (0.314)	*ANK2* (0.094)	*AKAP9* (0.094)
		TSC1 (0.564)	*RYR2* (0.417)	*AKAP9* (0.094)	*ANK2* (0.094)

[a]PHRED-scaled CADD (Combined Annotation Dependent Depletion). A score of >20 represents the top 1% deleterious substitutions in the human genome. [b]Minor allele frequency; only variants with a frequency below this threshold in both the exomes and genomes in the gnomAD database are included. [c]p-values are based on Fishers' exact test.

Tabel 6.5. Rare and predicted deleterious variants present in only relatively mildly or severely affected members of families with varying *SCN1A*-related phenotyes

	Family[a]	Gene	Established epilepsy gene[b]	Variant	MAF[c]	CADD-phred score[d]
Relatively severely affected family members	**1**	*PRKACA*		c.452T>C,p.Ile151Thr (missense)	0	26.1
		ITPR1		c.1435G>A,p.Val479Ile (missense)	0.0047	22.2
	2	*GPR98*		c.3151G>T,p.Asp1051Tyr (missense)	0.0021	26
	3	*DRD4*		c.1016G>A,p.Gly339Asp (missense)	0.0015	25
		DEPDC5	yes	c.3551T>A,p.Leu1184Gln (missense)	0	32
		DEPDC5	yes	c.3434C>T,p.Ser1145Phe (missense)	0.00007785	21.6
		CREB5		c.685C>A,p.His229Asn (missense)	0.0003	25.5
		DSG2		c.166G>A,p.Val56Met (missense)	0.0019	27
	4	*ATF2*		c.977C>T,p.Pro326Leu (missense&splice region)	0.0007	23.5
	5	*GNAS*		c.1648G>A,p.Ala550Thr (missense)	0.00002394	24.3
		OGDHL		c.2201T>C,p.Phe734Ser (missense)	0.0073	32
	6 (proband and father)	*CREB3*		c.359T>C,p.Leu120Pro (missense)	0.0003	23.6
		SNTA1		c.566C>T,p.Ser189Leu (missense)	0.0002	23.6
		GJA9		c.22G>A,p.Gly8Arg (missense)	0.0017	29.3
		LGI2		c.194C>T,p.Ser65Phe (missense)	0.000005	28.4
	6 (only proband)	*TSC2*	yes	c.275A>T,p.Glu92Val (missense)	0.001	25.9
		RAI1	yes	c.725C>T,p.Pro242Leu (missense)	0.003	24.3
		GABRA3	yes	c.766C>T,p.Arg256Trp (missense)	0	29.3

	Family[a]	Gene	Established epilepsy gene[b]	Variant	MAF[c]	CADD-phred score[d]
Relatively mildly affected family members	**1**	*KCNB1*	yes	c.2266A>C,p.Ile756Leu (missense)	0	23.3
		PKP2		c.76G>A,p.Asp26Asn (missense)	0.0084	33
		GPR98		c.9650C>T,p.Ala3217Val (missense)	0.0091	23.6
		SHANK3	yes	c.1379_1382delGAAT,p.Arg460fs (frameshift)	0.0021	25.4
		SYNGAP1	yes	c.3982_3983insCCCCCCCG,p.Arg1328fs (frameshift)	0	34
		DNM3		c.2171G>A,p.Arg724His (missense)	0.0059	23.3
		GABRA6		c.805G>A,p.Val269Ile (missens)	0.0025	28
	2 (father and brother of proband)	*RYR2*		c.4451A>G,p.Tyr1484Cys (missense)	0.000008183	25.5
	3	*PRRT2*	yes	c.647C>A,p.Pro216His (missense)	0.0005	26.2
		CHD5		c.5074G>T,p.Gly1692Trp (missense)	0.0002	34
	4	*SLC19A3*	yes	c.388G>A,p.Val130Met (missense)	0.000004062	21
		SZT2	yes	c.8384C>G,p.Thr2795Arg (missense)	0.000008129	27.5
	6	*JUP*		c.1165C>T,p.Arg389* (stop)	0.00003253	37
		GOSR2	yes	c.509A>G,p.Asn170Ser (missense)	0.0003	23.3
		DOCK3		c.5446G>A,p.Val1816Met (missense)	0	22.5
		SLC6A1	yes	c.1243C>A,p.Leu415Ile (missense)	0.0025	20.1

[a]Members from families 1-6, as described in supplemental data S6.5. The upper part of the table represents the patients who are relatively severely affected, compared to their other family members; the lower part of the table represents the participants who are relatively mildly affected, compared to their other family members. [b]Genes were considered established epilepsy genes when present in the diagnostic epilepsy gene panel of the University Medical Center Utrecht. [c]Highest frequency of the variant observed in both exomes and genomes in the gnomAD database. [d]Combined Annotation Dependent Depletion[264]: numbers represent PHRED-scaled CADD scores. CADD scores of >20 represent the top 1% deleterious substitutions in the human genome.

Table 6.6. Predicted most pathogenic, rare variants in extremely mild and extremely severe patients

		Established epilepsy genes[d]				Candidate modifier genes[d]			
	Patient (IQ at the age of 6)[c]	Gene	Variant	CADD[e]	MAF[f]	Gene	Variant	CADD[e]	MAF[f]
Mild patients[a]	**1 (89.1)**	*SCN8A*	c.1925C>T, p.Thr642Met	27.5	0	*EFHC1*	c.661C>T, p.Arg221Cys	33	0.0009
	2 (72.8)	*MOCS2*	c.367C>T, p.His123Tyr	31	0.0036	*AIFM3*	c.496C>T, p.Arg166Trp	34	0.0001
	3 (91.4)	*SCN9A*	c.3770A>G, p.Asn1257Ser	23.9	0.0044	*ARNTL*	c.1376C>T, p.Ser459Phe	29.3	0.00004
	Both 4 and 5 (brothers) (76.6 and 98.2)	*ACTL6B*	c.496G>A, p.Val166Met	25.2	0	-	-	-	-
	Both 6 and 7 (twins) (83.7 and 93.3)	*KPNA7*	c.1353T>G, p.Cys451Trp	26.7	0.00003964	*SCN10A*	c.4849G>T, p.Val1617Phe	28.8	0.00007
	8 (100)	*RAI1*	c.5036C>T, p.Ala1679Val	22.2	0.0006	-	-	-	-
	9 (75.7)	*NPRL3*	c.1123C>T, p.Arg375Cys	24.7	0.00003229	*AIFM3*	c.1123C>T, p.Arg375Cys	32	0.0044
	10 (81.9)	*KMT2A*	c.4972C>G, p.Arg1658Gly	29.6	0.0001	*CACNA1H*	c.2470G>T, p.Ala824Ser	22.8	0.00001
Extremely severe patients[b]	**1 (18.9)**	*KDM5C*	c.203G>A, p.Arg68Gln	34	0.000005615	*PLCB2*	c.2716G>A, p.Glu906Lys	28.8	0.0028
	2 (22.7)	*TSC2*	c.5383C>T, p.Arg1795Cys	31	0.0015	*PHTF1*	c.1779G>T, p.Trp593Cys	34	0
	3 (25.4)	*KCNQ3*	c.1706A>G, p.Asp569Gly	29.4	0.00002031	*BCAN*	c.2117G>T, p.Arg706Leu	32	0.0026
	4 (25.4)	*NBEA*	c.8350G>T, p.Val2784Phe	32	0.0025	*CACNA1H*	c.6048+2_6048+5 delTAGG	35	0.00003
	5 (26.7)	*BRAT1*	c.2353C>T, p.Arg785Trp	24.4	0.0029	*GJA9*	c.22G>A, p.Gly8Arg	29.3	0.0017
	6 (27.6)	-	-	-	-	*STXBP5L*	c.1135G>A, p.Val379Met	28.6	0.0054
	7 (28.3)	*SLC6A5*	c.1735A>G, p.Met579Val	25.1	0.0000488	*KCNH8*	c.1414A>G, p.Ile472Val	26.8	0.0001

[a] Patients with an interpolated IQ at the age of six years old >70 (all patients with an *SCN1A* variant that is predicted to cause complete LoF, or a variant that has been previously described in Dravet syndrome patients). [b]Patients with an interpolated IQ at the age of six years old <30. [c]IQ- and developmental assessment scores, conducted at different ages, were interpolated by linear regression, to obtain approximate scores at age 6 years of age as previsously described.[212,4] A distinction was made between established monogeneric epilepsy genes (when present in the diagnostic epilepsy gene panel of the University Medical Center Utrecht) and candidate genes (all other genes in the epilepsy group). [e]Combined Annotation Dependent Depletion:[264] numbers represent PHRED-scaled CADD scores (a score of >20 represents the top 1% deleterious substitutions in the human genome). [f]Highest frequency of the variant observed in both exomes and genomes in the gnomAD database.[156]

Table 6.7. Overview of previously described candidate modifier genes and variants in them detected in the current cohort

			Number of variants (CADD[a] >20) in current cohort				
			Variants in only mild or severe member of families with varying phenotypes (MAF<0.01, CADD>20)	Type A variants (MAF[b]<0.01, CADD>20)		Type D variants (MAF<0.5, all CADD scores)	
Previously described candidate gene	**Described effect**	**Reference**		**Mild patients (n)**	**Severe patients (n)**	**Mild patients (n)**	**Severe patients (n)**
SCN9A	Pathogenic variants present in multipe Dravet syndrome patients	Singh 2009[197]		1	2	2	3
SCN8A	SCN8A pathogenic variants rescue *SCN1A*-phenotype in mice; increased resistance for induces seizures in mice with GEFS+ variants	Martin, 2007[198]; Hawkins, 2012[200]		1		1	1
HLF	Decreased survival in HLF/ *SCN1A* double knockout mice	Hawkins, 2016[199]					
POLG	POLG variants may increase susceptibility to focal brain injury during prolonged seizures in Dravet syndrome	Gaily,2013[37]			1		4
CACNB4	Pathogenic variant in Dravet syndrome patient who died after status epilepticus	Ohmori, 2008[203]					
CACNA1G	Decreased Cacna1g expression led to partial amelioration in *SCN1A*+/– mice	Calhoun, 2017[204]			1	2	2
CACNA1A	More severe phenotype in Dravet syndrome patients who also have CACNA1A variants	Ohmori, 2013[202]			1		3
Gabra2	Potential candidate gene at locus linked to premature lethality in *SCN1A*+/– mice	Miller, 2014[206]					
Gabrg3	" "	Miller, 2014[206]				1	
Gabrb3	" "	Miller, 2014[206]					

Previously described candidate gene	Described effect	Reference	Number of variants (CADD[a] >20) in current cohort				
			Variants in only mild or severe member of families with varying phenotypes (MAF<0.01, CADD>20)	Type A variants (MAF[b]<0.01, CADD>20)		Type D variants (MAF<0.5, all CADD scores)	
				Mild patients (n)	Severe patients (n)	Mild patients (n)	Severe patients (n)
Gabra6	" "	Miller, 2014[206]		1		2	2
Gabrb2	" "	Miller, 2014[206]					1
Cacna1a	" "	Miller, 2014[206]					
Cacna2d1	" "	Miller, 2014[206]					1
Clcn3	" "	Miller, 2014[206]					
Kcnj11	" "	Miller, 2014[206]				1	
Atp1a3	" "	Miller, 2014[206]			1		2
Lgi2	" "	Miller, 2014[206]					2
Mapk10	" "	Miller, 2014[206]					
Reln	" "	Miller, 2014[206]					3
Slc7a10	" "	Miller, 2014[206]				1	
KCNQ2	Variants present in 3/12 severe Dravet syndrome patients, not in mild patients; More severe phenotype in GEFS+ mice that also carry KCNQ2 variant	Hammer, 2017[205]; Hawkins, 2012[200]				1	3
SCN2A	More severe phenotype in GEFS+ mice that also carry SCN2A variant	Hawkins, 2012[200]			1		1
Top ranking EE genes in common epilepsy							
DEPDC5	Enriched in common epilepsies	Epi4K consortium, 2017[266]	2 variants in more severe brother of family 3; both are however mildly affected			4	5
LGI1	" "	Epi4K consortium, 2017[266]					
PCDH19	" "	Epi4K consortium, 2017[266]					

Table 6.7. Overview of previously described candidate modifier genes and variants in them detected in the current cohort (continued)

			Number of variants (CADD[a] >20) in current cohort				
			Variants in only mild or severe member of families with varying phenotypes (MAF<0.01, CADD>20)	Type A variants (MAF[b]<0.01, CADD>20)		Type D variants (MAF<0.5, all CADD scores)	
Previously described candidate gene	Described effect	Reference		Mild patients (n)	Severe patients (n)	Mild patients (n)	Severe patients (n)
GRIN2A	" "	Epi4K consortium, 2017[266]					2
KCNA2	" "	Epi4K consortium, 2017[266]				2	1
GABRB3	" "	Epi4K consortium, 2017[266]					
GABRA1	" "	Epi4K consortium, 2017[266]					
KCNQ2	" "	Epi4K consortium, 2017[266]				1	3
GABRG2	" "	Epi4K consortium, 2017[266]					
SCN1B	" "	Epi4K consortium, 2017[266]					
SLC6A1	" "	Epi4K consortium, 2017[266]	1 variant in mildly affected grandmother of Dravet syndrome patients (family 6)				
EEF1A2	" "	Epi4K consortium, 2017[266]					

[a]PHRED-scaled CADD (Combined Annotation Dependent Depletion). A score of >20 represents the top 1% deleterious substitutions in the human genome. [b]Minor allele frequency; only variants with a frequency below this threshold in both the exomes and genomes in the gnomAD database are included.

alpha-subunit gene, was most often implicated in mild patients. One relatively severe patient carried a *GABRA3* variant (family 6); several GABA receptor genes have already been suggested as potential *SCN1A* modifiers [206]. Inhibition of *DOCK3*, in which a variant was found in a relatively mild patient (family 6), was previously shown to decrease epileptic activity.[268] Interestingly, frameshift variants in *SHANK3* and *SYNGAP1* were detected in a relatively mild patient (family 1). Both genes are associated with severe neurodevelopmental disorders.[269,270] The presence of these variants in a mildly affected patient may be explained by their location in the genes: the *SHANK3* variant resides in exon 11, which has previously been implicated to be absent from most or all *SHANK3* transcripts.[271] The *SYNGAP1* variant is at the 3' end of the gene, which may lead to less severe effects. Nevertheless, it remains a possibility that some of the presented variants are sequencing or calling errors, since it was not feasible to confirm all variants by Sanger sequencing. We however do not expect such variants to influence our main results, since similar error rates are to be expected between different groups of patients and categories of variants.

In conclusion, while the clinical outcomes of some patients affected by pathogenic *SCN1A* variants may be influenced by rare variants with a large effect size, our results indicate that relatively common variants in epilepsy genes play a large role in modulating phenotypes as well, in both severely and mildly affected patients. Studies in larger cohorts, combined with functional assessments, will be necessary to confirm or disprove the modifying effects of the genes implicated in this study, and to progress towards clinically meaningful testing of modifier gene variants in regular diagnostics.

6.5 Appendix

Table S6.2. Characteristics of epilepsy genes, ID genes and control sets 1-4

Gene set	Average z-score missense	Average pLI LoF	Average number of exons	Average number of coding basepairs	% of genes pLI >= 0.9	Total number of coding basepairs
Epilepsy genes	2.305	0.596	16.5	2559	47.5	1080053
Control 1	0.891	0.359	13.4	1962	26.4	700293
Control 2	1.409	0.469	16.9	2506	36.7	273194
Control 3	0.409	0.263	17.3	2679	21.1	597507
Control 4	0.635	0.297	14.3	2031	21.1	603202
ID genes	1.293	0.395	15.1	2404	32.3	1576863

Table S6.3. Coverage of epilepsy genes, ID genes and control sets 1-4 in the current cohort

Gene set	Average coverage complete cohort	% of basepairs >20X complete cohort	Average coverage extreme patients	% of basepairs >20X extreme patients	Average coverage intermediate patients	% of basepairs >20X intermediate patients
Epilepsy genes	98.0	95.6	102.9	95.7	85.7	94.8
Control 1	96.3	93.9	101.0	93.9	83.9	93.1
Control 2	99.2	97.0	104.1	97.0	86.8	96.4
Control 3	96.6	95.2	101.3	95.2	84.6	94.5
Control 4	98.6	96.7	103.5	96.8	86.2	96.0
ID genes	100.3	95.9	105.3	96.0	87.6	95.2

Table S6.4. Coverage of epilepsy genes, ID genes and control sets 1-4 in the ExAC database

Gene set	Average coverage ExAC	Median coverage ExAC
Epilepsy genes	53.2	55.0
Control 1	53.4	55.5
Control 2	55.5	58.7
Control 3	54.8	57.6
Control 4	54.9	57.1
ID genes	53.5	55.8

S6.5 Clinical descriptions of families with varying *SCN1A* phenotypes

Family 1

The proband of family 1, a 10 year old boy, started having febrile seizures at the age of 10 months. Soon after, afebrile seizures started occurring, and his epilepsy became intractable. He currently experiences multiple generalized tonic-clonic-, tonic-, absence seizures and myoclonias per week, while using valproate and clobazam. Status epilepticus has occurred twice. The longest period of seizure freedom since onset was 1.5 weeks. A developmental delay became evident at age 3 years old. At age 6.5 years old his developmental age was estimated to be around 2.5 years. He shows behavioral problems, is diagnosed with autism, and furthermore has walking difficulties caused by ataxia and hypotonia. At age 4 years old a pathogenic *SCN1A* variant was found (c.1186G>T (p.Gly396Trp)) and Dravet syndrome was diagnosed.

The father of this boy, a 43 year old man, was found to carry the same *SCN1A* variant. He has experienced seizures since the age of 18 months, at most 4 per year and in his childhood only occurring during fever. He was treated with valproate, which was discontinued between 12 and 16 years of age. At age 16 seizures reoccurred, after which valproate was prescribed again. With that, seizure freedom was established until the age of 28, after which 6-7 seizures occurred in 6 months. Currently, this patients uses levetiracetam and carbamazepine and has had no seizures for the last 11 years. He has only ever experienced generalized tonic-clonic seizures and there was never a status epilepticus. There has been no developmental delay and this patient has followed regular education.

Family 2

Family 2 is a large GEFS+ family in which multiple family members experienced febrile seizures, mild epilepsy or no symptoms at all, based on a pathogenic *SCN1A* variant (c.1217T>C (p.Val406Ala)). Three family members have been included in this study. Two of those, a father (age 64) and son (age 35), showed phenotypes comparable to most other affected family members. The father has never experienced any seizures, as far as he knows, and had a normal development. His son has experienced a febrile seizure two or three times between the age of 1-2 years, for which he was never treated. He has followed regular education also had a normal development. His sister however was severely affected compared to her other family members. Seizure onset was at age 18 months and tonic-clonic seizures occurred weekly. She soon also developed focal seizures with impaired awareness, at age 8 absences started to occur, and at age 15 she developed myoclonias. A status epilepticus has occurred 16 times. She had been treated with many anti-epileptic drugs, but became seizure free for 6 years using a combination of topiramate, oxcarbazepine and clobazam. Although she scored well on cognitive assessments (IQ 95 at age 29) she followed special education and was impaired on a social-emotional level, for which a developmental age of 2-4 years old was established. She showed behavioral problems and signs of ADHD. Ataxia was

present, leading to walking difficulties, and she furthermore suffered from Crohn's disease, psoriasis and asthma. She committed suicide at the age of 30.

Family 3

Family 3 consists of two brothers that both carry the same pathogenic *SCN1A* variant (c.1209delT (p.Phe403fs)). The oldest brother (age 37) had a first febrile seizure at the age of 9 months. Soon after he developed intractable generalized tonic-clonic seizures. During puberty he developed focal seizures with impaired awareness. He has never experienced myoclonias or a status epilepticus. He currently uses valproate and topriamate and is now free of seizures. There was a slowing of development and he has followed special education, although an IQ of 91 was assessed. He works in a protected environment.

His younger brother (33 years old) is more severely affected. He also experienced a first febrile seizure at the age of 9 months, and afterwards developed intractable epilepsy with mostly tonic-clonic seizures. He has experienced a status epilepticus 6 times and has recently developed focal seizures with impaired awareness, myoclonias and absences. He is diagnosed with an autism spectrum disorder, followed special education and an IQ of 56 was assessed. He attends day-care activities.

Family 4

The proband in family 4 is a 28 year old man that experienced 6 febrile seizures between the ages of 18 months and 5 years. Between the ages of 5 and 16 years tonic-clonic seizures became provoked by exercise, and seizures are currently still occurring 10 times per year. The longest period of seizure freedom was 5 months. He currently uses pregabalin, valproate, carbamazepine, clobazam an topiramate. He experienced status epilepticus as a young child, at 7-8 years old and at 10 years old. Although this patient had a normal development as a child, during puberty he was not able to keep up with classmates anymore and memory problems became an issue. He originally started high school at a high educational level, but had to drop down several levels and now works in a sheltered environment. A pathogenic *SCN1A* variant (c.1719C>A (p.Ser573Arg)) was detected at age 28. His father (age 67) carries the same variant, has never experienced any seizures and has followed regular education without any signs of developmental delay.

Family 5

Family 5 consists of two brothers that both carry a pathogenic *SCN1A* variant (c.2584C>T (p.Arg862*)). The oldest brother (8 years old) developed intractable seizures at the age of 12 months. He currently experiences tonic-clonic seizures twice per week, and has also developed focal seizures with impaired awareness, absences (twice per day), myoclonias and tonic seizures, while using valproate, clobazam and stiripentol. His longest period of seizure freedom was 3 months. He has experienced convulsive status epilepticus six times, and has had multiple occurrences of non-convulsive status epilepticus per year. His

development started slowing at age 18 months, and at 8 years old his developmental age was estimated to be 24 months. No behavioral problems are reported. He has difficulties walking, characterized by ataxia and hypotonia. His younger brother (age 4) also developed seizures at the age of 12 months, but experiences them much less frequently. He currently has around 6 tonic-clonic seizures and absences per year, while only using valproate. His longest period of seizure freedom was 7 months and he has experienced status epilepticus twice. He develops without any signs of delay and follows regular education. He shows no walking difficulties or behavioral problems.

Family 6

Family 6 consists of three members, all carrying a pathogenic *SCN1A* variant (c.2945T>C (p.Val982Ala)). The proband, a 9-year old boy, started having seizures at the age of 6 months, during a fever episode. He developed multiple seizure types (focal seizures with impaired awareness, atonic seizures, hemi-convulsions, absences, tonic seizures and myoclonias) but mostly suffered tonic-clonic seizures that could occur multiple times per day. He experienced status epilepticus once and has temporarily lived in an epilepsy center during weekdays. He has now however been seizure free since 2.5 years, while using valproate, topiramate and clobazam. A developmental delay was noticed at the age of 3 years and an IQ of approximately 70 was tested. He follows special education, shows behavioral problems and signs of ADHD. There are no walking disabilities. His father, a 32 year old man, has a less severe epilepsy phenotype. He experienced febrile seizure in his early youth and afebrile seizures as a child, but has also been seizure free for long periods of time. He only ever experienced tonic-clonic seizures and has never had a status epilepticus. He currently uses valproate and clobazam, but does not take his medication regularly and lives an unhealthy irregular life, which has caused an increase in seizure frequency to once a month. He has followed normal education although some support was necessary, and works as a cook. He is however diagnosed with an autism spectrum disorder, shows signs of ADHD, has problems with social functioning, struggles with addiction, has debts, is homeless and can show aggressive behavior. His mother, the grandmother of the proband (age 55), has a much milder phenotype than her son and grandson. She has only experienced 3 tonic-clonic seizures in her life, for which she never used any medication, and has been seizure free for 35 years now. She is highly educated and shows no behavioral problems. The grandmother of the proband has a deceased brother for whom no DNA was available. He had a severe epilepsy phenotype with 12-13 seizures per day and intellectual disability. He drowned during an epileptic seizure. *SCN1A* testing was never performed.

Chapter 7

Influence of common *SCN1A* promoter variants on the severity of *SCN1A*-related phenotypes

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Submitted for publication

Abstract

Objective: Pathogenic variants in *SCN1A* cause variable epilepsy disorders with different disease severities. We here investigate whether common variation in the promoter region of the unaffected *SCN1A* allele could reduce normal expression, leading to a decreased residual function of Nav1.1, and therefore to more severe clinical outcomes in patients affected by pathogenic *SCN1A* variants.

Methods: Five different *SCN1A* promoter-haplotypes were functionally assessed in SH-SY5Y cells using Firefly and Renilla luciferase assays. The *SCN1A* promoter region was analyzed in a cohort of 143 participants with *SCN1A* pathogenic variants. Differences in clinical features and outcomes between participants with and without common variants in the *SCN1A* promoter-region of their unaffected allele were investigated.

Results: All non-wildtype haplotypes showed a significant reduction of luciferase expression, compared to the wildtype promoter-region (65%-80%, p=0.039 - 0.0023). No statistically significant differences in clinical outcomes were observed between patients with and without common promoter variants. However, patients with a wildtype promoter-haplotype on their unaffected *SCN1A* allele showed a non-significant trend for milder phenotypes.

Significance: The non-significant observed trends in our study warrant replication studies in larger cohorts to explore the potential modifying role of these common *SCN1A* promoter-haplotypes.

7.1 Introduction

Dravet syndrome is one of the most well-known genetic epilepsy syndromes. The main characteristics of the disease are early onset intractable epileptic seizures and a delayed psychomotor development that results in mild to severe intellectual disability (ID). Furthermore, many patients experience walking difficulties and/or behavioral problems.[4,60,62,92,255] Mutations in the *SCN1A* gene are the cause of disease and detected in the majority of Dravet syndrome patients.[118] *SCN1A* encodes for the α-subunit of a neuronal sodium channel, Nav1.1. The main disease mechanism in *SCN1A*-related Dravet syndrome is haploinsufficiency, caused by complete or partial loss of function of the channel, which leads to disturbances in neuronal excitability.[121,131]

Pathogenic variants in *SCN1A* are also found in patients with much milder phenotypes, such as Genetic Epilepsy Febrile Seizures Plus (GEFS+) syndrome or febrile seizures only.[108] The association of *SCN1A* with multiple phenotypes may be partly explained by the varying effects of different pathogenic variants: variants that cause a complete loss of function (LoF) of the channel are virtually always associated with severe phenotypes, whereas variants that cause milder disturbances are usually found in milder phenotypes.[117] However, this does not fully explain the variability that is observed in *SCN1A*-related phenotypes: varying phenotypes have been associated with the exact same variant, even within families, and Dravet syndrome patients with similar LoF variants may show very different clinical outcomes.[8,9,25,96,97,159–161,171] Several modifying factors have already been proven or suggested to have an influence on these outcomes, such as mosaicism for the pathogenic *SCN1A* variants, the presence of variants in modifier genes and environmental factors such as anti-epileptic treatment.[47,83,167,169,171,209,212]

Another factor that could potentially contribute to phenotypic variability is additional variation in the *SCN1A* gene itself. Genome-wide association studies (GWAS) have shown a significant association between *SCN1A* and genetic generalized, focal and unclassified epilepsies in general, and hippocampal sclerosis and febrile seizures.[181,182] This observation suggests that common, low risk variation may affect normal function and/or expression of *SCN1A*.

