Aus der Klinik für Pädiatrie mit Schwerpunkt Neurologie
sowie dem NeuroCure Clinical Research Center
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Molecular genetic elucidation of a novel neurocutaneous syndrome

zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

Noomi Müller

aus Heidelberg

Datum der Promotion: 18.12.2020

Inhaltsverzeichnis

1 Abkürzungsverzeichnis

A	Adenine
C	Carbon
C	Cytosine
(c)DNA	(complementary) deoxyribonucleic acid
CERS2	Ceramide synthase 2, *
CERS3	Ceramide synthase 3, *
ELOVL1	ELOVL Fatty Acid Elongase 1, *
ELOVL4	ELOVL Fatty Acid Elongase 4, *
ELOVL5	ELOVL Fatty Acid Elongase 5, *
ER	Endoplasmatic reticulum
G	Guanine
gnomAD	Genome Aggregation Database
IBS	Identity-by-state
ISH	*In situ* hybridization
ko	Knockout
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MRI	Magnetic resonance imaging
(m)RNA	(messenger) ribonucleic acid
NGS	Next-generation sequencing
OMIM	Online Mendelian Inheritance in Man
PCR	Polymerase chain reaction
PNS	Peripheral nervous system
PPAR	Peroxisome proliferator-activated receptor
PPRE	PPAR response elements
RNA-seq	RNA-sequencing
RT-qPCR	Quantitative real-time PCR combined with reverse transcription
SNP	Single-nucleotide polymorphism
T	Thymine
(VLC)FA	(Very long-chain) fatty acid
WES	Whole-exome sequencing

*italic for gene, non-italic for protein, capitalized for human

2 Abstracts

English

Background: Diagnosis of rare diseases is often challenging. The genetic etiology of at least one-third of rare diseases is unknown. We investigated two unrelated Polish children with a novel congenital syndrome affecting skin and central nervous system. We aimed to discover and evaluate the genomic sequence variant causing this neurocutaneous syndrome.

Methods: Using whole-exome sequencing we identified the pathogenic variant in the *ELOVL1* gene. Familial segregation was examined using Sanger sequencing and restriction length polymorphism analysis. We determined variant and wildtype ELOVL1 activities by enzymatic assays. Lipid concentrations from fibroblasts and skin samples were measured by liquid chromatography-tandem mass spectrometry. Subcellular localization of wildtype and mutant ELOVL1 was examined by immunofluorescence. *Elovl1* tissue expression was studied in mice by *in situ* hybridization and by quantitative real-time PCR combined with reverse transcription. Global gene expression was analyzed by RNA-sequencing of patient and control fibroblasts. As potential treatment option, we tested loading of fibroblasts *in vitro* with very long-chain fatty acids (VLCFAs) of various lengths.

Results: We identified the novel heterozygous *de novo* variant of the ELOVL fatty acid elongase 1 (*ELOVL1*) gene (c.494C>T, p.S165F) in both patients. *ELOVL1* encodes a VLCFA elongase, synthesizing (monoun)saturated VLCFAs with 24-26 carbons. Both patients presented the same phenotype from infancy including ichthyosis, central hypomyelination with progressive spasticity mainly of the lower limbs, nystagmus, dysarthria, high frequency hearing deficit, optic nerve atrophy with peripheral visual field restriction, and reduced visual acuity. The genomic sequence alteration abrogated ELOVL1 enzymatic activity, which we verified (**i**) by enzymatic assay, and (**ii**) by demonstrating a reduction of ELOVL1 catalytic products (sphingolipids carrying C24- or C26-VLCFAs) in patient fibroblasts. Variant and wildtype ELOVL1 proteins co-localized in the endoplasmatic reticulum, thereby excluding mistargeting of the variant protein. *Elovl1* mRNA was highly expressed in affected tissues, mainly cerebral white matter, eye, and skin. Transcriptome analysis of patient and control fibroblasts revealed upregulation of genes responsible for epidermal development and keratinization in patient cells. Genes important for neurodevelopment, myelination, and synaptic function were down-regulated. VLCFA-loading partly overcame the biochemical defect in patient fibroblasts, though competitive inhibition between saturated and monounsaturated VLCFAs blunted the desired effects.

Conclusion: A heterozygous *ELOVL1* variant causes a severe neuro-ichthyotic disease. VLCFAs generated by ELOVL1 are crucial for myelination, neurodevelopment, skin barrier and auditory function. Further research is needed to investigate effects of VLCFA-deficiency on gene expression. Therapy-directed studies are required to optimize VLCFA-treatment.

Deutsch

Hintergrund: Die Diagnostik seltener Erkrankungen ist oft eine Herausforderung. In 30% der Fälle bleibt die genetische Ursache ungeklärt. In dieser Studie untersuchten wir zwei polnische, nicht miteinander verwandte Kinder, erkrankt an einem unbekannten, kongenitalen Syndrom mit Beeinträchtigung von Haut und zentralem Nervensystem. Ziel der Arbeit war die Auffindung und Evaluation der diesem neurokutanen Syndrom zugrundeliegenden genetischen Variante.

Methoden: Mittels Gesamtexomsequenzierung fanden wir eine pathogene Variante im *ELOVL1*-Gen. Die familiäre Segregation dieser Variante untersuchten wir mittels Sanger-Sequenzierung sowie spezifischen Restriktionsenzymverdau. Die enzymatischen Aktivitäten der ELOVL1-Proteine (Wildtyp *versus* Variante) wurden mittels Enzymassays bestimmt. Aus Fibroblasten und Hautproben extrahierte Lipide wurden durch Flüssigchromatographie mit gekoppelter Massenspektrometrie quantifiziert. Die subzelluläre Lokalisation der ELOVL1-Proteine wurde mittels Immunfluoreszenz dargestellt. Die mRNA-Expression von *Elovl1* untersuchten wir im Mausgewebe mittels *in-situ* Hybridisierung sowie quantitativer Echtzeit-PCR nach reverser Transkription. Die globale Genexpression analysierten wir mittels RNA-Sequenzierung in Patienten- und Kontrollfibroblasten. Als *in-vitro* Therapieversuch behandelten wir Fibroblasten mit überlangkettigen Fettsäuren (VLCFAs).

Ergebnisse: In beiden Patienten identifizierten wir die gleiche heterozygote *de-novo* Variante im *ELOVL1*-Gen (ELOVL fatty acid elongase 1, c.494C>T, p.S165F). *ELOVL1* codiert für eine Fettsäuren-Elongase, welche die körpereigene Synthese (einfach un)gesättigter VLCFAs mit 24-26 Kohlenstoffatomen katalysiert. Die Erkrankung beider Patienten manifestierte sich in den ersten Lebensjahren mit ähnlichem Phänotyp: Ichthyosis, zerebrale Hypomyelinisierung mit progressiver Spastik insbesondere der unteren Extremität, Nystagmus, Dysarthrie, Hochtonschwerhörigkeit, Optikusatrophie mit peripheren Gesichtsfeldeinschränkungen und reduziertem Visus. Die Variante führte zum Verlust der ELOVL1-Enzymaktivität, wie wir im Enzymassay zeigten. Patientenfibroblasten enthielten signifikant niedrigere Konzentrationen enzymatischer ELOVL1-Produkte (Sphingolipide mit VLCFAs-Komponenten bestehend aus 24 oder 26 Kohlenstoffatomen). Subzellulär waren beide ELOVL1-Proteine (Wildtyp und Variante) im endoplasmatischen Retikulum lokalisiert, wodurch wir eine mutationsbedingte Fehllokalisation ausschließen konnten. Im Mausgewebe detektierten wir eine hohe Expression der *Elovl1*-mRNA in von der Erkrankung besonders betroffenen Organen: Haut, Auge und zentrale weiße Substanz. Eine globale Genexpressionsanalyse mittels RNA-Sequenzierung ergab eine Hochregulation von Genen der Hautentwicklung und Keratinozytendifferenzierung in Patientenfibroblasten. Einige für die Gehirnentwicklung, Myeli-

nisierung und synaptische Funktion wichtige Gene waren in Patientenfibroblasten schwächer exprimiert als in Kontrollen. Die Behandlung von Patientenzellen mit VLCFAs erlaubte ein teilweises Überwinden des enzymatischen Blocks, allerdings wurde der therapeutische Effekt wahrscheinlich durch Kompetition um die enzymatische Bindungsstelle zwischen gesättigten und einfach ungesättigten VLCFAs abgeschwächt.

