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A STUDY OF INCIDENCE, CAUSATIVE FACTORS, SYMPTOMS, AND PROGNOSIS IN EPILEPSY WITH ONSET IN THE FIRST TWO YEARS OF LIFE

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A STUDY OF INCIDENCE, CAUSATIVE FACTORS, SYMPTOMS, AND PROGNOSIS IN EPILEPSY WITH ONSET IN THE FIRST TWO YEARS OF LIFE. THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To my sons William and Martin

and their children…

and their children…

and their children…

May they take on the challenges of the future

while they sometimes remember us that are here

now

ABSTRACT

Background. The motivation to start this thesis work came from the meetings with infants just having presented with epileptic seizures and from speaking to their parents. I found two issues to be particularly urgent. The first was to give the parents, and myself, an overall map of the situation. A map that had to address questions like: What are the implications of the seizures in this specific infant? What should be done next? What are the alternative future scenarios and their respective probabilities? When the population-based studies in this thesis started, the knowledge-base to answer such questions was poor, studies were few and the numbers of infants studied were small. The second issue concerned the cause of disease in the individual child. For decades the cause of epilepsy has been revealed in only a minority of cases. Especially in severe disease, like drug-resistant epilepsy, not knowing the cause places a heavy psychological burden on the family. In addition, treatment will be symptomatic, not directed at the (unknown) disease mechanisms and often ineffective.

Methods. Two circumstances offered the opportunity to deal with these issues. The Stockholm Incidence Registry of Epilepsy (SIRE) registered all new cases of epilepsy in the Northern Stockholm region from September 2001 and onwards. All children with epilepsy in this area are managed at the Karolinska University Hospital. This provides a population-based perspective, which is the best way to study a disease and all aspects of it including etiology, clinical characteristics, and outcome. The other favorable condition was the development of genetic diagnostics, in particular massively parallel DNA sequencing, where Science for Life Laboratory was very early to establish whole exome and whole genome sequencing for both research and clinical healthcare purposes, in a close collaboration between the Clinical Genomic facility and the Karolinska University Laboratory.

Results. The population-based studies of this thesis include 116 children with onset of epilepsy during the first 2 years of life. A majority of the cases could be assigned to an epilepsy syndrome and have the etiology revealed. Massively parallel sequencing contributed substantially to reveal genetic etiologies. About half of the children were diagnosed with intellectual disability and half of the cases were in seizure remission for 2 years or longer at age 7 years. Type of etiology is the main predictor of outcome. Two new disease genes, closely related and both with a central role in neuronal inhibition-excitation, were uncovered and described as part of the thesis.

Significance. Together with a few other recent population-based studies, this thesis contributes to a firm knowledge-base when managing infants with epilepsy and counselling their parents. The important role of massively parallel DNA sequencing in revealing monogenic etiologies in early onset epilepsy has been clearly shown. In addition to enabling genetic counselling and prenatal diagnostics, the inclusion of genetic diagnostics in the work-up of children with epilepsy, will henceforth further the development of more effective and even curative precision medicine treatments of epilepsy.

LIST OF SCIENTIFIC PAPERS

I. **Epilepsy syndromes, etiologies, and the use of next-generation sequencing in epilepsy presenting in the first 2 years of life: A population-based study**.
Stödberg, T. Tomson, T. Barbaro, M. Stranneheim, H. Anderlid, B. M. Carlsson, S. Åmark, P. Wedell, A.
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II. **Outcome at age 7 of epilepsy presenting in the first 2 years of life. A population-based study.**
Stödberg, T. Tomson, T. Anderlid, BM. Andersson, T. Henry, O. Åmark, P. Wedell, A.
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III. **Mutations in *SLC12A5* in epilepsy of infancy with migrating focal seizures**.
Stödberg, T*. McTague, A*. Ruiz, A. J. Hirata, H. Zhen, J. Long, P. Farabella, I. Meyer, E. Kawahara, A. Vassallo, G. Stivaros, S. M. Bjursell, M. K. Stranneheim, H. Tigerschiöld, S. Persson, B. Bangash, I. Das, K. Hughes, D. Lesko, N. Lundeberg, J. Scott, R. C. Poduri, A. Scheffer, I. E. Smith, H. Gissen, P. Schorge, S. Reith, M. E. Topf, M. Kullmann, D. M. Harvey, R. J. Wedell, A#. Kurian, M. A#.
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IV. ***SLC12A2* mutations cause NKCC1 deficiency with encephalopathy and impaired secretory epithelia**.
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CONTENTS

LIST OF ABBREVIATIONS

ADNFLE	autosomal dominant nocturnal frontal lobe epilepsy
ASD	autism spectrum disorder
ASM	antiseizure medication
BECT	benign childhood epilepsy with centrotemporal spikes
BFIE	benign familial infantile epilepsy
BIE	benign infantile epilepsy
CAE	childhood absence epilepsy
CNS	central nervous system
CNV	copy number variant
CP	cerebral palsy
CSF	cerebrospinal fluid
CT	computed tomography
DD	developmental delay
DEE	developmental and epileptic encephalopathy
DNA	deoxyribonucleic acid
DRE	drug-resistant epilepsy
EEG	electroencephalogram
EIEE	early infantile epileptic encephalopathy
EIMFS	epilepsy of infancy with migrating focal seizures
GABA	gamma-aminobutyric acid
GEFS+	genetic epilepsy with febrile seizures plus
GoF	gain-of-function
HIE	hypoxic-ischemic encephalopathy
HPO	Human Phenotype Ontology
ID	intellectual disability
ILAE	International League Against Epilepsy

JAE	juvenile absence epilepsy
JME	juvenile myoclonic epilepsy
KCC2	potassium-chloride (K-Cl) cotransporter 2
LoF	loss-of-function
mRNA	messenger ribonucleic acid
MIP	Mutation Identification Pipeline
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
NDV	neurodevelopmental
NKCC1	sodium-potassium-chloride (Na-K-Cl) cotransporter 1
NGS	Next Generation Sequencing
OMIM	Online Mendelian Inheritance in Man
PCR	polymerase chain reaction
RNA	ribonucleic acid
SIRE	Stockholm Incidence Registry of Epilepsy
SNP	single nucleotide polymorphism
SNV	single nucleotide variant
SV	structural variant
UKISS	United Kingdom Infantile Spasm Study
VNS	vagal nerve stimulation
WES	whole exome sequencing
WGS	whole genome sequencing

1 INTRODUCTION

The incentive for this thesis came from my meetings with infants that had just presented with epilepsy and talking to their parents about the situation. There were, and still are, two issues that I find particularly challenging. The first is the need to give the parents some kind of order and predictability in a situation that can be experienced as chaotic and frightening. “Will my child die, have severe disabilities or recover and be healthy?” is an urgent question for most parents, whether uttered or not. Unfortunately, often this question cannot be answered at the onset of epilepsy in a child. The future is not known.

To deal with this uncertainty the parents, and I as the treating physician, need a “map” of what is going on, why we – the doctors, nurses, and other healthcare professionals in the epilepsy team - do what we do and what might or might not happen henceforth. This shared map is also a foundation for the necessary working alliance and communication between the epilepsy team and the parents. In pediatric epileptology the parents are crucial as information carriers and many medical decisions are based on what they convey. We need to trust each other.

When I started working with infantile-onset epilepsy, data on which to base an overall map of the disease and the different causes, potential clinical outcomes, and treatments, were scarce. Studies were few, methodological differences and flaws made comparisons difficult and data uncertain and the numbers of infants studied were usually small. The etiological panorama had not been studied in a population-based setting with the use of new genetic diagnostic tools. During the course of the work with this thesis several valuable studies on early-onset epilepsy have been published by the scientific community. My hope is that the first two papers in this thesis similarly will add to the knowledge-base clinicians need when they manage infants with epilepsy and counsel their parents.

The second challenging issue is the fact that, in a majority of individuals with epilepsy, the cause of the disease is not known. This has been an unchanged dilemma for decades, is true also for epilepsy in children and is the more troublesome since the cause of epilepsy is a main determinant of outcome and the best base for treatment choice. As a result of this, epilepsy drug treatment has been symptomatic, not specifically directed at disease mechanisms and not curative. In about one third of patients with epilepsy, antiseizure medications do not prevent seizures. The epilepsy is drug-resistant. In addition, adverse effects, sometimes disabling and intolerable, are common with antiseizure drugs due to their, in many cases, non-selective mechanisms of action.

Genetic factors have long been suspected to be important in epilepsy of hitherto unknown cause. Family and twin studies in the 1940s strengthened this notion. However, the means to explore this further have been lacking. The development of new genetic technologies is now changing this in a dramatic way. We are in the middle of a diagnostic revolution, and this will be followed by a treatment revolution that is still in its early phase. Precision medicine is the term used. With the knowledge of specific disease mechanisms comes the opportunity to

develop more effective treatments, including but not limited to gene therapy, targeted at these mechanisms. The third and fourth scientific paper in this thesis contribute to this development by describing the discovery of two new disease genes. The phenotype described in paper IV does not include epilepsy, but the etiology revealed has a close molecular relation to the disease mechanism described in the cases of paper III. Therefore, paper IV was included in this thesis. As it turned out, the proteins encoded by the two genes are closely related in a dynamic balance deciding the excitability of neurons and therefore are attractive targets for precision medicine drug development for epilepsy.

2 LITERATURE REVIEW

2.1 INTRODUCTION

Epilepsy is the most common chronic neurologic illness in children as well as in adults [1].The impact on the lives of affected individuals and on society is great [2-5]. Epilepsy is a heterogenic disease in terms of causes, symptoms, and outcome. Therefore, definitions of concepts and classifications are difficult but important. In his pioneering publication from 1959 on the first epidemiological study of epilepsy Kurland wrote: "There is no precise agreement on the criteria for epilepsy or how long a person must be seizure free to be excluded from the statistics". He then defined epilepsy as two or more unprovoked epileptic seizures more than 24 hours apart. This pragmatic definition was formally adopted by the International League Against Epilepsy (ILAE) in 1969 [6] and has been commonly used ever since. ILAE has published several documents on definitions and classifications of epileptic seizures, etiology of epilepsy and epilepsy syndromes with recent updates [7-10] and also guidelines for epidemiological studies on epilepsy [11, 12]. Future revisions due to expanded knowledge in the field are to be expected.

According to present language and definitions adopted by ILAE [8, 13] an epileptic seizure is a "transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain". Epilepsy is "a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures, and by the neurobiologic, cognitive, psychological, and social consequences of this condition. The definition of epilepsy requires the occurrence of at least one epileptic seizure". This means that under certain conditions epilepsy can now be diagnosed after a first single seizure. Examples of such conditions could be an underlying brain malformation mediating an increased risk of seizure recurrence or the diagnosis of an epilepsy syndrome. The seizures should be unprovoked which means that no acute brain injury or temporary disturbance elicited the seizures. Febrile seizures and seizures in the acute phase of stroke or encephalitis are examples of provoked epileptic seizures that are not epilepsy. Neonatal seizures are also considered to be provoked and thus not the basis for epilepsy diagnosis. The concept "epileptic encephalopathy", or more recently "developmental and epileptic encephalopathy", has been established but is debated. It implicates that seizure activity and interictal epileptiform activity in themselves cause damage in addition to the underlying etiology. Onset is early in childhood. The disease course could be progressive even when the underlying etiology is static, e.g. a perinatal stroke. This perspective puts emphasis on the possibility to improve neurodevelopmental outcome by treatment of seizures and in some cases also of interictal activity.

2.2 POPULATION-BASED STUDIES OF EPILEPSY

A population-based incidence approach is the best way to study the etiology, natural course, and prognosis of epilepsy. As cross-sectional data are easier to obtain prevalence studies are much more common but miss cases of early death and of remission. Since Kurland's study [14] more than 50 population-based studies on incidence of childhood epilepsy have been

conducted, primarily from Europe, USA and Canada although some studies from developing countries exist. Most studies collect cases in retrospect, and a few are prospective. Some studies include all ages and some exclusively children. Some include prevalence figures. Basic data on seizure types, epilepsy syndromes and etiology are sometimes included. Outcome and prognostic factors are more rarely considered in population-based studies and such data have mostly been derived from cohorts of patients from hospitals and/or clinics and therefore represent selected populations. The multiple studies from Rochester [14-18], Finland [19-24], Iceland [25-27] and northern Sweden [28-32] have contributed considerably to our understanding and also provide data on trends over time. The Finnish and Icelandic studies together with a study from Faroe Islands [33] and a very recent one from Scotland [34] are the only nation-wide ones.

The comparison of different studies is hampered by methodological differences [35]. Despite consensus discussions since the 1960s the case definition has varied (for example single seizures and provoked seizures included or not). Different case ascertainment methods have been used prospectively or retrospectively. Studied populations differ in age, race, educational level, and social class in different geographical areas. Some studies present age-adjusted incidence, others do not. The numbers of cases are often small, especially when divided into subgroups by age and in children. As mentioned above definitions and classifications of seizures, epilepsy syndromes and etiology have changed over time. Even studies of the same population, during the same time period, by the same authors and with similar methodology can have different results as exemplified by Kurland 1959 and Hauser 1975 and 1993 [14-16]. These studies all included the Rochester population during 1945-54 and found 89, 142 and 154 incident cases respectively!

2.3 INCIDENCE OF EPILEPSY

Despite the above-mentioned difficulties some conclusions can be drawn, and patterns seen. Table 1-3 list some of the most relevant population-based incidence studies for different age groups. Most figures are expressed explicitly in the publications, but some could be calculated from the data presented. Most studies from developed countries show overall incidence rates for epilepsy between 30 and 50 per 100,000 person-years. The seven important incidence studies on epilepsy across all ages listed in Table 1 illustrates this [14, 15, 23, 27, 33, 36, 37]. In these studies, incidence rates vary from 19,5 to 71,6 with five of the studies between 30 and 47. The low incidence rate in the Stockholm Incidence Registry of Epilepsy (SIRE) study [36] could at least partly be explained by the short follow-up only six months after the first unprovoked seizure. Hauser 1993 showed that time from a first unprovoked seizure exceeded six months in 50% of the patients [15]. Six of the studies show a higher all ages incidence rate for men than for women which is also supported by other studies and a meta-analysis [38]. The male predominance starts from the second decade in life and increases with age. The exception is Olafsson 2005 [27] who found no sex difference.