SCN1A has two major promoters that are simultaneously active in various brain regions including the cerebellum, cerebral cortex, putamen, hippocampus and thalamus.[192] Both promoters alone yield transcription activity in a neuronal cell culture assay, though the activity was greatly enhanced when 5' untranslated exons (UE) were added.[189] A total of five 5' UEs of *SCN1A* are currently known, all of them carrying multiple putative transcription factor binding sites.[189,190] Adding to the complexity of *SCN1A* transcription, the 5' untranslated region including both promoters are located 75Kb upstream of the first coding exon (Figure 7.1).[189,190] This region has not been studied extensively in Dravet syndrome patients, but may harbor mutations that could either be the cause of their epilepsy, or include variants that could modify the phenotype caused by another major mutation in the coding region of the

gene. So far, two reports have been published that suggested that pathogenic mutations in the regulatory 5' region of *SCN1A* were likely the cause of disease in two Dravet syndrome patients, as no *SCN1A* coding mutations could be detected. Interestingly, the novel promoter mutations were found to reduce transcription *in-vitro,* increasing the likelihood of their causality.[191,192] These findings stress the importance of the *SCN1A* promoter-regions for correct functioning of the Nav1.1 channel. It has previously been suggested that part of the 20-30% of Dravet syndrome patients in whom no coding variants in *SCN1A* could be detected, harbor mutations in its regulatory regions.[149] However, in most diagnostic centers the promoter regions are not routinely sequenced when analyzing *SCN1A*, so its exact role remains unclear.

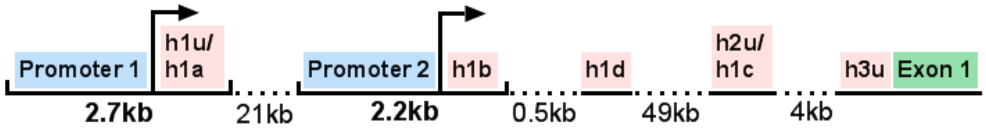

Figure 7.1. Overview *SCN1A* 5' UTR. *SCN1A* has a complex 5' UTR. Two major promoters (blue) and five 5' UE (pink) are currently known. The half-tick up lines indicates both promoter regions including the subsequent 5' UE with an Initiator element, transcription start site is indicated with an arrow. Dashed lines indicate the distance to the next element. Underlined elements indicate the remaining three 5' UEs and the first coding exon of *SCN1A*.

We hypothesize that not only pathogenic mutations, but also common variation on the promoter regions of *SCN1A* can interfere with normal expression. Although the effects of common variation are likely milder than those of a true pathogenic mutation in the promoter regions, a clinical effect might be detectable when common variation in the promoter regions coexists with a pathogenic mutation in the coding region of *SCN1A* on the other allele. A small decrease in expression of *SCN1A* could lead to a decreased residual function of Nav1.1 in patients that are already haploinsufficient, and therefore lead to more severe clinical outcomes. Previously, no significant differences in expression were observed for a group of common variants in the first *SCN1A* promoter region.[191] We have cloned a new set of haplotypes and used a slightly altered promoter region that includes the first 5' UE in the functional expression analysis. In this study, we analyze the first *SCN1A* promoter-region of 143 participants affected by pathogenic *SCN1A* variants, to investigate whether common variation in this region can affect phenotypic outcomes.

7.2 Materials and methods

7.2.1 Editorial Policies and Ethical Considerations

The study was approved by the Ethical Committee of the University Medical Center Utrecht. Informed consent was obtained from participants or their legal caretakers according to the Declaration of Helsinki.

7.2.2 Participants and clinical data

Participants

A cohort of 143 participants with *SCN1A* pathogenic variants was evaluated, of which most have previously been described.[209,212] Only participants with pathogenic variants (class V) or likely pathogenic variants (class IV) in *SCN1A* were included, according to the American College of Medical Genetics and Genomics criteria.[155] All variants had been detected and classified in genetic diagnostic laboratories. Patients who had previously been shown to be mosaic (n=4) for their pathogenic *SCN1A* variant were excluded from analyses, as mosaicism may greatly influence outcomes.[212] Our cohort comprised patients with Dravet syndrome, GEFS+, febrile seizures and also four participants who had been seizure free their entire lives, but did have a child with Dravet syndrome that carried the same pathogenic *SCN1A* variant. Dravet syndrome was diagnosed based on previously published criteria[217] and in line with recently published recommendations.[6] Our main statistical analyses of clinical outcomes were performed on patients with Dravet syndrome only. Non-Dravet syndrome patients remained included in the molecular analyses to separately investigate whether different promoter haplotypes could explain the inter-familial phenotypic variability of Dravet syndrome patients and their more mildly affected family members.

Clinical data

Detailed clinical data were collected from medical records for all participants, and a semi-structured telephone interview was conducted when possible (n=130). A classification of the developmental outcome was made, rated in a consensus meeting by a child neurologist, neuropsychologist, and clinical geneticist. Developmental outcome was rated on a five-point scale based on available data on IQ and developmental level (1= no ID (IQ or developmental quotient (DQ) >85), 2 = borderline ID (IQ or DQ 70-85), 3 = mild ID (IQ or DQ 50-70), 4= moderate ID (IQ or DQ 30-50), 5 = severe or profound ID (IQ or DQ <30)). When no (recent) IQ or DQ was available, the assessment was made based on school functioning, communication and adaptive behavior. Furthermore, approximated IQ/DQ scores after five years of disease were calculated, to obtain a cognitive outcome measurement unaffected by the influence of the different ages at assessment of the participants. For this, all IQ- and developmental assessment scores of each patient, conducted at different ages, were interpolated by linear regression as previously described.[209] When the first official assessment was made later than five years after seizure onset we used the age at which a developmental delay was first observed (by either parents or clinicians) as the first moment of decline, and IQ/DQ scores up until that age were estimated to be average (=100).

7.2.3 Molecular analyses

Functional characterization of common SCN1A promoter variants

The first *SCN1A* (RefSeq NM_001165963) promoter region including h1u was PCR amplified from human control DNA using primers with a 15bp extension arm used for cloning. Five different haplotypes of 2568bp were selected and ligated in Psicheck-2 plasmids using In-Fusion cloning (clontech). Plasmids were subsequently sequenced to confirm the haplotypes. SH-SY5Y cells were seeded in 24-well cell culture plates until 80% confluency was reached. Psicheck-2 plasmids carrying the *SCN1A* promoter haplotypes were transfected using polyethylenimine. After 48 hours, cells were lysed and the lysate transferred to a white opaque 96-well plate in which the luciferase recording took place. Firefly and Renilla luciferase activities were detected using the dual luciferase reporter assay system (Promega) in the Varioskan FLASH luminometer (Thermo Fisher Scientific). Read-out was performed twice as a technical replicate, and averaged values were taken as final mean. Luciferase experiments were replicated 8 times. Differences in expression between haplotypes was not normally distributed and therefore analyzed using the Mann-Whitney U-test. For primer sequences see supplemental data S7.1.

Reconstruction of SCN1A promoter-haplotypes of the unaffected allele in participants

SCN1A was re-analyzed in all participants as previously described.[212] In short, all *SCN1A* exons were captured by single molecule molecular inversion probes (smMIPs) and sequenced on a NextSeq500 (Illumina, San Diego, CA). The resulting data were analyzed using commercial software (SeqNext module of Sequence Pilot; JSI medical systems, Ettenheim, Germany). Reads with the same single-molecule tag were assembled into one consensus read, to correct for PCR and sequencing artefacts. *SCN1A* pseudogene reads were removed from alignment and analysis. The used smMIP design included the 5' promoter region to capture three common promoter-variants (-1964 (rs2212657), -1036 (rs4319946) and-52 (rs16851666)). The promoter-haplotypes of the unaffected *SCN1A* allele of each patient was reconstructed based on the genotypes on these positions when possible. Direct assignment of genotypes to the unaffected allele was only possible in the case of homozygous genotypes, when the same genotype is present on both alleles. In the case of heterozygous genotypes, assignment of genotypes to the affected and unaffected alleles was only possible if the following condition was met: the participant had an affected family member with a homozygous genotype at the same position, with whom they shared the same inherited pathogenic *SCN1A* variant. If so, the genotype present on the shared, affected allele is known and the genotype of the unaffected allele can be deducted. When the genotypes of the unaffected allele on all three positions could be reconstructed, one of the five described haplotypes could be matched and assigned.

7.2.4 Association of promoter variants with common epilepsies

A recent genome-wide association study (GWAS) of the epilepsies identified a strong association with SNPs in *SCN1A* (manuscript in press). We analyzed the association of the three common promoter variants with epilepsy using data from the latest epilepsy GWAS. We tested for independent associations of our promoter variants with epilepsy by performing a linear regression on each variant while conditioning on the most significant *SCN1A*-SNP (rs6432877) from the GWAS. Conversely, we then tested to see if the GWAS association with *SCN1A* could be explained by our promoter SNPs by conditioning in the opposite direction.

7.2.5 Statistical analyses of clinical outcomes

Differences in clinical features and outcomes between Dravet syndrome patients with and without common variants in the *SCN1A* promoter-region on their unaffected allele were investigated. Ordinal regression, corrected for age, was used to investigate cognitive outcome scores; the Mann-Whitney U test was used to investigate age at seizure onset, age at first notice of developmental delay, age at first afebrile seizure and interpolated IQ/DQ scores after 5 years of disease. A similar analysis was performed for Dravet syndrome patients with non-mosaic truncating pathogenic variants only, to limit the influence of different pathogenic *SCN1A* variants themselves on the results. All reported tests were performed 2-tailed with an alpha-level for significance of $p < 0.05$. We furthermore separately investigated whether family members, that carry the same pathogenic *SCN1A* variant but show varying disease severities, may have different promoter-haplotypes that could explain their different outcomes.

7.3 Results

7.3.1 Functional characterization of common *SCN1A* promoter variants

5 *SCN1A* promoter-haplotypes could be defined, based on eight common SNPs in the -2271 to 297 region (Figure 7.2). All non-wildtype haplotypes (2-5) showed a significant reduction in luciferase expression, compared to the wildtype promoter-region (haplotype 1) (Figure 7.2). The decrease of expression ranged from 65% (haplotype 3, p=0.039) to 80% (haplotype 5, p=0.0023).

7.3.2 Reconstruction of *SCN1A* promoter-haplotypes of the unaffected allele in participants

SmMIP-sequencing results were obtained for all participants. In all patients their known *SCN1A* pathogenic variant could be identified, except for variants undetectable by whole exome sequencing (e.g. deletions of the complete *SCN1A* gene), meaning no samples swaps had occurred. In 46 patients the promoter variant genotype of their unaffected *SCN1A*

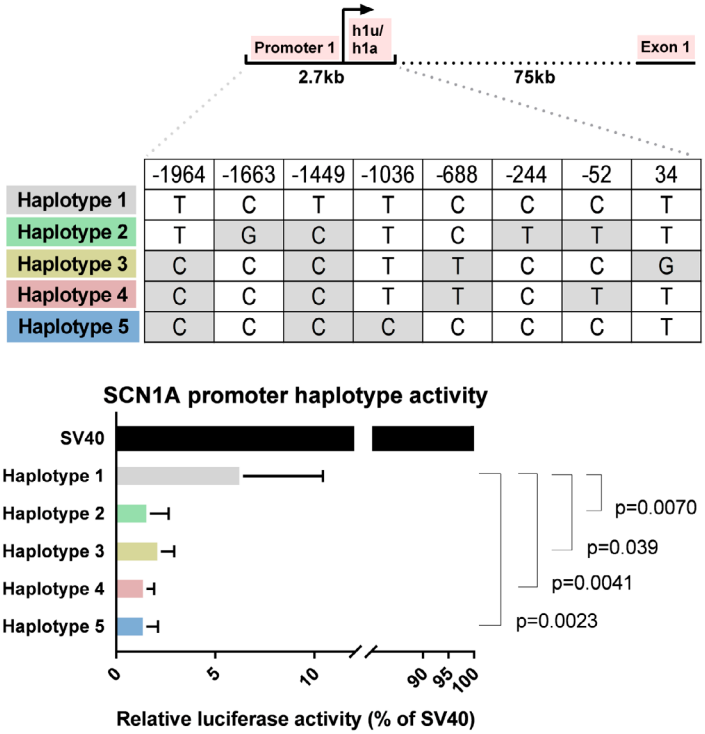

Figure 7.2. Functional effect of common promoter variants in *SCN1A*. Top: Simplified *SCN1A* 5' UTR, adapted from Figure 7.1. Middle: Promoter haplotypes tested in this study. Haplotype 1 depicts a promoter+h1u region without common variants. Haplotype 2,3,4 and 5 carry multiple common variants spread over the promoter region. Bottom: Luciferase expression analysis of *SCN1A* promoter haplotypes as depicted above. Empty vector (SV40) expression was set to 100%. Haplotype 1, without common variants was used as control haplotype of which the expression reduction of Haplotype 2,3,4 and 5 was measured.

allele could be reconstructed. All other patients had heterozygous genotypes at at least one of the three promoter variant locations, and had no included family members who could be used for haplotype phasing.

7.3.3 Epilepsy-GWAS associations of the common promoter variants

The strongest association with common epilepsy has been mapped to the *SCN1A* region in a recent GWAS of common epilepsy, with the top SNP being rs6432877. The -52 and -1036 promoter variants showed borderline genome wide significant with the "all epilepsy" phenotype, whereas the association of the -1964 variant was much weaker ($p=6{,}20E^{-8}$, $1{,}00E^{-7}$ and 0.68 respectively). In order to test whether the promoter variants were correlated to the *SCN1A* GWAS signal, we conditioned on the top GWAS *SCN1A*-SNP (rs6432877) and observed that the associations were no longer significant indicating that these promoter variants SNPs are in variable Linkage Disequilibrium with the top GWAS SNP ($r^2=0.21$,

0.63 and 0.12 respectively). Conversely, we also tested to see if the signal from the top GWAS *SCN1A*-SNP could be explained by one of the promoter variants by conditioning on each in turn. The strength of the GWAS signal diminished marginally when conditioning on the -52 and -1036 variants but was not affected by conditioning on the -1964 variant (p_{cond}=3.99E^{-08}, 1.16E^{-05} and 1.12E^{-13} respectively), indicating that the GWAS signal was not entirely dependent on the promoter variants.

7.3.4 Clinical outcomes

40 of the 46 participants with reconstructed promoter-haplotypes had been diagnosed with Dravet syndrome; the others had either GEFS+ syndrome or febrile seizures, and one participant had never experienced any seizures. Regarding the 40 Dravet syndrome patients: in 9 patients a wildtype promoter-region was detected (haplotype 1); none of the patients carried haplotype 2; haplotype 3 was identified in only one patient; haplotype 4 was present in 12, and haplotype 5 was found in 18 participants. An overview of the clinical outcomes of the 40 Dravet syndrome patients is shown in Table 7.1A. No statistically significant differences were seen between patients with and without the common promoter variants (Table 7.1, Figure 7.3-7.7). However, patients with a wildtype promoter-haplotype on their unaffected *SCN1A* allele showed a non-significant trend for milder phenotypes, when compared to patients that carried a variant promoter haplotype: on average, seizure onsets occurred at an older age (6.1 versus 5.1 months, p=0.746), as did developmental delays (median 36-47 months versus median 24-35 months, p=0.265). Furthermore, cognitive capacities declined slower (IQ after 5 years of disease 73 versus 65.9, p=0.566). More favorable cognitive outcome scores were also observed, although this is likely to be at least partly due to the wildtype-patients being younger than the other group. Similar outcomes were seen for Dravet syndrome patients with truncating variants only (Table 7.1B): although this group consisted of only 19 patients, leading to a lower detection power, a similar non-significant trend for milder phenotypes was observed in patients with wildtype promoters.

Table 7.1A. Clinical outcomes of patients with different promoter-haplotypes (all non-mosaic Dravet syndrome patients)

Promoter-haplotype unaffected allele[1]	**1**	**3**	**4**	**5**	**Any variant (haplotype 3, 4 or 5)**	**p-value (test)** [5]
Number of patients	9	1	12	18	31	
Age (years, mean/median)	12/7	14/14	14/13	16/14	15/13	
Cognitive outcome[2] (median)	3	5	4	4	4	0.859 (Ordinal regression corrected for age)
Age at seizure onset (months, mean)	6.1 (missing: 1)	5.0	5.7	5.2	5.4	0.746 (MWU test)
Age at first notice of developmental delay[3] (median)	3 (missing: 1)	1	2 (missing: 1)	2	2 (missing: 1)	0.265 (MWU test)
Age at first afebrile seizure[4] (median)	0 (missing: 1)	0	0 (missing: 1)	0 (missing: 2)	0 (missing: 3)	0.837 (MWU test)
Interpolated IQ/DQ score after 5 years of disease (mean)	73.0 (missing: 3)	33.0	64.5 (missing: 5)	68.9 (missing: 4)	65.9 (missing: 9)	0.566 (MWU test)

Table 7.2B. Clinical outcomes of patients with different promoter-haplotypes (non-mosaic Dravet syndrome patients with truncating *SCN1A* variants)

Promoter-haplotype unaffected allele[1]	**1**	**3**	**4**	**5**	**Any variant (haplotype 3, 4 or 5)**	**p-value (test)**[5]
Number of patients	5	1	5	8	14	
Age (years, mean/median)	14/7	14/14	11/13	22/24	17.3/14.5	
Cognitive outcome[2] (median)	3	5	4	4.5	4	0.547 (Multiple regression corrected for age)
Age at seizure onset (months, mean)	6.6	5.0	6.0	5.25	5.5	0.823 (MWU test)
Age at first notice of developmental delay[3] (median)	4	1	2	2.5	2	0.298 (MWU test)
Age at first afebrile seizure[4] (median)	0	0	0	0 (missing: 2)	0 (missing: 2)	0.712 (MWU test)
Interpolated IQ/DQ score after 5 years of disease (mean)	75.1 (missing: 2)	33.0	65.7 (missing: 3)	74.9 (missing: 1)	68.9 (missing: 4)	0.973 (MWU test)

[1]1: Wildtype (no variants). 2: variant at -52. 3: variant at -1964. 4: variant at -52 and -1964. 5: variant at -1964 and -1036. [2]Based on available data on IQ and developmental level, adjusted for age at assessment (1 = no ID (IQ or developmental quotient (DQ) >85), 2 = borderline ID (IQ or DQ 70-85), 3 = mild ID (IQ or DQ 50-70), 4= moderate ID (IQ or DQ 30-50), 5 = severe or profound ID (IQ or DQ <30). When no (recent) IQ or DQ was available, the assessment was made based on school functioning, communication and adaptive behavior. [3]By parents or physicians. 0 = <12 months, 1= 12-23 months, 2= 24-35 months, 3= 36-47 months, 4= >48 months, 5= no developmental delay. [4]0= <12 months, 1= 12-23 months, 2= 24-47 months, 3= >48 months, 4= never had afebrile seizures. [5]p-values are based on statistical analyses of differences between group 1 (wiltype) and all other haplotypes combined (any variant). All reported tests were performed 2-tailed with an alpha-level for significance of p <0.05. MWU-test=Mann Whitney U-test.

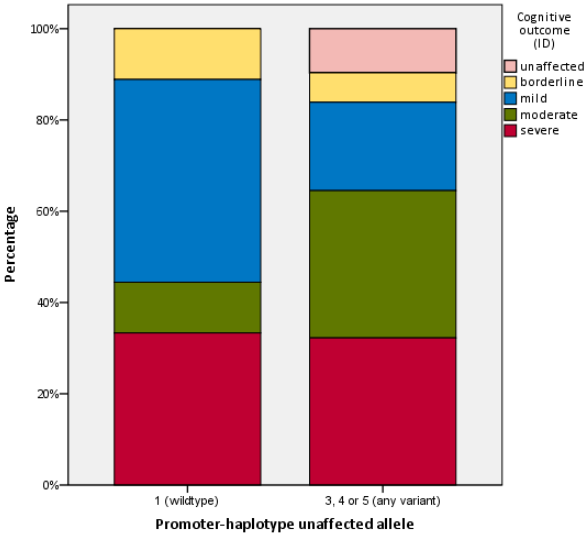

Figure 7.3. Distribution of different cognitive outcome scores between patients with and without variants in the promoter-region of their unaffected *SCN1A* allele.

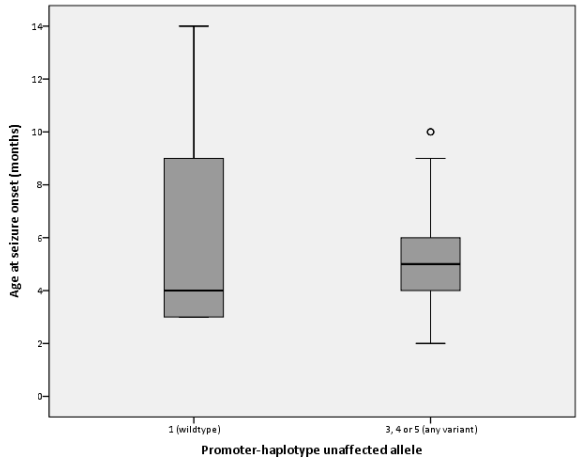

Figure 7.4. Distribution of age at seizure onset between patients with and without variants in the promoter-region of their unaffected *SCN1A* allele.

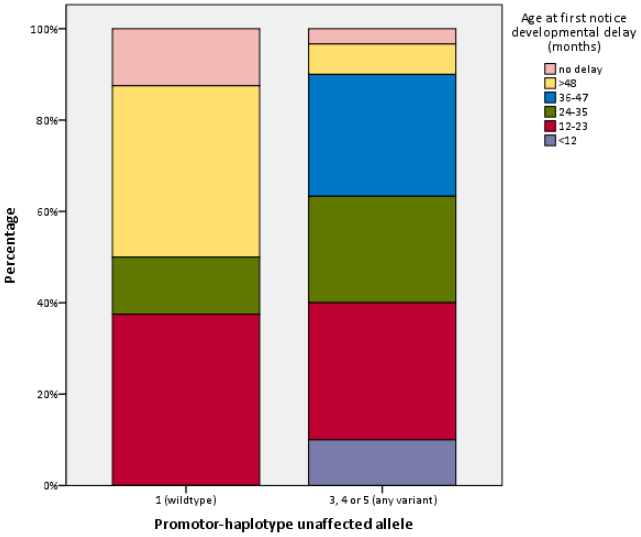

Figure 7.5. Distribution of onset of developmental delay between patients with and without variants in the promoter-region of their unaffected *SCN1A* allele.

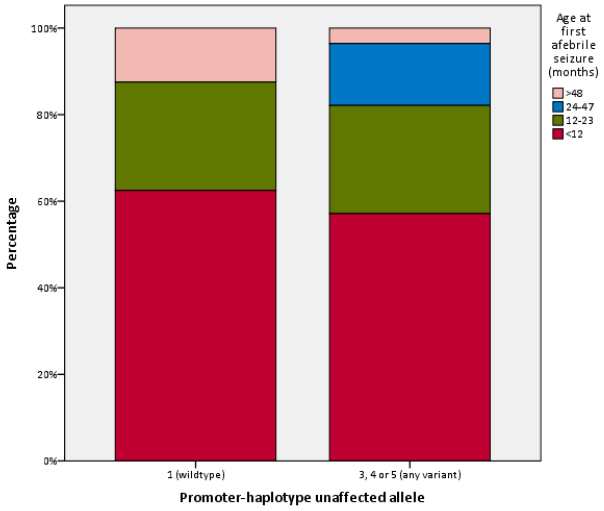

Figure 7.6. Distribution of age at first afebrile seizure between patients with and without variants in the promoter-region of their unaffected *SCN1A* allele.

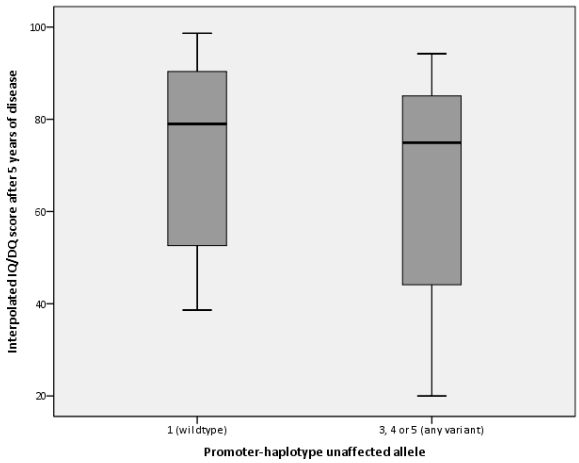

Figure 7.7. Distribution of IQ/DQ scores after five years of disease between patients with and without variants in the promoter-region of their unaffected *SCN1A* allele.

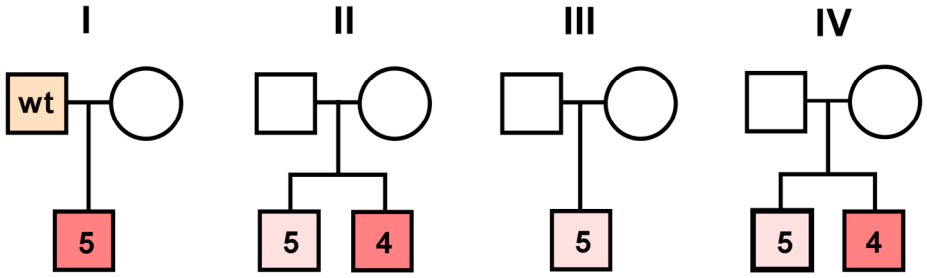

Figure 7.8. Familly tree I-IV. Orange box indicates mild epilepsy phenotype with normal cognitive functioning. Light red box indicates a mild DS, or borderline DS/GEFS+ phenotype. Dark red boxes indicate severe DS. Numbers in the boxes correspond to promoter haplotype number or wildtype haplotype from Figure 7.2.