Schlussfolgerung: Die heterozygote *ELOVL1*-Variante verursacht ein schweres neurokutanes Syndrom. ELOVL1-synthetisierte VLCFAs sind wichtig für Myelinisierung, Gehirnentwicklung, Hör-, Seh- und Hautbarrierefunktion. Weitergehende Untersuchungen zum Effekt des VLCFA-Mangels auf die Genexpression sind erforderlich. Außerdem benötigen wir systematische Studien zur Behandlung mit VLCFAs unterschiedlicher Länge in Zellkultur und Tierversuch.

3 Manteltext

3.1 Rare diseases

A rare disease is a health condition of particularly low prevalence. There is no uniform internationally recognized definition of a rare disease. In the European Union, rare diseases are defined as those affecting less than 5 per 10,000 persons.[1] 6000-8000 rare diseases have been described so far,[2,3] with rising numbers as novel diseases are reported regularly in the medical literature. In some rare diseases, only a few individuals are known to be affected worldwide, while in others it may be hundreds. Despite their individual low prevalence, collectively, they account for an important part of the global burden of disease, with an estimated 6-8% of human population suffering from rare diseases.[4] Around 400 million individuals worldwide[5] and 30 million in Europe[6] live with a rare disease, more than half of them are children.[4] Rare diseases regularly display a chronic and severe course, consuming a disproportionately high fraction of health care budgets.[4]

Rare diseases are often monogenic inherited diseases (also called Mendelian diseases),[7] resulting from a single defective gene. Due to gene pleiotropy, one third of disease genes causes more than one rare genetic disease.[8] Notably, around 6% of patients have more than one rare disease.[8]

The diagnostic process of rare diseases is challenging, often translating into delayed diagnosis and impeded access to tailored care. Frequently, patients embark on a diagnostic odyssey that may take years until the diagnosis is found eventually, if it is found at all. Difficulties in the diagnostic process arise from genetic heterogeneity, complicated pathophysiology, and simply lack of expertise on the part of the diagnosing physicians.[9] Although the discovery rate of novel disease-causing genes increased during the past few years,[8] genetic etiology of at least one-third of diseases is elusive.[9,10] Timely molecular genetic diagnosis may obviate unnecessary diagnostic interventions and facilitate genetic counseling.

Importantly, knowledge on relevant proteins and biological pathways may enable development of effective targeted therapies. In the European Union and the United states, incentives have been launched for the development of so called “orphan” drugs.[11] Nevertheless, the number of medicines for rare diseases is still limited. Currently, approved treatments are available for only 6% of rare diseases,[8,12] of which less than 1% are curative.[9]

Scarcely represented in classifications such as the International Classification of Diseases (ICD), rare diseases are widely neglected by hospital health information systems.[2] The research community utilizes public databases, notably the Online Mendelian Inheritance in Man (OMIM)[3] and Orphanet[2], and the Human Phenotype Ontology[13] to share clinical and genetic knowledge on rare diseases.

3.2 A novel neurocutaneous syndrome

All information on the presented study given in this text is based on the so-called "Top-Journal-Publication" by Mueller et al.[14]

In this study, we investigated two unrelated individuals of Polish origin exhibiting a severe, not yet described neurocutaneous syndrome. Parents and siblings were healthy. Over years, the boys underwent extensive diagnostic procedures including imaging, biochemical investigations, electrophysiology as well as traditional genetic testing that did not elucidate the genetic diagnosis. Remarkably, the parents learned about each other *via* an internet platform and noticed the striking similarity of their children's distinct phenotype. In 2016, both patients presented to the neuropediatric outpatient clinic of the Charité University Hospital, Berlin and were enrolled in this research study. Written informed consent was provided by the patients' parents. The study was approved by the Institutional Review Board and followed the Declaration of Helsinki.

Both children were born at normal gestational age. Growth parameters were normal along their respective percentiles. Since infancy, both patients had thickened, dry and scaly skin involving the entire integument, but predominantly extensor surfaces of joints, hands, and feet. Skin biopsy confirmed ichthyosis with broadened epidermal layers. *Acanthosis nigricans*, a brownish-black hyperpigmentation of axillary, umbilical, and nuchal skin was observed, as well as a few hypopigmented skin areas. Furthermore, both patients had a delay of motor development and never reached the milestones of independent sitting or walking. They suffered from progressive spasticity mainly of the lower limbs. Today, both children are wheelchair-dependent. In our neurological investigation, we found spastic paraplegia with abnormal pyramidal signs, hyperreflexia, muscle atrophy, multiple joint contractures, ankle clonus, and equinovarus foot deformity. Although the upper limb was less severely affected, elbow contractures and hand muscle atrophy were noted. Cranial magnetic resonance imaging (MRI) sequences detected T_2 signal hyperintensities in temporal and occipital regions of the brain. Absence of contrast enhancement made an inflammatory etiology of the lesions unlikely. Central hypomyelination was diagnosed, with the state of hypomyelination static over time. Interestingly, hypomyelination was present only in the central nervous system, corresponding to the first motoneurons in the pyramidal tract. The peripheral nervous system (PNS) including the second motoneurons was found to be entirely unaffected. This finding was supported by normal spinal MRIs, normal motor and sensory conduction velocities and normal ultrastructure of peripheral nerves studied by electron microscopy. Moreover, a high frequency hearing impairment was diagnosed in both individuals during childhood. Auditory evoked potentials were abnormal, indicating central conduction defects. In addition, ophthalmological examination found low visual acuity, high astigmatism, and constriction of the peripheral visual field

secondary to optic nerve atrophy. Further, an early manifesting horizontal nystagmus was noticed. Both patients had spastic dysarthria. Cognitive and social development was normal. Laboratory investigations including mitochondrial diagnostics were normal.

The aim of this study was the elucidation of the molecular genetic cause of this novel congenital syndrome by identification of the disease gene. We further aimed to investigate the pathophysiological basis of the disease and characterize the clinical phenotype.

3.3 Novel disease-gene discovery

The discovery process of novel disease genes traditionally relied on methods such as linkage analysis combined with positional cloning and subsequent Sanger sequencing of candidate genes. This method worked also in cases without any pre-existing information about the responsible gene.[15] However, this approach required the availability of large pedigrees, several affected families, and numerous samples of affected and unaffected individuals. Consequently, linkage analysis proved to be inefficient to detect very rare diseases in small and few families, and unable to detect *de novo* variants.[16] In the beginning of the 21st century, linkage analysis was pushed back in favor of next-generation sequencing (NGS) as the preferred method for disease gene discovery.[17] In 2003, the first human genome was sequenced in the course of the Human Genome Project, with production costs of up to US$ 3 billion.[18] Over the past decade, NGS technologies have rapidly advanced, leading to increased throughput and lowered cost to below US$ 1,000,[19] making them suitable techniques for routine clinical testing.

3.3.1 Whole-exome sequencing

Amongst others, NGS approaches comprise whole-genome sequencing and whole-exome sequencing (WES). In 2010, WES was first successfully applied for gene discovery in a rare monogenic disease of unknown origin.[20] Since then, WES has become the leading tool for uncovering novel pathogenic germ-line variants.[9,10] WES comprises sequencing of all protein-coding regions of the genome (called “the exome”), representing roughly only 1% of the genome,[15] thus limiting the amount of genomic information to be produced. Importantly, most variants causing monogenic diseases are located in protein-coding gene sequences.[15,21] The patient’s exome sequences are aligned to the human reference sequence. Variants are detected as single nucleotides or stretches of nucleotides differing from the reference sequence. Per individual, around 20,000 sequence variants are usually identified in WES.[22] WES is thus providing vast amounts of data with extensive lists of variants. Interpretation of WES results remains challenging.[23]

3.3.2 Computational search for pathogenic variants

Computational filters are applied to support the prediction of disease-causing variants among all given variants. To assess variant pathogenicity, filters may integrate information on evolutionary conservation of the sequence, alterations in the resulting protein, phenotypical data, and possible hereditary patterns.[23] Moreover, WES data analysis annotates variant frequencies in large populations, with rare variants being more likely to be categorized as potentially damaging or "disease-causing".[24] An important population database is the Genome Aggregation Database (gnomAD), aggregating the allele frequencies of 15,000 genomes and 120,000 exomes including data from the 1000 Genomes Project, the Exome Aggregation Consortium (ExAC), and the Exome Sequencing Project (ESP).[10,25] Even after bioinformatic filtering, genomic variants may still be too numerous to be tested for segregation in the family. Additional, below described filtering strategies may be utilized to narrow WES results:

Autozygosity mapping: In patients sharing a common ancestral origin, an autozygosity map can be generated from WES data.[26] Shared regions of homozygosity are determined. Detection of variants in these regions of homozygosity may facilitate the identification of genes causing recessive diseases in consanguineous families.[27]

Identity-by-state analysis: Identity-by-state (IBS) analysis of WES data may be applied to reduce possible disease loci within one non-consanguineous family.[28] This analysis requires at least two affected individuals in one family and presumes that affected individuals share the same haplotype at the region of the disease gene. These IBS regions may harbor the pathogenic variant.[29]

Trio-based whole-exome sequencing: Trio-based WES of the patient including both parents may be employed. The number of candidate variants can be significantly reduced by comparison between variants found in an affected individual to those in each parent.[20] The trio test may permit more sensitive identification particularly of *de novo* variants.[30] These are variants that occur in the patient, but not in the parents. This option is more costly, however gains increasing popularity due to falling prices.

3.3.3 The search strategy of this study

The two patients investigated in our study were neither related, nor had they common ancestry. Therefore, we refrained from IBS analysis or homozygosity mapping. In this study, WES data were available only for both index patients, but not for parents or siblings. Trio-based WES could not be performed for financial reasons in 2016.

In a first step, we aimed to rule out already known genetic diseases. Thus, we analyzed the WES data of each child separately, using virtual gene panels to detect already known genes associated

with symptoms of the children's phenotype. However, no pathogenic variant in a known disease gene was detected.

Thereafter, we created a list of all genes containing variants predicted to be disease-causing by the MutationTaster2[31] software, for each patient separately. MutationTaster2 assessed the pathogenic potential of variants and predicted functional consequences of the deoxyribonucleic acid (DNA) sequence alterations.[31] The software provides annotation of all freely available single-nucleotide polymorphisms (SNPs), indels, and known disease variants from public databases. Evolutionary conservation of variants was scored. Amongst the variants marked as potentially disease-causing, we searched separately according to dominant and recessive mode of inheritance models. For the recessive model, we differentiated between compound heterozygous and homozygous recessive. The division between heterozygous *versus* homozygous was based on the percentage of reads covering the respective variant in the exome alignment file (BAM file, **B**inary **A**lignment **M**ap). For instance, we expected a relative read frequency of 35-75% for the dominant as well as for the compound heterozygous recessive model. Further, our sequential filter strategy contained evaluation of variant frequencies in gnomAD and the exclusion of synonymous sequence alterations or variants outside of splice sites. All variants were visually inspected by help of the Integrative Genomics Viewer (IGV) allowing to remove WES alignment artifacts.

In a next step, we determined the intersection of gene lists of both patients that contained potentially pathogenic variants. This was done under the assumption that due to the same phenotype the same gene would be altered in both patients. This approach narrowed the number of potential disease gene variants further down. Variant assessment also entailed search through the scientific and medical literature. We finally detected a novel heterozygous variant in the *ELOVL1* (ELOVL Fatty Acid Elongase 1) gene on chromosome 1, leading to an amino acid exchange of an evolutionary highly conserved amino acid position chr1:g43,830,119G>A (GRCh37), NM_001256399, c.494C>T, p.(S165F).

chr1:g43,830,119G>A in GRCh37	DNA single base exchange from guanine (G) to adenine (A), located at the genomic position 43,830,119 on chromosome 1, based on the human reference genome sequence, in our case the Genome Reference Consortium Human Build 37 (GRCh37). The chromosomal positions refer to the physical positions counted from the 5' end of the respective chromosome.
c.494C>T in NM_001256399	Alteration in the coding sequence of the messenger ribonucleic acid (mRNA) with substitution of a thymine

	(T) for a cytosine (C) at position 494 of the complementary DNA (cDNA) in the GenBank transcript with the identity number NM_001256399. The coding sequence position 1 refers to the A of the ATG initiation codon. The *ELOVL1* coding region is located on the reverse strand of the chromosomal DNA. This explains the difference in nucleotide exchange between genomic position and cDNA.
p.(S165F) in NP_001243328	The altered amino acid is located at position 165 of the amino acid chain and replaces a serine by a phenylalanine (position 1 refers to the first amino acid of the protein), in GenBank transcript with the identity number NP_001243328.

Since NGS technologies yield higher error rates and shorter read lengths than those of Sanger sequencing,[19] we confirmed the *ELOVL1* c.494C>T variant by Sanger sequencing.

The presence of the variant was further validated on the genomic DNA level using a specific enzymatic digest. While the wildtype *ELOVL1* had a specific enzyme restriction site (5'-GGGCT**C**-3') for the restriction enzyme *Ban*II, the sequence alteration of *ELOVL1* c.494C>T abolished this restriction site (5'-GGGCT**T**-3'). Consequently, no restriction digest took place on the polymerase chain reaction (PCR)-fragment generated from the variant allele.

Using genomic DNA of patients, parents, and siblings, we investigated the segregation of the pathogenic variant with the disease phenotype *via* Sanger sequencing and enzymatic digest. As all other family members were negative for the mutation, we confirmed the *de novo* occurrence of the mutation in both index patients.

Moreover, enzymatic digests of PCR-fragments from cDNA confirmed the presence of the *ELOVL1* variant on mRNA level, thereby ruling out a selective degradation of the variant *ELOVL1* mRNA.

Further, we aimed to rule out the unlikely case of a common founder allele (e.g. *via* a genomic mosaic of the parental germ cells). Therefore, we explored the surrounding (±1 Mio base pairs) of the *ELOVL1* c.494C>T variant position for the presence of SNPs shared by both patients. Such sharing would indicate a common founder haplotype inherited by both children. We ruled out a common founder haplotype, therefore confirming the notion that a "real" *de novo* mutation had occurred in both children exactly at the same position.

Generally, at least two unrelated individuals with an overlapping or like in our case almost identical clinical phenotype are needed to confirm a novel disease gene.[10] However, as we searched for genetic variants within an overlapping gene list of both patients in the first place, it might not be appropriate to claim case-based matching as validation of gene discovery at present. The identification of further patients will determine the genetic variability and the constant features of the newly described syndrome.

3.4 Evaluation of novel candidate gene

We found the identical heterozygous *de novo* missense variant in the *ELOVL1* gene of two unrelated children presenting with the same well-defined phenotype. At this point, no reports were available on individuals living with any pathogenic variant in this gene. It is not recommended to solely rely on computational predictions and sequencing results as validations of a novel disease gene variant.[24] Therefore, we performed a series of functional and biological studies to support the pathogenicity of the mutated candidate gene.

3.4.1 Tissue gene expression studies

In order to understand the involvement of the candidate gene in the pathogenesis of the patients' phenotype, expression patterns of this gene may be studied across different organs or tissues. Variation of candidate gene expression may thus explain the predominance of certain organ systems or cell types to be involved in a specific disease.

One method of choice to analyze tissue gene expression is quantitative real-time polymerase chain reaction (qPCR) combined with reverse transcription (RT-qPCR)[32], as a fast and economical approach. mRNA is isolated from examined tissues, followed by reverse transcription into cDNA. A predefined segment of the candidate gene is amplified in a PCR reaction under real time monitoring. A dye (SYBR® green) emits fluorescent light if it intercalates with the DNA double strand of the emerging PCR-products. Increasing fluorescence intensity will be measured with each cycle of the PCR, thereby indicating the increase of the PCR-product concentration. This allows quantification of specific sequences in the cDNA transcripts, here of the *Elovl1* gene in different murine tissues.[33]

In situ hybridization (ISH) is another option to investigate the expression of the candidate gene with local resolution. A specific radioactively or digoxigenin (DIG)-labeled ribonucleic acid (RNA) probe detects complementary nucleic acid sequences in frozen tissue sections. In contrast to RT-qPCR or other PCR-based techniques, ISH enables visualization of sequences of interest in paraformaldehyde-fixated tissue sections.[34]

In our project, we utilized mouse tissue to investigate tissue gene expression. RT-qPCR was performed to quantify *Elovl1* expression across different mouse tissues. A ubiquitous expression pattern of *Elovl1* mRNA was found, with highest expression in eye, spinal cord, peripheral nerve, brainstem and white matter of the brain. Moreover, ISH was performed on E16.5 mouse embryos, with ubiquitous, but particularly high *Elovl1* mRNA detection in the epidermis, brain cortex, and spinal cord. ISH of adult mouse brain revealed high expression in hippocampus, cerebellar cell layers, and neocortical neurons. Remarkedly, adult cerebral myelin (white matter) did not reveal higher abundances of *Elovl1* mRNA. One possible explanation may be the generally lower concentration of gene transcripts in white matter due to its much lower cell density. While in the RT-qPCR we normalized the measured quantity of mRNA for each tissue to a stably expressed reference gene or mRNA quantity, this was impossible to do for ISH.