Table 1. Incidence studies across all ages					
Author	**Study region**	**Study years (onset)***	**Type**	**Incident cases (n)**	**Incidence rate#**
Kurland 1959	Rochester, USA	1945-1954	re	89	30,8
de Graaf 1974	Northern Norway	1968-1972	re	749	32,8
Joensen 1986	Faroe Islands	1970-1980	re	194	42
Hauser 1993	Rochester, USA	1935-1984	re	880	44
Olafsson 2005	Iceland	1995-1999	pro	294	32,5
Sillanpää 2006	Finland	1986-2002	re	51767	71,6-52,9
Adelöw 2009	Stockholm, Sweden	2001-2004	pro	585	19,5

Table 1. Seven important incidence studies from developed countries. *time period during which new onset cases were ascertained (follow-up could be later). re=incident cases were identified in retrospect. pro=cases were identified prospectively. #incident cases per 100,000 person-years.

Studies from low- and middle-income countries in Africa and Latin-America have shown higher incidence rates [38-41] . Part of these discrepancies could be due to methodological differences. One study from Ecuador [42] reported an incidence rate of 190 but included single seizures and acute symptomatic seizures. Still there is reason to believe that acquired epilepsy, e.g. after central nervous system (CNS) infection like neurocysticercosis and cerebral malaria, is more common in developing countries. However, a few studies from China and India have incidence rates in the range of developed countries [43, 44]. Possibly this could be due to a lower case ascertainment rate.

Almost all studies show that age-specific incidence is highest during infancy and among the elderly. Between approximately 15 and 60 years of age the incidence is relatively stable on a lower level around 25-30 [15, 27, 33]. From around 70-80 years-of-age incidence exceeds infancy´s [15, 27, 36]. The older studies from northern Norway and Faroes [33, 37] differ by lacking the increase among the elderly, probably due to lower case ascertainment rates among the elderly in a rural setting [37].

Data on the influence of ethnicity on the risk of epilepsy are very scarce. A study from USA found a doubled risk of epilepsy in black people compared to whites but how much of this was due to socioeconomic factors was not analyzed [45]. A small Scottish study found an increased incidence of epilepsy early in life related to ethnicity but not to socioeconomic status [46]. However, in a national Scottish cohort of early onset epilepsy socioeconomic deprivation was associated to increased incidence and this is supported by other studies [34, 47]. The increased incidence already in early childhood supports a cause-and-effect relation with low socioeconomic status influencing the risk of epilepsy in a multifactorial way. It's probable though that the opposite is also true with epilepsy having negative socioeconomic consequences. One study showed that poor children with epilepsy had the same remission rate but worse socioeconomic outcome than rich children [48].

A few studies have analyzed incidence time trends. Sillanpää showed a total decrease over the years 1986-2002 in the Finnish population [23] with a decrease in children and adults and an increase in the elderly. Hauser found a decrease in children and an increase in the elderly but

no total change in Rochester 1935-84 [15]. Other studies confirm the decrease of childhood epilepsy over time [20, 49, 50].

Table 2. Incidence studies with >250 epilepsy cases with onset in childhood and adolescence					
Author	**Study region**	**Study years (onset)***	**Type**	**Incident cases**	**Incidence rate#**
Shamansky 1979	New Haven,USA	1960-1970	re	557	56<15y
Hauser 1993	Rochester,USA	1935-1984	re	278	53<15y
Camfield 1996	Nova Scotia,Canada	1977-1985	re	693	41<16y
Sillanpää 2006	Finland, nationwide	1986-2002	re	13397	117-76,5<16y
Wirrell 2011	Connecticut,USA	1980-2004	re	359	44,5<18y
Åndell 2015	Stockholm,Sweden	2001-2006	pro	513	45<19y
Saarinen 2016	Finland, nationwide	1968-2012	re	29567	63<15y

Table 2. Seven incidence studies in children and adolescents with >250 cases. *time period during which new onset cases were ascertained (follow-up could be later). re=incident cases were identified in retrospect. pro=cases were identified prospectively. #incident cases per 100,000 person-years. y=years, upper age limit for inclusion.

As described above the incidence of epilepsy in infancy is higher than later in childhood and in adults and is exceeded only by the incidence in the very old. Table 2 lists the seven identified studies of overall childhood incidence, including two of the across all ages studies from Table 1, with more than 250 cases [15, 17, 20, 23, 45, 51, 52]. The nine identified incidence studies with more than 35 cases of onset during the first year of life listed in Table 3, show incidences from 82 to 144 per 100.000 person-years [15, 17, 19, 20, 34, 52-55]. Camfields study from Nova Scotia [52] shows a representative pattern. Incidence peaks during the first year of life with 118 cases per 100.000, is relatively stable from 1-10 years on 43-48 and decreases after 10 years-of-age to 21. This pattern is supported by other studies [15, 17, 36].

Table 3. Incidence studies with >35 epilepsy cases with onset during the first year of life					
Author	**Study region**	**Study years (onset)[2]**	**Type**	**Incident cases**	**Incidence rate#**
Hauser 1993	Rochester,USA	1935-1984	re	36	86
Camfield 1996	Nova Scotia,Canada	1977-1985	re	112	118
Rantala 1999	Oulu, Finland	1976-1986	re	49	89
Wirrell 2011	Connecticut,USA	1980-2004	re	50	102
Eltze 2013	London,UK	2005-2006	pro	45	82
Saarinen 2016	Finland	1968-2012	re	2369	85
Gaily 2016	Helsinki,Finland	1997-2006	re	158	124
Aaberg 2017	Norway[1]	1999-2012	pro?[3]	162	144
Symonds 2021	Scotland	2014-2017	pro	225	136

Table 3. Nine incidence studies of infantile-onset epilepsy with >35 cases. [1]this study is a cohort study rather than a national population-based study, with risk of selection bias. [2]time period during which new onset cases were ascertained (follow-up could be later). [3]cases were identified through a national patient registry and questionnaires for parents at ages 5, 7 and 8 years. re=incident cases were identified in retrospect. pro=cases were identified prospectively. #incident cases per 100,000 person-years.

Four Swedish studies show overall childhood incidence rates between 40 and 82 for epilepsy [28, 51, 56, 57] and two other present incidence rates for a first unprovoked seizure between 73 and 134 [31, 32]. The differences between studies that are seen could be explained by different methodology and case ascertainment and actual difference in incidence due to different time periods, populations and health care conditions affecting diagnosis and treatment. The relatively small numbers of cases in several studies also leave room for chance to affect results.

Proportions of incident cases with onset at different ages depend on the age distribution of the population studied. An overall estimation from several studies is that epilepsy with onset during the first year of life represents approximately 5% of incident cases across all ages and 15% of cases with onset <15 years of age [15, 27, 36, 52]. Onset <15 years of age constitutes 30% of all incident cases [15, 23, 27, 36].

2.4 PREVALENCE OF EPILEPSY

As prevalence studies are easier to perform, they are more common in the literature than incidence studies. Figures vary widely depending on the age-distribution of the studied population and the definition of "active epilepsy" among other things. In Europe most studies have shown overall prevalence between 3.5 and 7.8 per 1000 people [25, 33, 37, 58-61] with figures from USA and Canada within the same range [14, 16, 62-64]. Overall childhood prevalence appears to lie between 3.2 and 4.3 in European studies [21, 55, 56, 58, 65, 66]. Studies from developing countries tend to show higher figures [38, 67]. The age-distribution of prevalence rates are not u-shaped like the incidence as it does not show increased rates in the very young and the elderly despite high incidence in these groups. This is due to lower survival rates. In prevalence cohorts cases <10 years of age constitute roughly around 20% and cases <20 years around 40% [14, 16, 25, 33, 37].

2.5 SEIZURE TYPES IN INCIDENCE COHORTS

ILAE has published proposals and revisions for the classification of seizure types in 1964, 1969, 1981, 2010 and 2017 [6, 7, 9, 68, 69]. This has influenced the studies from different time periods. Two recent incidence studies have used the 2017 seizure classification [34, 70]. The distinction between focal (previously termed partial) and generalized seizures has been kept over the years. Of the 17 different studies in Table 1-3, 10 have analyzed seizure type at onset of epilepsy [14, 15, 17, 19, 27, 33, 34, 36, 51, 52]. Focal seizures including focal to bilateral tonic-clonic (previously termed secondary generalized) seizures constitute the majority (48-68%) of onset seizures in epilepsy across all ages as well as in childhood and infancy. In most studies the incidence of focal seizures has a u-formed curve being highest in the first year of life and in the elderly and gradually decreasing during childhood parallel to the overall incidence. Hauser 1993 is an exception where the peak in infants is missing, probably due to small numbers of patients [15]. Generalized seizures constitute 38, 38,5 and 39% of onset seizures in three all ages studies [14, 15, 33]. Olafsson's study from Iceland presents only 9% generalized seizures but excludes generalized tonic-clonic seizures without obvious focal or generalized

features from this category and keeps them in a separate category ("GTCS only" 41%) of which a substantial part probably belongs to the generalized type [27]. With a few exceptions the incidence of generalized seizures seems to be relatively stable in childhood and young adults and fades after age 30. Infantile spasms constitute 2-3% of incident cases across all ages [33, 36] and 20-40% of cases before age 1 year [53, 54, 70].

2.6 TYPES OF EPILEPSY AND EPILEPSY SYNDROMES IN INCIDENCE COHORTS

There are only two population-based studies of epilepsy across all ages reporting the occurrence of epileptic syndromes according to the 1989 ILAE classification of epilepsies and epileptic syndromes in incident cohorts [18, 27]. The epilepsy types are grouped into three main categories: localization-related, generalized, and undetermined whether localization-related or generalized. On the next level grouping is by etiology: idiopathic, symptomatic, and cryptogenic. On a third level specific electroclinical syndromes are distinguished. In Zarrelli's study [18] localization-related epilepsies constitute 67%, generalized epilepsies 15% and undetermined 18% of all cases. For children <15 years of age the fractions are 55%, 29% and 16% with a higher fraction of generalized epilepsies. Idiopathic epilepsies constitute 7% of all cases and 18% in children [18]. Olafsson [27] and two studies of children [71, 72] show similar figures apart from higher figures for idiopathic syndromes in children (31-47%). Five studies in children and infants [17, 34, 53, 54, 70] focus on specific syndromes according to the 2010 ILAE report. To summarize these studies, West syndrome constitutes around 2% of all incident cases (3-8% of childhood cases and 18-37% in infants), BECT 3% (12-22% in children) and CAE 2% (3-12% in children). In the two most recent studies 35% and 54% of cases respectively, could be assigned to a classified syndrome [34, 70].

2.7 ETIOLOGY

Before the ongoing genetic revolution, the proportion of epilepsy cases with known etiology was unchanged for decades on around a quarter to a third of incidence (and prevalence) cases all ages included [15, 25, 27, 36, 37, 53, 54, 60, 73]. In children the proportion has been a bit lower in the range of 13-30% [15, 27, 36, 71] with known cause. In the 1989 ILAE nomenclature "symptomatic" epilepsy was used for the cases with known underlying etiology as opposed to "cryptogenic" (= underlying but not revealed etiology) and "idiopathic" (= presumed to be genetic with no underlying other condition). The symptomatic category included malformation syndromes due to chromosomal abnormalities, e.g. trisomy 21 as a cause of West syndrome. Otherwise revealed genetic causes have been absent. The percentage of idiopathic epilepsy consisting of the idiopathic focal and generalized epileptic syndromes (BECT, CAE, JAE, JME…) range from 30-47% of all childhood onset cases [71, 72]. In infants the proportion of symptomatic cases is higher. One study showed 54% symptomatic cases in infants [71]. Four later incidence studies using the 2010 or 2017 ILAE etiological classifications, reported known etiology in 52-70% of first year onset cases [17, 34, 54, 70]. Confirmed molecular genetic etiologies have become more frequent as genetic diagnostics are more employed. The very recent nationwide Scottish incidence study reported a

confirmed genetic diagnosis in 133/390 (34%) cases with epilepsy onset before age 3 years [34], of which 33/133 (25%) also had a structural or metabolic etiology. Across all ages as well as in adults and the elderly stroke is the most common structural etiology constituting around 10% of all incident cases followed by brain tumor and head trauma in adults and degenerative disease in the elderly [15, 27, 36]. In children and infants, cerebral malformations and perinatal injury dominate. Hypoxic-ischemic encephalopathy (HIE) is the most common perinatal injury.

Few population-based incidence studies include etiological data on the most important infantile epileptic encephalopathy West syndrome. In five studies the cause is unknown in 22-50% of cases [17, 34, 53, 54, 71].There are quite a few studies of West syndrome etiology in hospital-based cohorts with 9-47% of cases having an unknown cause, most of them within the 20-40% range [74-91]. Cerebral malformations, perinatal injury (HIE, stroke, infection), tuberos sclerosis and Down syndrome are the most common etiologies. The unknown etiology cases have been a focus of the new epilepsy genetics with massively parallel DNA sequencing. This will be discussed below.

2.8 OUTCOME AND EARLY PREDICTORS IN CHILDHOOD EPILEPSY

There are population- as well as hospital-based studies of outcome and prognostic factors in childhood epilepsy. Comparing studies is not easy. Differences in patient selection, outcome variables, definition of seizure remission and drug-resistance, follow-up times for outcome evaluation (at a specific age or at "last follow-up" or at specific times after onset (2, 5, 10, 20 years…)), how patients who died or are lost prior to follow-up are handled in the analysis, choice of potential prognostic factors and how mortality and survival are analyzed and expressed are examples of difficulties. However, multiple studies from Rochester, Connecticut, Nova Scotia, Finland, and the Netherlands give important information on various aspects of short and long-term outcome. Other studies have contributed as well.

Mortality is increased in people with epilepsy. In adults, death rates per 1000 person-years are 2-4 times higher than in the general population [92, 93]. In children with epilepsy the increase is 5-10 times compared to children in general [94-99]. The increased mortality mainly affects patients with neurological disability, drug-resistant epilepsy and epileptic encephalopathies and is high in infantile onset epilepsy [19, 54, 100]. In the idiopathic epileptic syndromes and other epilepsies with seizure control and without comorbidities, mortality is not consistently increased compared to the general population.