7.3.5 Anecdotal family studies

Among the complete group of 46 participants with reconstructed promoter-haplotypes were eight participants, belonging to four different families that showed a clear intra-familial variability (Figure 7.8): family 1 consists of a severely affected 10 year old proband with Dravet syndrome, and a father with mild epilepsy and normal cognitive functioning. Family 2 consists of two brothers with Dravet syndrome, one of whom is more severely affected than the other. Family 3 consists of a proband with a phenotype on the border of Dravet syndrome and GEFS+, with regression over the years. His father has never had any seizures. Family 4 consists of two brothers of whom the oldest has severe Dravet syndrome and the youngest has a much milder phenotype. In family 2, 3 and 4, each of the milder participants carried haplotype 5, and each of the more severely affected participants carried

haplotype 4. Since only very small, insignificant differences in luciferase expression between haplotype 4 and 5 were observed, the different promoter-haplotypes are unlikely to explain the clinical differences between these patients. However, in family 1, the severely affected patient carried haplotype 5, whereas the milder patient had a wildtype promoter, for which we did observe a large difference in luciferase expression.

7.4 Discussion

Our experiments showed that the presence of common variants in the promoter-region of *SCN1A* cause a significant decrease in luciferase activity, compared to the wildtype promoter. This indicates that *SCN1A* expression and function may be negatively influenced by such variants, likely due to disturbance of RNA polymerase II and/or transcription factor binding. Although this reduced expression cannot cause epilepsy independently, since a large part of the healthy populations carries these common variants as well, it may modulate the effect of other variants that are present.

Our results can only in part be compared to those of Gao et al.,[272] who found no differences in expression between the most common promoter-haplotypes. These different results may be attributed to three factors. First, we are measuring a different group of common variants, which results in different expression levels. Second, we have cloned a slightly altered promoter region that is shorter on the 5' side, but extended on the 3' side to include h1u. H1u, the first 5' UE contains transcription factor binding sites such as EBF and the Initiator element that is required to form the transcription complex. Third, we use the Promega dual-assay luciferase plasmid, which has both the renilla and firefly luciferase gene incorporated. In single-assay luciferase assays, using two plasmids, normalization of luciferase data could be less sensitive. In general, the luciferase reporter assay is currently the fastest tool to measure gene expression at the transcriptional level. Nevertheless, it should be noted that *in-vitro* assays can never fully mimic an *in-vivo* state, especially in complex structures such as the brain. While for this study a neuronal cell line was used to perform the expression studies, this can be improved by introducing the luciferase constructs in the brain of an animal model. In this way, the interactions between cell types in the brain are included, approaching the *in-vivo* state more accurately. Nevertheless, we found that a combination of common variants in the *SCN1A* promoter reduced expression on transcription level.

The reduced luciferase expression was in line with our hypothesis that common *SCN1A* variants may affect expression of the gene and thus lead to more severe phenotypes, when present on the unaffected *SCN1A* allele of a Dravet syndrome patient. However, the clinical consequences of these different haplotypes were less convincing: no statistically significant differences were seen between patients with and without the common promoter variants, although we did observe a minor trend of more severe outcomes on multiple clinical variables

in patients with common promoter variants. There may be several reasons for this. First, it is likely that common variants in the promotor region only have small phenotypic effects, since they otherwise would have been subject to negative selection. This limited effect was also illustrated by Gao et al.;[191] although a pathogenic point mutation in the *SCN1A* promoter-region led to an *in vitro* decrease of expression and mild epilepsy in a proband, the same variant was found in the asymptomatic mother of the patient. This indicates that promoter-variants by themselves may only have a limited influence on phenotypes. To detect such small effects, large sample sizes are prerequisite. Our study sample is likely too small to reliably detect any phenotypic consequences. Second, other (stronger) modifiers may simultaneously modulate the effect of promoter-variants. Although we excluded patients with mosaic pathogenic variants, we cannot eliminate the influence of variants in modifier genes and environmental factors on outcomes. If these other factors are strong influencers, they may override any effects the promoter-variants have.

Besides a small sample size, our study has several other limitations. While the luciferase plasmids were fully sequenced, the *SCN1A* patient promoter haplotypes were reconstructed based on three common SNPs. No sequencing of the complete promoter-region was performed in the participants. The patients' haplotypes may therefore not fully correspond to the haplotypes tested during the luciferase experiments. Theoretically, patients may harbor additional promoter-variants that could either rescue or aggravate impaired expression. This could have large effects on outcomes in a sample size as small as ours. Furthermore, different primary pathogenic *SCN1A* variants may influence outcomes; however, a trend for milder phenotypes in patients with wildtype promoters was seen for the group of patients with truncating mutations only as well, which indicates that this effect is limited.

We also analyzed four families of which multiple members were affected by the same pathogenic *SCN1A* variant, but showed different phenotypes nonetheless; in these cases the effect of the primary mutation on the resulting phenotype is expected to be equal. Since in three of the four families both members had variant-haplotypes, our hypothesis could not explain their phenotypic differences. This is however not surprising, since in two of these families both members were affected by different clinical syndromes; as stated before, the modifying effect of promoter-variants is likely not strong enough to cause this independently. In only one family, consisting of two brothers with Dravet syndrome, the milder brother carried a wildtype promoter on his unaffected allele, whereas the more severe brother carried a variant-promoter. According to our hypothesis, this might explain their phenotypic differences; however, as mentioned previously, we cannot exclude other influencers and definitive conclusions are not possible based on only one family.

In conclusion, we found that common variants in the *SCN1A* promoter reduce transcription in neuronal cell culture, which may indicate that promoter haplotypes can act as a disease modifier in epilepsy. We however only found a small, non-significant effect of the *SCN1A* promoter on clinical outcomes of Dravet syndrome patients. These results are inconclusive due to a limited detection power; however, the observed trends in our cohort

warrant replication studies in larger cohorts to explore the potential modifying role of these common *SCN1A* promoter-haplotypes. The inclusion of large numbers of Dravet syndrome patients, ideally all with similar primary LoF variants, is essential to detect the likely small effect these haplotypes might have on phenotypes. Sequencing of both complete *SCN1A* promoter-regions, preferably including the 5'-UEs, would be required to obtain conclusive results.

7.5 Appendix

S7.1 Functional characterization of common SCN1A promoter variants

Construction of luciferase expression plasmids

The *SCN1A* promoter region -2271 to 297 (numbering with respect to the transcript start site of h1u) was PCR amplified from patient DNA using the following primers: 5'-CTCACTATAGGCTAGCGAGCTTAGGCCTCTGAAGACTG-3' and 5'-AGCCATGGTGGCTAGCCAGATGATGTCCGATAAGCAA-3' with the underlined region being a 15bp extension. The Psicheck-2 vector (Promega) was linearized using NheI and the PCR products carrying the 5' extension arms were inserted using the In-Fusion cloning kit (clontech). All constructs were confirmed by Sanger sequencing using the original *SCN1A* promoter region primers.

Cell culture

Human female neuroblastoma cell line SH-SY5Y was purchased from ATCC (ATCC® CRL-2266™). Cells were grown in a 1:1 mixture of DMEM/F12 supplemented with 10% FCS and 1X Penicillin and Streptomycin. For subculturing the cells were rinsed once with PBS and incubated with trypsin (0.25%) and .53mM EDTA for 2-5 minutes. As the cells detach from the culture flask, fresh culture medium was added, mixed and dispensed in new culture flasks. Regular, uncoated T25 and T75 Corning® plastic culture flasks (Sigma Aldrich CLS430372) were used for maintaining the cell line. The passage number of SH-SY5Y cells during experiments varied between 8 to 12.

Transfections

$8*10^4$ cells were seeded in 24-well Corning® Costar® cell culture plates (Sigma Aldrich CLS3527) so that 80% confluency is reached on day 2. Two transfection reaction tubes were prepared, one containing up to 1µg of Psicheck-2 DNA diluted in Opti-MEM and the second containing 3µL polyethylenimine (PEI) (1mg/mL) diluted in Opti-MEM. Reactions were vortexed briefly and then incubated for 20 minutes at room temperature. PEI-DNA complexes were pipetted dropwise to cells and incubated for two days. On day four 100µL passive lysis buffer (Promega) was added to the transfected cells and incubated for 15 minutes on a shaking platform. 10 µL of the lysed cell mixture was transferred to a white opaque 96-well plate (Biotek).

Dual Luciferase reporter assay

Firefly and Renilla luciferase activities were detected using the dual luciferase reporter assay system (Promega) in the Varioskan FLASH luminometer (Thermoscientific). Two alterations to the manufacturer's instructions were made; 1) luciferase and renilla substrates were diluted 1:1 with MilliQ water and 2) 50µL instead of 100µL of the substrates was

injected to the wells. Read-out was performed twice as technical replicate and averaged values were taken as final mean. Luciferase experiments were replicated 8 times but repeated when too many failed samples or outliers were present (see statistics).

Removal of failed samples

Empty well controls were included with each luciferase reading and the relative renilla or firefly luminescence measured in these wells was used to remove failed samples. Experimental wells that showed a lower renilla or firefly value compared to the empty well renilla or firefly value were removed from the dataset.

Outlier removal

For each haplotype dataset, outlier removal was performed using the IQR/Tukey's method. The difference between the higher quartile (Q3) and the lower quartile (Q1) was set as the interquartile range (IQR). Data was regarded an outlier when lower than Q1-1,5*IQR or higher than Q3+1,5*IQR, according to Tukey's method.

Statistical analyses

Correlation coefficients were calculated for each haplotype dataset separately. None of the datasets were found to be normally distributed. As the results are from a small dataset and they are not normally distributed, the Mann-Whitney U-test was chosen for statistical analysis.

Part 3

X-linked genetic epilepsy syndromes

Chapter 8

Male patients affected by mosaic *PCDH19* mutations: 5 new cases

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Published: Neurogenetics. 2017 Jul;18(3):147-153.

Abstract

Background: Pathogenic variants in the *PCDH19* gene are associated with epilepsy, intellectual disability (ID) and behavioral disturbances. Only heterozygous females and mosaic males are affected, likely due to a disease mechanism named cellular interference. Until now, only four affected mosaic male patients have been described in literature. Here, we report five additional male patients, of which four are older than the oldest patient reported so far.

Methods: All reported patients were selected for genetic testing because of developmental delay and/or epilepsy. Custom targeted next generation sequencing gene panels for epilepsy genes were used. Clinical data were collected from medical records.

Results: All patients were mosaic in blood for likely pathogenic variants in the *PCDH19* gene. In most, clinical features were very similar to the female phenotype, with normal development before seizure onset, which occurred between 5 and 10 months of age, clustering of seizures and sensitivity to fever. Four out of five patients had mild to severe ID and behavioral problems.

Conclusions: In five male patients, mosaic, likely pathogenic *PCHD19* variants were identified by Next Generation Sequencing. We reaffirm the similarity between male and female *PCDH19*-related phenotypes, now also in a later phase of the disorder (ages 10-14 years).

8.1 Introduction

Pathogenic variants in *PCDH19* are associated with early onset, clustered epileptic seizures often provoked by fever, intellectual disability (ID) that can be present in variable degrees, and behavioral disturbances such as autistic features, attention deficit, hyperactivity and aggression. The clinical features may resemble those of Dravet syndrome (phenotype MIM # 300088).[146,147,273–275]

PCDH19 is located at Xq22.1 and codes for protocadherin-19, a transmembrane protein involved in neuronal organization and migration, and cell-cell and cell-matrix adhesion [194,276–279]. It is highly expressed in the central nervous system.[147] *PCDH19*-related epilepsy shows a remarkable inheritance pattern: generally, females carrying heterozygous pathogenic variants are affected, whereas hemizygous male carriers are asymptomatic or only show psychiatric or behavioral symptoms.[146,147,280,281] However, in 2009 the first affected male mosaic for a *PCDH19* pathogenic variant was described.[146] This finding gave rise to a theory of cellular interference as disease mechanism: disease occurs when two different cell populations exist (cells expressing the normal *PCDH19* protein and cells expressing a mutant form of the protein), as is true for heterozygous and mosaic pathogenic variants, but not for hemizygous pathogenic variants in males. These non-homogeneous cell populations are likely to disrupt cell-cell interactions, leading to disease.[146]

Only four affected mosaic male patients have been described in literature until now, the oldest being 7 years old.[146,282,283] Here, we report five additional male patients with mosaic *PCDH19* mutations, of ages 2, 10, 13, 13 and 14 years old. We compared the male and female phenotypes.

8.2 Methods

8.2.1 Patients and molecular analysis

All five patients were selected for diagnostic genetic testing because of developmental delay and/or epilepsy. Genetic testing on DNA from lymphocytes was performed using a custom targeted next generation sequencing (NGS) gene panel for epilepsy genes (see online resource 1 for details). Mosaicism of *PCDH19* variants was determined based on the simultaneous presence of a variant allele and the reference allele, as *PCDH19* is located on the X-chromosome and all patients were male. The percentage of mosaicism was based on the percentage of reads that showed the alternate allele. *PCDH19* mosaic male patients from different centers were collected through personal communication between authors, meaning no structured cohort was tested. Detailed clinical data were collected from medical records. The parents of all patients gave informed consent for the publication of clinical data.

8.2.2 Literature search

A literature study was carried out in the PubMed database to identify previously described patients with *PCDH19* pathogenic variants.

8.3 Results

8.3.1 Molecular analysis

All five patients carried a *PCDH19* variant in various degrees of mosaicism (patient A: c.1864G>C, p.Gly622Arg, 60%; patient B: c.840C>G, p.Tyr280*, 22%; patient C: c.462C>G, p.Tyr154*, 65%; patient D: c.1682C>G, p.Pro561Arg, 78%; patient E: c.799G>T p.Glu267*, 20%, RefSeq NM_001184880). According to ACMG (American College of Medical Genetics and Genomics) criteria, the variant of patient A was classified as likely pathogenic, and the variants of patient B-D were classified as pathogenic. The variants of patient B, C and E lead to a premature stop codon; the variants of patient A and D were both predicted probably damaging and damaging by PolyPhen and SIFT respectively. None of the variants was present in control databases (Database of Single Nucleotide Polymorphisms (dbSNP), NHLBI Exome Sequencing Project (ESP) and the the Exome Aggregation Consortium (ExAC) database). The Pro561Arg variant has been previously described in two affected female siblings with ID, microcephaly, and seizures, and was paternally transmitted;[284] the others variants are novel. All variants were confirmed *de novo*, except for the variant in patient D, whose parents were not tested. No other variants that could explain the phenotypes were found.

8.3.2 Clinical characteristics

Clinical characteristics of the newly reported and previously described male patients with *de novo PCDH19* pathogenic variants are summarized and compared to those of previously published females in Table 8.1. See online resource for extensive clinical descriptions (online resource 2). Overall, all patients had normal development before seizure onset, occurring between 5 and 10 months of age, except for patient E. This patient had a delay in speech development, like his father, before onset of seizures and seizure onset occurred later, at 31 months. First seizure types were generalized clonic or clusters of focal or complex partial seizures. Later seizures were mainly complex partial seizures and primary or secondary generalized clonic and tonic-clonic seizures. In all five patients seizures tended to cluster and could be provoked by fever. Four out of five patients had mild to severe ID, autism and additional behavioral problems.

8.4 Discussion

We here report five male patients with mosaic *PCDH19* likely pathogenic variants, which raises the total number of described male patients to nine.[146,282,283] Four of the currently described male patients are the oldest reported so far (ages 10, 13 twice and 14 years old), which gives the opportunity to investigate whether *PCDH19* related phenotypes evolve the same way in male and female patients. Our current findings confirm previously reported observations of similar clinical features in male and female patients, also for older children.[146,282,283] Focal seizures with affective symptoms (fearful screaming) are very common in female patients and become more prevalent with an increasing age.[285] This distinctive seizure type is also reported in three male patients.[283] These similarities suggest that there are no differences in clinical consequences between phenotypes caused by postzygotic *PCDH19* pathogenic variants in mosaic males, and phenotypes caused by heterozygous *PCDH19* pathogenic variants in females. This lends further support to the hypothesis that cellular interference is the main disease mechanism, as proposed by Depienne et al.[146] The increasing number of affected mosaic male patients undermines the theory of a compensating effect by the nonparalogous *PCDH11Y* gene in male patients, as proposed by Dibbens et al.[147] It is highly unlikely that this gene would only compensate for a complete absence of *PCDH19* in hemizygous affected males, but not for a partial loss of *PCDH19* in mosaic males.

In female patients, seizure frequency often diminishes around puberty,[273,275,280,286–288] possibly due to hormonal changes.[288] Our 13-year old patient has been seizure free since the age of ten years old; our 10-year old patient only has one cluster of seizures per year. However, our other 13- and 14-year old patient still have ongoing seizures. Although four of our male patients are the oldest reported until now, none of them has reached adolescence yet and the numbers are still small, making it hard to draw definite conclusions. Nevertheless, since some of the few reported male patients already show a reduction in seizure frequency with increasing age, it seems unlikely that a declining seizure frequency is exclusively occurring in females and is related to female specific hormones.

We hypothesized that males with a mutated allele percentage around 50% in brain, who would have an inherent high level of cellular interference, may show a more severe clinical picture than males with a lower or higher percentage of mosaicism. High or low percentages of mosaicism would resemble skewed X-inactivation in female patients, which has also been suggested to lead to a milder phenotype,[275,284] although no clear correlation has been shown.[286,297] Indeed, in our cohort, the three patients with the lowest and highest percentages of mosaicism in blood are the least severely affected (patient B, D and E), and two previously described patients, one with borderline ID and one with normal intelligence showed percentages of mosaicism of 10% and 90%, respectively (patients H and I). However, patient G shows an equal number of mutated and wildtype alleles in blood and is also mildly affected, and patient F shows no mosaicism in blood cells at all, only in

Table 8.1. Clinical description of male and female patients carrying *PCDH19* likely pathogenic variants

Patient	A	B	C	D	E	F[146]	G[282]	H[283]	I[283]	Female patients[c]
Age at inclusion (years)	10	13	14	24 months	13	7	6	4	3,5	1-54
Variant[a]	c.1864G>C, p.Gly622Arg	c.840C>G, p.Tyr280*	c.462C>G, p.Tyr154*	c.1682C>G p.Pro561Arg	c.799G>T, p.GIu267*	del *PCDH19*	c.605C>A p.Ser202*	c.918C>G, p.Tyr306*	c.1352 C>T, p.Pro451Leu	*PCHD19* deletions, duplications, missense and nonsense variants
% of mosaicism	60% (blood)	22% (blood)	65% (blood)	78% (blood)	20% (blood)	100% (lymphocytes), 47% (fibroblasts)	50% (blood), 70% (buccal cells), 100% (urine sediment)	10% (lymphocytes, saliva, hair)	90% (lymphocytes, urine)	-
Technique used (number of alternate alleles/ total read depth at base position)	NGS gene panel (92/153 reads)	NGS gene panel (77/380 reads)	NGS gene panel (38/59 reads)	NGS gene panel (351/450 reads)	NGS gene panel (19/93 reads)	Detection by microarray, estimation of % mosaicism by FISH (100 cells)	Detection by exome sequencing, estimation of % mosaicism by Sanger	NGS gene panel (157 reads)	NGS gene panel (135 reads)	Various
Sex	Male	Male	Male	Male	Male	Male	Male	Male	Male	Female
Exam at birth	Meconium stained amniotic fluid, bradycardia	Normal	Normal	Umbilical cord around neck, quick recovery	Normal	?	Normal	?	?	Normal
Development prior to sz onset	Normal	Normal	Normal	Normal	Speech delay	Normal	Normal	Normal	Normal	Normal
Sz onset age (months)	5	10	10	7	31	12	9	9	10	3-70 (most <12)
Sz type at onset[b]	Generalized, clonic (fs)	Generalized clonic	Cluster of CP seizures	Clusters of focal sz, generalizing to GTC and tonic seizures	Clusters of focal sz, generalizing to one hemisphere	GTC (fs, prolonged, repetitive)	Focal myoclonic, tonic–clonic, FSsG	Afebrile hypotonic seizure with hypopnea (40 min)	24 hour cluster of febrile sz with fixed gaze, loss of contact, upper limb hypertonia, and jerks (30–40 sec.)	Febrile, febrile SE, afebrile; GTC, generalized tonic; hemiconvulsion; focal, FSsG; CP

Patient	A	B	C	D	E	F[146]	G[282]	H[283]	I[283]	Female patients[e]
Later sz types[b]	CP, secondary generalized. Focal with affective symptoms, secondary generalized. Primairy generalized	Generalized clonic	CP, secondary tonic clonic, tonic, status	Focal, febrile, GTC, tonic, CP	CP, febrile	Hemiclonic, GTC, myoclonic jerks	Focal myoclonic, tonic–clonic, rapid secondary generalization	FS, focal tonic-vibratory	FS and afebrile seizure clusters, tonic. Often fearful screaming at start	FS or afebrile; generalized clonic, tonic , tonic-clonic or atonic; hemiclonic; focal, CP, FSsG, myoclonic; absences; SE
Clusters of sz	+ (fever related)	+	+	+	+	+	?	+	+	+
Focal sz with affective symptoms	+	-	+	-	+	?	?	?	+	+
Fever sensitivity	+	+	+	+	+	+	?	+	+	+
AEDs used and response[c]	VPA: -, LTG: -, CBZ:-	VPA: +	VPA: ?, LEV: +/-, LZP (during clusters): -, TPM, CBZ, OXC, PHT, PHB, LTG, CLB, VGB: ?	LEV:+/-, TPM: -, OXC:-, VPA:+, PHB:+, LSM:-, Diazepam:-	VPA:+; CBZ: +/-; OXC: +/-	VPA, CLB, CLN, TPM, STP: ?	LEV: +/-, ZNS: +, multiple AEDs: -	VPA:?	PHB: ?, VPA:	LEV, VPA, CLB, CLN, TPM, STP, LTG, PHB, CBZ, OXC, ZNS, NTZ, VGB, KBR, LZP: different responses
Current AEDs[c]	LEV, OXC, CLB, TPM	VPA	LEV	OXC, VPA, PHB, LEV	OXC	?	ZNS	-	PHB, VPA	Many different AED's, some cases no AED's
Sz outcome	1 cluster of sz per year (5-10 sz/cluster)	Last sz at age 10 years	Ongoing sz	Ongoing sz	Ongoing sz during febrile illness	Persistence of febrile sz in spite of treatment	Seizure free for 20 months	Seizure free age 14-42 months (no AEDs), since then 1 cluster of sz	4-5 clusters per year	Often seizures less frequent or seizure free at certain age (4-36 years)

Table 8.1. Clinical description of male and female patients carrying *PCDH19* likely pathogenic variants (continued)

Patient	A	B	C	D	E	F[146]	G[282]	H[283]	I[283]	Female patients[e]
EEG at onset	7-8 months: normal. 10-11 months: asymmetrical background activity, non-specific high voltage delta-activity occipital right>left.	Frequent generalized epileptic discharges	2 years: normal	Multiple focal discharges, right centroparietal, secondary generalization. Mild diffuse background slowing	Focal epileptic discharges left parieto-temporal with generalization to one hemisphere (ictal)	?	Slower rhythm for age, mild diffuse disturbance. Infrequent right frontal, and rare left temporal sharp wave discharges, suggestive of epileptiform activity	Rare right frontotemporal sharp waves	Normal	Mostly normal
EEG at follow up	3,5 years: normal	-	3 years: No epileptic activity, generalized and focal slow activity	-		?	?	?	Bilateral centroparietal onset of seizures	Normal, interictal spikes, generalized poly-spike waves, slow waves, slow background
Last EEG	6 years: normal background activity, diffuse fast activity mainly frontal. No epileptic activity.	-	4 years: No epileptic activity, generalized and focal slow activity	-	32 months: interictal EEG normal	?	?	?	Normal interictal	-
ID[d]	++ (estimated: slowed PMD, special education)	+ (IQ 66 at 5 years)	++ to +++	- (only delayed speech development, no true ID yet, based on clinical evaluation)	+ (IQ 55 at 9 years)	++ to +++	+/- (IQ = 76)	+/- (GDQ 78 at 46 months, 72 at 52 months)	- (GDQ 101 at 30 months and 103 at 40 months)	- to +++
Developmental stagnation/ regression	-	-	-	-	-	?	?	-	-	Regression in some cases
Language (words/ sentences)	Delayed: words, sentences	Not delayed. First words at 12 months. Two-word sentences at 16 months	Sentences, stereotyped phrases	Mildly delayed speech development	Mildly delayed speech development	delay: words-sentences	?	?	?	Sometimes normal, often delayed, words-sentences, in rare cases absent

Patient	A	B	C	D	E	F[146]	G[282]	H[283]	I[283]	Female patients[e]
Behavioral/ psychiatric disturbances	Autism, aggression, behavioral problems, ADHD	Autism, mood disorder	Autism spectrum disorder, anxiety	-	Behavior problems resembling autism spectrum disorder , short attention span	Behavioral problems, autistic features	Irritability, aggression, rigidity, poor sleep, ADHD, anxiety, OCD, ODD	Compulsive and stereotyped behaviors	-	Prominent behavioral problems in most cases (autism, attention deficit, hyperactivity, aggression, emotional lability, impulsiveness, anxiety, jealousy, obsession, depression psychogenic nonepileptic sz)
Neurological examination	Crouched gait	Hypotonia	Motor delay and balance problems	Balance problems (medication induced), improving	Reduced coordination	Motor delay, ataxia	?	?	?	Mostly normal; hypotonia, dyspraxia, ataxia or motor delay in some
MRI images (age)	Expanded perivascular spaces (8 years)	Normal (7 years)	Widened peripheral subarachnoid spaces	Normal (7 months)	Normal (2,5 years)	?	Normal	? (CT: normal)	Normal (10 months)	Usually normal (mild atrophy/cortical dysplasia is rarely reported)
Additional comments	-	Pes plano valgus, obesity (BMI 23.3; +2.3 SD)	-	Hand food mouth disease			dysmorphic features: plagiocephaly occipital/ parietal area, cupped ears, intradigital webbing of phalanges. Severe myopia			

SZ=seizure(s); AED=anti-epileptic drug; PMD=psychomotor development. [a]RefSeq NM_001184880. [b]GTC=generalized tonic-clonic, CP=partial complex, FS=fever sensitive, SE=status epilepticus, FSsG=focal seizure with secondary generalization. [c]CBZ=carbamazepine, CLB=clobazam, CLN=clonazepam, KBR=potassium bromide, LEV=levetiracetam, LSM=lacosamide, LTG=lamotrigine, LZP=Lorazepam, NTZ=nitrazepam, OXC=oxcarbazepine, PHB=phenobarbital, PHT=phentytoin, STP=stiripentol, TPM=topiramate , VGB=vigabatrin, VPA=valproic acid, ZNS=zonisamide, -=not effective, +/-=slight effect, +=good effect. [d]-=absent, +/-=borderline, +=mild, ++=moderate, +++=severe, GDQ=Griffiths Developmental Quotient.[e146,148,273–275,280,281,284–287,289–298]

fibroblasts, but has moderate to severe ID and ongoing seizures. It is thus not possible to predict the phenotype based on the percentage of mosaicism in blood, most likely because it does not necessarily equal the percentage of mosaicism in brain.