Interestingly, these gene expression patterns largely correspond to the pattern of phenotypic involvement of our two patients carrying the *ELOVL1* variant. In both boys, hypomyelination of the central white matter was observed in cranial MRI. Optical coherence tomography (OCT) scanning showed thinning of the retinal nerve fiber layer, as a correlate of proximal optic atrophy. Further, the boys displayed ichthyosis with an abnormal epidermal keratinization involving their entire body.

3.4.2 Subcellular localization studies

Candidate gene expression may further be studied on the cellular level in order to determine the subcellular localization of a gene product. Investigation of the subcellular localization thus contributes to the understanding of gene product function.[35] We restricted our intracellular assessment to protein-detecting methods.

Using immunofluorescence, we were able to visualize ELOVL1 protein and localize it in the endoplasmatic reticulum (ER) *via* co-staining with an organelle marker. We utilized calnexin as specific ER marker. This finding was in line with published data.[36]

Next, we compared the subcellular localization of wildtype ELOVL1 and variant ELOVL1(p.S165F) protein. We aimed to detect a mislocalization of the mutant protein, possibly due to misfolding that might have been caused by replacement of the polar hydrophilic side chain of serine by the non-polar aromatic one of phenylalanine.

For this experiment, we planned a co-transfection of two protein expression plasmids containing either wildtype or variant *ELOVL1* cDNA into the same cell. During the transfection process this nucleic acid material would be introduced into eukaryotic cells. We thus needed to generate two plasmids carrying either wildtype or variant *ELOVL1* cDNA, each fused to a different fluorescence

marker gene. The generation of such fusion proteins was necessary in order to distinguish wildtype from mutant protein by its different fluorescence (e.g. red *versus* green fluorescence). For generation of a gene fragment containing the coding sequence of wildtype and mutant *ELOVL1*, RNA was isolated out of patient fibroblasts and reversely transcribed into cDNA.

Cloning step 1*:* The *ELOVL1* gene holding the heterozygous variant was amplified in a PCR reaction with a proofreading DNA polymerase (PhusionTaq) with two engineered primers each containing one specific enzyme restriction site (*Xho*I and *Kpn*I). The *ELOVL1* PCR-product was purified by agarose electrophoresis. As PhusionTaq produces a blunt-ended DNA strand, an adenine overhang was added at the 3'-end of the DNA-strand using "classic" Taq DNA polymerase, preparing the DNA for the TA-cloning protocol. Following this protocol, the *ELOVL1* inserts with the A-overhang (wildtype and variant separately) were inserted into a linearized vector with a 3' T-overhang at both ends. This vector (pGEM®-T Easy, Promega) carries an ampicillin resistance gene. Ligation products were transformed into heat shock competent JM109 (Promega) *Escherichia coli* bacteria. Only bacteria transformed by a plasmid that had been successfully re-ligated by incorporation of an *ELOVL1* insert were able to express the antibiotic resistance gene and grow on selective (ampicillin 100 mg/ml) agar. Plasmid DNA was then prepared by mini-prep from bacterial pellets after mini (5 ml) subculture of single cones.

Cloning step 2: Next, the purified plasmid DNA underwent restriction enzyme digestion with *Xho*I and *Kpn*I to excise the *ELOVL1* insert with the desired 5' and 3' overhangs. The excised *ELOVL1* insert was purified by agarose electrophoresis and cloned into the linearized and dephosphorylated targeting vectors encoding a C-terminal EGFP or RFP-tag. This targeting vector had been linearized before using the same restriction enzymes *Xho*I and *Kpn*I. We thus obtained two plasmids, expressing a fusion protein of wildtype or variant *ELOVL1* together with a green or red fluorescent tag. The correctness of all cloned plasmids was verified by automatic DNA sequencing using the Sanger method.

Using the calcium phosphate precipitation method, COS-1 cells were subjected to double-transfection with the two generated plasmids. After transfection, the cells expressed wildtype and variant ELOVL1 protein fused to a C-terminal fluorescence marker: enhanced green fluorescent protein (EGFP) for wildtype or red fluorescent protein (RFP) for variant. *Via* confocal immunofluorescence microscopy, we found wildtype and variant ELOVL1 protein to fully co-localize in COS-1 cells. Therefore, we concluded that the genetic variant in *ELOVL1* did not cause a visible effect on subcellular localization of the mutant protein.

3.4.3 Protein structure prediction

In order to enhance our understanding of ELOVL1 secondary structure, we used bioinformatic tools to predict protein topology. ELOVL1 localizes in the ER. The protein of 279 amino acids was predicted to exhibit seven transmembrane domains, with the variant ELOVL1(p.S165F) located at the border between ER lumen and lipid bilayer of the ER. This finding was in accordance with recently described dominant *ELOVL4* (OMIM #133190) and *ELOVL5* (OMIM #615957) mutations. Likewise, these mutations were predicted to be located at the border between ER lumen and lipid bilayer, leading both to spinocerebellar ataxia.

3.4.4 Protein function studies

The *ELOVL1* gene encodes for the protein ELOVL1 (***EL***ongation ***O***f ***V***ery ***L***ong-chain fatty acids protein 1). ELOVL1 is one of seven mammalian fatty acid (FA) elongase isoenzymes, responsible for the elongation of very long-chain fatty acids (VLCFAs). VLCFAs are FAs with carbon (C) chains of at least C21.[37] Following their activation to acyl-CoA, VLCFAs are elongated by four enzymes forming the elongase complex in the membrane of the ER.[38] The VLCFA elongation proceeds in a four-step cycle consisting of repeated biochemical reactions (condensation, reduction, dehydration, and reduction), resulting in the sequential addition of two carbon units to the carboxyl end of the growing chain.[39] ELOVL enzymes catalyze the rate-limiting step of VLCFA elongation, the condensation of malonyl-CoA with an acyl-CoA precursor to 3-ketoacyl-CoA. Each member of the ELOVL family has a distinct substrate specificity, differing in carbon chain length and the preferred number of double bonds.[40] ELOVL1 exhibits activity especially towards saturated and monounsaturated C20- and C22-VLCFAs, producing saturated and monounsaturated C24- and C26-VLCFAs.[37]

VLCFAs are indispensable for functioning of membranes and particularly important for the synthesis of myelin and photoreceptors, as well as for skin permeability barrier, cell signaling, and spermatogenesis.[37,39] In the organism, VLCFAs are mainly found as building blocks of lipids, namely sphingolipids and glycerophospholipids.[41] Being the backbone of all sphingolipids, ceramide is composed of a sphingosine linked to a FA of varying length. Sphingomyelin is generated from ceramide by addition of a polar head group (phosphocholine).

Ceramides carrying ≥C26-VLCFA are crucial for the epidermal permeability barrier, as they represent the principal component of lipid lamellae, a hydrophobic mixture situated between the epidermal corneocytes.[42] ELOVL1 is responsible for epidermal C26-VLCFA production, further utilized for C26-ceramide synthesis by ceramide synthase 3 (CERS3). *Elovl1* knockout (ko) mice died during their neonatal period due to epidermal barrier defects with transcutaneous water loss.