Morbidity is usually measured as rates of seizure remission and drug-resistance and neurological and cognitive outcome. In population-based incidence cohorts of childhood-onset epilepsy 60-70% of patients have been seizure free for 5 years or more at follow-up after 10-20 years [22, 98, 101, 102]. About 50-60% are seizure free and off antiepileptic drugs and around a quarter is drug-resistant according to the ILAE definition [103, 104]. There are a few population-based studies of infantile onset epilepsy and seizure outcome, however with differing definitions of remission (6 months to 2 years) and follow-up after various times [19, 34,

[54, 100, 105]. These studies show seizure remission in 48-58% of cases with seizure onset in the first year of life and drug-resistance in 42-43%. In hospital-based cohorts of infantile epilepsy, remission rates are usually lower due to selection of the severest cases [106-111]. Data on West syndrome from incidence cohorts show seizure remission in 28-55% of cases and drug-resistance in 52-63% [19, 34, 105]. In the multicenter United Kingdom Infantile Spasm Study (UKISS) 51% of patients were free of seizures at 4 years-of-age [112].

The most reported negative predictive factor for seizure remission is identified etiology [19, 34, 54, 98, 100-102, 105]. Traditionally this has meant "symptomatic" or "structural-metabolic" etiology, as opposed to when no underlying brain disease or lesion causes the epilepsy. Today genetic etiologies are revealed to an increasing extent [34, 70, 113]. The negative association between known etiology and outcome still stands but has become more complex. Some genetic epilepsies are benign and self-limited, and some are severe developmental and epileptic encephalopathies with a high rate of drug-resistance. Other negative predictive factors mentioned in the scientific literature are developmental delay and/or neurological abnormalities at presentation, multiple seizure types, certain seizure types (atonic, tonic, atypical absence, spasms), high initial seizure frequency, early pharmacoresistance and onset in infancy or after 10 years of age. However, conclusions vary and most of these factors are more or less dependent on etiology.

Cognitive outcome is normal after infantile onset epilepsy in around half of all patients and half of cases have intellectual disability or global developmental delay [19, 34, 54, 100]. For West syndrome cognitive development were normal in 23-43% of cases in two incidence studies [19, 54]. Hospital-based studies have reported normal cognitive outcome in 11 to 46% of all infants and 18 to 39% of West syndrome [74, 82, 84, 87, 89, 90, 114]. As for seizure outcome, identified etiology is the foremost independent factor predicting worse cognitive outcome [19, 34, 54, 100, 115]. Many other factors have been studied with varying results. Whether seizure control and possibly control of interictal epileptiform activity affect cognitive outcome is under debate and relates to the concept of epileptic encephalopathy. Some studies support this notion [34, 54, 100, 112, 116]. The UKISS study suggests that lead time to treatment and treatment choice affects cognitive outcome in West syndrome without proven etiology [112, 116]. Neurological impairments other than intellectual disability are common in infantile onset epilepsy. 22 to 33% of West syndrome cases developed cerebral palsy in hospital-based cohorts [108, 117].

2.9 GENOMICS SURVIVAL KIT FOR NON-GENETICISTS

This is a summary of basic concepts for us non-geneticists. Others, please move on to page 14. As reference see Human Molecular Genetics by Strachan and Read (5th edition), published by Garland Science 2018 [118].

DNA. Deoxyribonucleic acid. The macromolecule that contains and transfers all the inherited information of an organism between generations of living cells and from parent to child. DNA is built of only four different but similar building blocks, *nucleotide bases*. These bases - A, G, C and T - are the only four "letters" in the "alphabet" of the genetic code. All cells of an organism harbor an identical copy of the DNA. The complete inherited information contained in the DNA is referred to as the *genome*. DNA consists of protein coding and non-protein coding parts.

The human genome. The complete inherited information of humans. It consists of 6 billion letters of genetic code, 3 billions inherited from each parent, stored as an equal number of nucleotide bases in the DNA. *Haplogenome* refers to the 3 billion nucleotide bases that we inherit from one parent.

Chromosomes. The human DNA is organized in 23 chromosome pairs (and small amounts in the mitochondria of the cells), one chromosome in each pair from each of the parents. The single sex chromosome pair differs between sexes – females have two X chromosomes and males have one X and one Y chromosome. The 22 autosomal chromosome pairs, number 1 to 22, do not differ between sexes.

Gene. The human genome contains around 20,000 genes. The mean length of our genes is 10,000 nucleotide bases (10 kb). A gene is a blueprint of a protein, encodes a protein, and consists of *exons* – protein coding segments – and *introns* – non-coding segments. The proteins make up the structure of our bodies, regulates all biochemical and physiological processes, give us our characteristics as species and individuals and can cause disease if altered.

Allele. Both our haplogenomes contain all 20,000 genes, which means we have two versions – referred to as alleles – of each gene. The exception is that males have only one allele of the genes on the X chromosome.

Codon. A sequence of three nucleotide bases in the exons of genes that codes for an amino acid or a stop signal. The number of bases divided by 3 make up x number of codons encoding x number of amino acids. Chains of amino acids are the main constituents of proteins.

Exome. The part of the genome that is protein coding. Consists of all the exons of all genes. The human exome makes up only 1-2% of the genome, the rest is non protein coding intergenic and intronic regions.

Non protein coding DNA. Constitutes >98% of the human genome. Contains segments that regulate gene expression and codes for different types of RNAs but most of its function is unknown.

mRNA. Messenger RNA. Contains the information of the exons of a gene and carries this information to the ribosomes of cells where the proteins are built based on this information. *Transcription* is the process by which DNA is read and mRNA synthesized. *Translation* is the process by which mRNA is read and protein synthesized.

Reference genome. A fixed sequence of nucleotide bases that is the most likely version of an organism's genome.

Variant. A position or segment where an individual's nucleotide bases differ from the reference genome. A variant can consist of only one base change – a *single nucleotide variant* (*SNV*), or involve insertion and/or deletion of several bases – referred to as *indels*. SNVs can be *silent* where the same amino acid is encoded, *missense* where the encoded amino acid is changed to another and *truncating* where the codon is changed to a stop codon, which causes the reading of the gene to stop, and the protein becomes incomplete or degraded. *Frameshift* variants are another type of variants that are often truncating. Frameshift means a deletion or insertion of a number of nucleotides that are not dividable by three which means that the reading frame of the chain of nucleotides will be shifted. As a consequence, the protein will be abnormal downstream of the variant and a stop codon is likely to appear. Variants engaging very large segments of DNA are termed *structural variants* (*SV*). A *copy number variant* (*CNV*) is the most common type of SV and means a deletion or duplication of a longer segment (>1 kb) of DNA. *Single nucleotide polymorphisms* (*SNP*s) are SNVs that occur in >1% of the population. The genome of a human individual typically differs from the reference genome in 3 million positions/nucleotide bases, which means we are similar to 99.9% !

Pathogenic variant. A variant that causes disease. Previously termed mutation. Most variants are not pathogenic but part of the normal variation.

Functional effects of variants. *Loss-of-function* (*LoF*) means that the encoded protein has lost its effect. LoF usually implies complete loss of function but sometimes there is a partial loss and reduced function. *Gain-of-function* (*GoF*) means an increased effect of the protein. There can also be an altered biological function of the protein. LoF is the most common mechanism of pathogenic variants, but GoF variants can also be pathogenic. Several severe genetic epilepsies are examples of when GoF variants cause disease.

Inheritance patterns. Autosomal dominant inheritance means that a pathogenic variant in one of the alleles of a gene on chromosome 1-22 can cause disease. For some dominant diseases *de novo* pathogenic variants are a common cause. *De novo* means that a variant has not been confirmed in the parents but is assumed to have occurred in a parental germline cell or early in the fertilized egg. In some autosomal dominant diseases, an individual can carry a

pathogenic variant without developing the related disease. This is called reduced or incomplete penetrance and means that a sick individual can have inherited his/her dominant disease from a healthy parent. The fact that a genetic disease can have different symptoms or severity, even within the same family with a common pathogenic variant, is referred to as variable expression.

For some genetic diseases, both alleles of a gene have to carry a pathogenic variant in order for the individual to have the disease. This is called autosomal recessive inheritance. X-linked inheritance is caused by pathogenic variants in genes on the X chromosome. In a typical X-linked disease mainly males are affected while females are healthy or show milder symptoms.

How to decide whether a variant is pathogenic or not? Is the gene a known disease gene? Does the patient phenotype resemble previously reported cases? Are the gene and gene functions relevant to the patient phenotype? Does the segregation of the variant in the family fit with the inheritance pattern of the disease? What is the population frequency of the variant? What is the variant effect (type of variant), LoF or GoF? Is the variant conserved through evolution? What does variant prediction tools say? Is it a previously described pathogenic variant?

These, and other questions, need to be asked when evaluating a potential pathogenic variant. However, this is a complex and fascinating story of its own and for this I have to refer the interested reader to other sources, like the book used as reference for this survival kit [118].

2.10 THE GENETIC REVOLUTION IN EPILEPTOLOGY

Our basic knowledge of the genetic influence on epilepsy risk stems from epidemiological studies predating the era of molecular genetics. Already in the 1940s Lennox studied epilepsy genetics in a population-based cohort by assessing the risk in relatives of patients and in twins [119]. He showed that there is a significant genetic component behind epilepsy in general and showed that there are genetic factors of different effect size. Even in epilepsy after acquired brain injury (stroke, brain tumor, trauma etc.) the risk in a relative of a patient with epilepsy is increased although it´s higher in non-acquired cases. Early presentation, generalized seizures and female sex of the patient also increase the risk for relatives. A recent epidemiological study from Rochester presented the so far most solid estimates of epilepsy risk in relatives of patients [120]. The cumulative risk at age 40 is increased by 3,3 in epilepsy in general, 7,3 in idiopathic epilepsy and by 7,3 in idiopathic generalized as opposed to 2,0 in idiopathic focal epilepsy.

A DNA sequencing technology was described by Frederic Sanger in Cambridge in 1975 [121]. The first epilepsy gene discovered, and Sanger sequenced in 1994 was *CHRNA4* as a cause of autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) [122]. In the following ten years additional causative genes, mainly ion channel genes, were discovered in monogenic familial epilepsy syndromes [123-125]. This was achieved by sequencing candidate genes derived from linkage analysis in large families, a relatively slow and arduous task [126].

Two new genetic diagnostic tools have entered clinical research and clinical practice during the last 10-15 years: chromosomal microarray technology and massively parallel DNA sequencing, the latter also termed Next Generation Sequencing (NGS). Both increase our capacity to reveal genetic etiologies in epilepsy in a dramatic way. They allow the investigation of sporadic cases and small families as well as large number of patients and/or genes. Also, genetic variants of various effect size, weak risk factors as well as strong variants like in monogenic disease with full penetrance, can be explored.

Through different chromosomal microarray methods like SNP microarray and oligonucleotide microarray, copy number variants (CNVs) have been explored. CNV means a deletion, duplication (or even triplication) or insertion of a small part (>1kb) of a chromosome comprising part of a gene or several genes. CNVs contribute to the genetic variation in humans [127-129]. Some CNVs can also cause disease, usually by haploinsufficiency, as has been shown in neurocognitive and neuropsychiatric disease as well as in epilepsy [130, 131]. In epileptic encephalopathy (mainly West and Lennox Gastaut syndromes), early onset epilepsy and pharmacoresistant epilepsy of previously unknown etiology, rare causative *de novo* CNVs are found in around 5-8% of patients [132-134]. In addition, three recurrent CNVs, microdeletion of 15q13.3, 15q11.2, and 16p13.11, are established as risk factors in 0,5-1% of idiopathic generalized epilepsy [131, 135]. The term structural variant (SV) includes CNVs but also inversions and balanced translocations that can also cause epilepsy by disrupting epilepsy-related genes.

With NGS technology large amounts of DNA can be sequenced in a short time. Today a sequencing instrument can have the capacity of many human genomes per day. Rather than the sequencing speed, the bioinformatic analysis and evaluation of sequencing data have become the time limiting steps. NGS has moved from selected gene panels to whole exome sequencing (the protein coding regions of the DNA is extracted and sequenced) to whole genome sequencing (both coding and non-coding DNA is sequenced) in a few years, all three strategies being clinically available at present.

The main focus of NGS studies so far has been on epileptic encephalopathies and other severe early onset epilepsies. A large number of causative genes have been discovered in recent years. *ARX* [136], *CDKL5* [137], *STXBP1* [138], *PCDH19* [139] and *SCN1A* [140] are examples and the list is growing. Several of the genes encoding GABA receptor subunits are on the list [141-143] as well as genes encoding glutamate receptor subunits [144-147]. At present 100 genes implicated in developmental and epileptic encephalopathies, DEEs (previously termed early infantile epileptic encephalopathies, EIEEs) are listed on the public database Online Mendelian Inheritance in Man (OMIM). The diagnostic yield in epileptic encephalopathy varies between 20 and up to 50% in different studies [88, 132, 148-158] which means a revolution of diagnostics in these severely sick patients. Autosomal dominant *de novo* segregation is the most common pattern in DEE but autosomal dominant inheritance with incomplete penetrance or variable expression also exists as well as recessive, x-linked and mitochondrial inheritance. In *de novo* cases the recurrence risk is slightly increased due to potential parental mosaicism [159-161].

Also, in less severe autosomal dominant familial epilepsies a number of disease genes have been described [162, 163], usually with incomplete penetrance. Some of these genes are shared with the DEEs, which can make prognostication difficult with phenotypes of highly variable severity occurring even within the same family [164-166].

Some studies have also been performed on the more common and less severe idiopathic epilepsies with mostly complex/polygenic etiology. Quite a few susceptibility loci and genes have been described in idiopathic generalized epilepsy and recently also in idiopathic focal epilepsy [167-171]. Monogenic etiology is rare in idiopathic generalized epilepsy but seems to be more common in idiopathic focal epilepsy, with *GRIN2A* being the most evident example so far [172-174]. The capacity to investigate very large cohorts of several thousands of patients have begun to reveal a complex genetic architecture with an increased burden of both ultrarare and common variants in common as well as rare epilepsies including in epilepsies considered to be monogenic or acquired [131, 168-170, 175].