The number of identified male patients mosaic for *PCDH19* pathogenic variants increases,[146,282,283] probably due to our improving abilities to detect mosaicism in general by using NGS techniques. It is now clear that *PCDH19* pathogenic variants can cause epilepsy both in males and females and that mosaicism for *PCDH19* pathogenic variants in males might be more common than previously thought. Because our five described patients were gathered through personal communication between authors from different diagnostic centers, no structured cohort with clearly defined inclusion criteria was tested, which makes it difficult to estimate the frequency of mosaic pathogenic *PCDH19* variants in male patients. Since the *PCDH19* and Dravet syndrome phenotypes show many similarities, testing a cohort of *SCN1A*-negative, male Dravet syndrome patients for mosaic *PCDH19* pathogenic variants could give more insight in the true incidence. In this cohort and in males with clinical features characteristic of a *PCDH19*-related disorder, NGS techniques with high coverage should be used to look for *PCDH19* pathogenic variants, as traditional Sanger Sequencing is not sensitive enough to reliably detect mosaicism. This overview helps create more knowledge about the disease course in male patients, which is extremely relevant for counseling those affected and their families. Reporting on more (older) patients in the future is essential for establishing a good understanding of prognosis in male patients.

8.5 Appendix

S8.1 Molecular analyses

Patient A, C and E were tested at University Medical Center Utrecht, the Netherlands, using Agilent Sure Select Enrichment. Base positions were genotyped using an 'in house' developed variant calling pipeline for NGS sequencing data. At an informative coverage of >15X, the genotyping sensitivity and specificity is > 99% (FN and FP << 1%, NGS versus SNP-array genotyped control DNA). The required horizontal coverage of target sequence (coding region including 20 bp flanking intron sequence) is > 95% at 15X. Patient B was tested at University Medical Center Groningen, the Netherlands. Base positions were genotyped using an 'in house' developed variant calling pipeline for NGS sequencing data. At an informative coverage of >20X, the genotyping sensitivity and specificity is > 99% (FN and FP << 1%, NGS versus genotyped control DNA). The required horizontal coverage of target sequence (coding region including 20 bp flanking intron sequence) is > 98% at 20X. Patient D was tested at Ambry Genetics, Aliso Viejo, USA. Sequence enrichment of the targeted coding exons and adjacent intronic nucleotides was carried out by a bait-capture methodology using long biotinylated oligonucleotide probes, and was followed by polymerase chain reaction (PCR), NGS sequencing with an analytical sensitivity of >99%. All mutations were confirmed by conventional Sanger sequencing.

S8.2 Clinical descriptions

Patient A

Patient A is a ten year old boy, who had generalized clonic seizures starting at the age of five months. These seizures typically occurred in clusters of 20 to 30 seizures during one day every month. From age three years most clusters were triggered by infections with fever; before that there was no association with fever. He was initially treated with valproic acid, lamotrigine and carbamazepine, which was unsuccessful. Best epilepsy control was achieved with a combination of clobazam, topamirate, oxcarbazepine and levetiracetam, although he still has fever related clusters of seizures. His brain MRI showed enlarged perivascular spaces without other abnormalities. His first EEG at the age of 7 months was normal, whereas his second EEG at age 10 months showed an asymmetrical background pattern with nonspecific high voltage delta-activity in the occipital region, predominantly at the right side. He has a global developmental delay and attends a special school for children with learning and behavioral problems. He has been diagnosed with autism. At age nine years he developed a crouched gait.

Patient B

This 13-year-old boy had generalized clonic seizures since the age of 10 months. Seizures were triggered by fever, mostly due to middle ear infections and infections of the upper

respiratory tract or urinary tract. Only once a seizure occurred in the absence of infection or fever. Seizures tended to cluster, with multiple seizures occurring during disease episodes. He has been treated with valproic acid, and had his last seizures at the age of 10 years. An EEG at the age of 10 months showed generalized epileptic discharges. Brain MRI at the age of 7 years was normal. He has a global developmental delay and a mild to moderate intellectual disability (at age 6 years his TIQ was 50, with a VIQ of 55 and a PIQ of 55 (SON-R)). He attends a special school for children with learning problems. He has mood swings and was further diagnosed with a pervasive developmental disorder not otherwise specified (PDD-NOS). His mother had one febrile seizure during infancy; family history was otherwise negative for epilepsy. He has pes planovalgus and mild obesity, but no particular dysmorphic facial characteristics.

Patient C

Patient C is a 14-year-old boy who started having seizures at the age of 10 months. Clusters of complex partial and tonic-clonic, or tonic seizures occurred every 1 to 2 months, with seizure free intervals of several months to one year. Clusters were provoked by fever and stress. They were refractory to antiepileptic drugs, leading to admittance to the intensive care unit at least nine times each year. The first interictal EEG was normal; later interictal EEGs showed generalized and focal slow activity, compatible with atypical diffuse encephalopathy. Developmental delay was 1.5 year at age three years, both on mental and motor scales. At age 5;11 years, the Bayley's Scales (BSID-II-NL) revealed a developmental level of 2;2 and 2;3 years on the mental and motor scales, respectively. At age 14 years old, he makes short, two to three-word sentences, spoken mostly in a high pitched tone or a whispering voice. Words and sentences may be repeated over and over again. He is unable to do basic school tasks like reading or writing. Behavior is characterized by rigidity, fixations and perseverations, diagnosed as an autistic spectrum disorder. Anxiety is present as well. He attends a school for children with epilepsy and learning problems and has a moderate to severe intellectual disability.

Patient D

This 24 month old boy presented at age 7 months with a cluster of febrile focal and generalized tonic seizures which was stabilized with valproic acid. He was admitted to the hospital following a second episode of seizure clusters precipitated by fever two months later. Three more admissions with clustered seizures and fever followed. Seizures always occur out of sleep. An EEG showed multiple focal discharges, right centroparietal, with secondary generalization and mild diffuse background slowing. Current medication consists of oxcarbazepine, levetiracetam, phenobarbital and valproate. Speech development is mildly delayed. There is no known family history of febrile seizures or epilepsy.

Patient E

This 13 year old boy was known with a delay in speech development, when, at the age of 2.5 years he had his first cluster of seizures with gazing, head turn to the left, blue lips and pale face, during a period of febrile illness. An ictal EEG was asymmetrical with background slowing at the left with epileptic discharges at the left parieto-temporal side with spreading to both hemispheres. He was treated with valproic acid for 6 months. MRI of the brain was normal. At the age of 4;7 years a developmental age of 2.6 years was measured (SON-R, 2.5-7 years). After discontinuation of valproic acid, seizures reoccurred during febrile illnesses and valproic acid was restarted until the age of 5.5 years, when it was discontinued because of behavioral problems. At age 9 years his IQ score was 55 (WISC), and he showed some autism characteristics but did not fulfil diagnostic criteria of autism spectrum disorder . At the age of 11 years he had multiple episodes with recurrent seizures during febrile illnesses and after BMR vaccination. He was treated with clobazam during periods of fever, resulting in reduced seizure frequency and duration. At the age of 13 years oxcarbazepine treatment was started because of a further increase in seizure frequency with moderate effect. Currently he has a mild to moderate, mainly cognitive, developmental delay. He is not able to speak sentences fluently. His reading, writing and calculating skills are comparable to those of a 7-year old. There is no known family history of febrile seizures or epilepsy, but his father had a stutter during childhood and experienced difficulties with learning how to read.

Chapter 9

De novo mutations of *KIAA2022* in females cause intellectual disability and intractable epilepsy

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Published: J Med Genet. 2016 Dec;53(12):850-858.

Abstract

Background: Mutations in the *KIAA2022* gene have been reported in male patients with X-linked intellectual disability and related female carriers were unaffected. Here, we report 14 female patients who carry a heterozygous *de novo KIAA2022* mutation and share a phenotype characterized by intellectual disability and epilepsy.

Methods: Reported females were selected for genetic testing because of substantial developmental problems and/or epilepsy. X-inactivation- and expression studies were performed when possible.

Results: All mutations were predicted to result in a frameshift or premature stop. Twelve out of 14 patients had intractable epilepsy with myoclonic and/or absence seizures, generalized in 11. Thirteen patients had mild to severe intellectual disability. This female phenotype partially overlaps with the reported male phenotype which consists of more severe intellectual disability, microcephaly, growth retardation, facial dysmorphisms, and, less frequently, epilepsy. One female patient showed completely skewed X-inactivation, complete absence of RNA expression in blood, and a phenotype similar to male patients. In the six other tested patients X-inactivation was random, confirmed by a non-significant two- to threefold decrease of RNA expression in blood, and consistent with the expected mosaicism between cells expressing mutant or normal *KIAA2022* alleles.

Conclusions: Heterozygous loss of *KIAA2022* expression is a cause of intellectual disability in females. Compared to its hemizygous male counterpart the heterozygous female disease has less severe intellectual disability but is more often associated with a severe and intractable myoclonic epilepsy.

9.1 Introduction

KIAA2022 is a known X-linked intellectual disability (XLID) gene, with pathogenic variants causing severe intellectual disability (ID) in males. Other, more variable features include epilepsy, postnatal growth retardation, autistic behavior, strabismus and dysmorphic facial features. The first description of alterations in this gene causing ID was in two related male patients where both *KIAA2022* and *P2YR8* were interrupted by a pericentric inversion of the X chromosome (Inv X(p22;p13.2)), as reported by Cantagrel et al.[299] One breakpoint was mapped to the first intron of *KIAA2022* and was predicted to disrupt the gene. A complete loss of *KIAA2022* expression in lymphocytes was shown. Other reported *KIAA2022* pathogenic variants include a microduplication of exon 1, a duplication of the entire gene and several truncating mutations, all leading to reduced *KIAA2022* expression.[300–303] Limited data about *KIAA2022* gene function is available, but it is thought to have an important role in early brain development.[299,300,304–306]

Female relatives carrying the *KIAA2022* disruptions identified in reported males were all unaffected.[299,300,303] Nevertheless, a few affected female patients have recently been described.[307–309] Here, we report 14 female patients with heterozygous *de novo* mutations of *KIAA2022*. All mutations result in a frameshift or premature stop codon, predicting complete loss of function of the protein. Twelve patients had epilepsy, of which 11 generalized, and all but one patient had mild to severe ID. These data strongly suggest that pathogenic *KIAA2022* variants can lead to a phenotype not only in males, but also in females.

9.2 Methods

9.2.1 Patients

All 14 reported females were selected for genetic testing because of their substantial developmental problems and/or epilepsy. In 10 patients exome sequencing was performed, using methods that are described previously,[232,310,311] of which 9 were diagnostic and one was in a research setting (patient 4). In patient 9 whole genome sequencing was performed in a research setting, as previously described.[312] Patient 5 was part of a research series of 209 cases with Dravet(-like) syndrome or myoclonic atonic epilepsy who were selected for candidate gene screening with a gene panel using molecular inversion probes (MIPS) as described previously.[313] Patient 8 was diagnosed after a diagnostic array CGH (Agilent 180 k chip) indicated a microdeletion disrupting *KIAA2022*. Patient 11 was diagnosed after a *KIAA2022* mutation was identified with her two sons with ID, through sequencing of a diagnostic intellectual disability related-gene panel. X-inactivation- and expression studies were performed when possible (XCI: patient 1, 2, 3, 4, 6, 8, 9; expression studies: patient 1, 3, 4, 5, 6). See supplemental data S9.1 for extensive details on the molecular analyses.

Detailed clinical data were collected from medical records. The study was approved by the Ethical Committees of the respective local institutions.

9.2.2 Literature search

A literature study was carried out in the PubMed database to identify previously described patients with *KIAA2022* mutations. Resulting articles and their references were screened for reported patients with *KIAA2022* mutations.

9.3 Results

9.3.1 Clinical description

Clinical characteristics of the 14 new and three previously described female patients with *de novo KIAA2022* mutations are summarized and compared with the clinical characteristics of previously reported males in Tables 9.1A and 9.1B. Table 9.2 gives further details with respect to the epilepsy phenotype of the female patients. See supplemental material S9.2 for extensive clinical descriptions of each patient. In summary, twelve out of the 14 new patients had intractable epilepsy with myoclonic and/or absence seizures. In eleven patients, epilepsy was generalized. Thirteen patients had mild to severe intellectual disability. Developmental delay preceded epilepsy onset in six individuals. Behavioral problems, like autism, aggression and hyperactivity were present in ten patients. Other findings were hypotonia, neonatal feeding difficulties, microcephaly, and mild dysmorphic facial features.

9.3.2 Molecular analysis

KIAA2022 *mutations*

Thirteen patients carried a *de novo* truncating mutation in *KIAA2022* (NM_001008537.2) (Table 9.1A). In one patient, a deletion of the complete non-coding exon 1was identified by array CGH, which is likely to affect expression of the protein. Figure 9.1 represents the genomic organization of the *KIAA2022* gene, as well as the location of the mutations reported previously and those presented here. All mutations were absent from the Exome Aggregation Consortium (ExAC) database (URL: http://exac.broadinstitute.org) [accessed October 2015].[156] All sequence alterations are in the large exon 3 (coding exon 2) and are predicted to activate nonsense-mediated decay (NMD), with one recurrent mutation, p.Arg322*, that is present in one of the currently described females (patient 4), one male patient[301] and one previously reported female.[309] Overall, there seems to be no particular domain in which mutations cluster, consistent with the hypothesis that these truncating mutations all cause loss of function of *KIAA2022* regardless of their position in the protein.

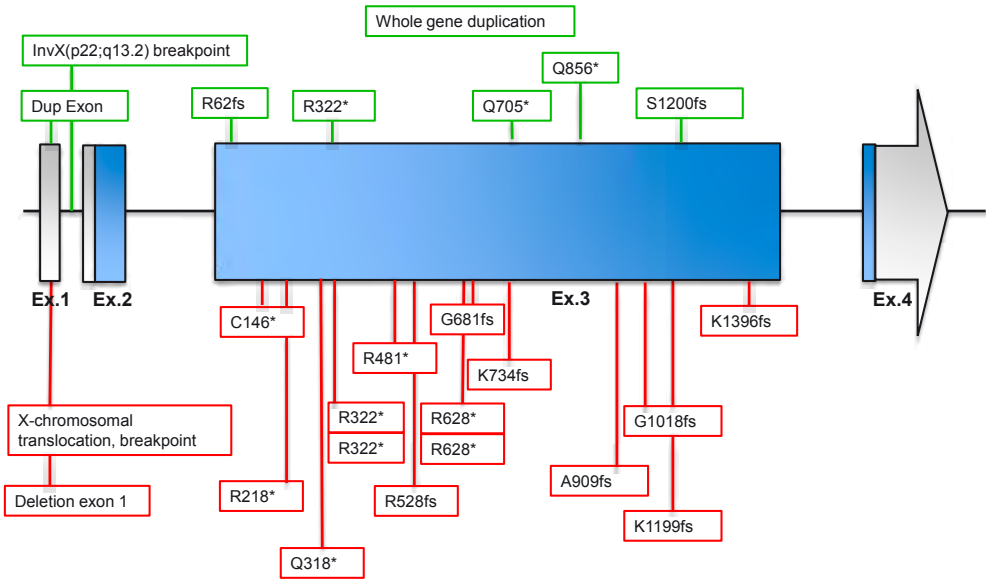

Figure 9.1. Schematic presentation of known exon-intron organization of *KIAA2022.* Exon size is at scale apart from exon 4, for which the arrow indicates the continued size. Untranslated regions are indicated by grey colour, coding regions are indicated by blue colour. The boxes indicate the location and identity of the (previously) observed mutations in female (lower/red boxes) and previously reported mutations in male patients (upper/green boxes).

Chromosome X-inactivation studies and KIAA2022 *expression*

X-inactivation was tested in seven patients and was found to be random in patients 1, 2, 3, 4, 8 and 9. One patient (patient 6) showed 100% skewing. We further tested *KIAA2022* expression using digital droplet PCR of RNA derived from whole blood of 4 patients (patient 1, 3, 4 and 5), and compared it with 4 healthy female controls. *KIAA2022* expression in blood was expressed as a ratio of *KIAA2022* mRNA copies compared to *GAPDH* and was found to be low in both cases and controls. Cases had on average a 2-3 times lower expression than female controls, but this was not statistically significant (non-parametric test on ratio cases vs controls p=0.486; Figure 9.2). Expression of *KIAA2022* in patient 6 was tested using qPCR and was found to be completely absent.

9.3.3 Previously described patients

A literature search resulted in 13 publications on *KIAA2022*. Of these, seven reported patients with sequence alterations of *KIAA2022* or structural X-chromosome abnormalities affecting *KIAA2022,*[299–301,303,307–309] whereas the other six did not describe patients. In these first seven publications, 15 male patients were described. Clinical characteristics are given in Table 9.1B. Five affected males from one family[303] are excluded from the table because only limited clinical data were available. Three female patients affected by *KIAA2022*

Table 9.1A. Clinical description of female cases presented carrying *KIAA2022* mutations

Patient	1	2	3	4	5	6	7	8	9	10	11	12	13	14	307	308	309
Age (years)	26	9	25	11	36	2,5	9	18	5,5	2,3	53	11	51 (deceased)	8,5	26	13	17
Mutation	c.4185del, p.Lys 1396fs	c.438C>A, p.Cys146*	c.2042del, p.Gly681fs	c.964C>T, p.Arg322*	c.2201_2202 delAA, p.Lys734 Serfs*24	c.1441 C>T, p.Arg481*	c.3053-3066del14, p.Gly1018 Aspfs*2	del exon1	c.1582delA, p.Arg 528Glufs*4	c.1882 C>T, p.Arg 628*	c.2725del, p.Al-a909Profs*13	c.652C>T, p.Arg218*	c.952C>T, p.Gln318*	c.3596_3597insA, p.Lys 1199Asnfs	46,X,t(X;3) (q13;q11)	c.1882C>T, p.Arg628*	c.964C>T, p.R322*
XCI	57:43 (random)	70:30 (random)	52:48 (random)	63:37 (random)		100% skewing		62:38 (random)	64:36 (random)						100% skewing	65:35 (random)	73:27 (borderline skewed)
***KIAA2022* expression** (ratio)	0.000624456		0.00029598	0.000355424	0.000256076	absent									absent		
Walking age (months)	24	18	19	15	14,5	-	12	24	14	19	12	15	24	18	?	?	12-18
Language skills	sentences	sentences	sentences	sentences	sentences	absent	full sentences	absent	150 words	5 words	normal	2 word phrases	single simple words, no sentences	simple sentences	absent	?	2 words
ID	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
Degree of ID[a]	+	+	+/-	+	+ to ++	+	+/- to +	+ to ++	+	+/-	-	+	++	+	++	+/-	++
Age first notice of delay (months)	18	8	from birth	30	12-24	3	35	15	12-18	7	-	<20	12-24	12-18	?	?	18
Autistic behavior	+	+	-	+	-	-	+	+	-	-	-	+	-	-	+	?	+
Other neurobehavioral problems	-	agression hyper-active	tantrums hyper-active	ADHD	attention deficit hyperactive	-	+	tantrums hyper-active	hyperactive, impulse control difficulties	-	-	severe ADHD, impulse control disorder	-	opposition	-	?	repetitive behaviors, aggression, hyperactive
Seizures	+	+	+	+	+	-	+	+	+	-	+	+	+	+	-	-	+
Neurological exam	normal	normal	normal	normal	normal	hypotonia	normal	normal	normal tone ataxic gait	normal	normal	hypotonic, broad based gate	normal	normal	?	?	normal
Growth retardation prenatal	-	-	-	<-2SD	-	-2 SD	-	-	-	-	-	-	-	-	p3-p10	?	-
Growth retardation postnatal	-	-	-	-	-	+	-	-	-	-	-	-	+	-	+	-	+
Obesity	+	+	+	-	-	-	-	+	-	-	-	-	+	-	-	?	-
Microcephaly	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	?	+

Patient	1	2	3	4	5	6	7	8	9	10	11	12	13	14	[307]	[308]	[309]
Dysmorphisms	-	+	-	-	-	+	-	-	-	-	-	-	+	-	+	-	+
Joint laxity	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	?	
Hypotonia	-	-	-	-	-	+	very mild	-	-	+	-	+	-	mild	-	?	-
Additional medical problems[b]	hip dysplasia	GER	neonatal feeding difficulties	-	-	GER	-	-	otitis media, PFO	-	IDDM, horse-shoe kidney	cardiac rhabdomyoma, TSC 1 and 2 negative	-	pulmonary stenosis	primary amenorrhea, hyperglycemia	?	-
MRI brain	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	?	normal	normal	frontal atrophy (3 years)	morphological alterations of temporal lobes	?	status after corpus callosotomy surgery

Table 9.1B. Clinical description of previously reported males carrying *KIAA2022* mutations

Family	**Family 1**[299,300]		**Family 2**[300]		**Family 3**[300]	**Family 4**[300]			**Family 5**[301]	**Family 6**[301]
Patient	1	2	3	4	5	6	7	8	9	10
Age (years)	13	20	6	4	8	14	10	40	3	5
Mutation	InvX	InvX	Ser1200fs	Ser1200fs	Exon 1 dup	Arg62fs	Arg62fs	Arg62fs	Gln705*	Arg322*
XCI	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
***KIAA2022* expression**	absent	absent	?	?	40%	?	?	?	?	?
Walking age (months)	36	36	34	48	17	18	18,5	14	-	48
Language skills	absent	absent	rudimentary	absent	rudimentary	delayed	poor	poor	absent	absent
ID	+	+	+	+	+	+	+	+	+	+
Degree of ID[a]	++	++	++	++	+/-	+	++	+	++	++
Age first notice of delay (months)	0-12	0-12	0-12	0-12	?	?	?	36	0-12	3
Autistic behavior	+	+	+	+	+	-	+	-	-	+
Other neurobehavioral problems	self biting hyperactive	agressive anxiety	-	-	+	hyperactive attention deficit	agressive, attention deficit, hyperactive	hyperactive	-	-
Seizures	-	+	+	+	-	-	+	+	-	-
Syndrome diagnosis							Lennox-Gastaut			
Neurological exam	hypotonia	spastic quadriplegia	axial hypotonia, lower limb spasticity	hypotonia, lower limb spasticy	normal	normal	normal	normal	hypotonia	hypotonia
Growth retardation prenatal	-	-	-	-	-	-	-	-	-	-
Growth retardation postnatal	+	+	+	+	-	-	-	-	+	+
Obesity	-	-	-	-	-	-	+	+	-	-
Microcephaly	+	-	+	+	-	-	-	-	-	+
Dysmorphisms	+	+	+	+	-	-	-	?	+	+

Family	Family 1[299,300]		Family 2[300]		Family 3[300]	Family 4[300]			Family 5[301]	Family 6[301]
Joint laxity	-	-	-	-	-	-	-	?	-	-
Hypotonia	+	+	+	+	-	-	-	?	+	+
Additional medical problems[b]	GER	GER, gastric ulcer	GER	GER gastrostomy	-	bulimia	GER	bulimia	GER	nephrotic syndrome, central hypothyroidism
MRI brain	small brain, mild enlargement of sulci in frontal lobes	moderate brain atrophy	?	?	normal	normal	normal	?	normal	normal

n.a. = not applicable. [a]- = absent, +/- = mild, + = moderate, ++ = severe. [b]GER=gastroeosophageal reflux, PFO=patent foramen ovale, IDDM=insulin dependent diabetes mellitus
Note: if features are not described in the original article, we assume they are not present. Note that more details on the female phenotypes were available in some cases.

Table 9.2. Clinical description of female cases presented; detailed epilepsy phenotypes

Patient	1	2	3	4	5	6	7	8	9	10	11	12	13	14	307	308	309
Age	26	9	25	11	36	2.5	9	18	5,5	2,3	53	11	51 (deceased)	8,5	26	13	17
Mutation	c.4185del, p.Lys 1396fs	c.438C>A, p.Cys146*	c.2042del, p.Gly681fs	c.964 C>T, p.Arg322*	c.2201_2202 delAA, p.Lys734 Serfs*24	c.1441C>T, p.Arg481*	c.3053-3066 del14, p.Gly1018 Aspfs*2	del exon1	c.1582del,A p.Arg528 Glufs*4	c.1882C>T, p.Arg628*	c.2725del, p.Ala909 Profs*13	c.652 C>T, p.Arg218*	c.952C>T, p.Gln318*	c.3596_3597 insA, p.Lys 1199Asnfs	46,X,t (X;3) (q13;q11)	c.1882 C>T, p.Arg 628*	c.964C>T, p.R322*
XCI	57:43 (random)	70:30 (random)	52:48 (random)	63:37 (random)		100% skewing		62:38 (random)	64:36 (random)						100% skewing	65:35 (random)	73:27 (borderline skewed)
***KIAA2022* expression** (ratio)	0,000624456		0,00029598	0,000355424	0,000256076	absent									absent		
Degree of ID[a]	+/- to +	+	+/-	+/-	+ to ++	+	+/- to +	+ to ++	+	+/-	-	+	++	+	++	+/-	++
Seizures	+	+	+	+	+	-	+	+	+	-	+	+	+	+	-	-	+
Age seizure onset (months)	8	8	72	30	36		24	24	14		192-216	24	84	36			18
Generalized	+	+	+	+	+	-	+	-	+	-	+	+	+	+	-	-	+
Myoclonic	+	+	+	+	+	-	+	-	+	-	-	+	+	+	-	-	
Typical absence	-	+	+	-	+	-	+	-	+	-	+	+	-	-	-	-	+
Myoclonic absence	-	-	+	-	-	-	+	-	+	-	-	-	-	+	-	-	
Tonic	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	+
Atonic	-	+	-	+	-	-	-	-	+	-	-	-	+	+	-	-	+
Clonic	-	-	-	-	-	-	-	+	-	-	-	+	-	+	-	-	-
GTCS	+	+	+	-	+	-	-	-	+	-	-	+	+	-	-	-	+
Focal	-	+	-	+	probably not	-	-	+	-	-	-	+	-	-	-	-	-
Spasms	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Status epilepticus	-	+	-		+	-		+		-	-	+	+	-	-	-	
Other seizure types	-	-	-	-	-	-	automa-tisms (focal seizures?)	-	-	-	-	-	-	-	-	-	
Photo-sensitivity	-	+	+	?	-	-	-	-	?	-	-	-	?	-	-	-	?