Epidermal ceramide (≥C26-VLCFAs) and sphingomyelin (≥C24-VLCFAs) levels of these animals were decreased, as was the case for levels in brain and other tissues.[37]

Myelination and neurodevelopment depend on well-balanced VLCFA supply. A surplus of VLCFAs due to lack of degradation may lead to white matter lesions, seen in X-linked adrenoleukodystrophy (OMIM #300100). The lack of VLCFAs due to impeded synthesis may cause hypomyelination and severe neurodevelopmental defects, seen in homozygous *ELOVL4* mutations (OMIM #614457). VLCFAs synthesized by ELOVL1 are converted to sphingolipids essential for myelin synthesis and maintenance.[43] Myelin-producing cells mostly utilize C24-sphingolipids for myelin formation.[43,44] C24-ceramides are produced by ceramide synthase 2 (CERS2) from ELOVL1-generated C24-VLCFAs.[41] *Cers2* ko mice exhibited impaired myelin formation due to lack of C24-sphingolipids.[43]

3.4.4.1 Enzymatic assay

To determine the functional consequences of the ELOVL1(p.S165F) variant on protein function, we employed an *in vitro* enzymatic assay. Enzyme assays are amongst the most accepted tools in support of variant pathogenicity.[24] The experiments using enzymatic assays were performed by our collaborators at Hokkaido University, Sapporo, Japan.

They performed a FA elongation assay to examine the enzymatic activity of wildtype and variant ELOVL1(p.S165F). HEK 294T cells were transfected with plasmids containing the coding sequence of either wildtype or variant *ELOVL1*. The cloning procedure is briefly described in 3.2. As negative controls, they utilized a plasmid carrying an engineered loss-of-function mutation in *ELOVL1*(p.H144A/p.H145A) and an empty vector. Western blot analysis confirmed adequate ELOVL1 protein expression in the cells. The total membrane fraction containing the ELOVL1 protein of each transfected cell line was incubated with [^{13}C]malonyl-CoA as the C2 (two carbons) donor and C20:0-CoA or C22:0-CoA as the acyl-CoA substrate. Later, lipids were extracted and elongation products [^{13}C]C22:0-CoA or [^{13}C]C24:0-CoA converted to [^{13}C]C22:0 and [^{13}C]C24:0 FAs, to be analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Variant ELOVL1(p.S165F) exhibited a significantly lower activity towards both substrates compared with wildtype ELOVL1. ELOVL1(p.S165F) activity was comparable to ELOVL1(p.H144A/p.H145A) or empty vector control activity. Hence, we concluded that ELOVL1(p.S165F) has no enzymatic activity.

Evidence for pathogenicity of the mutation might have been stronger had the enzymatic activity been assayed directly on patient tissue, rather than on transfected cells.[24] However, our patient cells were heterozygous for the mutation and consequently had one allele expressing wildtype

ELOVL1. We presumed that by performing enzymatic assays directly on patient tissue, measurements of enzymatic activity might have been difficult to interpret: The wildtype transcript could have been upregulated in compensation, or there might have occurred a negative interference between wildtype and variant protein (e.g. in multiprotein complexes). In order to simply assess the enzymatic activity of the wildtype *versus* the variant ELOVL1(p.S165F) version of the protein, we overexpressed either of them in cells that were then utilized for the *in vitro* elongation assay. By this assay, an effect would be clearly attributable to the alteration in the protein. Thus, by using transfected cells instead of patient cells we were able to study solely the effect of the *ELOVL1* c.494C>T genomic sequence variation on ELOVL1 enzymatic activity.

3.4.4.2 Lipid analyses in patient cells

Using the enzymatic assay, we were able to demonstrate that the mutation in *ELOVL1* most likely causes a severe functional defect at the molecular level. Now, we were interested to investigate whether the ELOVL1 enzyme deficiency would derange the composition of the VLCFAs in the cytosol of our patients' cells. The experimental work of these lipid analyses was again performed by our collaborators in Hokkaido University, Sapporo, Japan.

They examined the steady state levels of sphingolipids in cells of affected individuals and controls. They determined ceramide and sphingomyelin composition of patient (n=2) and control (n=3) fibroblasts, as well as in skin specimen/keratinocytes. Using LC-MS/MS, they found a decrease of ELOVL1 enzymatic products (ceramide and sphingomyelin carrying C26:1 and C26:0) behind and accumulation of ELOVL1 educts (C22:0-sphingomyelin) in patient *versus* control cells. This was reflected by significantly lower ratios of C24:0/C22:0, C24:1/C22:1, C26:0/C22:0, and C26:1/C22:1 FAs as lipid components of the sphingolipids in patient fibroblasts and skin samples. Hence, this indirect functional assessment showed reduced levels of ELOVL1 catalytic products in patient cells compared with controls. Together with the results of the *in vitro* enzymatic assay – confirming absent ELOVL1(p.S165F) enzymatic activity – we conclude that the measured lipid alterations derive from the pathogenic effect of the ELOVL1(p.S165F) protein variant.

3.4.5 Transcriptome studies

In order to identify genes whose RNA transcript levels are differentially regulated in response to the presence of the pathogenic ELOVL1 variant, we conducted an RNA transcriptome study. The transcriptome is the total of all RNA transcripts present in a cell at a certain time point.[45] Currently, there are two widely used methods to study the transcriptome: microchip arrays or RNA-sequencing (RNA-seq).

RNA microarrays measure large numbers of predefined transcripts simultaneously. Fluorescently pre-labeled mRNA-transcripts hybridize to complementary nucleic acid sequences that are fixed on the array and are quantified by fluorescence read-out.[46]

RNA-seq is based on NGS technology and aims to sequence all RNA transcripts currently present in cells.[47] Transcript abundance correlates with the number of obtained RNA sequences.[48] RNA-seq is becoming increasingly important for analysis of global gene expression. Compared with microarray technologies, RNA-seq detects larger dynamic ranges of transcript expression levels and requires lower amounts of input RNA.[47,48]

In our study, we performed transcriptome studies *via* RNA-seq in control (n=4) and patient (n=2) fibroblasts. Our aim was to investigate whether global mRNA-expression would be different between heterozygous patient cells and age-matched controls. Due to the lack of tissue access our analyses were performed in fibroblasts instead of neuronal tissue. However, genome-wide expression data from patient fibroblast have been shown to be, within limits, representative for the human brain transcriptome.[49] In order to make gene expression data comparable among samples, we calculated the FPKM values for each gene. FPKM is the acronym for ***F***ragments ***p***er ***k***ilobase of exon per ***m***illion reads mapped. This value normalizes the measured reads per gene for sequencing depth and lengths of genes.[50]

In patient fibroblasts, we found consistent up- or down-regulation of various gene transcripts in comparison with controls. Interestingly, the *ELOVL1* expression level was unaltered in patients' cells. Notably, *CERS3* was found to be compensatory upregulated in patients. ELOVL1 and CERS3 functionally interact closely in the skin: C26-VLCFAs produced by ELOVL1 are further processed by CERS3, thus generating C26-ceramides.[37,41] *Cers3* ko mice died neonatally due to defects of their epidermal permeability barrier.[51] In humans, recessive *CERS3* mutations are associated with ichthyosis.[52] Overall, we detected the most excessive upregulation in patient cells for genes involved in epithelial differentiation, development of epidermis and keratinization, which may correspond to thickened epidermal layer noted in our patients.

In contrast, we found a downregulation of genes important for brain development and myelination in patient cells. One explanation may be the lack of myelin building blocks (especially of ≥C24-sphingolipids) due to the ELOVL1 enzyme defect. With limited number of building blocks available, myelination and neurodevelopment may become deranged. However, the building blocks for epidermal development were likewise reduced, while we measured involved genes to be up-regulated. This tissue specific reaction towards lack of VLCFAs needs further study.

We observed an altered expression level of entire gene modules in patient cells with VLCFA deficiency due to ELOVL1 enzymatic block. Therefore, we considered the possibility of VLCFAs

directly affecting the gene transcription machinery. VLCFAs are known to interact with peroxisome proliferator-activated receptors (PPARs) which are nuclear receptors. PPARs bind to specific DNA sequence elements in the nucleus called PPAR response elements (PPREs), thereby up- or downregulating gene transcription. Consequently, PPARs control the transcription of genes carrying a PPRE in their promotor region. The effect of C16-FAs on PPARγ activity was recently studied in the peripheral nerve system (PNS) of mice.[53] Those genetically modified mice were unable to perform *de novo* FA synthesis and therefore lacked C16-FA. The C16-FA deficiency downregulated the PPARγ transcriptional network and led to defective PNS myelination. Interestingly, restoring PPARγ-activity by application of a PPARγ-agonist ameliorated PNS myelination in those C16-FA deficient mice.[53] In another study, PPARα was described to perform a conformational change upon C24:1-VLCFA binding, leading to suppression of gene transcription.[54] Lack of VLCFAs might therefore upregulate PPARα-pathways.