To summarize the present knowledge, an autosomal dominant *de novo* pattern is the most common in DEEs. Therefore, trio sequencing (of proband and parents) increases sensitivity and specificity. Interpretation of variants is complicated by the existence of incomplete penetrance, variable expression, and parental mosaicism. Idiopathic generalized epilepsies usually have a complex and oligogenic etiology with CNVs, single nucleotide variants (SNVs) and indels as risk factors, but rare monogenic families have been described. The

same goes for idiopathic focal epilepsies but with monogenic inheritance, with incomplete penetrance being more common. Heterogeneity from genotype to phenotype (the same gene being implicated in different epilepsy phenotypes of varying severity) and from phenotype to genotype (one phenotype having several alternative genetic etiologies) is pronounced. There is also a significant overlap of genetic variants causing neurocognitive, neuropsychiatric, and epileptic disorders which is reflected in the high rate of comorbidity between these disorders.

A confirmed molecular genetic diagnosis in epilepsy has value in relation to information, prognosis, genetic counselling, and prenatal genetic diagnostics. In some cases, there are treatment implications in terms of specific diets or choice of antiepileptic drugs and the list of genetic etiologies affecting treatment choice is growing [158, 176-178]. A genetic diagnosis can also inform decisions related to epilepsy surgery [179]. Last but not least, the development of new more effective treatments, targeted at specific disease mechanisms and including but not limited to gene therapy, is ongoing. This development, referred to as precision medicine, promises to make life better for the one third of all epilepsy patients that are drug-resistant and for those who suffer from adverse effects of antiseizure medications [180, 181].

3 RESEARCH AIMS

The overall aim of this thesis was to describe and analyze the incidence, phenotypes, etiologies, and outcome of infantile-onset epilepsy from a population-based perspective and to investigate if new disease genes could be uncovered.

The specific aims of the individual studies were:

- To describe the phenotypes in a population-based cohort of children with epilepsy onset during the first 2 years of life, according to the latest ILAE classifications of epileptic seizures and epilepsy syndromes (**paper I**).
- To calculate incidence rates, overall and for specific epilepsy syndromes, in the same population-based cohort (**paper I**).
- To analyze etiologies in this same cohort and to explore to what degree the use of massively parallel DNA sequencing in cases of unknown cause can increase the diagnostic etiological yield (**paper I**).
- To describe outcome at age 7 years in terms of neurodevelopmental comorbidities and seizures in the same population-based cohort, and to analyze factors associated to outcome (**paper II**).
- To uncover and describe new disease genes by the use of massively parallel DNA sequencing (**paper III-IV**).

4 PATIENTS AND METHODS

For more detailed information on methodologies please see Papers I-IV.

4.1 STUDY COHORT

The study cohort of **paper I-II** was defined as all children having a first unprovoked epileptic seizure before age 2 years, between September 1, 2001 and December 31, 2006, while living in the study area of Northern Stockholm and who met epilepsy criteria before age 7 years.

For **paper III** the two Swedish patients with epilepsy of infancy with migrating focal seizures (EIMFS) came from the population-based cohort of paper I-II and have the first author of paper III as the main responsible pediatric neurologist. The two British patients were identified through an international collaboration and had previously been recruited to a United Kingdom EIMFS genetic study.

The proband described in **paper IV** was managed at the Karolinska University Hospital with the first author of paper IV as the responsible pediatric neurologist. Together with her deceased older sister she was recruited for the paper IV case study.

4.2 DATA COLLECTION

Paper I-II. Potential incident cases for the study cohort were retrieved from the prospective Stockholm Incidence Registry of Epilepsy (SIRE). As has been previously described SIRE aimed to register all cases in Northern Stockholm of a first unprovoked epileptic seizure leading to medical attention (termed the index seizure) from September 1, 2001[36]. The electronic medical records of all children with their index seizure before age 2 years and between September 1, 2001 and December 31, 2006, were reviewed in detail. In cases where information was lacking, parents were contacted by letter and after consent had been received, a structured telephone interview was conducted. Of all potential study subjects retrieved from SIRE, the cases who met epilepsy criteria before age 7 years were defined as the study cohort. Seizures during the neonatal period (the first 4 weeks of life), typical febrile seizures and symptomatic seizures during acute illness like the acute phase of encephalitis, stroke etc. were considered provoked and therefore not the basis for epilepsy diagnosis. Data on potential risk factors, work-up, seizures, epilepsy syndromes, etiologies, treatments, and comorbidities up to age 7 years were compiled as well as whether the child attended mainstream or special education school at age 7. Genetic etiologies were included also when revealed after 7 years of age. In cases of unknown etiology, additional genetic work-up was offered when clinically indicated and results were included in the study.

For the study subjects of **paper III** clinical data were compiled from medical records and multiple hospital visits at the Karolinska University Hospital and the Royal Manchester Children's Hospital.

For **paper IV** clinical data were gathered from medical records and multiple visits at the Karolinska University Hospital.

4.3 DIAGNOSIS AND CLASSIFICATION

The diagnosis of epilepsy and the classification of seizure types, epilepsy syndromes and etiologies in the study subjects of **paper I-II** were done according to the recommendations of the International League Against Epilepsy (ILAE) by two experienced pediatric neurologists among the authors [7-10]. Cases were discussed until consensus was reached.

The diagnoses and classification of the rare epilepsy syndrome EIMFS in the four patients of **paper III** were done by the two first authors.

The phenotype of the two subjects of **paper IV** did not match any known published phenotype at the time when investigations were performed and data for this paper were compiled. The study subjects did not have epilepsy but due to the revealed etiology's close molecular relation to the etiology and disease mechanism described in paper III they were included in this thesis.

4.4 MOLECULAR GENETIC ANALYSES

For **paper I**, children in the study cohort with unknown etiology and drug-resistant epilepsy or neurological comorbidities were offered *whole exome* (*WES*, until 2014) or *whole genome* (*WGS*, from 2015) *sequencing*. Cases with structural or metabolic etiology with a suspected but not confirmed molecular genetic etiology were also included. WES and WGS were performed on the Hiseq X Ten or the Illumina NovaSeq 6000 platforms after sequencing libraries had been prepared using a PCR–free paired-end protocol (Illumina TruSeq DNA PCR-free for >1000 ng input). Trios (child and parents) were sequenced. Sequencing data were analyzed with the in-house developed Mutation Identification Pipeline (MIP) that ranks variants and their potential pathogenicity based on multiple factors including inheritance pattern, population frequency, conservation, and protein damage prediction [182]. In a first step pre-compiled lists of known genes for epilepsy and metabolic disease were analyzed. If no genetic diagnosis was revealed, individualized Human Phenotype Ontology (HPO) generated gene lists were created and analyzed, and in some cases the whole exome/genome was explored for new disease genes after consent had been obtained from parents. Findings were confirmed using *Sanger sequencing*.

The two Swedish patients in **paper III** were investigated with WES as part of the population-based study in paper I. The whole exome was explored for pathogenic variants. On the two British patients from a consanguineous family, homozygosity mapping using SNP genotyping was undertaken and WES was performed. Exome data in the homozygous regions were explored for homozygous pathogenic variants. The findings in all four patients and their parents were confirmed with Sanger sequencing.

Paper IV. WGS was performed on the proband, her diseased older sister, her healthy older brother and both their parents, using a HiSeq X sequencing instrument and a PCR-free library preparation method. Data were analyzed using MIP and findings were confirmed with Sanger sequencing. PCR amplification and Sanger sequencing of complementary DNA were used to

assess the effect of the variants on RNA level, using RNA obtained from patients' and parents' fibroblasts.

4.5 FUNCTIONAL STUDIES OF *SLC12A5* VARIANTS (PAPER III)

Homology modelling of the potassium-chloride cotransporter 2 (KCC2). To predict damaging effects on the structure-function properties of KCC2 of the variants found in *SLC12A5*, a homology model of KCC2 was created using multiple resources and software (HHPRED, MODELLER-9v10, ConSurf, DOPE, ProSA, Chimera, Dunbrack backbone-dependent rotamer library).

Electrophysiological voltage-clamp studies. To assess the chloride export function of wildtype and mutant KCC2, HEK293 cells were transfected with enhanced green fluorescent protein (eGFP) and the glycine receptor (GlyR) α2 subunit, which forms homopentameric glycine-gated chloride channels. Voltage clamp recordings were performed on eGFP positive cells while glycine was applied at different holding potentials. Recordings were also done on cells transfected with plasmids encoding wildtype KCC2 or any of the three mutant KCC2 from the patients.

Immunoblotting studies. To compare the expression of cell surface vs total cell KCC2 and glycosylated vs unglycosylated KCC2 in wildtype and mutant KCC2, an *in vitro* heterologous expression system for immunoblotting and surface protein biotinylation was used. LLC-PK cells were transfected with plasmids encoding Myc-tagged wildtype and mutant KCC2. After 24 hours surface proteins were labelled with biotin and cells were lysed. Biotinylated fraction and total cell lysates were eluted, proteins were separated on a gel and blotted with anti-myc antibodies.

Immunofluorescence microscopy studies. To further visualize the expression and localization of wildtype and mutant KCC2, HEK293 cells were transfected with plasmids encoding FLAG-tagged KCC2 (wildtype and mutant), incubated with anti-FLAG antibodies and studied by immunofluorescence microscopy.

Zebrafish KCC2 knock-out model and behavioral analysis. Genome editing was undertaken to generate KCC2 knockout zebrafish embryos. At day two post fertilization the *escape swimming response* of the wildtype and KCC2 knockout embryos was studied.

4.6 STATISTICS

Paper I. Descriptive statistics were applied to describe the study cohort and differences between age groups were analyzed with the chi-square test at a significance level of $p<0.05$. Population figures on December 31, 2001-2006 were retrieved from Statistics Sweden. Northern Stockholm is an urban area with approximately one million inhabitants during the study period. Of these, 2.6% were below age 24 months and 1.3% were below 12 months. Approximately 13 000 children were born each year in the area. Overall and subgroup incidences were calculated by dividing the number of index cases by the number of person-

years at risk. The 95% confidence intervals (CI) were calculated by applying the exact method for single proportions.

Paper II. The outcomes for different subgroups of the study cohort were analyzed as proportions in percentage. Factors potentially associated to outcome were univariably analyzed with relative risks (RR) using likelihood-ratio test and 95% confidence intervals (CI). A multivariable log binomial regression analysis was performed using R free software to assess the independent effects of factors showing significance in the univariable analysis.

In **paper III-IV** statistical analyses were not performed.

4.7 ETHICAL APPROVALS AND CONSIDERATIONS

The studies in this thesis were approved by the Regional Ethical Review Board at the Karolinska Institutet, Stockholm (diary numbers 212/533-31/1 (paper I-II) and 2008/351-31 (paper III-IV)).

Ethical considerations in everyday pediatric neurology healthcare. When reflecting upon ethical aspects of the studies in this thesis, it can be of value to consider the ethics of everyday pediatric neurology as a background. Ethical questions and dilemmas are frequent in clinical pediatric neurology and epileptology. There are several ethical issues related to treatment. One is the balance between treatment effect and adverse reactions. Antiseizure medications commonly have both good and bad effects. To evaluate this can be challenging, especially in young children and older children and adolescents with impairments affecting cognition and communication. The frequent use of medications off label in pediatric neurology and epileptology is a related treatment issue. In very difficult situations like sometimes in the more severe epilepsy syndromes, with drug-resistance and high seizure frequency, both healthcare and parents for understandable reasons tend to be more prone to try experimental treatments. Sometimes this is successful but sometimes unexpected adverse effects occur. And the patients often cannot express their own view. Another ethical treatment issue concerns treatment restrictions and when to change focus to palliative care and quality of life in progressive diseases. Severe neurological conditions with causes that in themselves are static, commonly show a slowly progressive course which sometimes make decisions on appropriate “aggressiveness” of care difficult.

Also, when deciding on investigations it has to be considered whether the results will actually benefit the care of the patient in a meaningful way. The fact that the cause of disease hitherto has been unrevealed in a majority of all individuals with epilepsy, including in children, can be a chronic stress factor. Especially in severe disease like the epileptic encephalopathies this can result in longstanding crisis, feelings of guilt and lack of trust. From this perspective searching for and finding the cause of disease can be a relief for the affected children and their families. On the other hand, the chronic stress of “not knowing” can make it difficult to “accept things as they are” and result in an endless journey of investigations with very little chance of revealing the disease cause or affecting treatment.

Dependency is a factor to consider from ethical perspective. Children are dependent on their parents/caregivers and sick or neurodevelopmentally impaired children are even more dependent. Parents of sick children are dependent in relation to healthcare. This must be treated with sensitivity and respect. The health and needs of the child patient are the main focus of healthcare but the parents, and other caregivers, are main sources of information on which medical decisions are based and they are main resources when it comes to realizing plans for nursing and medical treatment. A working alliance between healthcare and parents and other caregivers is necessary for the best possible treatment and quality of life outcome. When deciding on investigations and treatment the ambition is always consensus with well-informed parents, and the child if he/she is able to understand. However, in the end it is the responsibility of healthcare to respectfully decide what is the appropriate treatment. The few times when one as a healthcare worker experiences that parents are not able to see or meet the needs of their child in a reasonable way pose ethical and other challenges that can be very difficult to deal with. Fortunately, in my own experience, these situations are rare, and more than often I have been touched and impressed by how parents of severely sick children manage.

Ethical considerations in relation to the studies of the thesis. Coming back to the studies of this thesis they do not include any therapeutic interventions and thus pose no ethical problems related to that. On the contrary, a main objective of this thesis has been to explore etiology and disease mechanisms. This will hopefully make possible more specific treatments directed at these disease mechanisms, and therefore more effective and less prone to cause adverse effects. In terms of investigations performed as part of the studies they have been recommended fully on clinical grounds and include mainly genetic diagnostics through a simple blood sample.

Genetic diagnostics carry the risk of getting to know something that one has not asked for and do not wish to know. This is managed in several ways. First, genetic work-up has only been offered and performed in cases of severe disease where it is already a fact that something "bad" has happened. Getting a genetic confirmation that one's child has a progressive disease can of course cause severe distress and sorrow, but in the end, it is usually better to know the cause of what is going on. Second, in the clinical test only genes related to the phenotype of the patient are analyzed. This reduces the risk of unwanted information, for example confirmation of a high penetrance risk variant for a severe disease with onset later in life and for which there is no preventive or effective treatment. In cases like those described in paper III-IV where the genetic analysis is expanded to include the whole exome or genome with genes not known to be related to the patient's phenotype or not known to be disease-related at all, the families must be well informed beforehand about the risk of coincidental genetic findings. Most families with a severely sick child are more than willing to take that risk.