Patient	1	2	3	4	5	6	7	8	9	10	11	12	13	14	307	308	309
EEG[b]	PSW	PSW, ECS	PSW, ECS	PSW, focal discharges	generalized SWC and PSW	normal	PSW	PSW, right focal dis-cha-rges	background slowing and gener-alized and multifocal epileptiform discharges	normal	?	mixed gen-era-lized (PSW and slow wave) and focal dischar-ges	interictal: featureless; ictal: bifrontal PSW, fast activity, evolving into diffuse slow rhythmic activity; ECS	Interictal: generalized SWC and PSW; ictal:PSW concomittant with palpe-bral clonia or atonic fall.	?	?	Suggestive of multi-focal and generalized epilepsy
Seizure out-come[c]	Ongoing despite AED	Ongoing despite AED	Ongoing despite AED	Ongoing despite AED	Ongoing despite AED	n.a.	Ongoing despite AED	Ongoing despite AED	Ongoing despite AED	n.a.	Ongoing despite AED	Ongoing despite AED	Ongoing until death despite AED	Ongoing despite AED	n.a.	n.a.	Ongoing despite AED

n.a. = not applicable. [a]- = absent, +/- = mild, + = moderate, ++ = severe. [b]PSW = polyspike waves, ECS = eye closing sensitivity, SWC = spike wave complex. [c]AED = anti-epileptic drugs

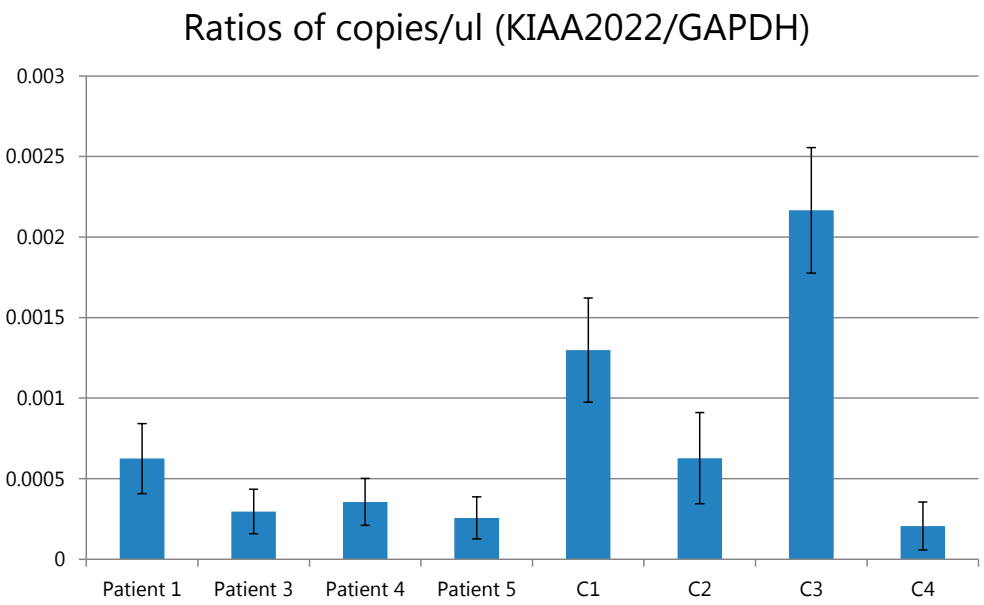

Figure 9.2. Relative expression of *KIAA2022* in 4 cases versus female controls. Y-axis gives the ratio of positive droplets for *KIAA2022* versus GAPDH; experiment was done in triplicate. 95% confidence intervals are indicated by error bars.

disruptions have previously been reported,[307–309] two with a phenotype comparable to that in males, and one with mild intellectual disability. Clinical characteristics are given in Table 9.1A and 9. 2.

KIAA2022 *mutations in public databases*

All mutations reported in the currently described females were absent from public databases. However, a few other truncation variants have been reported. In the ExAC database, three female and one male subjects out of a total of 60706 subjects are reported to have heterozygous apparent loss of function (LOF) variants in *KIAA2022* (hg19, NM_001008537.2), namely: chrX:g.73959335T>C, (c.4458-2A>G; one female, resulting in a putative disruption of the canonical splice acceptor site for the last exon), chrX:g.73959987G>A, (c.4405C>T or p.Arg1469*, two females, located right at the end of the encoded protein), and chrX:g.73961129_73961139delCTCTCACATCT (p.Arg1085TyrfsTer45, one hemizygous male).

9.4 Discussion

We report 14 female patients with a heterozygous *de novo* mutation of the X-chromosomal *KIAA2022* gene. Thirteen mutations resulted in a frameshift or premature stop codon that can elicit NMD, predicting a complete loss of function of the protein. The fourteenth mutation is a complete deletion of the non-coding exon 1, most likely affecting expression of the protein. *KIAA2022* mutations and alterations have previously been reported in males with severe intellectual disability,[299–301,303] and it is an established XLID-gene. We show that *KIAA2022* mutations can also cause a phenotype in females. Overall, eleven of our female patients with *KIAA2022* loss-of-function mutations showed a similar clinical phenotype, which added to our belief that these mutations were indeed responsible for their phenotype. This phenotype was characterized by intractable epilepsy with predominant myoclonic seizures and/or absences, with onset in infancy or early childhood, behavioral problems and mild to severe intellectual disability. Developmental delay preceded epilepsy onset in six individuals. Hypotonia, neonatal feeding difficulties, microcephaly, and mild dysmorphic facial features were less frequent findings.

Three female patients affected by *KIAA2022* disruptions were previously reported. Moyses-Oliveira et al. reported a female patient with a balanced X-autosomal translocation disrupting *KIAA2022* at intron 1.[307] X-chromosome inactivation studies showed inactivation of the normal X-chromosome in all cells, leading to complete absence of *KIAA2022* expression. The reported phenotype was comparable to the previously described male phenotype, with severe ID, microcephaly, autistic behavior, growth retardation and facial dysmorphia, without epilepsy. Athanasakis et al.[308] reported a 13 year old girl with mild intellectual disability and a *de novo* nonsense mutation of *KIAA2022* (p.Arg628*) as part of a larger study which included patients with ID and absence of dysmorphic features, normal growth parameters and no seizures or malformations. The X-inactivation pattern was 65:35. Farach et al.[309] reported a 17-year old girl with a recurrent *de novo* nonsense mutation of *KIAA2022* previously reported in a male (p.Arg322*) and a phenotype comparable to previously reported male patients, with severe ID, hypotonia, behavioral problems, microcephaly, growth retardation, mild dysmorphisms and seizures. An X-inactivation pattern of 73:27 was reported. These previously reported female patients seem to be on the extreme ends of the phenotypic spectrum in comparison to our new cases, with one patient being relatively mildly affected[308] while the other two have a phenotype comparable to our most severely affected cases.[307,309] Although we observe a comparable clinical picture in most female cases, this illustrates that the phenotypes of females affected by *KIAA2022* mutations can be very variable. Moreover, unaffected female carriers of *KIAA2022* disruptions have been reported[300] and the ExAC database includes three females with heterozygous apparent loss of function (LOF) variants in *KIAA2022*.[156]

No inheritance or clinical data is available for the females in the ExAC database, but it does not include patients with pediatric onset intellectual disability.[156] However, the

presence of these variants in the ExAC database might be explained by the nature of the variants. Regarding the truncating alteration (Arg1469*), it appears that this alteration will not undergo nonsense mediated decay, as it is located at the end of the second to last exon. Therefore a truncated protein, lacking the last 48 amino acids will be produced, which may not be a complete loss-of-function alteration. The splice alteration is in the splice acceptor in the last exon and it might have minimal functional impact since it is localized near the 3'end of the protein. The apparent hemizygous frameshift variant (Arg1085fs) looks like an in-frame indel that was miscalled (c.3245_3265del21insCCT; deletion of 21bp, insertion of 3bp) upon manual visualization. Therefore, these reported variants are likely non-pathogenic. Furthermore, it is worth noticing that ExAC variants are not validated and some of them could be false positives. On the other hand, it is possible that these mutations are pathogenic but that they occur in asymptomatic female carriers, as previously described.[300]

Although both males and females with *KIAA2022* mutations have intellectual disability and frequent autistic behavior, males tend to have a more severe phenotype. They have more severe intellectual disability (as opposed to mild to severe in females) and more frequently microcephaly, hypotonia, growth retardation, feeding difficulties and facial dysmorphisms. Epilepsy was reported for five of ten male patients whereas it was seen in thirteen of seventeen females, making it a more frequent clinical finding in females.[299–301] It should be noted, however, that some of our female patients (1, 2, 3 and 5), were selected for genetic testing because of their epilepsy, which may have introduced a selection bias.

A reduced penetrance and variable expression in females and a more severe phenotype in males versus females is a common observation in many X-linked disorders, and may be explained by X-chromosome inactivation (XCI) patterns in females.[314–318] Our female patient 6 and the patient previously reported by Moyses-Oliveira et al.[307] support this theory. Both had 100% skewed X-inactivation patterns and complete loss of expression, leading to a phenotype closely resembling the more severe male phenotype. Conversely, a previously reported male with the least severe phenotype among reported males was shown to have a duplication of exon 1, resulting in a decrease in expression of 60%.[300] This is in contrast to several other male patients that lack expression completely. So far, the degree of *KIAA2022* loss thus seems to correlate with the severity of the phenotype, with complete loss of expression predicting a severe phenotype. The patient reported by Farach et al.[309] had a borderline skewed X-inactivation (73:27) and was also severely affected. In six out of seven tested female patients a random XCI was found, and expression of *KIAA2022* was on average 2-3 times lower than in female controls, although this was not statistically significant. These female patients all showed a similar clinical phenotype, characterized by intractable myoclonic epilepsy and an intellectual disability that is less severe than in male patients. The very mildly affected patient described by Athanasakis et al.[308] also had a random X-inactivation according to our criteria (65:35). These results indicate that partial loss of *KIAA2022* expression explains the phenotype in those females who are more mildly

affected than males. Unfortunately, data on X-inactivation patterns is lacking in patient 11, who has a very mild phenotype compared to other affected females, and in the unaffected female carriers of familial *KIAA20022* disruptions. However, expression in at least one unaffected female carrier was reported to be normal compared to controls, suggesting XCI skewing towards the wild type allele as an explanation for their lack of symptoms.[303] No clinical data on unaffected female carriers is available.

Next to X-inactivation patterns in blood, other factors might also explain the clinical variability in females with *KIAA2022* mutations. First, X-inactivation and expression in blood might not reflect what is occurring in the brain. Second, other factors may modify expression, such as variants in regulatory sequences of *KIAA2022* and related genes, or a parent of origin effect. Finally, mechanisms other than loss of expression might also play a role in affected females. The random XCI found in several female patients predicts a mosaic population of cells with either normal or absent expression of *KIAA2022*, in contrast to male patients, where all cells have the defective allele. Although this mosaic cell population in females may have similar effects as the defect in males, an additional disease mechanism might be cellular interference, as is the proposed disease mechanism in *PCDH19*-related epilepsy.[147,177,178,319] In females, *PCDH19*-mutations cause variable degrees of epilepsy, behavioral problems and intellectual deficits, while male carriers are unaffected. However, one reported male with a female phenotype was found to be mosaic for a *PCDH19*-mutation, just as affected females.[319] Brain mosaicism in females, as a result of random X-inactivation, was proposed to disrupt cell-cell interactions between the two different cell populations (the cells with normal *PCDH19* on the one hand, and the cells with mutated *PCDH19* on the other).A similar disease mechanism is plausible, since the *KIAA2022* protein seems to be involved in the same processes of cell-cell and cell-matrix adhesion and neuronal migration as the *PCHD19* protein, by influencing expression of N-cadherin.[305] Additional X-inactivation and expression studies of asymptomatic mothers of affected males carrying *KIAA2022* mutations could further clarify the disease mechanism in females with *KIAA2022* related disease.

9.5 Conclusion

In conclusion, we show that *de novo* truncating mutations in the X-chromosomal *KIAA2022* gene can lead to a phenotype not only in males, but also in females. While males had more pronounced intellectual disability and dysmorphic features, females with *KIAA2022* mutations show variable symptoms, and some are even asymptomatic. Females with 100% XCI skewing and absent *KIAA2022* expression show a phenotype similar to the male phenotype. Females with random XCI patterns tend to have a more prominent epilepsy phenotype, with predominant generalized myoclonic and/or absence seizures. Mechanisms underlying the female phenotype may be both cellular mosaicism and reduced protein expression.

9.6 Appendix

S9.1 Molecular analysis

Three patients (patient 1, 2 and 3) and their parents were sequenced using SureSelect XT Human All Exon V5 kit (Agilent) enrichment and the HiSeq2500 sequencing system (Illumina) at a mean target depth of 100X. Reads were aligned to Hg19 using BWA (BWA-MEM v0.7.5a) and variants were called using the GATK haplotype caller (v2.7-2). Detected variants were annotated, filtered and prioritized using the Bench lab NGS v.3.1.2 platform (Cartagenia, Leuven, Belgium). Sequencing and analysis was performed at the diagnostics section of the Department of Medical Genetics, University Medical Center Utrecht, The Netherlands.

Whole exome sequencing and data analysis of patient 4 and her parents was performed as described previously,[310] at the Wellcome Trust Sanger Institute (Hinxton, Cambridgeshire). In brief, genomic DNA (~3 mg) was fragmented by sonication, and fragments with a length of 150–200 bp were purified. After a paired-end DNA library was prepared from the DNA fragments (with the TruSeq DNA Sample Preparation Kit from Illumina), targeted enrichment was performed with the SureSelect Human All Exon 50Mb Kit (Agilent Technologies). Captured DNA was then sequenced on a HiSeq2000 (Illumina) as paired-end 75 bp reads according to the manufacturer's protocol.

Whole exome sequencing of patient 6 and her parents was conducted through Ambry Genetics using paired-end, 100 cycle chemistry on the Illumina HiSeq 25000. Enrichment was performed using SeqCap EZ VCRome 2.0 (Roche NimbleGen, Madison, WI). Data were annotated with the Ambry Variant Analyzer tool, including nucleotide and amino acid conservation, biochemical nature of the amino acid substitutions, population frequency, and predicted functional impact as previously described.[232] Approximately 94% of the bases sequenced had a base calling accuracy of 99.9% and 95% of patient's exome was covered at 10x or higher.

Clinical whole exome sequencing of patient 7 and 12 was performed by GeneDx. Genomic DNA was extracted from whole blood from the affected children and their parents. Exome sequencing was performed on exon targets isolated by capture using the Agilent SureSelect Human All Exon V4 (50 Mb) kit (Agilent Technologies, Santa Clara, CA). The sequencing methodology and variant interpretation protocol used has been previously described.[311] The general assertion criteria for variant classification are publicly available on the GeneDx ClinVar submission page (http://www.ncbi.nlm.nih.gov/clinvar/submitters/26957/).

Diagnostic whole exome sequencing of patient 10 and her parents was performed at Mendelics Genomic, Sao Paulo Brazil) on a DNA sample extracted from peripheral blood. The Extended Nextera Rapid-Capture Exome kit was used and sequencing was performed on the Illumina HiSeq2500 system (Illumina, San Diego, CA, USA). Exome reads were analyzed in a standard Bioinformatics pipeline, using BWA for sequence alignment to the

GRCh37 reference, Broad Institute GATK for genotyping, SnpEff for variant annotation, and ExomeDepth for CNV detection.

Patient 13 and her parents were exome sequenced at the Epilepsy Society, Chalfont, using the Ion AmpliSeq Exome RDY Kit (ThermoFisher) and the Ion Proton sequencing system (LifeTechnologies). Mean target depth of >100X was obtained for each member of the trio (Mother 139X, Father 163X, Proband 101X). Variants were called and Trio analysis was undertaken using Ion Reporter software Ampliseq Exome Trio v5.0 workflow.

For patient 14 and her parents, library generation, exome enrichment and clinical WES were performed at the French National Center for Genotyping (CNG, Evry, France). Briefly, libraries were prepared from 3 µg genomic DNA extracted from whole blood using an optimized SureSelect Human Exome kit (Agilent) following the manufacturer's instructions. Captured, purified and clonally amplified libraries targeting the exome were then sequenced on a HiSeq 2000 (Illumina) according to the manufacturer's recommendations. Obtained sequence reads were aligned to the human genome (hg19) using BWA software. Downstream processing was carried out with the Genome analysis toolkit (GATK),[263,320] SAMtools.[321] and Picard Tools (http://picard.sourceforge.net/). Single-nucleotide variants and indels were subsequently called by the SAMtools suite (mpileup, bcftools, vcfutil). All calls with a read coverage ≤5x and a Phred-scaled SNP quality of ≤20 were filtered out. Substitution and variation calls were made with the SAMtools pipeline (mpileup). Variants were annotated with an in-house Paris Descartes bioinformatics platform pipeline based on the Ensembl database (release 67).[322]

Whole genome sequencing of patient 9 was done in a research setting at Complete Genomics (Mountain View, CA) as described previously.[312] Variants were annotated using Annovar and custom script at The Center for Applied Genomics (TCAG, Toronto, Canada).

Sequencing for patient 5 was performed in a research setting at the University of Washington (Seattle, WA, USA). All library preparation, data analysis, and variant calling were performed as described previously.[313] Briefly, all coding *KIAA2022* exons and at least five base pairs of flanking intronic sequences were captured by using molecular inversion probes (MIPs); next-generation sequencing was performed used a 100 paired-end protocol on the Illumina HiSeq, reads were aligned to Hg19 using BWA (v0.7.8) and variants were called with GATK UnifiedGenotyper (v2.4-9).

All mutations were confirmed by standard Sanger sequencing.

Patient 8 was diagnosed by Array-CGH using the Agilent 180 k chip (Arr[hg19] Xq13.3(74,108,610-74,178,971)x1

Patient 11 was diagnosed by targeted analysis of the *KIAA2022* mutation identified in her two sons.

X-inactivation ratios were determined in patient 1, 2, 3, 4, 6 and 9 by methylation-sensitive restriction digest (CfoI) of genomic DNA followed by PCR and fragment analysis of the highly polymorphic trinucleotide repeat within the first exon of the human androgen receptor gene (AR), to distinguish between the maternal and paternal alleles

and simultaneously determine their methylation status. A methylation ratio of up to 1:4 is considered normal.

Expression of *KIAA2022* was evaluated in patient 1, 3, 4 and 5, with digital PCR using Taqman probe Hs02339405_m1 (Invitrogen) spanning exon 2-3. GAPDH dHsaCPE5031597 (Bio-Rad) was used as an endogenous control. Droplets were generated in a QX100TM and thermal cycling was done using a Bio-rad C1000 Touch Thermal Cycler. A QX200 Droplet Reader and QuantasoftTM Software were used for data acquisition and analysis. Expression levels of cases and female controls were compared using the Poisson corrected ratios of copies/ul (*KIAA2022*/GAPDH) as implemented in the QuantasoftTM Software.

S9.2 Clinical descriptions

Patient 1 (Dutch)

This 26 years old female was diagnosed with epilepsy at the age of 8 months, but in retrospect she may have had seizures already in the first months of life. She has therapy resistant myoclonic seizures, that used to occur up to 30 times per day, but seizure frequency has gradually decreased to 4 per day. Except for two tonic-clonic seizures, she has had no other seizure types. EEGs showed generalized (poly)spike-wave complexes. Brain MRI at age 20 showed no abnormalities. Psychomotor retardation became evident at age 18 months. She could walk independently at age two years and speech development started at age three years. She currently has a mild to moderate intellectual disability (IQ ~50) with an estimated developmental age of 3 years. An autism spectrum disorder has been diagnosed. Furthermore, her medical history includes congenital hip dysplasia, joint laxity and obesity. No facial dysmorphisms were observed.

Patient 2 (Dutch)

Seizure onset in this 9 years old female patient was at age 8 months. She presented with myoclonic seizures, but later also developed absences, myoclonic atonic seizures, atonic seizures and focal dyscognitive seizures, refractory to pharmacological treatment. She also had a single tonic-clonic seizure. In the first weeks of life she had 2 episodes with loss of muscle tone, apnea and cyanosis, which in retrospect might have been seizures too. At the age of 2 years and 9 months she developed a myoclonic status epilepticus. She self-induces seizures by looking at lights. Her EEG at age 5 showed slight background slowing with sporadic generalized spike wave complexes when awake. During sleep frequent spike wave complexes in the temporal regions, and frequent generalized polyspikes were seen. Her last EEG at age 8 also showed eye-closure related discharges. Brain MRI at age 5 showed no abnormalities. Her psychomotor development slowed after the onset of her seizures. At age 5 years and 8 months she had a developmental age of 28 months. She shows hyperactivity, aggression and autistic behavior. In addition, her medical history includes severe esophageal

reflux during her infancy, and obesity. She has a narrow forehead and a hypotonic face with an open mouth.

Patient 3 (Dutch)
This 25-years old female patient was diagnosed with epilepsy at age 6 years. In retrospect, her parents reported that she might have had myoclonic seizures already in her infancy. She developed myoclonic absences, absences and tonic clonic seizures, refractory to pharmacological treatment. Her EEG showed generalized polyspikes, epileptic discharges over the frontal regions and eye closure sensitivity. Brain MRI at age 19 was normal. Motor and speech development were delayed. She could walk independently at age 19 months. She had an IQ score of around 55. Her behavior is problematic with hyperactivity and tantrums. Additional medical problems consisted of neonatal feeding difficulties with persistent vomiting and obesity. No facial dysmorphisms were observed.

Patient 4 (Danish)
This patient is an 11 years old female with myoclonic atonic epilepsy, with onset of atonic seizures at age 2.5 years. She was born at 38 weeks with a birth weight of 2065 gram (<-2 SD). She had multiple seizure types, including myoclonic seizures, atonic seizures, focal seizures and tonic seizures, refractory to multiple antiepileptic drugs. She currently has daily atonic and tonic seizures. Her EEG showed generalized spike-wave complexes mixed with focal discharges. Her development was normal prior to seizure onset, except for a slight language delay. She now has a moderate developmental delay. Repetitive behavior and ADHD have been reported.

Patient 5 (Belgian)
This patient is a 36 years old female who presented with myoclonic seizures at age 3 years. She soon developed frequent therapy resistant seizures consisting of myoclonic seizures, tonic clonic seizures, absences and non-convulsive or myoclonic status epilepticus. Seizure frequency often increased during the premenstrual period. EEG showed 2 Hz generalized spike waves, spikes and polyspikes. MRI of the brain was normal, as was ophthalmologic examination, skin biopsy, and evoked potentials. Early motor development was normal and she walked independently at the age of 14.5 months. Her first words were spoken at the age of 3 years. Since then, developmental delay became clearer and she now has a moderate to severe intellectual disability with hyperactive behavior.

Patient 6 (US)
This patient is a 2 years and 8 month old girl of Mexican descent, without any symptoms of epilepsy. Routine EEG was normal. Concerns regarding her development were first noted at 3 months of age, and at 2 years and 8 months of age she was non-verbal and not yet walking. She currently has a moderate developmental delay. She was born full term with a

birth weight of 2600 gram. She experienced poor feeding with gastroesophageal reflux in the neonatal period and has since had poor growth and failure to thrive. Her height is below the 2nd percentile and weight is at the 6th percentile for her age. She has dysmorphic features characterized by microcephaly, hypotonic facies, narrow forehead, anteverted nares, open mouth, large ears, upslanting palpebral fissures, hypertelorism, and overriding second toes bilaterally. Furthermore, she has severe generalized hypotonia. No behavioral problems are reported, especially no autistic features.

Patient 7 (US)

Patient 7 is a 9 year old girl of unrelated Caucasian parents. She showed a normal developmental profile until the age of 2 years when her epilepsy started and her development started slowing. Her seizures were initially absence seizures and myoclonic seizures that have been difficult to control on multiple antiepileptic medications. At the age of 9 years, she mainly has absence seizures with associated head nods, but no myoclonic seizures or atonic seizures. She has an intellectual disability and pervasive developmental disorder with significant behavioral issues, currently at the developmental level of a 5 year old in all developmental domains. She never regressed or lost skills. Her general pediatric exam and neurological exam at the age of 9 years is unremarkable. She does not show dysmorphic features.

Patient 8 (French)

Patient 8 is a 17 year-old girl born to unrelated parents originating from Portugal, Italy and France. Delivery occurred at term and parameters were normal. Development was normal until the age of 15 months when she entered hospital for a febrile illness. Subsequently her developmental milestones started slowing. It is during a psychomotor delay work-up at two years that an abnormal EEG was noticed. She was placed on Valproate that was discontinued when aged 8 years. She walked without assistance at 24 months but did not develop language. Her stereotypic movements and behavioral disorders during infancy suggested an autism spectrum disorder (ASD). Seizures recurred at 11 years with poor response to antiepileptic drugs. She had an episode of status epilepticus at the age of 16 years. She currently has a moderate to severe intellectual disability. Toilet training is not obtained and she expresses herself with grunting. Behavioral problems are reported with fits of hyperactivity. At clinical examination, there is an open mouth, overweight and no dysmorphic features. Her length and OFC are normal.

Patient 9 (Canadian)

This patient is a 6 year-old girl who developed normally until 14 months of age, when atypical absences were noticed as well as tonic seizures that led her to fall while attempting to walk. Subsequently, she had mainly myoclonic jerks and atypical absences until around age 3 when the predominant seizures became atonic seizures of the head and the whole

body with frequent drop attacks. At the peak of her epilepsy she was having a jerk, stare or drop every 3 minutes. Presently, she has 2-10 of the above every 5 hours. She takes multiple AED's and follows a ketogenic diet, which substantially improved the seizures. She also takes cannabis oil, which was also associated with improvement. EEG shows background slowing and generalized and multifocal epileptiform discharges. She has continued to develop though very slowly, without regression. She walks and is potty-trained. She speaks approximately 150 words and is able to make 5-word phrases. Her thinking and speech are regularly interrupted by absence spells. She is hyperactive and has impulse-control difficulties.