Using computational analysis, we searched for PPREs in the promotor regions of all genes that were differentially expressed in patient fibroblasts compared with controls. We detected PPREs in multiple up- or downregulated genes. Notably, *CERS3* and keratin differentiation associated protein (*KRTDAP*) genes revealed the highest probability for PPARα binding. The deficiency of C24-VLCFAs may contribute to disinhibition of PPARα, translating into higher expression levels of genes involved in epidermal proliferation and keratinization. However, we did not capture causal relationships. Therefore, our data do not permit to draw firm conclusions. We need further experimental investigations in various tissues to determine the effect of VLCFAs on the gene transcription machinery, particularly on PPAR-mediated action. Of special interest would be the search for PPAR-modulating drugs with a potential therapeutic effect.

3.5 Treatment approaches and perspectives

Having biochemically characterized the ELOVL1(p.S165F) enzymatic block and subsequent VLCFA deficiency in patient cells, we were interested to explore options to treat the disease. Biochemical fatty acid loading experiments were conducted by our collaborators in Hokkaido University, Sapporo, Japan. Transmembrane transport of VLCFAs is challenging because cells lack a mechanism to take up VLCFAs from their surroundings. We thus settled for *in vitro* treatment of the patient cells using shorter chain FAs that have the capability to overcome the cell membrane. We tested treatment of patient fibroblasts with C22- and C24-VLCFA molecules separately. Loading with C22:0-VLCFAs augmented levels of ceramides and sphingomyelins carrying C22:0- and C24:0-VLCFAs. However, levels of ceramides and sphingomyelins holding C26:0-VLCFAs remained unaltered. Notably, we observed a decrease in monounsaturated C24:1- and C26:1-

ceramides and -sphingomyelins in C22:0-VLCFA treated cells, probably mediated by competitive inhibition between saturated and monounsaturated FAs. While loading with C22:1-VLCFAs led to cell death, loading with C24:0-VLCFAs did not show any effect. Moreover, supplementation with C24:1-VLCFAs led to an increase in C24:1-sphingomyelins and decrease in sphingomyelins and ceramides carrying C24:0-VLCFAs. In summary, VLCFAs of smaller size (C22:0) were found to be successfully imported into fibroblast cells. Imported C22:0-VLCFAs were then elongated as ELOVL1 enzymatic educts to C24:0-VLCFAs, both in patient and control cells. However, the FA elongation cycle did not proceed the next step towards C26:0-VLCFAs. Our findings show that adequate VLCFA supplementation is a complex endeavor. Importantly, we should consider competitive inhibition between saturated and monounsaturated substrates of elongases. We need to conduct systematic studies of VLCFA repletion as a therapeutic approach. Our data calls for further research to determine the optimum mixture of saturated and monounsaturated VLCFAs for therapy, whereby reducing competitive inhibition effects. For skin-related symptoms like ichthyosis, topical applications of VLCFA-mixtures could be beneficial. VLCFA-deficiency in the central nervous system would be more difficult to target, because diet derived VLCFAs may not cross the blood-brain-barrier.

Currently, we are not able to determine the exact pathomechanism of this dominant disease, which might be based either on haploinsufficiency or a dominant negative effect.[55] In haploinsufficiency, the presence of only one wildtype allele instead of two copies leads to a lack of protein dosage, which is deleterious to the organism. In a dominant negative model, the variant protein disturbs or competes with the wildtype protein (e.g. in multi-protein complexes). In order to distinguish between these different mechanisms, the following experiments could be conducted: Several co-transfections of cells with variant and wildtype *ELOVL1* constructs could be performed using varying concentrations of transfected variant *ELOVL1* c.494C>T. The bioavailability of ELOVL1-products (sphingolipids carrying C24- and C26-VLCFAs) could then be measured. In haploinsufficiency, we would expect no alteration of ELOVL1 product levels with higher dosages of variant *ELOVL1*. In a dominant negative model, we might observe a decrease in ELOVL1 enzymatic products in parallel with a rising amount of transfected *ELOVL1* variant.

During preparation of our manuscript which we submitted in August 2018[56] we became aware of a publication by Kutkowska-Kazmierczak *et al.* in June 2018[57], who had – unknown to us – been working on the same two families. Patients with rare diseases sometimes move internationally, searching for diagnosis and therapy. Regarding the finding of the heterozygous *de novo* mutation in *ELOVL1*, we confirm the work of this group. In other aspects, our results are important for better understanding of disease pathophysiology, molecular mechanism, clinical phenotype, and first

treatment approaches. We believe that closer cooperation of research groups worldwide working in the field of rare diseases would be favorable for the optimization of diagnostic processes and therapeutic advances. Recently developed matching tools such GeneMatcher[58] help to connect researchers around the globe, but also patients and clinicians, interested in a certain gene. In particular, the project aims to help finding additional individuals affected by a rare genetic disease. In order to secure a genetic defect according to the criteria of the American Journal of Human Genetics at least two patients with the same phenotype and alterations in the same gene should be described delineating the phenotype of the disease.[10] Recently, the International Rare Disease Research Consortium (IRDiRC), uniting national and international government and non-profit organizations, announced several goals for the period 2017-2027 that included (i) entry of all currently undiagnosed patients into a global research database, (ii) diagnosis of patients within one year for already known rare diseases, and (iii) development of 1000 new therapies.[12]

In conclusion, we identified the novel *ELOVL1* c.494C>T genomic sequence alteration in two unrelated individuals as disease-causing variant for a novel neurocutaneous syndrome. We described the clinical phenotype and molecular mechanisms of this heterozygous *ELOVL1* variant. The missense mutation completely abrogated ELOVL1(p.S165F) activity as verified by functional enzyme assays. As ceramides and sphingomyelins are the important building blocks made from VLCFAs, we assessed their composition in the skin as one of the primarily affected tissues. C24/C22-VLCFA- and C26/C22-VLCFA-ratios for sphingomyelins and ceramides were significantly reduced in patients' fibroblasts and skin specimens. Moreover, *Elovl1* mRNA was highly expressed in tissues impaired by the disease, such as cerebral white matter, eye, and skin. Our analysis of the patients' fibroblast transcriptome may help to understand the regulatory network involved in this multifaceted disease. Further investigations of the PPAR transcriptional network and its dependency on adequate VLCFAs supply could be valuable. In the future, PPAR-modulating drugs may be studied as possible treatment options. Along these lines, we explored VLCFA-treatment of patient fibroblasts. While this therapeutic approach showed some first promising results, adverse effects such as competitive inhibition amongst enzymatic substrates demand caution. We need systematic testing of VLCFA-supplementation before initiation of clinical orphan drug trials using topical ointments.