A genetic diagnosis makes possible genetic counselling, carrier screening and prenatal diagnostics. In some cases, the nature of the pathogenic variant brings ethical issues in

relation to this. How to view prenatal diagnostics when the revealed disease has significantly reduced penetrance or highly variable expression is one such situation.

There are overall ethical aspects of being a study subject. The families of the children in the study cohort of paper I-II were not asked for informed consent, when they were not specifically contacted about the study and data were compiled only from the medical records. This is in accordance with the ethical approval and is based on the notion that the potential value of the research for other and future patients overrides the subjects' need to know that they are part of a study. All families that were contacted about clinical information to complement the medical records and those who were offered genetic testing were first asked for informed research consent. Considering the dependency that families might experience in relation to the healthcare, steps were taken to make it very clear that participation was voluntary and to make it easy to say NO, for instance by emailing the investigator before any telephone contact. In some cases where the children in the population-based cohort were dead since many years and the parents had not had contact with the hospital for long, the decision was made not to contact the parents to not risk creating emotional distress. These decisions were not easy since it could have been that these parents would have experienced a contact from the investigator as something positive and meaningful.

5 RESULTS

5.1 PAPER I-II: A POPULATION-BASED STUDY OF INCIDENCE, PHENOTYPES, ETIOLOGIES AND OUTCOME IN EARLY-ONSET EPILEPSY

5.1.1 The study cohort: incidences, background characteristics and work-up

In SIRE, 163 children were registered as having had their index seizure before age 2 years during the study period. After review of all available data in electronic medical records and from complementary telephone interviews with six families, 116 cases were assessed to fulfill diagnostic criteria of epilepsy before age 7 years, of which 88 had their first seizure during the first year of life. The remaining 47 children did not fulfill epilepsy criteria (see Figure 1). Calculated incidences per 100,000 person-years were 139 (95% CI 112-171) for first year seizure onset, 42 (95% CI 29-61) for second year onset and 88 (95% CI 72-107) for the whole cohort with onset before age 2 years. The age distribution is shown in Figure 2.

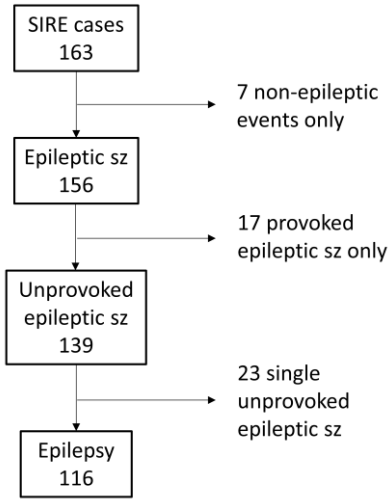

Figure 1. Classification of 163 cases retrieved from the Stockholm Incidence Registry of Epilepsy (SIRE) after review of all available information up to age 7 years.

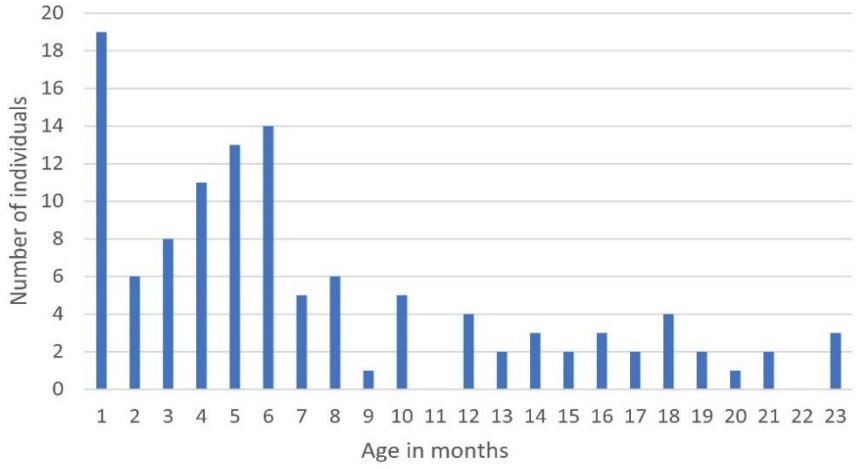

Figure 2. Age distribution of seizure onset.

Background characteristics are displayed in Table 4. Males and females were equally represented. Having a febrile seizure before the first afebrile seizure and having a first degree relative with febrile seizures were more common in second year onset cases. Developmental delay at epilepsy onset was present in 40% of all cases and in 52% of children with West syndrome.

Table 4. Background characteristics in 116 cases of epilepsy with onset before 2 years				
	Onset age <12 months, n (%)	Onset age >12 months, n (%)	Age difference, p-value, significance at p<0.05	All cases, onset age <24 months, n (%)
All	88/88 (100)	28/28 (100)		116/116 (100)
Gender, female	45/88 (51)	12/28 (43)	Ns	57/116 (49)
Epilepsy 1st degree relative	18/88 (20)	1/27 (3.7)	ns, p=0.08	19/115 (17)
Febrile sz 1st degree relative	2/83 (2.4)	22 (5/23)	p=0.005	7/106 (6.6)
Type of birth: sectio	24/86 (28)	5/28 (18)	Ns	29/114 (25)
Gestation: <37 weeks	7/88 (8.0)	2/28 (7.1)	Ns	9/116 (7.8)
Birth weight: <2,5 kg	7/83 (8.4)	2/24 (8.3)	Ns	9/107 (8.4)
Acute symptomatic seizures	16/88 (18)	4/28 (14)	Ns	20/116 (17)
Febrile before afebrile seizure	8/88 (9.1)	15/28 (54)	p<0.001	23/116 (20)
Developmental delay at onset	34/88 (39)	12/28 (43)	Ns	46/116 (40)

Table 4. Onset refers to the first unprovoked seizure. n = number of cases (% of all cases). Difference between age groups: Chi2-test with Yates correction. ns = non-significant =p>0.05.

In terms of work-up, MRI of the brain was performed in 49% of cases, CT in 78% and any neuroimaging in 90%. WES or WGS was done in 26/116 cases (22%) and 47% of children had some kind of genetic investigation. All children went through at least one wake or sleep EEG. The highest diagnostic yields were seen in MRI 65% and WES/WGS 58%.

5.1.2 Seizure types and epilepsy syndromes

The seizure registered as index seizure in SIRE was the first unprovoked seizure (= onset seizure) in 102/116 cases. The remaining 14 children had their first unprovoked seizure on average 9 weeks before the index seizure. The onset seizure type was focal in 46%, generalized in 6%, spasms in 21% and unclassified in 28% and the mean age at onset was 7.4 months (median 6, range 1-23). The second unprovoked seizure occurred on average 0.5 months after the onset seizure. In seven children, the onset seizure was a status epilepticus and in six of these cases it was febrile. Another nine children had status epilepticus later in the disease course. Periods of high seizure frequency were common. At least one period of at least 4 weeks of daily seizures occurred in 64% of cases before age 7 years.

An epilepsy syndrome was diagnosed before 7 years of age in 54% of children as displayed in Table 5. This corresponds to a birth prevalence of 1/1100 live born children. West syndrome was the most frequent epilepsy syndrome constituting 35% of first year onset cases and having a birth prevalence of 1/2100. The next common syndrome was self-limited (*benign* according to previous nomenclature) infantile epilepsy. Lennox-Gastaut syndrome was seen in 10 cases of which 8 had previously had West syndrome.

Table 5. Epilepsy syndromes diagnosed before age 7 years				
Epilepsy syndrome	Onset age <12 months n (%)	incidence (95% CI)	All cases, onset age <24 months, n (%)	Birth prevalence
West syndrome	31 (35)	49 (34-69)	33 (28)	1/2,100
Lennox-Gastaut syndrome	10 (11)	16 (8-29)	10 (8.6)	1/6,900*
EIMFS	2 (2.3)		2 (1.7)	
Dravet	1 (1.1)		1 (0.9)	
GEFS+	0		4 (3.4)	
Myoclonic atonic epilepsy	0		1 (0.9)	
Benign familial infantile epilepsy	5 (5.7)	7.9 (3.3-19)	5 (4.3)	1/13,900
Benign infantile epilepsy	13 (15)	20 (12-35)	13 (11)	1/5,300
Benign neonatal epilepsy	1 (1.1)		1 (0.9)	
Myoclonic epilepsy in infancy	1 (1.1)		1 (0.9)	
Any epilepsy syndrome	56 (64)	88 (68-115)	63 (54)	1/1,100*
No syndrome	32 (36)	50 (36-71)	53 (46)	
Total	88 (100)	139 (112-171)	116 (100)	

Table 5. Epilepsy syndromes diagnosed before age 7 years. Onset refers to the first unprovoked seizure, not necessarily syndrome onset. CI, confidence interval; EIMFS, epilepsy of infancy with migrating focal seizures; GEFS+, genetic epilepsy with febrile seizures plus; n, number of cases (% of all cases). Incidence: cases/100 000 person-years. Birth prevalence rounded to nearest hundred. *Potentially underestimations because Lennox-Gastaut syndrome and some other syndromes may have seizure onset >24 months of age.

5.1.3 Etiologies

The etiologies revealed in 59% of the study cohort were diverse and are displayed in Table 6. The most prevalent types of etiology were structural (34%) and confirmed genetic (29%) with some overlap since 10/39 structural cases also had a confirmed genetic cause of the structural lesion. Cerebral malformations (14%), perinatal asphyxia (8,6%), metabolic disease (8,6%) and monogenic epilepsy without a structural or metabolic disorder (10%) were the most frequent more specific types of etiology. As shown in Table 4 of paper I, monogenic etiology was confirmed in 28 cases from 23 families with pathogenic variants spread across 21 genes. Only two genes had disease causative variants in more than one family (*STXBP1* and *TSC2* in two families each). Of the 28 cases, nine were also structural and seven were metabolic. The most common inheritance pattern was autosomal dominant in 11 families (*de novo* in nine) while inheritance was recessive in eight and X-linked in four. Six children had chromosomal abnormalities as etiology. If presumed genetic etiology was included (cases of tuberous sclerosis complex, metabolic disease, and familial epilepsy syndromes without molecular confirmation) altogether 40% of the study cohort had a genetic etiology and any type of cause was known in 65% of all cases.

In West syndrome, the etiology was revealed in 73% of cases, being structural in 42% and confirmed genetic in 33%. West syndrome occurred in 4/4 Down syndrome cases, 3/5 cases of tuberous sclerosis and in 4/10 children with perinatal asphyxia.

Table 6. Etiology in 116 cases of epilepsy with onset before 2 years				
Etiology	Onset age <12 months, n (%)	Onset age >12 months, n (%)	All cases, onset age <24 months, n (%)	West syndrome n (%)[%][9]
Structural[1]	29 (33)	10 (36)	39 (34)	14 (42)[36]
Cerebral malform.[2]	11 (13)	5 (18)	16 (14)	4 (12)[25]
Tuberous sclerosis[3]	5 (5.7)	0	5 (4.3)	3 (9.1)[60]
Asphyxia	8 (9.1)	2 (7.1)	10 (8.6)	4 (12)[40]
Stroke	4 (4.5)	1 (3.6)	5 (4.3)	2 (6.1)[40]
PVL/PVH preterm	1 (1.1)	0	1 (0.9)	1 (3.0)[100]
Trauma	0	2 (7.1)	2 (1.7)	0
Metabolic disease[4]	9 (10)	1 (3.6)	10 (8.6)	3 (9.1)[30]
Infectious	3 (3.4)	0	3 (2.6)	1 (3.0)[33]
Immune	0	0	0	0
Underlying condition[5]	41 (47)	11 (39)	52 (45)	18 (55)[35]
Genetic, confirmed and no underlying condition[6]	14 (16)	3 (11)	17 (15)	6 (18)[35]
Monogenic epilepsy	9 (10)	3 (11)	12 (10)	2 (6.1)[17]
Chromosomal syndr.	5 (5.7)	0	5 (4.3)	4 (12)[80]
Known, total	55 (62)	14 (50)	69 (59)	24 (73)[35]
Unknown	33 (38)	14 (50)	47 (41)	9 (27)[19]
Total	88 (100)	28 (100)	116 (100)	33 (100)[28]
Genetic, confirmed with structural/metabolic	16 (18)	1 (3.6)	17 (15)	5 (15)[29]
Genetic, all confirmed	30 (34)	4 (14)	34 (29)	11 (33)[32]
Genetic, presumed[7] Structural/metabolic Familial syndrome	7 (8.0) 5 (5.7) 2 (2.3)	5 (18) 1 (3.6) 4 (14)	12 (10) 6 (5.2) 6 (5.2)	2 (6.1)[17] 2 (6.1)[33] 0
Genetic, confirmed and presum. no underlying[8]	16 (18)	7 (25)	23 (20)	6 (18)[26]
Genetic, all confirmed and presumed	37 (42)	9 (32)	46 (40)	13 (39)[28]
Known total, including presumed genetic	57 (65)	18 (64)	75 (65)	24 (73)[32]

Table 6. [1]10/39 confirmed genetic cause. [2]8/16 confirmed genetic. [3]2/5 confirmed genetic. [4]7/10 confirmed genetic. [5]Structural+metabolic+infectious+immune. [6]no underlying structural or metabolic condition. [7]cases of tuberous sclerosis (3), metabolic disease (3), BFIE (2) and GEFS+ (4), without revealed mutations. [8]confirmed genetic without underlying structural or metabolic condition plus familial cases of BFIE and GEFS+, for comparison with other studies. [9][%] = % of etiology.

5.1.4 Treatment and seizure outcome

At age 7 years a majority (91%) of the children had been treated with on average 3.8 (median 3, span 1-14) antiseizure medications (ASMs). Half (51%) of all children had tried more than two ASMs and in 41% of the cases polytherapy with 3-6 drugs simultaneously was tried at some point. Half (49%, 51/105) of the children being alive at age 7 years were still on one or more ASM. In terms of non-pharmacological treatment five children had tried ketogenic diet, two had VNS treatment and in two cases epilepsy surgery was performed.