Patient 10 (Brazilian)
This 2 year-old female, born to non-consanguineous Brazilian parents, presented with hypotonia and joint laxity in the context of global developmental delay. There are no signs of epilepsy. She walked without assistance at 19 months and her first words were pronounced at 18 months. Currently she speaks around 5 words without elaborating phrases. Brain MRI and EEG were unremarkable

Patient 11 (French)
This 34 year-old female came to medical attention after a diagnosis of *KIAA2022*-related epileptic encephalopathy was made in her two sons with ID, through sequencing of an intellectual disability related-gene panel. She has had absences-type epilepsy starting at age 17 years, that responded, although incompletely, to levetiracetam and lamotrigine. Currently, she describes persistent occasional absence episodes, about once per month. She has no intellectual disability; she has a university degree. She also has insulin-dependent diabetes mellitus and horseshoe kidneys.

Patient 12 (US)
Patient 12 is a 12 years old child of unrelated parents. She was noted to have developmental delays (gross and fine motor skills, decreased eye contact, stereotypies) before age 20 months. Her first seizures were clusters of myoclonic seizures at age 2 years, 3 days after the MMR vaccine. She subsequently developed myoclonic and absence as well as focal and generalized clonic seizures. She has intellectual disability and developmental delay. She has had developmental regression in the setting of a marked increase in seizure activity. EEG shows mixed generalized (polyspike-and-slow wave) as well as focal discharges. Her seizures have remained refractory to more than ten antiepipleptic drugs, the ketogenic diet, and vagus nerve stimulation. Her currently behavioral function is at a 4 year age level in all domains. Her general physical examination is normal. Apart from her cognitive and behavioral deficits, her neurological exam is remarkable for impaired fine and gross motor and coordination skills. There are no focal neurological findings.

Patient 13 (UK)

This lady died at the age of 51 years. Following a normal pregnancy, she was born at term by breech delivery and was thought to have suffered a degree of hypoxic brain injury. Her development was delayed: she walked at the age of 2, her language development was markedly impaired and she was never able to speak in sentences. Her seizure onset was around the age of 7 years, with myoclonic seizures that responded well to treatment with ethosuximide and phenytoin for about 16 years. At the age of 23, her seizure control deteriorated with increased frequency of myoclonic seizures, often occurring in clusters with onset of atonic and generalized tonic-clonic seizures. Her seizures became soon drug-resistant and she experienced status epilepticus aged 38 years. She had severe intellectual disability and, from the age of 40, was confined to a wheelchair because of concerns about falls. She had facial dysmorphism, with small hands and feet. Her latest brain MRI scan, aged 37, was unremarkable. Her latest EEG video-telemetry recording, aged 50, showed frequent subtle seizures, of the order of seventy per day, mostly myoclonic (associated on EEG with an increase in frontal fast activity, sometimes followed by rhythmic slow activity) and atonic seizures (associated with bifrontal polyspike evolving into diffuse rhythmic slow activity). The most likely syndromic classification was myoclonic-astatic epilepsy.

Patient 14 (French)

The patient is an 8years old girl born from unrelated parents of French and Portuguese origins. All developmental milestones were acquired with moderate delay: she walked steadily around age 18 months, acquired fine motor skills after 3 years and presented with a marked language delay. The first words appeared at 3 years of age, and simple sentences are possible since age 7 years. She attends school in a specialized structure and has moderate intellectual deficiency. She has some autonomy in daily life. She does not have dysmorphic features, and her physical examination is normal, except for hyperlaxity. Epilepsy started at the age of three years, by the association of atypical absences, with drooling sometimes followed by atonic fall of the head. Myoclonic and astatic falls were also described. The epilepsy was resistant to more than 10 antiepileptic drugs, to steroids and to a ketogenic diet. Currently, the child still suffers from weekly seizures and some rare tonic seizures during sleep. Electroencephalograms (EEG) showed slowing of occipital basal rhythm (6-7 Hz), and nearly continuous generalized spike-waves and poly-spike-wave complexes. This pattern is asymptomatic or may be concomitant with eye-lid clonia and/or atonic head falls. Lately, tonic seizures were recorded. Some physiologic sleep patterns appeared in the last EEG (at age 8). MRI shows a mild frontal atrophy.

Chapter 10

General discussion

The main aim of the research presented in this thesis was to investigate whether an early screening of *SCN1A* in children with febrile seizures would benefit children who turn out to have Dravet syndrome. For this, two questions have to be answered: can we accurately predict the clinical consequences of a detected genetic variant, and does an early diagnosis improve the overall outcome and counseling of patients with *SCN1A*-related epilepsy? This chapter discusses the main findings of the presented studies, their implications for patients and clinicians, and the challenges that remain.

10.1 Can we reliably predict the associated phenotype of a detected *SCN1A* variant?

10.1.1 Determining the pathogenicity of an *SCN1A* variant

Understanding the meaning of *SCN1A* test results is essential to accurately counsel parents: results should not cause unnecessary worry, and false hopes should be avoided as well. Once an *SCN1A* variant is detected, the first question should be whether it has a clinically relevant negative effect on Nav1.1 function, i.e. whether it is a pathogenic or a neutral variant. Approximately 80% of detected pathogenic variants is unique, which means that literature supporting the pathogenicity of a specific variant is often not available.[117] Furthermore, even when a variant has previously been classified as pathogenic, laboratory specialists and clinicians have to remain cautious; a study that re-evaluated the pathogenicity of *SCN1A* variants has shown that several variants may have been misclassified according to current insights.[323]

Classification is straightforward in the case of a frameshift- or a nonsense mutation. However, interpreting a missense variant can be challenging. When an *SCN1A* missense variant is detected in a patient with febrile seizures it might be tempting to assume it is causal, but this may not always be true: 351 different non-synonymous exonic *SCN1A* variants have been described in the ExAC database (Figure 10.1).[156] The ExAC database includes exome data from 60,706 unrelated healthy participants and can be considered representative of the general population. Absence or a very low frequency in such databases is a strong argument for the pathogenicity of a variant, as common variants are unlikely to have damaging effects. However, although participants affected by severe pediatric disease were excluded from the ExAC database, it likely includes a number of participants with for example mild febrile seizures. This means that some variants in the database may actually be pathogenic. Other helpful instruments are *in silico* prediction tools, such as SIFT, Polyphen-2, MutationAssessor and FATHMM.[324–327] A relatively new tool that was used in **Chapter 6** is the CADD-score, which integrates many diverse annotations into a single, quantitative score.[264] However, different prediction tools may provide contradicting results, and do not provide sufficient evidence for pathogenicity by themselves.[323,328] Segregation studies of a variant in a family may provide additional information. They may however be

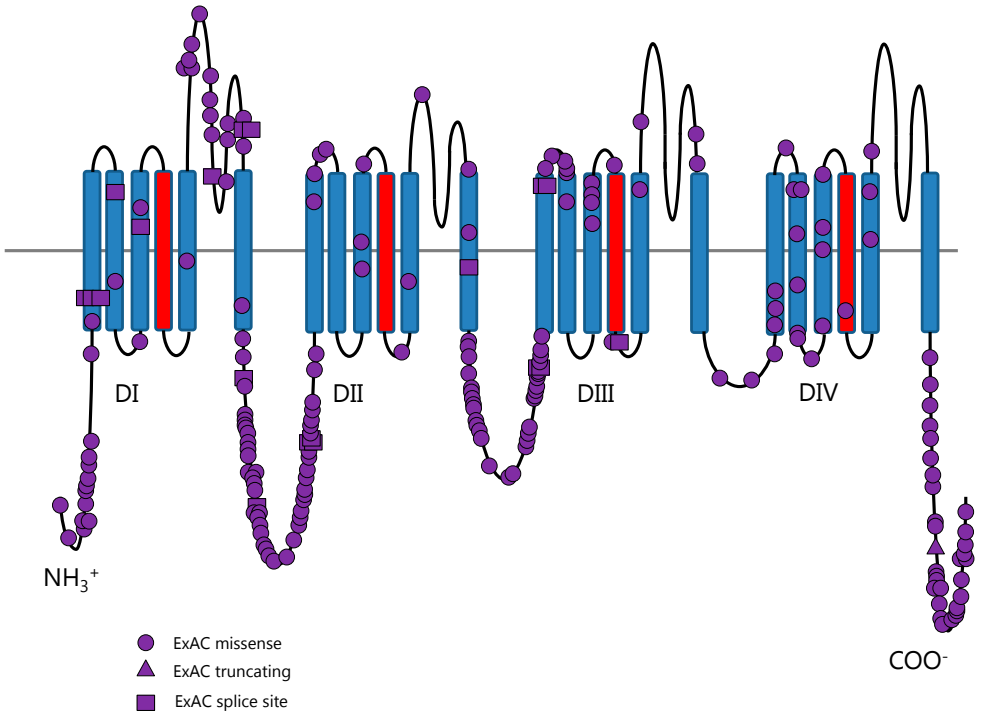

Figure 10.1. *SCN1A* variants described in the ExAC database.

inconclusive in several scenarios. Firstly, mosaicism of a variant may influence the results. For example, in **Chapter 5**, we describe two fathers, who were found to carry the same pathogenic variant as their severely affected sons but showed no symptoms of epilepsy themselves. They were unaffected due to being mosaic for the shared pathogenic variants. However, if mosaicism remains undetected, such a variant may be dismissed as causal. Deep sequencing, to investigate mosaicism, may therefore provide essential information for variant classification. Secondly, a variant detected in an asymptomatic parent may still be a non-fully penetrant risk factor; its negative effects on Nav1.1 functioning may only become clinically relevant in the presence of additional genetic variants or environmental factors that are present in the proband but not in his or her parents.[329] Thirdly, some variants are only disease-causing when both *SCN1A* alleles are affected. This is illustrated by two pairs of siblings with consanguineous parents were reported to have epilepsy caused by homozygous *SCN1A* variants, with asymptomatic carrier parents.[330]. These recessive cases are however extremely rare; only these two families have been described. Collaborations between diagnostic laboratories, to share all detected *SCN1A* variants that have been classified using standardized criteria and the associated phenotypes, may contribute to the molecular diagnoses of new patients. Several *SCN1A* databases are already available, such as the database described by Meng et al. (http://www.caae.org.cn/gzneurosci/*SCN1A*database/

index.php),[117] the Antwerp Variation Database of *SCN1A* (http://www.molgen.vib-ua.be/*SCN1A*mutations/Mutations/Default.cfm) and the *SCN1A* section of the Leiden Open Variation Database 3.0 (https://databases.lovd.nl/shared/genes/*SCN1A*), although not all of these are updated regularly and phenotypes of patients are not always described. A similar approach is essential in rarer syndromes as well, such as *KIAA2022*-related epilepsy, which was described in **Chapter 9.** Since *KIAA2022* variants were first reported to be associated with X-linked mental retardation and only a few female cases were known, many *KIAA2022* variants in female patients have likely been dismissed so far. Sharing data will hopefully lead to a more complete comprehension of associated phenotypes and more correctly diagnoses.

10.1.2 Determining the clinical effects of a pathogenic *SCN1A* variant

Once it is established that a variant is (likely) pathogenic, its clinical effects may still not be obvious. Especially when a child is diagnosed at a young age it is not always possible to predict whether he or she will develop Dravet syndrome or a milder phenotype, such as GEFS+ of FS, as these diseases can have similar symptoms at onset.[17] This distinction is obviously of extreme importance to parents, as it will greatly impact their future perspectives. The most important differences between the syndromes are the presence or absence of intellectual disability (ID) and the frequency and severity of epileptic seizures, which both significantly influence the level of care an affected child requires. Furthermore, we have shown in **Chapter 2** that Dravet syndrome patients are often affected by walking disabilities and severe behavioral problems, and suffer from a lower health-related quality of life, whereas these comorbidities are rarely or never seen in GEFS+ patients.

Virtually all truncating variants are expected to cause Dravet syndrome,[117] although disease severity may vary between patients (see **section 1.5.5**). In the cohort described in this thesis we indeed found truncating variants to be much more common in Dravet syndrome patients (in 52% of patients, in contrast to in 4% of non-Dravet syndrome patients; **Chapter 3**). The effects of missense variants are harder to predict, as these can be associated with both mild and severe phenotypes. The location of a missense variant in the gene is a strong predictor for the corresponding syndrome: patients with severe phenotypes more often have missense variants in important functional regions of Nav1.1, such as the pore region, voltage sensor and inactivation gate.[117,118,129,130,157,208] In our cohort however, the localization of missense variants did not clearly differ between patients with Dravet syndrome and patients with milder phenotypes, possibly due to the small number of non-Dravet syndrome patients (Figure 10.2, **Chapter 3**). Similarly, larger changes in amino acid polarity may be observed in Dravet syndrome patients,[130,157] but these cannot fully predict phenotypes in single patients either. Although these genotype-phenotype associations are strong when assessed in large cohorts, exceptions are common and a completely reliable syndrome prediction based on variant characteristics is not possible in individual patients.

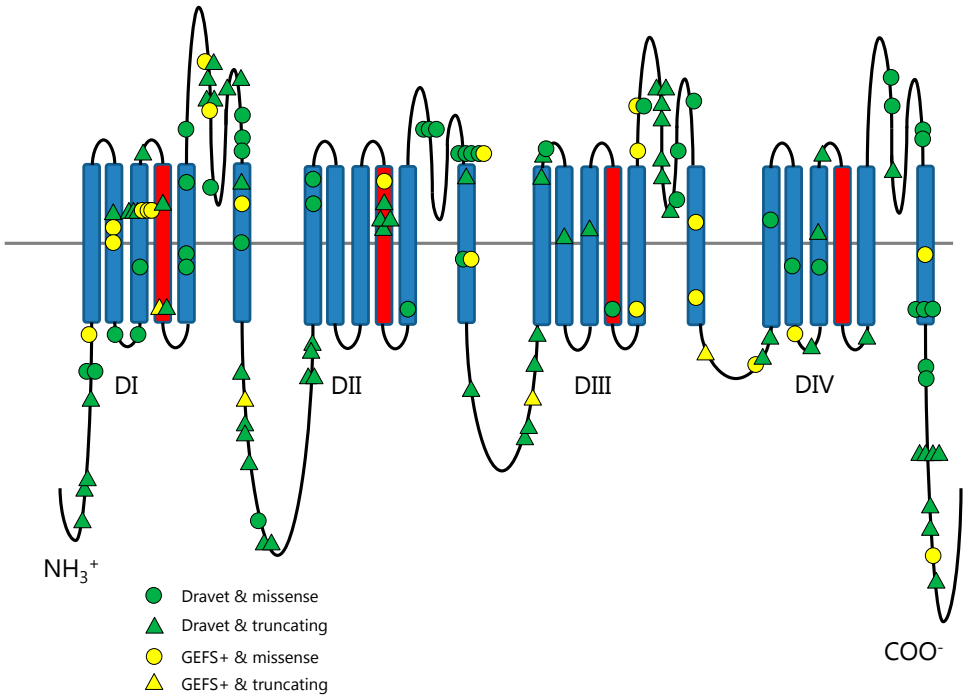

Figure 10.2. *SCN1A* mutations in described cohort. Only one mutation shown when present in multiple family members. Splice site mutations are annotated as truncating. Large structural variants are not shown.

Often, the clinical course of the disease will have to guide clinicians in diagnosing a patient.[36] Previous research has suggested that Dravet syndrome theoretically can be diagnosed definitively at the end of the second year of life, since most disease characteristics have manifested themselves by then.[16] However, no studies have investigated the actual average age at (correct) syndrome diagnosis in a cohort of *SCN1A*-positive patients, in which genetic testing was performed at an early age. Several clinical predictors have been suggested to be informative on clinical outcomes: Dravet syndrome patients have been shown to have an earlier onset of seizures, and none of the patients with a seizure onset of >12 months developed Dravet syndrome in previous studies.[17,227] Furthermore, Dravet syndrome patients more often experienced focal seizures and seizures lasting >5 minutes. Additionally, in their first year of life, they had a higher seizure frequency, more hot water induced seizures and more frequently experienced hemiconvulsions and myoclonic seizures.[227] In **Chapter 3**, we identified age at a first afebrile seizure as an important additional predictor. Although both Dravet syndrome patients and non-Dravet syndrome patients may experience afebrile seizures, their first occurrence is much earlier in Dravet syndrome patients. We have furthermore shown that more severely affected Dravet syndrome patients tend to experience a first afebrile seizure at an earlier age than relatively mildly affected Dravet syndrome patients, which makes the first afebrile seizure a very informative

parameter that can be of great use during counseling. As we have shown in **Chapter 2**, severe behavioral problems and walking disabilities are common in Dravet syndrome (71% and 43%, respectively) and almost never occur in non-Dravet syndrome patients. Once these comorbidities are observed in a patient with *SCN1A*-related epilepsy, the likelihood of Dravet syndrome increases significantly (positive predictive values: walking difficulties 100%; behavioral problems 92%), indicating that they should be included as diagnostic criteria for patients in which an *SCN1A* pathogenic variant has been detected.

Although almost all adult patients can easily be classified as having Dravet syndrome or not, a small minority of patients may show a phenotype on the border of Dravet syndrome and GEFS+ (a handful of cases in our cohort). This raises the question to what extent Dravet syndrome and GEFS+ are part of a continuous spectrum of disease severity, as has been previously suggested.[59,101,104,208] If there is a truly sliding scale in severity, an equal distribution of outcome scores ranging from mild to severe would be expected; if Dravet syndrome and the non-Dravet phenotypes are distinct disorders, then a dichotomous distribution is presumed. In **Chapter 2**, we have analyzed multiple outcomes of Dravet syndrome patients together with patients showing milder *SCN1A*-related phenotypes. Our results showed that the large majority of outcomes was distributed at the different ends of the spectrum, meaning that in general a clear distinction between the syndromes can be made.

10.1.3 Advanced genotyping

As previously discussed, merely the characteristics of a pathogenic *SCN1A* variant are not enough to confidently predict phenotypes. No clinical or genetic predictor is completely accurate, and patients with unexpected phenotypes are common. Furthermore, even when a distinction between Dravet syndrome and a non-Dravet *SCN1A* phenotype can be made, there are large differences in disease severity between Dravet syndrome patients themselves. It is overly clear that *SCN1A*-related phenotypes are influenced by genetic modifiers (see **section 1.4.5**). However, investigating these modifiers can be challenging for several reasons. It is likely that multiple factors simultaneously exert modulating effects, as different potential modifiers have already been identified.[24,26,33,37,42–45,166,171,197–206] These different factors comprise both ameliorating and deteriorating factors that may have opposite effects in single patients, which can hamper their identification[331]. Furthermore, knowing the primary effect of a specific *SCN1A* variant is essential to be able to observe modulation of it, which remains difficult in the case of *de novo* missense variants. This is illustrated by the families described in **Chapter 6**, in which family members have different disease severities, but the same *SCN1A* missense variant. This individual variant may cause a mild phenotype, in which case the more severe phenotypes are influenced by negative modifiers; conversely, their variant could cause a severe phenotype, which means that in the milder patients compensating factors play a role. Consequently, only patients with mutations of which the primary effects can reliably be predicted (for example only truncating variants)

may be included in studies to identify modifying factors,[205] which excludes a significant number of cases. In addition, to observe the strongest effects, only patients that are most likely to have modified phenotypes should be included.[258] These are patients that exhibit the most extreme and unexpected phenotypes, which by definition represent a small percentage (~1%) of cases. These requirements combined lead to small sample sizes and low detection power, whereas large sample sizes are required to demonstrate the effects of single modifiers, especially those with small effects.[332] As Dravet syndrome is a rare disease, international collaborations are imperative in order to establish large cohorts of patients in which modifying factors can be studied accurately. Nonetheless, considering these concerns, we have investigated three potential genetic modifiers to assess whether advanced genotyping could improve the prediction of *SCN1A*-related phenotypes.

Mosaicism

In **Chapter 4**, we have demonstrated that mosaicism for a pathogenic *SCN1A* variant can have a major ameliorating effect on phenotypes of affected patients. These results were not unexpected, as similar results have been described for other genetic disorders.[164,165,246] Mosaicism may well be the strongest modifier of *SCN1A*-related epilepsies. In addition to causing milder forms of Dravet syndrome, it can alter Dravet syndrome into GEFS+ syndrome, or even fully eliminate any symptoms that a variant could have caused had it been in a heterozygous state, as was demonstrated by several families in our cohort. We and others have shown that symptoms of Dravet syndrome or GEFS+ generally arise when the variant allele fraction is at least 0.125 to 0.25.[117,241] However, patients with very low-grade mosaicism may still have a higher risk for mild seizure phenotypes than the general population (**Chapter 5**).[176] It is likely that the percentage of mosaicism positively correlates with disease severity as well above 25%, although no such trend was observed in our cohort. However, our sample size was small and the percentages of mosaicism in blood do not necessarily correspond to those in brain tissue. Additional research in larger cohorts is necessary to confirm this hypothesis and strengthen our detected associations.

In addition to showing that mosaicism can significantly modify clinical outcomes, another main finding was that both high-grade (**Chapter 4**) and low-grade (**Chapter 5**) mosaicism for pathogenic *SCN1A* variants is common. We found mosaicism in 12% of investigated families, in either the proband or in one of the parents. This percentage is likely to be an underestimation, since no reliable results could be obtained for several participants due to low coverages. Furthermore, ultra-low-grade mosaicism can never be completely excluded, especially in the case of gonadal mosaicism. However, we have shown that the use of smMIPs in combination with high coverage NGS performs significantly better than regular diagnostic procedures in detecting both high- and low-grade mosaicism. Its costs are low, and implementing it in standard diagnostics will improve counseling for a significant number of families (12%), either by detecting mosaicism in parents (higher recurrence risks) or in a proband (recurrence risk of virtually zero, and a likely milder disease in the

proband). As it is likely that mosaicism is not only a major modifier for *SCN1A*-related phenotypes, our results have implications for other disorders as well. Similar studies could be performed in patients with other genetic syndromes, especially in those for which variable phenotypes are common. If comparable results are observed, smMIP-analyses can be implemented for a range of different genes.

Surprisingly, mosaicism does not guarantee a favorable disease course: we observed three patients with severe outcomes, despite being mosaics (**Chapter 4**). To our knowledge, mosaicism in severely affected patients has never been described before. This may however be due to a selection bias; since mosaicism is not expected in severely affected patients, it might not specifically be investigated. Since we systematically included patients with *SCN1A*-related phenotypes, without a bias for milder phenotypes, we were able to also identify these patients with unexpected results. These patients may have a discrepancy in levels of mosaicism between blood and brain tissue, which could explain our results. It is also possible that other factors modulate the phenotypes of severely affected mosaic patients. Investigating modifiers in this (small) group of patients could be worthwhile. Conversely, not all mild phenotypes could be explained by mosaicism, indicating that this group is subject to other disease modifiers as well.

Mosaicism in general may even have negative effects, as is illustrated by the patients described in **Chapter 8**. X-linked *PCDH19*-related epilepsy, which may resemble *SCN1A*-related Dravet syndrome, shows a counterintuitive inheritance pattern: males with hemizygous pathogenic variants are unaffected, while mosaic males do show symptoms. In these cases, the presence of two different cell populations is detrimental. Instead of ameliorating phenotypes, cell-cell interactions are disrupted by the simultaneous presence of affected and unaffected cells.[146] A similar disease mechanism affects female *PCDH19* patients. Female patients have two different cell populations in practice as well, due to random inactivation of one of their X-chromosomes (either with or without the pathogenic variant) in each cell,[179,180] and can therefore be considered mosaics too. This is true for all X-linked genetic diseases in women. The female patients affected by X-linked *KIAA2022*-related epilepsy, described in **Chapter 9**, are possibly affected by both positive and negative effects of X-inactivation-related mosaicism. Overall, female patients showed a milder phenotype than male patients, which could be explained by their partial loss of *KIAA2022* compared to a complete loss of *KIAA2022* in males.[314,316,317,333] However, female patients more often experienced epileptic seizures than male patients. We hypothesize that a mechanism similar to cellular interference in *PCDH19*-related epilepsy may affect seizure susceptibility in these patients. This is not unlikely, since *KIAA2022* is possibly involved in the same processes of cell-cell and cell-matrix adhesion and neuronal migration as *PCHD19*.[305] Additional studies in larger cohorts of female and male patients are necessary to further define the *KIAA2022* phenotype and to investigate the true incidence of epilepsy in both sexes.

Postzygotic mutations may arise through different mechanisms than those occurring during gametogenesis. Most mechanisms known to cause DNA mutation involve cell replication.[334] *De novo* mutations originating from gametogenesis more often occur on the paternal allele than the maternal allele, since spermatogenesis requires more cell divisions than oogenesis.[335] *De novo* mutation rates also increase with higher parental age.[336] However, if a mutation occurs postzygotically, it is expected that parental age and the parental origin of the mutated allele are irrelevant. For postzygotic mutations, one would therefore expect a) similar mutation rates on the paternal and maternal allele, and b) an on average higher parental age in non-mosaic participants compared to mosaic participants. However, regarding the second statement, no large differences were observed in our *SCN1A*-cohort (fathers of mosaic families: 32.14 years versus fathers of non-mosaic families: 32.97 years; mothers of mosaic families: 29.50 years versus mothers of non-mosaic families: 30.85 years). Studies to investigate whether specific risk factors and mutational patterns are associated with postzygotic mutations could contribute to an increased understanding of the mechanisms underlying the occurrence of such mutations during embryogenesis and aid the identification of patients that are more likely to carry mosaic variants.

Modifier genes

In contrast to our clear hypothesis of the effects of mosaicism, our theories on the effects of modifier genes were less straightforward. Variants in genes associated with neuronal excitability and other known epilepsy genes have been implicated to affect *SCN1A*-related phenotypes.[37,197,206,198–205] However, both rare variants with large effects and combinations of common variants with small effects may influence phenotypes. Furthermore, variants can either deteriorate or ameliorate phenotypes, and different variants in the same gene may even have opposing effects. In Chapter 6, we have explored these different possibilities.