3.6 Literature

1. The European Parliament and the Council of the European Union. Regulation (EC) No. 141/2000 of the European Parliament and of the Council of 16 December 1999 on orphan medicinal products. http://data.europa.eu/eli/reg/2000/141/oj.
2. Rath, A., Olry, A., Dhombres, F., Brandt, M. M., Urbero, B. & Ayme, S. Representation of rare diseases in health information systems: The orphanet approach to serve a wide range of end users. *Human Mutation* **33**, 803–808 (2012).
3. Amberger, J., Bocchini, C. & Hamosh, A. A new face and new challenges for Online Mendelian Inheritance in Man (OMIM®). *Hum. Mutat.* **32**, 564–567 (2011).
4. Yáñez-Muñoz, R. J. 10 Years of Rare Disease Day. *Gene Therapy* **24**, 67 (2017).
5. World Health Organization. Rare diseases. Priority diseases and reasons for inclusion. http://www.who.int/medicines/areas/priority_medicines/Ch6_19Rare.pdf?ua=1. (2018).
6. Commission of the European Communities. Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the committee of the regions on Rare Diseases: Europe's Challenges. https://ec.europa.eu/health/ph_threats/non_com/docs/rare_com_en.pdf. (2008).
7. Melnikova, I. Rare diseases and orphan drugs. *Nat Rev Drug Discov* **11**, 267–268 (2012).
8. Boycott, K. M. & Ardigó, D. Addressing challenges in the diagnosis and treatment of rare genetic diseases. *Nat Rev Drug Discov* **17**, 151–152 (2018).
9. Dawkins, H. J. S., Draghia-Akli, R., Lasko, P., Lau, L. P. L., Jonker, A. H., Cutillo, C. M., Rath, A., Boycott, K. M., Baynam, G., Lochmüller, H., Kaufmann, P., Le Cam, Y., Hivert, V., Austin, C. P. & International Rare Diseases Research Consortium (IRDiRC). Progress in Rare Diseases Research 2010-2016: An IRDiRC Perspective. *Clin Transl Sci* **11**, 11–20 (2018).
10. Boycott, K. M., Rath, A., Chong, J. X., Hartley, T., Alkuraya, F. S., Baynam, G., Brookes, A. J., Brudno, M., Carracedo, A., den Dunnen, J. T., Dyke, S. O. M., Estivill, X., Goldblatt, J., Gonthier, C., Groft, S. C., Gut, I., Hamosh, A., Hieter, P., Höhn, S., Hurles, M. E., Kaufmann, P., Knoppers, B. M., Krischer, J. P., Macek, M., Matthijs, G., Olry, A., Parker, S., Paschall, J., Philippakis, A. A., Rehm, H. L., Robinson, P. N., Sham, P.-C., Stefanov, R., Taruscio, D., Unni, D., Vanstone, M. R., Zhang, F., Brunner, H., Bamshad, M. J. & Lochmüller, H. International Cooperation to Enable the Diagnosis of All Rare Genetic Diseases. *Am. J. Hum. Genet.* **100**, 695–705 (2017).

11. Giannuzzi, V., Conte, R., Landi, A., Ottomano, S. A., Bonifazi, D., Baiardi, P., Bonifazi, F. & Ceci, A. Orphan medicinal products in Europe and United States to cover needs of patients with rare diseases: an increased common effort is to be foreseen. *Orphanet J Rare Dis* **12**, 64 (2017).
12. Austin, C. P., Cutillo, C. M., Lau, L. P. L., Jonker, A. H., Rath, A., Julkowska, D., Thomson, D., Terry, S. F., de Montleau, B., Ardigò, D., Hivert, V., Boycott, K. M., Baynam, G., Kaufmann, P., Taruscio, D., Lochmüller, H., Suematsu, M., Incerti, C., Draghia-Akli, R., Norstedt, I., Wang, L., Dawkins, H. J. S. & International Rare Diseases Research Consortium (IRDiRC). Future of Rare Diseases Research 2017-2027: An IRDiRC Perspective. *Clin Transl Sci* **11**, 21–27 (2018).
13. Köhler, S., Carmody, L., Vasilevsky, N., Jacobsen, J. O. B., Danis, D., Gourdine, J.-P., Gargano, M., Harris, N. L., Matentzoglu, N., McMurry, J. A., Osumi-Sutherland, D., Cipriani, V., Balhoff, J. P., Conlin, T., Blau, H., Baynam, G., Palmer, R., Gratian, D., Dawkins, H., Segal, M., Jansen, A. C., Muaz, A., Chang, W. H., Bergerson, J., Laulederkind, S. J. F., Yüksel, Z., Beltran, S., Freeman, A. F., Sergouniotis, P. I., Durkin, D., Storm, A. L., Hanauer, M., Brudno, M., Bello, S. M., Sincan, M., Rageth, K., Wheeler, M. T., Oegema, R., Lourghi, H., Della Rocca, M. G., Thompson, R., Castellanos, F., Priest, J., Cunningham-Rundles, C., Hegde, A., Lovering, R. C., Hajek, C., Olry, A., Notarangelo, L., Similuk, M., Zhang, X. A., Gómez-Andrés, D., Lochmüller, H., Dollfus, H., Rosenzweig, S., Marwaha, S., Rath, A., Sullivan, K., Smith, C., Milner, J. D., Leroux, D., Boerkoel, C. F., Klion, A., Carter, M. C., Groza, T., Smedley, D., Haendel, M. A., Mungall, C. & Robinson, P. N. Expansion of the Human Phenotype Ontology (HPO) knowledge base and resources. *Nucleic Acids Res.* **47**, D1018–D1027 (2019).
14. Mueller, N., Sassa, T., Morales-Gonzalez, S., Schneider, J., Salchow, D. J., Seelow, D., Knierim, E., Stenzel, W., Kihara, A. & Schuelke, M. De novo mutation in ELOVL1 causes ichthyosis, acanthosis nigricans, hypomyelination, spastic paraplegia, high frequency deafness and optic atrophy. *J. Med. Genet.* **56**, 164–175 (2019).
15. Botstein, D. & Risch, N. Discovering genotypes underlying human phenotypes: past successes for mendelian disease, future approaches for complex disease. *Nat. Genet.* **33 Suppl**, 228–237 (2003).
16. Ballabio, A. The rise and fall of positional cloning? *Nat. Genet.* **3**, 277–279 (1993).
17. Shendure, J. & Ji, H. Next-generation DNA sequencing. *Nature Biotechnology* **26**, 1135–1145 (2008).

18. Schwarze, K., Buchanan, J., Taylor, J. C. & Wordsworth, S. Are whole-exome and whole-genome sequencing approaches cost-effective? A systematic review of the literature. *Genetics in Medicine* **20**, 1122 (2018).
19. Goodwin, S., McPherson, J. D. & McCombie, W. R. Coming of age: ten years of next-generation sequencing technologies. *Nat. Rev. Genet.* **17**, 333–351 (2016).
20. Ng, S. B., Buckingham, K. J., Lee, C., Bigham, A. W., Tabor, H. K., Dent, K. M., Huff, C. D., Shannon, P. T., Jabs, E. W., Nickerson, D. A., Shendure, J. & Bamshad, M. J. Exome sequencing identifies the cause of a mendelian disorder. *Nat. Genet.* **42**, 30–35 (2010).
21. Biesecker, L. G. Opportunities and challenges for the integration of massively parallel genomic sequencing into clinical practice: lessons from the ClinSeq project. *Genet. Med.* **14**, 393–398 (2012).
22. Bamshad, M. J., Ng, S. B., Bigham, A. W., Tabor, H. K., Emond, M. J., Nickerson, D. A. & Shendure, J. Exome sequencing as a tool for Mendelian disease gene discovery. *Nat. Rev. Genet.* **12**, 745–755 (2011).
23. Cooper, G. M. & Shendure, J. Needles in stacks of needles: finding disease-causal variants in a wealth of genomic data. *Nat. Rev. Genet.* **12**, 628–640 (2011).
24. Richards, S., Aziz, N., Bale, S., Bick, D., Das, S., Gastier-Foster, J., Grody, W. W., Hegde, M., Lyon, E., Spector, E., Voelkerding, K. & Rehm, H. L. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genetics in Medicine* **17**, 405–423 (2015).
25. Lek, M., Karczewski, K. J., Minikel, E. V., Samocha, K. E., Banks, E., Fennell, T., O'Donnell-Luria, A. H., Ware, J. S., Hill, A. J., Cummings, B. B., Tukiainen, T., Birnbaum, D. P., Kosmicki, J. A., Duncan, L. E., Estrada, K., Zhao, F., Zou, J., Pierce-Hoffman, E., Berghout, J., Cooper, D. N., Deflaux, N., DePristo, M., Do, R., Flannick, J., Fromer, M., Gauthier, L., Goldstein, J., Gupta, N., Howrigan, D., Kiezun, A., Kurki, M. I., Moonshine, A. L., Natarajan, P., Orozco, L., Peloso, G. M., Poplin, R., Rivas, M. A., Ruano-Rubio, V., Rose, S. A., Ruderfer, D. M., Shakir, K., Stenson, P. D., Stevens, C., Thomas, B. P., Tiao, G., Tusie-Luna, M. T., Weisburd, B., Won, H.-H., Yu, D., Altshuler, D. M., Ardissino, D., Boehnke, M., Danesh, J., Donnelly, S., Elosua, R., Florez, J. C., Gabriel, S. B., Getz, G., Glatt, S. J., Hultman, C. M., Kathiresan, S., Laakso, M., McCarroll, S., McCarthy, M. I., McGovern, D., McPherson, R., Neale, B. M., Palotie, A., Purcell, S. M., Saleheen, D.,

Scharf, J. M., Sklar, P., Sullivan, P. F., Tuomilehto, J., Tsuang, M. T., Watkins, H. C., Wilson, J. G., Daly, M. J., MacArthur, D. G. & Exome Aggregation Consortium. Analysis of protein-coding genetic variation in 60,706 humans. *Nature* **536**, 285–291 (2016).