The outcomes at age 7 for the most common etiologies and major etiology types are summarized in Table 7. Half (53%) of the study cohort had been seizure free for 2 years or more at age 7 and a third (32%) for less than 6 months. West syndrome hade a similar seizure

outcome with the corresponding figures 58% and 33%, respectively. Of all cases, 47% were seizure free and off ASMs at age 7 and for West syndrome this proportion was 45%. Perinatal asphyxia had the worst seizure outcome and unknown etiology the best.

Table 7. Outcome at age 7 years by etiology						
	Intellectual disability	ASD	Cerebral palsy	No NDV	Seizure free>2y[2]	Sz free and no NDV
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Cerebral malformation, n=16	13 (81)	2 (12)	11 (69)	1 (6)	3 (19)	2 (12)
Perinatal asphyxia, n=10	8 (80)	0 (0)	9 (90)	0 (0)	1 (10)	0 (0)
Metabolic disease, n=10	10 (100)	0 (0)	10 (100)	0 (0)	2 (20)	0 (0)
Tuberous sclerosis, n=5	3 (60)	3 (60)	0 (0)	0 (0)	1 (20)	0 (0)
Stroke, n=5	3 (60)	0 (0)	3 (60)	2 (40)	3 (60)	2 (40)
Struct/Met/Inf etiology, n=52	40 (77)	5 (10)	36 (69)	6 (12)	13 (25)	6 (12)
Genetic etiol, no s/m[1], n=17	12 (71)	2 (12)	2 (12)	4 (24)	12 (71)	4 (24)
Unknown etiology, n=47	5 (11)	6 (13)	0 (0)	32 (68)	36 (77)	30 (64)
All cases, n=116	57 (49)	13 (11)	38 (33)	42 (36)	61 (53)	40 (34)

Table 7. ASD= autism spectrum disorder. NDV=neurodevelopmental problems, includes intellectual disability, ASD, cerebral palsy, ADHD, other learning difficulties, other motor impairments. [1]molecularly confirmed genetic etiology without structural or metabolic abnormality. [2]Seizure free for 2 years or longer at age 7.

5.1.5 Outcome in terms of comorbidities and educational support needs

Altogether at death or 7 years of age, 42 children (36%) had no documented neurodevelopmental problems and in the remaining majority the comorbidities diagnosed were intellectual disability (ID) in 49% (57/116), autism spectrum disorder (ASD) in 11%, cerebral palsy in 33% and other neurodevelopmental problems (ADHD, learning difficulties, motor impairments) in 9%. The corresponding figures for West syndrome were 70%, 18%, 39% and 9%. Table 7 shows the outcomes by etiological subcategories. One third (34%) of all cases and 15% of the West syndrome cases were "healthy" from a neurological viewpoint, meaning they had no neurodevelopmental problems and were seizure free for 2 years or more at age 7.

The educational needs of the children at 7 years of age were met by special education school in 37% (43/116) of cases and mainstream school with (19 cases) or without (43 cases) extra individualized support in 53%. In the West syndrome subgroup, 58% (19/33) attended special education and 36% mainstream school.

5.1.6 Factors associated to outcome

To assess what factors affect or can predict outcome, 18 potential predictors and eight outcome variables were analyzed in a univariable analysis (RR using likelihood-ratio test and 95% CI). For details see Table S2 of paper II for all variables and case numbers and Table 2 of paper II for the results. Six of the potential predictors, *Developmental delay at epilepsy onset*, *Daily seizures for >4 weeks*, *Status epilepticus*, *Early EEG abnormality*, *Structural/metabolic/Infectious etiology* and *Known etiology* (also including confirmed and presumed genetic etiology), showed statistical significance ($p<0.05$) for the highest number (six to seven out of eight) of outcome variables. Due to small case numbers, specific

individual etiologies were not included as predictors. *Onset<4 months* was significantly associated only to cerebral palsy and *Male sex* only to ASD.

A multivariable log binomial regression analysis was performed on nine predictors (showing significance in the univariable analysis) in relation to six outcome variables. For further explanation and detailed results see Table 4 of paper II. *Developmental delay at epilepsy onset*, *Daily seizures for >4 weeks* and *Structural/metabolic/infectious etiology* showed the strongest overall association to outcome as displayed in Table 8. In addition, *Status epilepticus* was significantly associated to seizure outcome. *Neonatal disease*, *Onset<4 months* and *West syndrome* did not show independent association to any of the six outcome variables.

Table 8. Results of multivariable log binomial regression analysis for 3 major predictors						
Predictor →	**DD at ep onset**		**Daily sz >4 weeks**		**Struct/metab/inf etio.**	
Outcome↓	RR (CI)	p	RR (CI)	p	RR (CI)	p
ID	1.67 (1.17-2.81)	0.001	2.56 (1.27-5.56)	0.008	1.49 (1.09-2.44)	0.006
CP	2.93 (1.69-6.48)	<0,001		ns	13.78 (4.35-83.96)	<0,001
Sz free 2y		ns	0.76 (0.52-0.96)	0.02	0.53 (0.31-0.81)	<0,001
Sz free 2y, no ASM	0.57 (0.25-0.96)	0.03	0.71 (0.45-0.93)	0.01	0.55 (0.26-0.94)	0.02
No NDV, sz free	0.08 (0.00-0.36)	<0,001	0.57 (0.25-1.00)	0.048		ns
Mainstream school	0.43 (0.22-0.73)	<0,001	0.60 (0.38-0.86)	<0,001		ns

Table 8. Multivariable regression analysis. Significant predictors. ASM=antiseizure medication. CI=95% confidence interval. CP= cerebral palsy. DD=developmental delay. Ep=epilepsy. ID=intellectual disability. ns=not significant. NDV=any neurodevelopmental problem. RR=relative risk. Sz=seizure. y=years.

5.2 PAPER III-IV: TWO NEW CLOSELY RELATED DISEASE GENES

5.2.1 Mutations in *SLC12A5* in epilepsy of infancy with migrating focal seizures (paper III)

Two years apart and at around the same age of 3-4 months, two siblings, a boy and his younger sister (Figure 3) presented at the Karolinska University Hospital with focal seizures. Within a couple of weeks from onset both siblings developed a highly drug-resistant epilepsy (DRE) with an electroclinical phenotype compatible with epilepsy of infancy with migrating focal seizures (EIMFS). The parents were healthy, non-consanguineous and of Caucasian ethnicity.

Figure 3. Photo of the two Swedish siblings (Family A II:1 and II:2) used with consent from the parents.

The neurodevelopment of the siblings was normal prior to seizure onset but then stagnated. They were both later diagnosed with intellectual disability, are non-verbal and have motor impairments.

Whole exome sequencing (WES) was performed in the siblings and both parents. After known disease genes had been found negative for pathogenic variants, the rest of the genome was analyzed. Two potentially disease-causing missense variants were detected in *SLC12A5* as both siblings were compound heterozygotes for c.1277T4C (L426P) and c.1652G4A (G551D) and the parents were heterozygous carriers (Figure 4). *SLC12A5* encodes the potassium-chloride cotransporter 2 (KCC2) which is exclusively expressed in neurons.

Through an international network of pediatric neurologists and researchers a pair of siblings from a UK EIMFS cohort were identified. These two children from a consanguineous Pakistani family were found to be homozygotes for the variant c.932T4A (L311H) in *SLC12A5* (Figure 4).

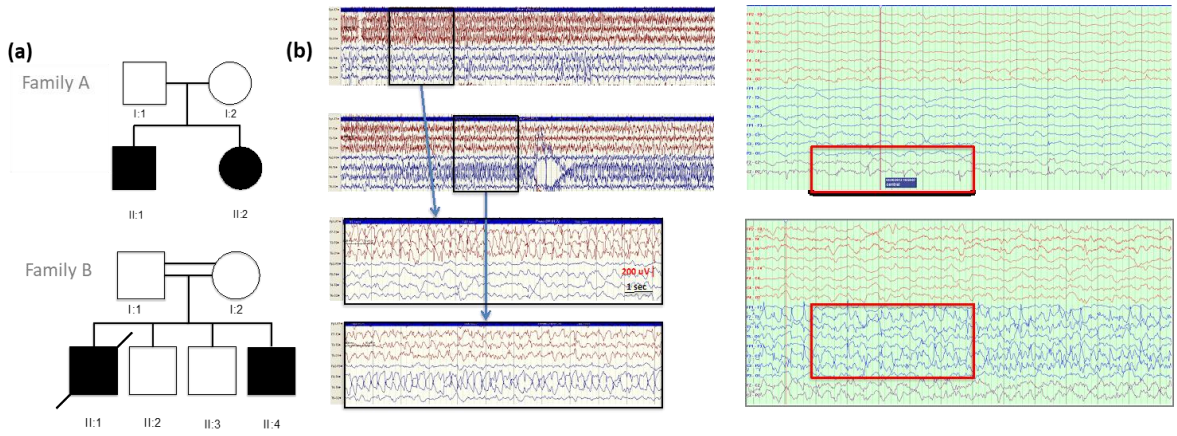

Figure 4. a: Family trees of EIMFS kindreds. Family A (Caucasian origin) and Family B (Pakistani origin). Squares represent males. Circles represent females. Affected individuals are represented by black shading. Double parallel horizontal bars indicate consanguinity in Family B. b: EEG recordings from affected patients. *Left upper and lower EEG recording:* Two consecutive EEG segments, each 1 minute long, from the ictal EEG of proband A-II:1. Seizure activity is boxed in black with rhythmic spike-waves evident initially on the left hemisphere (red), then fading and seizure activity starting up in the right (blue) temporal area. Expanded segments below (10 sec) show the spike-wave activity in more detail. Montage: Longitudinal bipolar according to the 10-20 system. Only lateral channels shown. *Right upper and lower EEG recording:* Two examples revealing central/vertex epileptiform activity (purple and boxed in red, upper right figure) associated with unresponsiveness that wanes and is immediately followed by left centro-temporal ictal onset associated with facial twitching (blue and boxed in red, lower right figure).

To support the variants as the cause of EIMFS in the patients and to establish *SLC12A5* as a new gene related to Mendelian disease in humans, functional studies were performed in an international collaboration. Homology modelling predicted L426P and G551D to be deleterious but could not model the L311H variant. Voltage-clamp studies in transfected cells showed reduced chloride export capacity for all three mutant KCC2 compared to wildtype. Immunoblotting and immunofluorescence microscopy studies revealed reduced cell surface localization and reduced glycosylation of mutant KCC2. Reduced motility was seen in KCC2

knockout zebrafish embryos. Together the functional studies provided a firm support for the pathogenic role of the *SLC12A5* variants. The postulated disease mechanisms are schematically represented in Figure 5.

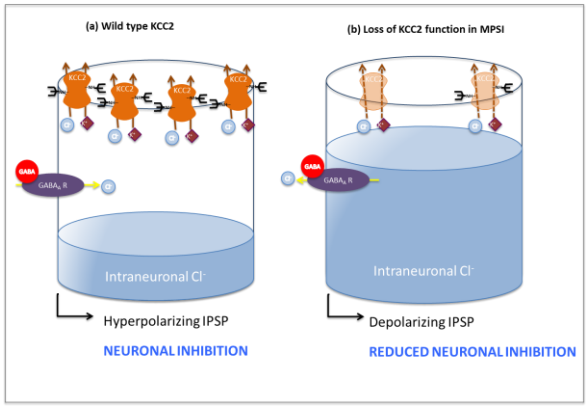

Figure 5. Schematic representation of postulated disease mechanisms in a post-synaptic neuron in KCC2-EIMFS. Wildtype (a) and mutant (b) KCC2. Reduced cell surface expression and reduced glycosylation of mutant KCC2 (b) lead to loss of KCC2 function, impaired chloride export, increased intraneuronal chloride concentration and impaired inhibition, and potentially excitation, when GABA binds its receptor. IPSP (inhibitory post-synaptic potential).

5.2.2 *SLC12A2* mutations cause NKCC1 deficiency with encephalopathy and impaired secretory epithelia (paper IV)

The proband (II:3) (Figure 6) of this study was born full term after a normal pregnancy, as the third child of non-consanguineous parents of Swedish descent. Delivery was uneventful but she was transferred to the neonatal ward at the local hospital due to muscle hypotonia and difficulties breastfeeding. At day 5 she showed symptoms of parotitis, and *S aureus* septicemia was diagnosed. She had recurrent central and obstructive apneas and was admitted to the pediatric intensive care unit at the Karolinska University Hospital at day 9. The Moro reflex that was initially present vanished during the second week of life. At this point it became obvious that the phenotype of the patient was strikingly similar to the phenotype of an older sister (II:2) who had died at 22 days of age due to recurrent apneas and aspiration. The proband was treated with antibiotics, continuous positive airway pressure, oxygen, caffeine citrate, and frequent inhalations of sodium chloride and survived the neonatal period. MRI and MRS of the brain revealed abnormalities in white matter and the basal ganglia and CSF analyses showed increased damage biomarkers.

Figure 6. Photos of the proband (II:3) used with consent from the parents. Photographs at age 8 years, showing dysmorphic facial features and strabismus. She has a broad and square lower face, broad chin with mandibular prognathia, wide mouth, and narrow forehead.

During the continued course she has been diagnosed with intellectual disability and has a hearing impairment. She is nonverbal and nonambulatory. She does not have epilepsy. A severe secretory impairment is a main symptom engaging the respiratory mucosa as well as sweat, lacrimal and salivary glands. She needs frequent inhalations with hypertonic sodium chloride to prevent life-threatening airway obstruction by mucus plugs. She has had surgery for intestinal malrotation.

The combination of symptoms in the proband and her older sister could not be found in the scientific literature of human disease. After an extensive work-up that did not reveal any underlying etiology, whole genome sequencing was performed on blood samples from the proband, the healthy older brother and both parents and on DNA from preserved fibroblasts from the deceased older sister. No pathogenic variants were detected in known disease genes but both patients were found to be compound heterozygous for two truncating variants in the gene *SLC12A2* encoding the transport protein sodium-potassium-chloride cotransporter 1 (NKCC1) expressed in neurons and secretory epithelia where it is the main chloride importer. Parents and the healthy sibling were carriers of one of the variants (Figure 7).