To date, no single modifier gene has been described that significantly influences phenotypes in a substantial number of patients, like mosaicism does. Our study could not identify such a gene either. This could be due to our small sample size, but it is also very likely that such a single gene does not exist, and that many different genes are capable of altering phenotypes. Single variants in such genes may still exert large effects in individual patients; various studies have observed dual genetic diagnoses in 1-7% of patients.[232,337–342] These "second hits" could affect genes in the *SCN1A* pathway: they may worsen the phenotype by a further loss of function of inhibitory neurons (such as GABA-receptor variants[206]) or by a gain of function of excitatory neurons (for example by gain of function variants in SCN8A).[343] Conversely, a loss of function of excitatory neurons, for example by loss of function variants of SCN8A, could balance out the effects of an *SCN1A* variant, leading to amelioration of the phenotype.[198,201] Gene variants in unrelated pathways may also have significant effects, for example by influencing the susceptibility to focal brain injury during prolonged seizures,[37] or by influencing cognitive abilities (second hits in unrelated ID genes). When variants are easy to interpret, such as LoF variants in well-known genes,

their identification may help in counseling patients. The rapid adoption of whole exome sequencing (WES) in regular diagnostics may aid in the prediction of *SCN1A*-related phenotypes. If WES is performed earlier in the diagnostic trajectory, before specific *SCN1A* testing, it may yield additional variants that are easy to interpret and can help predict disease severity. However, in most cases additional variants will likely be of unknown significance, which requires functional testing to support their modulating effects. This may especially be the case for variants in modifier genes that can harbor both LoF variants and gain of function variants, and that can therefore have either ameliorating or deteriorating effects, such as variants in SCN8A.[343] Potentially interesting variants should be shared between researchers, so that trends in the cumulative data may be discovered and groups of patients with similar genotype-phenotype correlations may be assembled.

In addition to investigating rare, pathogenic variants, we have shown that patients on the extreme ends of the phenotypic spectrum carry an excess of slightly more common variants in epilepsy-related genes (118%, p=0.000). These may simultaneously tip the balance over to a milder or more severe phenotype, but a combination of such variants is even harder to interpret in a single patient. Interestingly, we did not observe an excess of variants in ID-related genes, which further implicates variants in epilepsy genes as the main modifying factors. However, epilepsy-related genes and ID genes are likely not the only genes in which modifying variants can reside. Future research should ideally investigate exome-wide variants, to also include genes of which little is known yet. This strategy however requires unrealistically large groups of patients (>1000)[331] for relatively common variants with weak effects to become statistically significant. A strategy to circumvent this would be to first analyze whether exome-wide rare, damaging variants with a large effect size in certain genes are more prevalent in patients with extreme phenotypes, and then afterwards investigate the effects of more common variants in this subset of new candidate genes. Another approach to identify new potential candidate genes could be to first identify modifier genes in other, more common epilepsy syndromes, for which larger sample sizes are available. To maximize sample sizes, not only patients with absolute extreme phenotypes should be included, but also other patients with likely strongly modified (unexpected) phenotypes. Examples include severely affected mosaic patients and family members with phenotypes that clearly differ from their affected relatives that carry the same *SCN1A* variant.

Another important measure is the co-sequencing of healthy individuals from the same population, to enable direct, reliable comparisons between cases and controls that have been sequenced and processed using the exact same methods. Direct comparisons between cases and control databases such as gnomAD, instead of co-sequenced controls, can lead to incorrect results, as differences in sequencing procedures, coverage and variant calling may introduce biases.[265] However, if certain modifying variants only have a phenotypic effect when combined with a pathogenic *SCN1A* mutation, a comparison between mildly and severely affected patients may be more informative than a comparison between affected patients and healthy controls without pathogenic *SCN1A* variants.

Regulatory regions

Many genetic studies focus on variation in the coding regions of genes, as these are crucial in the assembly of proteins. However, many more factors are involved in the correct function of proteins, mainly by regulating gene expression. A decreased expression of a gene may cause disease independently,[344] but could perhaps also modify the phenotypes associated with pathogenic variants in the coding region. Dravet syndrome patients are affected by heterozygous variants that lead to haploinsufficiency; the single unaffected copy of *SCN1A* alone does not produce enough gene product for a normal function. We hypothesized that if expression of the single functional allele is higher, a less severe phenotype would be expected, since more residual Nav1.1 would be present. Conversely, a lower expression of the functional allele could be associated with more severe phenotypes. In **Chapter 7**, we have investigated the role of one of the factors involved in *SCN1A* expression: the 5' promoter-region of *SCN1A*, which is located 75 kb upstream of the first coding exon.[189,190] Two reports have been published of Dravet syndrome patients who were affected by pathogenic variants in the regulatory 5' region of *SCN1A*, while no coding variants could be detected.[191,192] Furthermore, GWAS studies have implicated that common, low-risk variation in *SCN1A* may affect normal function and/or expression of *SCN1A*.[181,182] Based on these observations, we hypothesized that common variation of the *SCN1A* promoter of the unaffected allele could interfere with normal expression, leading to a decreased residual function of Nav1.1, and therefore to more severe clinical outcomes. Although common variants are not expected to have large effects by themselves, since they are present in a large part of the healthy population, they may modulate the effect of pathogenic *SCN1A* variants. We indeed observed a lower *in vitro* expression of *SCN1A* when common promoter variants were present (65%-80%, p=0.039-0.0023), implicating that these variants indeed negatively affect *SCN1A* expression. In patients we observed a weak trend for more severe phenotypes when common promoter-variants were present, although these results were not statistically significant. It is therefore likely that promoter-variants only have a limited role as modifiers. Studies with much larger sample sizes are therefore necessary to reliably determine the true effects of common promoter-variants. In these large patient cohorts, pathogenic variants and promoter-haplotypes will have to be phased (i.e. determine which promoter variants exist on the unaffected *SCN1A* allele). This may be aided by upcoming techniques such as Nanopore sequencing, which can process reads long enough to contain both the promoter region and the pathogenic variant.[345] Furthermore, future studies should not only focus on common variants, like ours did, but also investigate the presence of rarer promotor region variants that may have more substantial effects on phenotypes. Additionally, not only the *SCN1A* promoter-region should be sequenced, but also its 5' untranslated exons, that have been shown to greatly enhance transcription,[189] and its 3' untranslated regions.

With the increasing use of whole genome sequencing, more knowledge will become available on variants in these regions and in other non-coding regulatory elements that are essential for expression, such as enhancers and silencers.[346] Variants in such regions, not

only in those associated with *SCN1A* but perhaps also in those associated with modifier genes, may influence *SCN1A*-related phenotypes.

Another scarcely explored field in *SCN1A*-related epilepsy is epigenetics, which describes changes in gene expression that do not involve changes in the DNA sequence itself, but are due to, for example, DNA methylation, histone modification and non-coding RNA based regulation. Epigenetic changes, which may be induced by modifier genes, repetitive seizures, treatment or other environmental factors may be important in the clinical manifestation of epilepsies in general.[347–350] Although knowledge of epigenetic mechanisms is currently still limited, differences in epigenetic signatures between mildly and severely affected patients might turn out to be useful as prognostic biomarkers in the future.

10.2 Does an early diagnosis improve clinical outcomes?

10.2.1 Clinical determinants of cognitive outcomes

Dravet syndrome has long been regarded as an epileptic encephalopathy, i.e. a syndrome in which epileptiform activities may contribute to a progressive cognitive dysfunction.[24,26,33,42–46] This classification was based on several studies that found an association between seizure severity and cognitive outcomes,[24,33,52,92] and that an improved seizure control has been associated with cognitive improvement.[26,59] A similar correlation has been observed in mice carrying a GEFS+ variant.[45] Furthermore, a developmental regression may occur after repeated status epilepticus.[36] An extreme example of this is acute encephalopathy, in which a status epilepticus is followed by a coma, significant regression and additional neurological sequelae.[37–41] These findings indicate that an optimal seizure control is essential to best preserve cognitive abilities.

However, there is emerging evidence that the widespread Nav1.1 channel dysfunction throughout the brain also contributes to the cognitive disabilities in Dravet directly, instead of merely through seizures.[42] For example, in several cohorts no correlation between seizure severity and ID was observed.[16,47,52,228] Furthermore, selective downregulation of Nav1.1 in the forebrain region of mice caused cognitive impairment without the presence of seizures,[49] and autism-like behavior that could be fully rescued by a low dose of clonazepam.[128] The earlier established associations between seizures and cognition may therefore not represent a causal relationship (cognitive impairment due to seizures), but rather an expression of disease severity by two distinctive symptoms (both cognitive impairment and seizures worsen when a more detrimental Nav1.1 dysfunction is present). Overall, it is most likely that both direct Nav1.1 dysfunction and seizure activity contribute to the cognitive phenotype of Dravet syndrome patients.

In **Chapter 3**, we have investigated the role of sodium channel blockers on cognitive outcomes in Dravet syndrome patients. This group of anti-epileptic drugs is contra-indicated in Dravet syndrome, as they block the already impaired Nav1.1 channel and are reported

to exacerbate seizures.[14,26,47,63,83,92] Nevertheless, sodium channel blockers are prescribed frequently to Dravet syndrome patients, often before their diagnosis is established.[56,76,86,228] We observed a significantly higher probability of a worse cognitive outcome, as well as a more severe cognitive decline in the first five years of the disease, when sodium channel blockers were used for longer periods of time. However, our data did not allow us to answer whether the results were caused by a higher seizure frequency (either by deprivation of indicated treatment or by further impairment of Nav1.1 function), or by a direct negative effect of aggravated Nav1.1 channel dysfunction. Future studies could include both detailed data on seizure severity and on cognitive functioning during periods of sodium channel blocker-use to elucidate this. Furthermore, not only the effects of sodium channel blockers on cognitive outcomes, but also on comorbidities such as behavioral problems and walking disabilities are worth investigating. Nevertheless, we have shown that avoiding sodium channel blockers in children with Dravet syndrome is essential in preserving cognitive abilities.

Although significant negative effects of sodium channel blockers on cognition were observed in our cohort, not all patients responded similarly. Many patients had barely used sodium channel blockers and were still severely impaired, indicating that merely avoiding this group of drugs does not guarantee positive outcomes. Conversely, several patients had used sodium channel blockers for years and were still only mildly affected. (Genetic) modifiers may therefore not only influence disease outcomes directly, but also through modification of anti-epileptic drug responses.[230,351,352] Investigating these effects in Dravet syndrome patients may improve our understanding of their drug resistance and could aid the development of precision medicine treatments.

Virtually no clearly positive effects of treatment with sodium channel blockers in Dravet syndrome have been described in young patients. However, contradicting reports exist for older patients. Although some older patients respond favorably to sodium channel blocker withdrawal,[26] some also experience an increase of seizure frequency when sodium channel blockers are discontinued after long term use.[229] This may be due to the occurrence of secondary lesions or compensatory mechanisms after a long disease course, leading to seizures that do respond to sodium channel blockers.[229] Ideally, a larger cohort of adult Dravet syndrome patients that uses sodium channel blockers at the time of inclusion should be analyzed prospectively, with cognitive assessments before and after withdrawal, to properly investigate whether a medication change in older patients is advisable. An improvement in function or a reduction in cognitive decline may even be worth the downsides of a medication change in patients that have an acceptable seizure control while using sodium channel blockers.

Although the detrimental effects of sodium channel blockers are probably most important in Dravet syndrome patients, they may also occur in patients with milder *SCN1A* phenotypes. Presumably, these patients are prescribed sodium channel blocker less frequently; only 15 of the 48 non-Dravet syndrome patients in our cohort had used sodium

channel blockers at some point. Although in most of these cases no clear effect of the medication was reported by patients, parents or doctors, seven (parents of) participants mention a negative effect on either seizure severity or cognition (unpublished data). A systematic retrospective study in large groups of *SCN1A*-positive GEFS+ patients could offer more insight into the effects of sodium channel blockers in milder cases.

10.2.2 Benefits and disadvantages of early *SCN1A*-testing

A correct diagnosis can end the uncertainty that many parents experience after seizures have been observed in their child. It also prevents the use of further unnecessary diagnostic tests. Additionally, once Dravet syndrome is diagnosed, parents and caretakers can be alert for the first signs of comorbidities that are common in this disease, as described in **Chapter 2**. Furthermore, the discovery of a pathogenic *SCN1A* variant enables providing parents an accurate recurrence risk, especially when both parents and the proband are assessed using smMIPs to detect low-grade mosaicism in parents (**Chapter 5)** or high- grade mosaicism in a proband (**Chapter 4**). Diagnosing a child as early as possible maximizes these benefits.

It has furthermore been suggested that an early diagnosis can positively influence the disease course.[14,25,26,47,59,63,83–85,92] This may be achieved by starting indicated treatment earlier, both for seizures and comorbidities, but perhaps most importantly by avoiding sodium channel blockers. As we have shown in **Chapter 3**, a longer use of sodium channel blockers was associated with more severe cognitive outcomes. We furthermore found the strongest negative effect of sodium channel blockers in the second year of disease, indicating that the young, developing brain might be particularly sensitive to negative influences in that timeframe, during which it could be crucial to avoid sodium channel blockers. The earlier a diagnosis of Dravet syndrome is established, the earlier these (commonly used) drugs can be avoided, and optimal treatment can be initiated. An early diagnosis may therefore indirectly lead to optimal clinical outcomes in these patients. Directly investigating the effects of an early diagnosis on cognitive outcomes is complicated. Diagnostic *SCN1A* testing at an early age has only been available for the younger patients in our cohort; for older patients, the test only became available at a later age. Because of the strong correlations between a higher age and both cognitive deterioration and later *SCN1A* testing, it is difficult to calculate the exact influence of age at diagnosis on cognitive outcomes. Re-investigating this in a younger cohort, in which all patients could have been tested at a young age, may provide more insight in the effects of an early diagnosis on cognition and epilepsy severity. It would furthermore be worthwhile to investigate the effects of an early diagnosis on comorbidities such as behavioral problems, as these have been shown to have important negative effects on the patients' quality of life.

Despite the clear advantages of an early diagnosis, *SCN1A* testing may also have negative consequences. As discussed before, it is still not always possible to accurately predict the effects an identified *SCN1A* variant. Parents might be told that their child could have Dravet syndrome, whereas he or she may actually develop a mild *SCN1A* phenotype. This

may cause unnecessary anxiety and fear. The research described in this thesis can help improve counseling, for example by investigating possible mosaicism (**Chapter 4**), but as discussed, a completely accurate phenotypic prediction cannot be made yet.

Weighing the advantages and disadvantages of early *SCN1A* testing, it may be at least advisable to exclude the presence of a pathogenic *SCN1A* variant before maintenance treatment is started, to avoid a lengthy use of sodium channel blockers and limiting their negative effects. The benefits of this may outweigh any possible disadvantages parents may experience from the prognostic uncertainty associated with the discovery of an *SCN1A* variant at an early age. Furthermore, if sodium channel blockers are shown to have negative effects on the disease course of GEFS+ patients as well, it might also be beneficial to detect milder *SCN1A* variants at an early age. Prediction of the effects of *SCN1A* variants and disease modifiers will likely become more accurate in the future, in which case the main arguments against an even earlier screening (for example in every child with more than one febrile seizure) may become inapplicable.

10.2.3 Future perspectives regarding treatment

Currently, medication changes that clinicians can make, based on a newly established diagnosis of Dravet syndrome, mostly encompass the withdrawal of sodium channel blockers and prescribing indicated anti-epileptic drugs (AEDs). Although such changes likely ameliorate clinical outcomes, seizures in Dravet syndrome often remain therapy resistant. These perspectives may however change in the future. Several AEDs in development, such as cannabidiol and fenfluramin, show promising results.[78–81] Furthermore, an atypical sodium channel blocker (GS967), has been described that may restore the excitatory/inhibitory balance in the brain by reducing Nav1.6 expression.[353] However, all these agents are used to treat symptoms of *SCN1A*-related disease, and do not resolve the underlying defect. Although gene therapy is still in its infancy, it offers exciting outlooks.[354] For example, ataluren is an agent that enables ribosomal read-through of premature stop codons, diminishing the effects of nonsense mutations. Trials in patients with, cystic fibrosis and Duchenne muscular dystrophy have been performed, in which minor positive effects were observed for subgroups of patients.[355–357] A phase 2-trial is currently being performed among Dravet syndrome patients with nonsense mutations (https://clinicaltrials.gov/ct2/show/NCT0.758626). Other strategies could be to increase expression of the unaffected *SCN1A* allele by manipulating involved micro-RNAs,[358,359] to upregulate the activity of already existing unaffected Nav1.1 channels,[360] to deliver a healthy copy of the *SCN1A* gene to affected neurons using modified virus vectors (a mouse model study is currently in progress), or to eliminate the genetic defect altogether using CRISPR-Cas9 technology.[354] Although these developments are promising, many challenges will have to be overcome before Dravet syndrome patients will be able to benefit from these potential treatments. Nevertheless, when an inevitable breakthrough happens, an early diagnosis will be more important than ever: patients will have to be treated before repetitive seizure cause irreversible damage.[354]

Accurate predictions of phenotypes will become less important when more successful therapies become available.

10.3 Implications from a patient's perspective

This thesis only exists because 176 (parents of) patients affected by *SCN1A*-related epilepsy were willing to volunteer their valuable time to answer the many questions we had about the course of their disease. In turn, we hope to answer some of the questions that parents often have after realizing that their child is affected by a serious disease.

What is wrong with my child and what can we expect from the future?
Having a diagnosis and knowing the cause of their child's symptoms is obviously very important to almost all parents. However, even when a pathogenic *SCN1A* variant is found many questions about their future perspectives remain, for which clinicians do not have satisfying answers. The results presented in this thesis show that testing for mosaicism can lead to a better estimation of future disease severity for at least 7.5% patients **(Chapter 4)**. Patient 1, described in **Chapter 1**, turned out to be mosaic for her pathogenic *SCN1A* variant, which explained her relatively mild phenotype. When she was first diagnosed, her parents were told that she likely would have severe, untreatable epilepsy, due to her frameshift variant. Instead, she now only has 2 seizures per year and has previously been seizure free for over a year. If mosaicism was assessed earlier in her disease course, a more favorable outlook could have been given to parents. Hearing back from us that their child was mosaic for their pathogenic *SCN1A* variant was appreciated by all parents. Even though a prediction of phenotypes was not necessary anymore at their age, an explanation for their milder phenotypes was very valuable to most of them.

The assessment of mosaicism is also crucial for male patients with pathogenic *PCDH19* variants. When mosaicism is present, a diagnosis may be established; if not, it is unlikely that this variant is the cause of disease (**Chapter 8**). SmMIPs, as used in **Chapter 4** and **5**, could be implemented for *PCDH19* as well, which may lead to a higher number of correct diagnoses. Due to our research, parents of these boys can be told that their future perspectives are likely similar to those of girls with *PCDH19* mutations, of which much more was already known.

Family two, described in **Chapter 1**, provides an interesting example of variable phenotypes associated with the same pathogenic *SCN1A* variant. The proband's grandmother wondered how it was possible that she differed so much from her son and grandson. We discovered that she was not mosaic for the pathogenic *SCN1A* variant, so other modifying factors are likely to have an effect. Unfortunately, we do not yet have a definitive answer for her. The research presented in **Chapter 6** and **7** may however guide future research to progress towards clinically meaningful testing of modifier gene and promoter variants in

regular diagnostics, so that we can hopefully improve the counseling of families like this.

Even without a complete understanding of genotype-phenotype relations, our research presented in **Chapter 3** shows that clinical variables, such as the onset of afebrile seizures, can also help to predict phenotypes. For example, a child may experience afebrile seizures in the first two years of life, in which case parents may be told that it is very likely that he or she will develop Dravet syndrome (positive predictive value: 96%), even when no developmental delay is obvious yet. Alternatively, parents may be informed that the longer a child only experiences febrile seizures, the less they have to worry about a severe phenotype developing, with a negative predictive value of 80% at the age of two years. Furthermore, in **Chapter 2** we demonstrate strong relationships between different outcome measurements, on which parents may also rely; if a child is doing well cognitively, it is unlikely that he or she will, for example, develop severe walking disabilities. Alternatively, parents with severely affected children may be extra alert for these comorbidities early in the disease course. When a milder *SCN1A* diagnosis is established, our findings in **Chapter 2** can give parents an idea about the general disease course, including at which age seizure and/or medication freedom on average can be established, and the chances of developing subtle learning and behavioral problems.

Although most of the research described in this thesis focuses on *SCN1A*-related epilepsy or Dravet-like phenotypes, the results presented in **Chapter 9** have important implications for female patients affected by *KIAA2022*-related epilepsy. By describing the first cohort of female patients with this disease, we have made clinicians aware that this diagnosis can also be established in female patients, and not just in males. This has already shown to aid the diagnosis of many new cases. Since the publication of this chapter, many clinicians and even patients themselves have informed us about additional diagnoses. When more patients are diagnosed, more knowledge will become available on the clinical picture of *KIAA2022*-related epilepsy, which assists the counseling of patients. We have furthermore been able to establish contact between several patients, and even an active Facebook parent support group now exists.

What can we do?

Obviously, parents want the best possible treatment for their child. Patient 3, who was introduced in **Chapter 1**, had used sodium channel blockers for almost four years at a very young age, which probably negatively affected his severe disease course. Had he been diagnosed sooner, sodium channel blockers could have been avoided. Our results may lead to guidelines in which *SCN1A* testing is advised before maintenance treatment is started, to avoid an increase seizure severity and optimally preserve cognitive functioning in as many patients as possible.

What are the chances of this happening again?

When a genetic disease is diagnosed, parents may worry about the risk of having another child that may be affected by the same syndrome. Our research in **Chapter 4** and **5** shows that by assessing both the affected child and both parents for high- and low-grade mosaicism, recurrence risks can be more accurately estimated. Patient 1 was shown to be high-grade mosaic for her pathogenic variant. This means it has arisen *de novo* during embryonic development, and that her parents therefore do not carry the variant in their germline. This reduces their recurrence risk to virtually zero, and they will not have to undergo invasive prenatal testing to be sure of an unaffected child in a following pregnancy. The detection of mosaicism has additional implications for mildly affected patients: if they are only mildly affected because they are mosaic for a pathogenic variant, their children could be more severely affected when they inherit the same variant in a heterozygous state, which is important knowledge. Parents in which low-grade mosaicism has been detected may also make different choices regarding family planning, since they have an increased recurrence risk. When informing parents with low-grade mosaicism of our results, their reactions differed. One parent was relieved to carry the mutation, because it meant he could ensure his wife that she was not to blame for the disease of her son. Another parent expressed guilt, because she felt responsible for having transmitted the mutation to her son.

Overall, the comment we most heard from patients was that although our results did not have any direct implications for themselves anymore, it was very fulfilling to be able to help future generations with the struggles they had endured in the past.

10.4 Main conclusions

The main question of this thesis was whether early genetic screening of *SCN1A* would benefit children with febrile seizures. A first requirement for this is that we need to be able to interpret the clinical consequences of a detected *SCN1A* variant. Accurately predicting the effects of such a variant remains a challenge, even when using advanced genotyping. We have shown that many modifying factors have a role in determining disease severity, which suggests that Dravet syndrome may not merely be a simple monogenic disease, but could rather be seen as a multifactorial disorder. Although we currently cannot identify all of these factors in individual patients, the counseling of affected patients could be improved by implementing deep sequencing to detect mosaicism in regular diagnostics, both for phenotype prediction and recurrence risk estimations. Future research will hopefully lead to similarly informative essays for the detection of variants in modifier genes and regulatory regions, to optimize the accuracy of future perspectives that are presented to parents. In addition to counseling based on genotype-phenotype correlations, clinical predictors remain very informative.

A second requirement for early *SCN1A* screening is that an earlier diagnosis improves clinical outcomes in these patients, which we have shown to be true. *SCN1A* testing is probably most beneficial when performed before the prescription of maintenance treatment, to avoid the lengthy use of contra-indicated sodium channel blockers and to optimally preserve cognitive abilities. When better treatment options become available, a younger age at diagnosis will be even more important. A genetics first-approach, in which extensive genetic testing will be performed early in the disease course, will likely prove to be most advantageous for many patients in the future, including those with other forms of genetic epilepsy.

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Nederlandse samenvatting
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Dravetsyndroom en* SCN1A-*mutaties

Het Dravetsyndroom is een van de bekendste genetische epilepsiesyndromen. Het belangrijkste kenmerk van de aandoening is het optreden van moeilijk behandelbare epileptische aanvallen vóór de leeftijd van 1 jaar, bij een voorheen gezond kind. Daarnaast treedt er bij alle kinderen met deze aandoening een vertraging van de psychomotore ontwikkeling op, vaak vanaf het tweede levensjaar. Dit resulteert uiteindelijk in een milde tot ernstige verstandelijke beperking bij het grootste deel van de patiënten. Veel patiënten ontwikkelen ook ernstige gedrags- en loopproblemen.

Bij het grote merendeel van de patiënten met het Dravetsyndroom wordt de aandoening veroorzaakt door een DNA-afwijking (mutatie) in één van beide kopieën van het *SCN1A*-gen. Het *SCN1A*-gen bevat de genetische code voor een eiwit dat onderdeel is van een natriumkanaal, Nav1.1, dat zich in de zenuwcellen bevindt. Dit heeft een belangrijke rol bij de prikkeloverdracht (communicatie) tussen de hersencellen. Ernstige mutaties in het *SCN1A*-gen zorgen ervoor dat het natriumkanaal te weinig, of niet (goed) gevormd wordt, waardoor het Dravetsyndroom kan ontstaan.

Mutaties in het *SCN1A*-gen kunnen naast het Dravetsyndroom ook mildere aandoeningen veroorzaken, zoals genetische epilepsie met koortsstuipen (febrile seizures) plus (GEFS+), koortsstuipen plus (FS+) en koortsstuipen (FS), waarbij de epilepsie minder ernstig verloopt en patiënten geen verstandelijke beperking ontwikkelen. Het feit dat mutaties in het *SCN1A*-gen tot meerdere aandoeningen kunnen leiden, wordt deels verklaard door het wisselende effect dat verschillende typen mutaties hebben: patiënten met het Dravetsyndroom hebben meestal een mutatie die tot een ernstigere verstoring van het *SCN1A*-gen leiden dan patiënten met mildere ziektebeelden. Toch zijn er tussen patiënten met *SCN1A*-gerelateerde epilepsie ook verschillen die nog niet volledig verklaard kunnen worden op basis van alleen het type mutatie: er kunnen grote verschillen in het ziekteverloop zijn tussen patiënten met vergelijkbare mutaties. Zelfs exact dezelfde *SCN1A*-mutaties leiden niet altijd tot eenzelfde ziektebeeld. Als een *SCN1A*-mutatie wordt vastgesteld bij een jonge patiënt met koortsstuipen of epilepsie is het voor zijn of haar ouders uiteraard van groot belang om te weten hoe ernstig de aandoening zal verlopen. Het is daarom belangrijk om het effect van *SCN1A*-mutaties beter te kunnen voorspellen dan op dit moment mogelijk is.

Het doel van dit proefschrift was om verschillende potentiële modificerende factoren (zogeheten *modifiers)* te onderzoeken, die de ernst van *SCN1A*-gerelateerde epilepsiesyndromen kunnen beïnvloeden. Dit kunnen bijvoorbeeld varianten in het DNA zijn, maar ook het gebruik van verschillende medicijnen. Als bekend is welke factoren effect

hebben op het ziekteverloop kunnen deze worden geëvalueerd bij toekomstige patiënten. Hierdoor kan de zorg aan patiënten en ouders worden verbeterd, vooral als er *modifiers* worden geïdentificeerd die beïnvloed kunnen worden om de aandoening minder ernstig te laten verlopen. Als de consequenties van een *SCN1A*-mutatie nauwkeurig kunnen worden voorspeld, én als een vroege diagnose ertoe leidt dat de aandoening minder ernstig verloopt, dan is screening van *SCN1A* bij jonge kinderen met koortsstuipen mogelijk aan te bevelen. In dit proefschrift worden de bovenstaande vraagstukken onderzocht in een cohort van 176 patiënten met een *SCN1A*-mutatie. De resultaten hiervan worden beschreven in **Deel 1** en **2** van dit proefschrift. In **Deel 3** van dit proefschrift worden twee epilepsiesyndromen met een andere genetische achtergrond beschreven.

Klinische kenmerken van patiënten met SCN1A-gerelateerde epilepsie

Deel 1 van dit proefschrift richt zich op de klinische kenmerken van de 176 deelnemers aan deze studie. In **Hoofdstuk 2** analyseren we de verschillende klinische uitkomsten van patiënten met Dravetsyndroom en patiënten met mildere *SCN1A*-gerleateerde aandoeningen. We laten zien dat patiënten met het Dravetsyndroom vaak al op jonge leeftijd loop- en gedragsproblemen ervaren. Tevens is er in deze groep een lage gezondheid-gerelateerde kwaliteit van leven. Deze zogeheten comorbiditeiten komen (vrijwel) nooit voor bij patiënten met mildere *SCN1A*-gerelateerde epilepsie. Onze bevindingen suggereren dat het vaststellen van loop-en gedragsproblemen van belang kan zijn voor het stellen van de juiste klinische diagnose.

In **Hoofdstuk 3** onderzoeken we of verschillende klinische kenmerken de ernst van *SCN1A*-gerelateerde aandoeningen kunnen voorspellen. We tonen aan dat een langdurig gebruik van natriumkanaalblokkers (een specifieke groep medicijnen die voorgeschreven kunnen worden bij epilepsie) geassocieerd is met een ernstigere verstandelijke beperking bij patiënten met het Dravetsyndroom. Deze medicatie, die voor andere vormen van epilepsie wel goed kan werken, wordt echter vaak voorgeschreven aan deze patiënten, bijna altijd voordat bekend is dat deze kinderen het Dravetsyndroom hebben. Een vroege diagnose kan daarom indirect leiden tot een gunstigere uitkomst, door het vermijden van deze gecontra-indiceerde medicatie. Daarnaast tonen we aan dat hoe jonger de leeftijd waarop een eerste epileptische aanval zonder koorts optreedt, des te groter de kans is dat een kind met een *SCN1A*-mutatie het Dravetsyndroom ontwikkelt. Daarnaast verloopt het Dravetsyndroom ernstiger als een eerste aanval zonder koorts vroeger optreedt.

Genetische modifiers van SCN1A-gerelateerde epilepsie

In **Deel 2** van dit proefschrift onderzoeken we of we met geavanceerd DNA-onderzoek de ernst van *SCN1A*-gerelateerde aandoeningen beter kunnen voorspellen. Daarvoor onderzoeken we verschillende potentiële genetische *modifiers*. In **Hoofdstuk 4** en **5** bekijken we hoe vaak mozaïcisme van *SCN1A*-mutaties voorkomt. Bij mozaïcisme is de *SCN1A*-mutatie slechts in een deel van de lichaamscellen aanwezig, terwijl in een ander

deel van de lichaamscellen de mutatie niet voorkomt. De aandoening zou hierdoor minder ernstig kunnen verlopen dan bij patiënten die de mutatie in alle lichaamscellen hebben. We laten zien dat mozaïcisme van *SCN1A*-mutaties vaak voorkomt: bij tenminste 7,5% van de aangedane patiënten (**Hoofdstuk 4**), en bij tenminste 5% van de ouders van patiënten (**Hoofdstuk 5**). Mozaïcisme ging gepaard met minder ernstige ziekteverschijnselen, en kan daarom als een belangrijke genetische *modifier* beschouwd worden. Daarnaast heeft het aantonen van mozaïcisme in een klein deel van de cellen van niet-aangedane ouders belangrijke consequenties: de kans dat een volgend kind opnieuw Dravetsyndroom zal hebben is dan vergroot. Als technieken die mozaïcisme betrouwbaar kunnen aantonen in de reguliere genetische diagnostiek worden geïmplementeerd kan daarmee zowel een accuratere voorspelling van de ernst van de aandoening, als van de herhalingskansen voor ouders gemaakt worden.

In **Hoofdstuk 6** onderzoeken we of genetische varianten in andere genen dan *SCN1A* invloed kunnen hebben op de ernst van de aandoening. Deze genen worden dan *modifier genes* genoemd. We laten zien dat bij zowel relatief ernstig als relatief mild aangedane patiënten meer varianten aanwezig zijn in genen die betrokken kunnen zijn bij epilepsie, dan bij patiënten met een meer 'gemiddeld' ziekteverloop. De varianten in deze andere genen beïnvloeden dus mogelijk de ernst van de aandoening. Onze resultaten kunnen richting geven aan vervolgonderzoek naar *modifier genes,* om zo hopelijk in de toekomst hier DNA-diagnostiek naar te kunnen verrichten bij patiënten.

Hoofdstuk 7 richt zich op het natriumkanaal Nav1.1 zelf. Voor een goede werking van het natriumkanaal is niet alleen een correcte code van het *SCN1A*-gen nodig; het moet ook in voldoende mate aanwezig zijn in de cellen. Verschillende factoren reguleren deze zogenaamde expressie van *SCN1A*, waaronder de promotor-regio: een DNA-element vóór een gen dat de werking ervan controleert. In **Hoofdstuk 7** onderzoeken we het effect van veelvoorkomende varianten in de promotor-regio van de intacte kopie van het *SCN1A*-gen. Zulke varianten leiden er mogelijk toe dat het natriumkanaal in mindere mate wordt aangemaakt. Het is te verwachten dat zo'n lagere expressie zal leiden tot een verminderde restfunctie van Nav1.1, wat vervolgens kan leiden tot een ernstiger ziektebeeld bij patiënten met *SCN1A*-gerelateerde epilepsie. We observeerden in het laboratorium in cellen een lagere expressie van *SCN1A* als veelvoorkomende promotor-varianten aanwezig waren, hetgeen impliceert dat deze varianten inderdaad een negatieve invloed hebben op de expressie van Nav1.1. Alhoewel er geen statistisch significante klinische verschillen waren aan te tonen tussen patiënten met en zonder deze promotor-varianten, was er wel een kleine trend waarbij ernstigere ziekteverschijnselen voorkwamen als promotor-varianten aanwezig waren.

Andere genetische epilepsiesyndromen: PCHD19- en KIAA2022-gerelateerde epilepsie

In **Deel 3** van dit proefschrift onderzoeken we twee epilepsiesyndromen met een andere genetische achtergrond: *PCDH19*- en *KIAA2022*-gerelateerde epilepsie. Beide genen liggen

op het X-chromosoom. Mutaties in genen die op het X-chromosoom liggen hebben vaak verschillende consequenties voor mannen en voor vrouwen.

In **Hoofdstuk 8** beschrijven we vijf mannelijke patiënten met een mozaïek-*PCDH19* mutatie. *PCDH19* mutaties zijn een bekende oorzaak van epilepsie bij vrouwelijke patiënten. Mannelijke patiënten hebben geen epilepsie als in al hun lichaamscellen een *PCDH19*-mutatie aanwezig is; als een *PCHD19*- mutatie echter in mozaïek-vorm aanwezig is, zijn zij wel aangedaan. Voorheen waren wereldwijd slechts vier aangedane mannelijke patiënten bekend. Door het beschrijven van vijf extra Nederlandse patiënten laten we zien dat mozaïek-*PCDH19* mutaties mogelijk vaker voorkomen dan gedacht en overwogen dienen te worden als mogelijke oorzaak van epilepsie bij mannelijke patiënten. Daarnaast beschrijven we de kenmerken van de aandoening bij mannelijke patiënten en laten we zien dat deze vergelijkbaar zijn met die bij vrouwelijke patiënten.

In **Hoofdstuk 9** beschrijven we 14 vrouwelijke patiënten met een mutatie in het *KIAA2022*-gen. *KIAA2022*-mutaties zijn een bekende oorzaak van een verstandelijke beperking bij mannelijke patiënten. Voorheen waren wereldwijd slechts drie aangedane vrouwelijke patiënten beschreven. De mutaties bij deze vrouwelijke patiënten kunnen alle als mozaïek beschouwd worden, omdat bij vrouwen in elke cel één van beide X-chromosomen willekeurig geïnactiveerd wordt. Als resultaat is in sommige cellen de aangedane kopie van het gen actief, en in andere cellen de gezonde kopie. We tonen aan dat vrouwelijke patiënten in het algemeen milder aangedaan zijn dan mannelijke patiënten, hoewel zij wel vaker epilepsie ontwikkelen. Door het beschrijven van dit eerste cohort vrouwelijke *KIAA2022*-patiënten zal deze diagnose vaker bij vrouwelijk patiënten gesteld worden en kan informatie worden gegeven over de kenmerken van deze aandoening.

Conclusies van dit proefschrift

Hoofdstuk 10 omvat een algemene discussie over de resultaten van dit proefschrift. We concluderen dat veel verschillende *modifiers* een rol spelen bij de ernst van *SCN1A*-gerelateerde aandoeningen en dat we deze momenteel niet allemaal kunnen identificeren bij individuele patiënten. Wel kunnen we ouders beter informeren over de te verwachten ernst van de aandoening en de herhalingskans voor volgende kinderen, door in de reguliere diagnostiek technieken te implementeren die mozaïcisme betrouwbaar kunnen aantonen. Toekomstig onderzoek zal hopelijk leiden tot testen die net zo informatief zijn, gericht op het aantonen van varianten in *modifier genes* en in regio's van het gen die de expressie ervan reguleren. Bij het informeren van patiënten blijven, naast resultaten van genetische testen, klinische voorspellers zeer informatief, zoals een jonge leeftijd waarop een eerste aanval zonder koorts optreedt, en de aanwezigheid van loop-en gedragsproblemen. We concluderen verder dat het testen op *SCN1A*-mutaties waarschijnlijk het meest gunstige effect heeft wanneer dit gedaan wordt vóórdat onderhoudsmedicatie wordt voorgeschreven aan jonge kinderen met epileptische aanvallen die zouden kunnen passen bij Dravetsyndroom. Op die manier kan gecontra-indiceerde medicatie vermeden worden, waardoor gemiddeld

genomen minder ernstige cognitieve beperkingen zullen optreden. Wanneer er in de toekomst betere behandelmogelijkheden beschikbaar komen zal een vroege diagnose nog belangrijker worden.

Dankwoord / Acknowledgements

Als eerst wil ik graag de leden van de leescommissie, prof. dr. **Kees Braun**, prof. dr. **Peter Burbach**, prof. dr. **Jan Veldink**, dr. **Tjitske Kleefstra** en prof. dr. **Sarah Weckhuysen,** bedanken voor het lezen en beoordelen van dit proefschrift.

Ik heb het geluk gehad om met een aantal fantastische supervisoren te mogen werken. Mijn promotor, prof. dr. **Nine Knoers**, wil ik graag bedanken voor haar begeleiding. Ook nadat je zelf onze afdeling had verlaten bleef je altijd laagdrempelig bereikbaar en maakte je tijd om regelmatig af te spreken. Nine, bedankt voor je optimisme, vertrouwen en ondersteuning.

Eva Brilstra en **Bobby Koeleman**, ik had me geen beter team van copromotoren kunnen wensen. De combinatie van jullie creatieve ideeën met een praktische aanpak heeft ontzettend prettig gewerkt. Ondanks jullie vele andere taken kon ik altijd bij jullie terecht, en was er altijd tijd voor snelle en zorgvuldige commentaren op al mijn manuscripten. Jullie zijn veruit de belangrijkste reden dat mijn promotietraject zo voorspoedig is verlopen en dat ik er met zoveel plezier aan heb gewerkt. Bedankt voor het vertrouwen in mij!

Dit proefschrift bestaat alleen maar omdat 176 (ouders van) **patiënten met *SCN1A*-gerelateerde epilepsie** bereid waren om meerdere vragenlijsten in te vullen over het beloop van hun aandoening. Ontzettend bedankt voor jullie deelname aan onze studies. Veel dank ook aan alle artsen die hebben bijgedragen aan de inclusie van deelnemers.

De acht artikelen in dit proefschrift zijn tot stand gekomen met de hulp van een heleboel coauteurs, aan wie ik veel dank verschuldigd ben. Ik wil graag iedereen bedanken voor zijn of haar waardevolle bijdragen, en een aantal personen hier in het bijzonder noemen. **Nienke Verbeek**, wat fijn dat ik het Dravetonderzoek van jou kon overnemen! Ik heb weinig teksten zo vaak gelezen als jouw proefschrift, dat mijn favoriete boek van 2018 is geworden. Er zullen altijd snacks voor jou op mijn bureau liggen. **Marco Koudijs**, ontzettend bedankt voor al je hulp bij de MIP-assays en alle overleggen daarover. Ook veel dank hiervoor aan **Benjamin Kant**! **Carolien de Kovel**, bedankt voor je geduldige uitleg toen ik 4 jaar geleden begon met mijn promotieonderzoek maar de basics van genetisch onderzoek nog niet kende. **Ies Nijman** en **Robert Ernst**, heel erg bedankt voor jullie bioinformatische bijdragen. **Marjan van Kempen**, bedankt voor al het meedenken en het kritisch lezen van verschillende manuscripten. **Boudewijn Gunning**, erg bedankt voor je verschillende bijdragen aan meerdere van de papers. **Sanjay Sisodiya**, thank you for inviting me to Chalfont to work on the modifier project. My stay has been a challenging but great experience! **Claudia Sinoo**, wat fijn dat jij aan de slag bent gegaan met alle CBCL-data en wat is het een mooi paper geworden! Ik ben heel blij dat ik op mijn beurt het stokje weer aan jou kan doorgeven. Many of my coauthors are members of the illustrious **LEGOLAS** association. I would like to thank this group of very photogenic people for some great trips to several exotic places and some of the most fun meetings I have been to.

Elly, ontzettend bedankt voor je hulp bij het invoeren van de vele medische gegevens in de database!

Dan de **Koeleman-groep**! **Ruben**, ontzettend bedankt voor al het werk dat je in het lab hebt verricht voor onze projecten. Ik heb heel veel van je geleerd, hoewel ik nooit zo goed zal kunnen pipetteren als jij. Bedankt voor de gezelligheid tijdens de vele MIP-runs en onze ddPCR-lasso-avonturen! **Flip**, ik heb al vaker gezegd dat we eigenlijk net zo goed jouw naam op de voorkant van dit boekje hadden kunnen zetten. Zonder jou en de befaamde FlipTools hadden de meeste projecten niet alleen 10 keer zo lang geduurd, maar had ik waarschijnlijk ook al meerdere keren opgegeven. Het blijft bewonderenswaardig hoe je in ieder geval nooit hebt laten doorschemeren helemaal klaar te zijn met mij en al mijn problemen. Bedankt voor je optimisme! **Wout**, ik vind het zo leuk dat wij samen nog een paper hebben kunnen schrijven! Bedankt voor (het uitleggen van) alles wat je in het lab hebt gedaan, en je snelle acties toen voor mij de tijd begon te dringen. **Mark**, thank you so much for helping us out with the GWAS-part of this paper! **Sakshi**, thank you for cooking us the best Indian food I've ever had, and for being an awesome roommate during both trips to Antwerp. **Annabel**, bedankt voor de gezellige kletsmomenten, je wijze woorden en je nuchterheid! **Remi**, bedankt voor het op peil houden van mijn cafeïnegehalte en het feit dat je altijd bereid bent te helpen bij elk mogelijk probleem. **Anja**, jij bent onmisbaar geweest: zie 5 alinea's verder. Besides all of your contributions to several projects, I would like to thank all of you for being great, fun people to be around,

Dear **Room**, where to begin! Most people in these acknowledgements are being thanked for saving me a lot of time. This cannot be said about you. However, your distractions have led to some of the most fun times during my PhD, so all is forgiven. **Glen**, I admire your tolerance for this room full of women. I had so much fun with you and **Federico** during the 2017 ESHG! Thank you for being an overall awesome person. **Rozemarijn**, ik ben je eeuwig dankbaar voor het opzetten van het wekelijkse Heel-STR1.303-Bakt-schema. Bedankt voor alle gezelligheid, de kerstliedjes (toch wel), je goede relatie met Sinterklaas, je luisterend oor en je betrokkenheid. **Remi**, de laatste maanden zonder **Sanne** aan mijn zijde waren zwaar, maar jij hebt als nieuwe linkerbuurman de pijn enorm weten te verzachten. Bedankt voor je onverstoorbaar goede humeur! **Edith**, bedankt voor al je waardevolle adviezen op verschillende fronten: bakken, breien, tuinieren, schaatsen, labwerk… Je bent een van de meest veelzijdige personen die ik ken. **Karen**, het is bewezen dat de afdeling zou instorten zonder jou. Bedankt voor je hulp, uitleg en interesse. **Helen**, thank you for keeping me company during all the times bus 12 took hours to get us home. It has been fun getting to know you! **Sanne**, op jouw belangrijke rol komen we straks nog even terug. **Antoine** and **Alphonsine**, thanks for being the best 2/3 of our trinity. I would also like to mention several roommates from the past. **Nayia**, **Mirjam**, **Marijn**, **Chris** and **Christina**: you are still dearly missed! **Ellen C. Carbo**, ook al zijn we nooit officieel kamergenoten geweest, je regelmatige visites maakten dat het wel zo voelde en als rasechte MIP-Miep verdien jij hier ook een eervolle vermelding. Bedankt voor al je hulp met de MIPs en voor

alle avonturen buiten het werk om! Thank you all for so many great times during coffee breaks, sailing trips, scary Walibi-visits, festivals, retreats, the production of some very professional movies.. I could go on for pages.

To all members of the other research groups of the Genetics departments: thank you so much for a great working environment! I really appreciated the many lunches (shout-out to the 11:30 lunch group, including **Ivo**, **Robert**, **Martin**, **Joep**, **Ies** and **Ruben**), the mid-summer ice cream breaks, the fun retreats, CMM-borrels and other activities. To (former) members of the **Van Haaften** and **Renkema** groups, including **Gijs**, **Kirsten**, **Albertien**, **Anukrati**, **Maarten, Federico, Sanne, Edith, Karen, Rozemarijn, Marijn, Nayia, Helen** and **Glen**: thank you for the inspiring discussions during the SGM meetings. **Kirsten**, ik ben zo blij dat jij en **Lancelot** elkaar hebben gevonden. Veel dank aan **Kirsten, Sanne** en **Rozemarijn** voor het leveren van onze maandelijkse dosis schattige kattenfoto's. **Sander**, bedankt voor het zijn van, onder andere, een fantastische Kanye. **Terry**, bedankt voor het uitvoeren van de beste cilia-dans die ik ooit heb gezien.

Aan alle (voormalige) arts-assistenten, **Charlotte**, **Saskia**, **Nienke**, **Daniëlle**, **Noor**, **Claire**, **Remco**, **Aster**, **Marten**, **Jette**, **Maaike**, **Laura** en **Kyra**: bedankt voor het warme welkom terug in de kliniek. Ondanks mijn afwezigheid van zo'n 3 jaar hebben jullie mij toch altijd het gevoel gegeven nog onderdeel van de groep uit te maken. **Marijn**, ik vind het zo fijn dat we weer kamergenoten zijn! Je bent voor mij een groot voorbeeld wat betreft het combineren van onderzoek en kliniek. Bedankt dat ik je altijd mag storen met vragen over allebei die dingen. Ik vond het fantastisch om jouw paranimf te mogen zijn! **Saskia**, ontzettend bedankt voor het zijn van mijn buddy. **Evelien**, het is een hele tijd geleden dat wij kamergenoten waren, maar ik wil je graag bedanken voor je enthousiaste aanmoediging om aan dit promotietrajcet te beginnen. Dank ook aan alle stafleden en andere collega's voor de fijne werkomgeving! **Marie-José**, bedankt dat je mijn mentor wil zijn. **Paulien** en **Mieke**, jullie hebben mij tijdens mijn studie geneeskunde begeleid tijdens keuzecoschappen en wetenschappelijke stages op de afdeling Genetica. Dankzij jullie ben ik enthousiast geworden en gebleven over het vak. Bedankt!

Beste **Wilmy** en **Monique**, bedankt voor jullie hulp bij alle administratieve uitdagingen die komen kijken bij promotieonderzoeken. **Saskia**, bedankt voor je hulp met alle priksetjes en plaspotjes, het beantwoorden van mijn vele vragen en de gezellige praatjes!

Lieve **Sanne** en **Anja**, ontzettend bedankt dat jullie mijn paranimfen willen zijn. **Anja**, ik ben blij dat je na enige overreding "ja" hebt gezegd, want hoe had ik iemand anders kunnen kiezen dan jou! Zonder jouw hulp was ik nu nog steeds medische gegevens aan het verzamelen. Je bent ontzettend belangrijk geweest voor het onderzoek; inhoudelijk maar ook wat betreft alles er omheen. Heel erg bedankt voor de warme, gezellige sfeer in je kamer boven: even bijpraten met een kopje koffie was altijd mogelijk. Ik vond het ontzettend leuk om samen met jou naar Praag te gaan! **Sanne**, ik ben zo blij dat jij 3 jaar mijn linkerbuurvrouw bent geweest. Ik heb heel erg veel van je geleerd en zonder jou was dit proefschrift maar half zo dik geweest, omdat alle MIP-papers niet hadden bestaan. Ik ben

je erg dankbaar voor je hulp, uitleg en geduld bij alle experimenten, maar nog meer voor het zijn van zo'n lieve vriendin! Ik heb ontzettend genoten van je humor en creativiteit, je wilde feestjes en het zijn van slaapkamergenootjes op de retreats. Bedankt voor je betrokkenheid, en dat je mij **Noan** onbeperkt laat knuffelen (dat helpt overal bij).

Ik ben een heleboel mensen buiten het UMC dank verschuldigd voor de nodige afleiding en gezelligheid tijdens de afgelopen 4 jaar. **Wayne**, **Thom**, **Hugo**, **Robbert**, **Pim**, **Niek**, **Maarten**, **Stijn** en **Michel**, bedankt voor de vele gezellige avondjes, wintersportvakanties, roadtrips en feestjes! **Laura**, **Guy**, **Jeroen**, **Joop**, **Ralph**, **Robbert** en **Tom**, bedankt voor alle Zeistdagen en andere avonturen. Lieve **Jeanine**, onze gedeelde liefde voor eten en slapen heeft geleid tot een heleboel heerlijke vakanties, uitjes en avondjes. Bedankt dat je er altijd voor me bent! Dear **Hannah**, I'm so happy we stayed in touch, and that Skype also exists on the other side of the ocean. Thank you for your never-ending support!

Lieve **Hans** en **Monique**, **Stefan** en **Sophie**, **Pieter** en **Heleen**, bedankt dat jullie zo'n fijne schoonfamilie zijn. Ik ben heel blij met jullie gastvrijheid en interesse, en heb heel goede herinneringen aan alle bijzondere uitjes die we hebben gemaakt. Bedankt voor alle etentjes en gezellige avonden. Er is inmiddels een volgende generatie onderweg, en ik kijk er enorm naar uit om haar te ontmoeten!

Lieve **papa** en **mama**, zonder jullie steun, opvoeding en goede genen was ik nooit zo ver gekomen als nu. Jullie zijn er altijd van overtuigd geweest dat ik alles kan, waardoor ik ook in mezelf heb leren geloven. Volgens mij is het voor jullie geen verrassing dat ik hier nu sta, want ik heb mama vaak genoeg horen zeggen dat ze altijd al heeft gedacht dat ik "een wetenschappertje" zou worden. **Nick**, aangezien we onze ouders delen is het geen wonder dat jij ook zo goed terecht bent gekomen. Dank jullie wel voor het zorgen voor een warm (spreekwoordelijk) en gezellig thuis, waar een heleboel gelachen wordt, en waar ik altijd weer naar terug kan komen.

Lieve **Martijn**, ik zie op tegen het schrijven van deze laatste alinea omdat het me nooit gaat lukken om in een paar regels uit te drukken wat jij de afgelopen jaren voor me hebt betekend. Van het corrigeren van mijn teksten en het oplossen van mijn Excelproblemen, tot het samen vieren van overwinningen en me moed inpraten als het even niet lukte: jij was er altijd en je hebt me op alle mogelijke manieren geholpen. Ik hoop dat we nog heel lang samen blijven lachen.

Curriculum vitae

Iris Marie-Louise de Lange was born on May 13th, 1990 in Eindhoven. She obtained her high school diploma *cum laude* at Pleincollege Bisschop Bekkers and began studying Medicine at Utrecht University in 2008. During her study, she did several clinical and scientific internships at the department of Genetics. She graduated in 2014. The first three months of 2015 she worked as a resident not in training (ANIOS) at the department of Genetics of the University Medical Center Utrecht. In April 2015 she started her PhD research that has resulted in this thesis, at the same department. The project was entitled "*SCNIA*-related seizure disorders: prediction of clinical course based on advanced genotyping", and was carried out under supervision of prof. dr. Nine Knoers, dr. Eva Brilstra and dr. Bobby Koeleman. During her PhD, she spent two months at the Chalfont Epilepsy Society, affiliated to University College London, where she worked on a project on modifier genes in Dravet syndrome under supervision of prof. dr. Sanjay Sisodiya. In July 2018, she resumed her work as ANIOS. Iris lives in Utrecht with Martijn.

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*these authors contributed equally

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