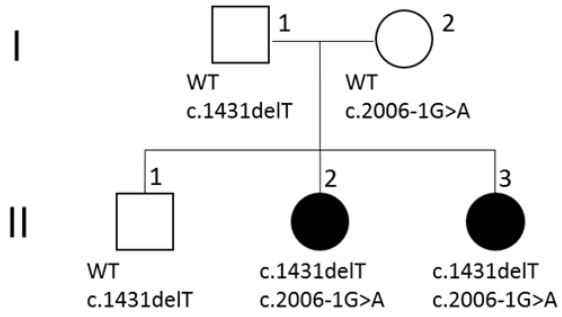

Figure 7. Pedigree of family with *SLC12A2* variants.

The variants, c.1431delT and c.2006-1G>A, and the segregation in the family were confirmed by Sanger sequencing. Both variants were absent from gnomAD and are expected to cause severe loss-of-function. The maternal variant c.2006-1G>A alters a canonical splice acceptor site and is predicted to affect normal splicing of exon 13. PCR amplification of complementary DNA (cDNA), obtained from RNA isolated from fibroblasts, from exon 12 to 14 followed by Sanger sequencing showed a lack of the entire exon 13. The paternal variant c.1431delT causes a frameshift in exon 8 and sequencing of cDNA did not show any expression of the mutated allele indicating nonsense mediated decay of the RNA. Thus, none of the variants are expected to be compatible with a functional NKCC1 protein.

The expression and function of the NKCC1 protein in neurons and multiple secretory epithelia supported the *SLC12A2* variants as disease causative in our patients. In addition, there was a close similarity between the phenotype in the patients and the phenotype

described in *SLC12A2* knockout mouse models, further supporting the pathogenicity of the variants and *SLC12A2* as a gene implicated in human disease.

6 DISCUSSION

6.1 THE POPULATIONS-BASED STUDY (PAPER I-II)

Our results confirm that the incidence of epilepsy peaks during the first 6 months and drops to a third from first to the second year of life. We show that an epilepsy syndrome can be diagnosed, and an etiology revealed in a majority of cases with epilepsy onset before age 2 years, with next generation sequencing contributing significantly to the etiological yield. Altogether 40% of cases had a confirmed or presumed genetic etiology. Seizure outcome was in line with the few comparable studies, with about half of the children being seizure free for 2 years or longer at age 7. Half of the children had intellectual disability and two thirds any neurodevelopmental impairment. The West syndrome subgroup had a similar seizure remission rate but worse cognitive outcome. Nevertheless, half of all children and a third of the West syndrome cases attended mainstream school at 7 years of age. Etiology is the main predictor of outcome but developmental delay at epilepsy onset, daily seizures for a period of 4 weeks or longer and status epilepticus seemed to have an independent impact when etiology was controlled for.

6.1.1 Strengths and weaknesses of the population-based study

The prospective ascertainment of incident cases is a strength of our study. The healthcare structure for children in the study area of Northern Stockholm is another strength. All children with epilepsy are managed at the Karolinska University Hospital which has the only neuropediatric inpatient ward and the only pediatric epilepsy outpatient clinic. All EEGs are interpreted at the Karolinska University Hospital. Together this promotes a high ascertainment rate but also a homogenous standard of care and readily accessible medical information through the electronic medical records of the hospital. The fact that clinical data were collected in retrospect from medical records is a weakness as is the lack of a fixed protocol for work-up. The work-up of each patient was decided by the responsible physician, although guided by our local written guidelines for work-up of epilepsy in infants. The use of next-generation sequencing (WES and WGS) in our cohort is a strength, although we did not offer this in cases of self-limited epilepsy. The relatively long follow-up until age 7 years is a strength compared to a few comparable reports on outcome of infantile epilepsy.

6.1.2 Incidences, background characteristics and work-up

Most incidence studies of pediatric epilepsy have identified cases retrospectively, with a few exceptions [34, 53, 55]. The incidence of epilepsy with onset during the first year of life in our study, 139/100,000 person-years, is higher than in older studies but similar to the findings in recent Nordic [54, 55] and British studies [34] (see Table 3 on page 6). There was no difference in incidence between genders in our cohort. A few other studies have shown some male predominance in this age group [17, 20, 34, 55] although this is more evident in adults where the difference increases with age [15]. The first seizure being febrile and febrile seizures in a first

degree relative showed significant differences between first- and second-year onset, the implication of which is unclear. MRI was performed in half of the cases which is low compared to other studies and compared to our present guidelines [17, 53, 54]. This was due to lack of resources during the study period, for general anaesthesia needed for MRI in young children, which led to the use of short CT scans as an alternative.

WES/WGS had not been used in previous population-based studies but was employed in 28 patients in the national Scottish study published after our paper I [34]. A genetic diagnosis was revealed in 6/28 (21%) cases investigated with WGS compared to 15/26 (58%) of children in our study where WES (n=7) or WGS (n=19) was performed. The lower yield in the Scottish study is probably explained at least partly by selection bias and the fact that their patients had previously been investigated with a panel of 104 genes with negative results. In addition, they were including also milder cases, potentially with complex oligogenic inheritance, for genetic work-up.

To summarize, the incidence of early onset epilepsy in our study was higher than in older reports but in line with three recent studies. Our use of next generation sequencing was comparable to only one other study and MRI was performed in a lower proportion of cases but with a higher yield than in previous studies.

6.1.3 Seizure types and epilepsy syndromes

Our study confirmed the findings from previous studies that focal seizures are the predominant onset seizure type in epilepsy presenting during the first few years of life and with spasms being the second most common type in first year onset [17, 19, 34, 52]. In many cases we found it difficult to classify the onset seizure as focal or generalized and 28% were left in the unclassified category. Also, when looking at seizure types up to age 7 years focal seizures predominated. The first and second unprovoked seizures occurred close in time, with less than one month apart on average, which is similar to previous reports [53].

In our study we diagnosed an epilepsy syndrome according to the 2010 and 2017 ILAE recommendations [7, 10] in 64% of first year onset cases and 54% of all cases. This is similar to or higher than in the five identified comparable studies [17, 34, 53-55] and most similar to the Finnish and Scottish figures [34, 54]. The birth prevalences in our cohort of the most common infantile syndromes, 1/2,100 in West syndrome and 1/3,800 in BIE/BFIE, are higher than in the Finnish (West syndrome 1/2,400, BIE/BFIE 1/4,300) and Scottish (West syndrome 1/3,300, BIE/BFIE 1/7,000) studies. The differences can reflect a high ascertainment rate in our study but also other methodological differences including follow-up time as well as coincidence due to relatively small case numbers. We only had one Dravet case in our cohort for which we have no other explanation than coincidence.

In summary, the seizure type frequencies in our cohort resemble previously known data. The proportion of cases with a diagnosed epilepsy syndrome are at the upper end of the published range and the birth prevalences of West syndrome and BIE/BFIE are higher than in comparable studies.

6.1.4 Etiologies

Due to our use of WES/WGS we have a higher proportion of genetic etiology than in older studies where genetic etiologies were scarce. Otherwise the spectrum of etiologies revealed in our study cohort are comparable to previous studies, structural etiology being the most common type and the proportion of first year onset cases with known etiology, 62% (65% if presumed genetic cases of familial epilepsy syndromes are included), being within the range of the 52-70% reported [17, 34, 54, 70, 71]. For known, structural/metabolic or genetic etiologies there were no differences between first and second year onset, but like in a Finnish study [54] structural/metabolic etiology was more common in onset before age 4 months compared to later (61% vs 38%, p = 0.04). In studies where older children are also included, both structural/metabolic and confirmed molecular genetic etiologies are clearly more common in infantile onset epilepsy than in toddlers or school children [70]. Of West syndrome cases in our study, around three quarters had a revealed etiology, a high yield comparable to only two other population-based studies and a few hospital-based reports [17, 34, 74, 76, 90]. One third had a confirmed genetic cause similar to the 42% in the only other study using genetics systematically, the national Scottish study published after our paper I [34].

The Scottish study reported a confirmed genetic diagnosis in 34% of all cases with epilepsy onset before age 3 years, of which a quarter also had a structural or metabolic diagnosis. Our use of WES/WGS in 26 patients raised the proportion of genetic etiology from 27% to 40% if cases with presumed genetic etiology and confirmed genetic etiology with underlying structural or metabolic etiology were included. Altogether 29% had a confirmed molecular and 11% a presumed genetic cause. The genetic heterogeneity with 21 different disease related genes in 23 families, confirms previous data as does the predominant autosomal dominant *de novo* segregation. A weakness of our study compared to the Scottish one, is that we did not offer WES/WGS in milder cases who were seizure free and otherwise neurologically healthy at follow-up. By this we probably missed genetic etiologies in cases of BIE/BFIE and GEFS+. In these epilepsy syndromes, the most common genetic etiologies in the Scottish study, pathogenic variants in *PRRT2* and *SCN1A*, are the predominant etiologies.

As shown in Table 4 of paper I, 13/15 patients who received a genetic diagnosis by WES/WGS had gone through extensive previous investigations, including various genetic tests, which could have been avoided by earlier WES/WGS. However, in none of the 10 children still alive treatment was affected by the genetic diagnosis. This will presumably change in the future as precision medicine evolves.

To conclude, the use of next generation sequencing in our study increased the overall and genetic diagnostic etiological yields considerably. Structural and genetic etiologies were the predominating types.

6.1.5 Treatment and seizure outcome

Polytherapy was common in our study cohort just as it is known to be in drug-resistant epilepsy (DRE) [183]. One third of the children had been seizure free for less than 6 months at

age 7 years. We did not directly address DRE according to the ILAE definition [103]. There are innumerable possible combinations of ASMs and little guidance on what constitutes a rational polytherapy [183-185]. As precision medicine evolves further, more precise, and even curative treatments will become available [176, 178, 180, 181]. An ongoing phase 1 and 2 study in Dravet syndrome (ClinicalTrials.gov: NCT04442295) is the first trial of gene expression therapy in epilepsy using antisense oligonucleotide (ASO) technology.

The hitherto only curative epilepsy treatment, epilepsy surgery, was performed in 1,7% (two cases) of our cohort. This is in line with two Scandinavian reports [54, 105] but lower than in two North American studies [48, 100]. It has been suggested that one out of 25 children (4%) could benefit from epilepsy surgery [186]. Surgery was probably underutilized in our cohort where one third had a structural etiology.

The seizure remission rates in our study were in line with previous studies and lower than the two thirds described in cohorts including older children [48, 98, 105, 187, 188]. About half of all cases had been seizure free for 2 years or longer at 7 years of age and 56% of the first-year onset cases were in remission, the latter within the 48-58% previously reported [19, 54, 105]. Onset age did not affect seizure remission. West syndrome had a remission rate similar to the rest of the cohort, similar to two other studies [19, 34] and higher than in another [105].

To sum up, the drug treatment pattern in our cohort reflects the high proportion of difficult to treat cases. Epilepsy surgery was probably underutilized. Seizure remission rates were within what has been previously described for infantile onset epilepsy and lower than in cohorts including older children. In view of the results, the need for more effective treatments targeting specific disease mechanisms becomes obvious.

6.1.6 Outcome in terms of comorbidities and educational support needs

Half of the children in our cohort were diagnosed with intellectual disability (ID). This is comparable to one previous study [19]. Two studies reporting a lower prevalence of ID in infantile-onset epilepsy, at 30-33%, either excluded cases with a high risk of ID (e.g. “acquired cerebral palsy”) [189], or had a short last follow-up time at age 24 months when mild to moderate cognitive impairments are difficult to detect [54]. Hospital-based studies usually report a higher rate of ID [109, 111, 117, 190]. In line with other studies our West syndrome cases had a worse outcome with ID in three quarters of children [114, 191].

As shown in Table 7, some etiologies had a more severe cognitive outcome than others, even though case numbers of specific etiologies are small. For most etiological categories a high risk of ID comes with a low chance of seizure remission, but interestingly genetic etiology without underlying structural or metabolic abnormality differs from this pattern with 71% ID and 71% seizure remission.

A study of early-onset seizures limited to focal epilepsy [192], as expected had a somewhat higher proportion of structural/metabolic/infectious etiology than our study (55% vs 45%). The seizure remission rates were similar (57% vs 53%). The cognitive outcome was worse

(68% IQ<80 vs 49% ID (IQ<70)). However, all 20/20 children who had a normal development at epilepsy onset and unknown etiology were in seizure remission at follow-up and were neurodevelopmentally normal to be compared to the less striking 29/41 (71%) in our study.

Children with epilepsy, and especially when associated to neurodevelopmental impairments, often have extra educational support needs. How this is met during school-age years will depend on non-medical factors like school system, availability of individual support within mainstream school and local attitudes. Studies on adults with childhood onset epilepsy show a considerable social and academic burden, not only on those with ID or DRE [193, 194]. In our cohort, half of all children and one third of West syndrome cases attended mainstream school at age 7, which could be viewed as encouraging. We did not find published population-based data on early-onset epilepsy to compare with.

To summarize, the prevalence of comorbidities in our study cohort is line with comparable studies including ID in about half of all cases and two thirds of children with West syndrome. Nevertheless, a considerable proportion of the children can attend mainstream school.

6.1.7 Factors associated to outcome

Etiology reported as a main predictor of both seizure remission and cognitive outcome in previous population-based studies of early-onset epilepsy [19, 54, 100, 105], is confirmed by our study. The association is complex with different specific etiologies having different outcomes. The "known etiology" category showing association to worse outcome in our and other studies [105, 192], will become even more heterogenous as monogenic causes are revealed to an increasing extent. The more genetic diagnostics are focused on epileptic encephalopathies and other severe cases, as in our study, the worse the outcome of cases with "known genetic etiology" will seem. From now on, focus on outcome of specific etiologies will be necessary, especially when developing and evaluating etiology specific precision treatments. For the rare genetic epilepsies, this means multicenter and multinational collaborations will be necessary to gather large enough cohorts.

Developmental delay at epilepsy onset and *age at epilepsy onset* were reported as associated to outcome in several reports, although mostly in univariable analysis, and are highly influenced by etiology [19, 54, 105, 189, 195]. Developmental delay at onset remained a significant predictor independent of etiology in our study (with stronger association to intellectual disability than to seizure remission). Age of onset did not show independent association to outcome in our cohort and results are contradictory in two previous studies, one population-based and one hospital-based, of epilepsy with onset during the first 3 years of life [100, 111].

Two seizure-related variables showed independent association to outcome in our study. *Status epilepticus* was the strongest negative predictor of seizure remission. Two comparable studies of early-onset epilepsy did not support this association [100, 105], and results from studies including older children are contradictory [187, 196]. *Daily seizures for >4 weeks* was independently associated to five of the six outcome variables in the regression analysis. This

strengthens the hypothesis that seizure burden in itself has a negative impact on outcome. There are no previous data on the same or a similar variable to support this, but DRE is associated to poorer cognitive outcome [100]. The impact of a high initial seizure frequency on outcome has been analyzed in a few studies with contradictory results [187, 196].

Male sex showed a strong overrepresentation among cases being diagnosed with ASD (12/13 cases, 92%, RR 11.59 (2.40-207.70)) but was not included in the multivariable analysis due to a small case number. A sex difference in ASD, but at a lower ratio, is previously well described in both epilepsy (RR 1,67:1) [197] and non-epilepsy cohorts (odds ratio OR 3:1) [198]. How much of this difference between genders and between epilepsy and non-epilepsy cohorts is explained by true prevalence differences due to biological factors or differences in diagnostics is under discussion [197, 198]. Our study suggests that the autism/ASD sex difference could be larger in early-onset epilepsy.

To conclude, type of etiology is a main predictor of outcome, but some other factors seem to have an independent association to outcome.

6.2 THE TWO NEW DISEASE GENES (PAPER III-IV)

In addition to the described patients, the lead roles of paper III and IV are played by the genes *SLC12A5* and *SLC12A2* encoding the proteins KCC2 and NKCC1. The close interaction of these two proteins and the dynamic balance between their functions in neurons are visualized in Figure 8 [199].

KCC2 is the main chloride exporter in neurons and NKCC1 is the main importer. Together they decide the intraneuronal chloride concentration on which the effect of GABA binding to its receptor depends. In the immature neuron and the prenatal brain NKCC1 is more active while KCC2 is downregulated resulting in a high intraneuronal chloride concentration. When GABA binds its receptor and opens the chloride channel, chloride ions flow into the neuron along the concentration gradient which leads to depolarization and excitation. When the neuron matures, NKCC1 becomes less active and KCC2 is upregulated. This mediates the so-called GABA switch by which GABA becomes the main inhibitory neurotransmitter in the postnatal brain. When the chloride concentration is low, like in the mature neuron, chloride ions flow into the neuron along the concentration gradient when GABA binds its receptor and opens the chloride channel. This leads to hyperpolarization and inhibition.

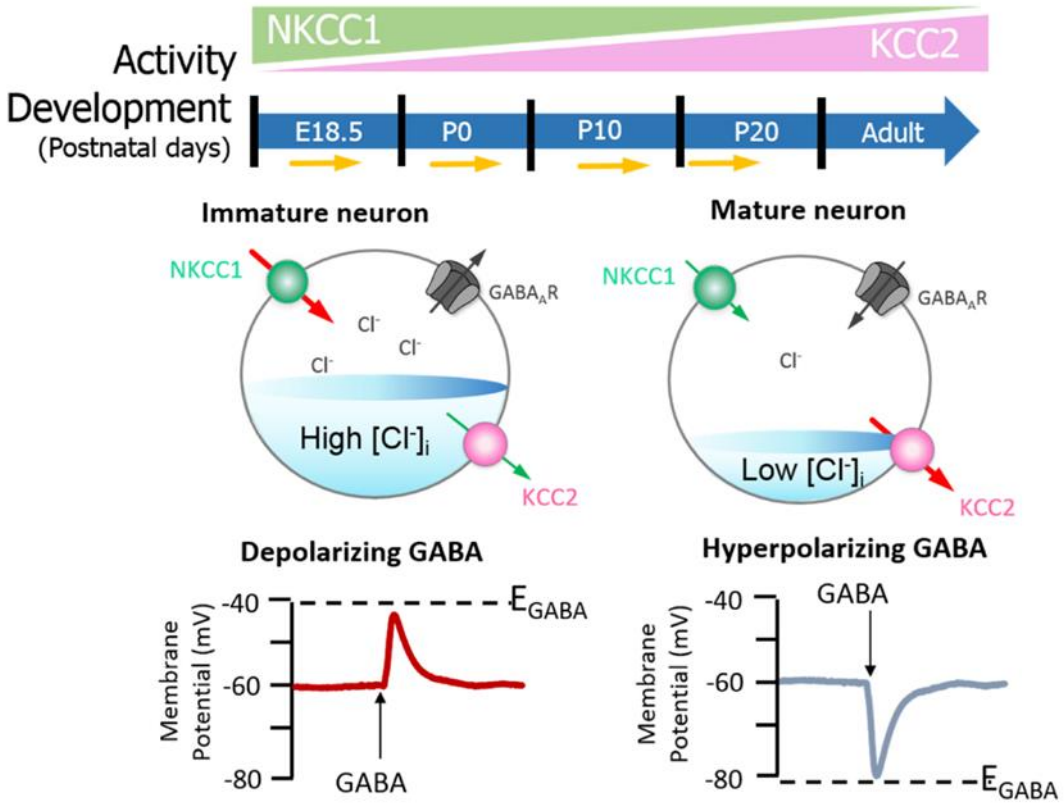

Figure 8. The maturation of the GABA system in rats causing a shift from excitation to inhibition. From Andrews K, Josiah SS, Zhang J. International Journal of Molecular Sciences. 2020; 21(23):9142 [199]

The balance between KCC2 and NKCC1 has been considered as a target for treating epileptic seizures in general and neonatal seizures specifically, more effectively by shifting the balance towards KCC2 and inhibition [200, 201]. An open-label study of an NKCC1 antagonist, the diuretic bumetanide, as add-on to phenobarbital in the treatment of seizures in newborn babies with hypoxic-ischemic encephalopathy was disappointing [202]. A recent pilot randomized, controlled, double-blind study of neonatal seizures was more promising in terms of antiseizure effect (but left a concern about hearing impairment as a common adverse effect) [203], as was a study on adults with drug-resistant temporal lobe epilepsy [204].

Despite, or perhaps because of, their crucial role in neuronal excitability, neither *SLC12A5* nor *SLC12A2* had been implicated in human disease when the work described in paper III and IV started.

6.2.1 The role of *SLC12A5*, encoding KCC2, in human epilepsy (paper III)

The epilepsy syndrome Epilepsy of Infancy with Migrating Focal Seizures (EIMFS) was first described by Coppola in 1995 under the name Migrating Partial Seizures in Infancy (MMPSI) [205]. It belongs to the severe epileptic encephalopathies with onset in early infancy and shows a characteristic pattern of seizure activity migrating between hemispheres during an ongoing seizure. The etiology of EIMFS was for long elusive but since 2011 multiple

monogenic causes have been identified with *de novo* variants in *KCNT1* and *SCN2A* being the most and second most common [206-208].

Since the function of KCC2 and its role in neuronal inhibition was revealed [209], there has been and still is much speculation about its relevance for epileptogenesis, seizure physiology and epilepsy treatment [210]. It was not until 2014 that the first reports implicating *SLC12A5* variants in human disease were published, describing polymorphisms associated to idiopathic generalized epilepsy [211] and febrile seizures [212]. Soon after this, in 2015, we published paper III and for the first time described a monogenic disease in humans caused by biallelic loss-of-function variants in *SLC12A5* in a recessive form of EIMFS. Based on our functional studies of the variants and previous experimental work, several disease mechanisms can be hypothesized: impaired KCC2-mediated chloride extrusion from defective transporter activity, decreased cell-surface KCC2 expression and reduced protein glycosylation contributing to loss of function and hyperexcitability. *SLC12A5*-EIMFS is a rare disease. To date a few more patients have been published [213, 214]. The ClinVar database at present (April 16, 2022) contains 41 variants in *SLC12A5* listed as pathogenic or likely pathogenic. *SLC12A5* has so far not been firmly established as a disease gene in any other monogenic epilepsy syndrome than EIMFS but a more general role in epileptogenesis is still under investigation as is its role as a target in epilepsy treatment.

6.2.2 The role of *SLC12A2*, encoding NKCC1, in human disease (paper IV)

Together with a similar case reported [215] just prior to our paper IV publication, our study of two siblings with a characteristic not previously described phenotype of encephalopathy and impaired secretory epithelia, established biallelic variants in *SLC12A2* as a monogenic cause of human disease. In a previous study of genetic causes of intellectual disability, *SLC12A2* had been proposed as a candidate gene based on a single recessive case without functional support [216].

GABA, while being the main inhibitory neurotransmitter in mature neurons and the postnatal brain, is excitatory in the prenatal brain due to high expression of NKCC1 and immature KCC2. Acting as a trophic factor it affects proliferation, migration, differentiation, and synapse maturation and therefore is important for brain development and network formation [217, 218]. Reduced NKCC1 function leading to reduced GABA excitation is expected to disturb the prenatal brain development and one can hypothesize that the severe encephalopathy and neurodevelopmental impairments in our proband were mainly of prenatal origin. However, there were also signs of ongoing postnatal neurodegeneration in the proband and the mechanisms of NKCC1 encephalopathy in humans have to be investigated further. The central apneas and the disappearance of the Moro reflex in our subjects could possibly be related to NKCC1 deficiency in brainstem nuclei and networks [219, 220].

Several of the non-neurological features of the proband's phenotype are mimicked by *SLC12A2* knockout mice showing reduced saliva secretion, impaired hearing (due to impaired secretion of endolymph in the inner ear) and gastrointestinal problems [221-223]. These

mice do not exhibit the cystic fibrosis-like lung problems seen in our proband, which could be explained by compensatory mechanisms not present in humans [224].

In addition to biallelic loss-of-function variants in *SLC12A*2 recently being reported in five more cases with similar features [225, 226], phenotypes with autosomal dominant inheritance have been described. One of these is non-syndromic hearing loss [227, 228], while other described autosomal dominant cases have had more complex phenotypes with neurodevelopmental abnormalities and symptoms from multiple organs [226, 229, 230]. The mechanisms behind the symptoms and the variability in phenotype in autosomal dominant *SLC12A2* disease are not fully understood.

As has been discussed above NKCC1 is an attractive target for epilepsy treatment. The NKCC1 antagonist bumetanide has been studied in rodent models not only of epilepsy but also of other neurological diseases and there have also been clinical trials in disorders like autism, schizophrenia, Mb Down and obsessive-compulsive disorder [231]. However, bumetanide has some drawbacks including a low penetration across the blood-brain barrier and a prominent diuretic effect and novel NKCC1 antagonists are under investigation [231].

7 CONCLUSIONS

Together with a few older and recent studies, this thesis provides a firm knowledge-base describing the phenotypical and etiological panorama, outcome and factors associated to outcome in infantile-onset epilepsy. Our population-based study shows that an epilepsy syndrome can be diagnosed, and an etiology revealed in a majority of epilepsy cases with onset during the first 2 years of life. Genetic diagnostics, and above all the use of massively parallel sequencing, can increase the etiological yield considerably and should be included in the work-up of early-onset epilepsy. Two new disease genes were discovered within this thesis work. This is of importance for genetic counselling and makes prenatal diagnostics possible. Since etiology is a main predictor of outcome, revealing etiology is also of value for prognostication. Outcomes in our study cohort, with half of the children being diagnosed with intellectual disability and half being seizure free for 2 years or longer at age 7, are in line with comparable studies. This is expected since no extra treatment options were available for the study cohort compared to other studies. However, due to the uncovering of novel disease genes, and thereby of specific disease mechanisms, by the use of massively parallel sequencing, the future looks promising in terms of more precise epilepsy treatments. Already today, a genetic diagnosis can affect treatment choice.

Despite the high rates of neurodevelopmental comorbidities, half of the children in the study cohort attended mainstream school at age 7 years. The further development of precision medicine will provide more effective and, in some cases, curative treatments which will much benefit the one third of epilepsy patients presently being drug-resistant. Precision treatments directed at specific disease mechanisms will also have the potential to normalize neurodevelopment if initiated early.

8 POINTS OF PERSPECTIVE

Two longstanding and related dilemmas of epilepsy management are about to change: the fact that the cause of epilepsy is known in only a minority of cases and the high rate around 30-40% of drug-resistance.

The development of massively parallel DNA sequencing technologies has revolutionized etiological work-up, revealing a monogenetic etiology in 30-50% of epileptic encephalopathies and other early-onset epilepsies of previously unknown cause. The work of developing new treatments directed at the related disease mechanisms are ongoing and many studies are on the way. Multicenter and international collaborations are a prerequisite for this work since many of these epilepsies are rare. This collaboration and data sharing is also necessary to explore etiology in the 50-70% of cases still being unsolved. In addition, new methods will have to be employed including long-read sequencing and ways to explore variants in non-coding regions that constitute more than 98% of human DNA and whose functions are mostly unknown.

In epilepsy presenting later in childhood as well as in the few studies of adult-onset cases the diagnostic yield has been much lower. In epilepsy presenting after the first few years of life monogenic etiology is less common and complex inheritance predominates, which makes it more challenging to entangle the genetic architecture behind the seizure susceptibility. Even in acquired epilepsy genetic factors are at play, impacting the risk of epileptogenesis after stroke, trauma, encephalitis etc. To explore the genetic influence in such scenarios, other approaches are needed than in the monogenic epilepsies, including genome wide association studies on very large cohorts and advanced mathematics. Probably the road to more effective and "precise" treatments will be longer and bumpier than in the monogenic epilepsies.

Another challenge is the transition from paediatric to adult care of the increasing number of genetic epilepsies. So far there has been little interest in genetics and precision medicine among adult epileptologists. They will however soon "inherit" large numbers of patients with rare genetic epilepsies with implications for management, from the paediatric epilepsy care. Adult neurologists will have to learn about the paediatric epilepsy syndromes and the genetic epilepsies and their treatments. Structured programs will be needed to make the transition to adult healthcare smooth.

Precision treatments for epilepsy will most probably become available at an increasing speed, especially for monogenic epilepsies. The financing of these expensive treatments and making them available to those in need will be a big challenge, not least in the less privileged parts of the world. Already today there are considerable global inequalities in terms of access to epilepsy care.

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