26. Belkadi, A., Pedergnana, V., Cobat, A., Itan, Y., Vincent, Q. B., Abhyankar, A., Shang, L., Baghdadi, J. E., Bousfiha, A., Consortium, the E., Alcais, A., Boisson, B., Casanova, J.-L. & Abel, L. Whole-exome sequencing to analyze population structure, parental inbreeding, and familial linkage. *PNAS* **113**, 6713–6718 (2016).
27. Wakeling, M. N., Laver, T. W., Wright, C. F., Franco, E. D., Stals, K. L., Patch, A.-M., Hattersley, A. T., Flanagan, S. E. & Ellard, S. Homozygosity mapping provides supporting evidence of pathogenicity in recessive Mendelian disease. *Genetics in Medicine* 1 (2018). doi:10.1038/s41436-018-0281-4
28. Knierim, E., Seelow, D., Gill, E., von Moers, A. & Schuelke, M. Clinical application of whole exome sequencing reveals a novel compound heterozygous TK2-mutation in two brothers with rapidly progressive combined muscle-brain atrophy, axonal neuropathy, and status epilepticus. *Mitochondrion* **20**, 1–6 (2015).
29. Nemesure, B. B., He, Q. & Mendell, N. R. A normalized identity-by-state statistic for linkage analysis of sib pairs. *Genetic Epidemiology* **17**, S673–S677 (1999).
30. Lee, H., Deignan, J. L., Dorrani, N., Strom, S. P., Kantarci, S., Quintero-Rivera, F., Das, K., Toy, T., Harry, B., Yourshaw, M., Fox, M., Fogel, B. L., Martinez-Agosto, J. A., Wong, D. A., Chang, V. Y., Shieh, P. B., Palmer, C. G. S., Dipple, K. M., Grody, W. W., Vilain, E. & Nelson, S. F. Clinical Exome Sequencing for Genetic Identification of Rare Mendelian Disorders. *JAMA* **312**, 1880–1887 (2014).
31. Schwarz, J. M., Cooper, D. N., Schuelke, M. & Seelow, D. MutationTaster2: mutation prediction for the deep-sequencing age. *Nat Meth* **11**, 361–362 (2014).
32. Wagner, E. M. Monitoring gene expression: quantitative real-time rt-PCR. *Methods Mol. Biol.* **1027**, 19–45 (2013).
33. Pfaffl, M. W. A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Res* **29**, e45 (2001).
34. Kadkol, S. S., Gage, W. R. & Pasternack, G. R. In situ hybridization-theory and practice. *Mol. Diagn.* **4**, 169–183 (1999).
35. Guo, X., Liu, F., Ju, Y., Wang, Z. & Wang, C. Human Protein Subcellular Localization with Integrated Source and Multi-label Ensemble Classifier. *Scientific Reports* **6**, 28087 (2016).

36. Ohno, Y., Suto, S., Yamanaka, M., Mizutani, Y., Mitsutake, S., Igarashi, Y., Sassa, T. & Kihara, A. ELOVL1 production of C24 acyl-CoAs is linked to C24 sphingolipid synthesis. *Proc Natl Acad Sci U S A* **107**, 18439–18444 (2010).
37. Sassa, T., Ohno, Y., Suzuki, S., Nomura, T., Nishioka, C., Kashiwagi, T., Hirayama, T., Akiyama, M., Taguchi, R., Shimizu, H., Itohara, S. & Kihara, A. Impaired Epidermal Permeability Barrier in Mice Lacking Elovl1, the Gene Responsible for Very-Long-Chain Fatty Acid Production. *Mol. Cell. Biol.* **33**, 2787–2796 (2013).
38. Denic, V. & Weissman, J. S. A Molecular Caliper Mechanism for Determining Very Long-Chain Fatty Acid Length. *Cell* **130**, 663–677 (2007).
39. Kihara, A. Very long-chain fatty acids: elongation, physiology and related disorders. *J Biochem* **152**, 387–395 (2012).
40. Guillou, H., Zadravec, D., Martin, P. G. P. & Jacobsson, A. The key roles of elongases and desaturases in mammalian fatty acid metabolism: Insights from transgenic mice. *Prog. Lipid Res.* **49**, 186–199 (2010).
41. Sassa, T. & Kihara, A. Metabolism of very long-chain Fatty acids: genes and pathophysiology. *Biomol Ther (Seoul)* **22**, 83–92 (2014).
42. Kihara, A. Synthesis and degradation pathways, functions, and pathology of ceramides and epidermal acylceramides. *Prog. Lipid Res.* **63**, 50–69 (2016).
43. Imgrund, S., Hartmann, D., Farwanah, H., Eckhardt, M., Sandhoff, R., Degen, J., Gieselmann, V., Sandhoff, K. & Willecke, K. Adult ceramide synthase 2 (CERS2)-deficient mice exhibit myelin sheath defects, cerebellar degeneration, and hepatocarcinomas. *J. Biol. Chem.* **284**, 33549–33560 (2009).
44. Becker, I., Wang-Eckhardt, L., Yaghootfam, A., Gieselmann, V. & Eckhardt, M. Differential expression of (dihydro)ceramide synthases in mouse brain: oligodendrocyte-specific expression of CerS2/Lass2. *Histochem. Cell Biol.* **129**, 233–241 (2008).
45. Transcriptomics technologies. Available at: https://journals.plos.org/ploscompbiol/article?id=10.1371/journal.pcbi.1005457. (Accessed: 7th December 2018)
46. Bumgarner, R. DNA microarrays: Types, Applications and their future. *Curr Protoc Mol Biol* **0 22**, Unit-22.1. (2013).
47. Finotello, F. & Di Camillo, B. Measuring differential gene expression with RNA-seq: challenges and strategies for data analysis. *Brief Funct Genomics* **14**, 130–142 (2015).
48. Wang, Z., Gerstein, M. & Snyder, M. RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet* **10**, 57–63 (2009).

49. Visser, W. E., Swagemakers, S. M. A., Ozgur, Z., Schot, R., Verheijen, F. W., van Ijcken, W. F. J., van der Spek, P. J. & Visser, T. J. Transcriptional profiling of fibroblasts from patients with mutations in MCT8 and comparative analysis with the human brain transcriptome. *Hum. Mol. Genet.* **19**, 4189–4200 (2010).
50. Conesa, A., Madrigal, P., Tarazona, S., Gomez-Cabrero, D., Cervera, A., McPherson, A., Szcześniak, M. W., Gaffney, D. J., Elo, L. L., Zhang, X. & Mortazavi, A. A survey of best practices for RNA-seq data analysis. *Genome Biol* **17**, (2016).
51. Jennemann, R., Rabionet, M., Gorgas, K., Epstein, S., Dalpke, A., Rothermel, U., Bayerle, A., van der Hoeven, F., Imgrund, S., Kirsch, J., Nickel, W., Willecke, K., Riezman, H., Gröne, H.-J. & Sandhoff, R. Loss of ceramide synthase 3 causes lethal skin barrier disruption. *Hum. Mol. Genet.* **21**, 586–608 (2012).
52. Radner, F. P. W., Marrakchi, S., Kirchmeier, P., Kim, G.-J., Ribierre, F., Kamoun, B., Abid, L., Leipoldt, M., Turki, H., Schempp, W., Heilig, R., Lathrop, M. & Fischer, J. Mutations in CERS3 cause autosomal recessive congenital ichthyosis in humans. *PLoS Genet.* **9**, e1003536 (2013).
53. De novo fatty acid synthesis by Schwann cells is essential for peripheral nervous system myelination | JCB. Available at: http://jcb.rupress.org/content/217/4/1353. (Accessed: 17th December 2018)
54. Hostetler, H. A., Kier, A. B. & Schroeder, F. Very-long-chain and branched-chain fatty acyl-CoAs are high affinity ligands for the peroxisome proliferator-activated receptor alpha (PPARalpha). *Biochemistry* **45**, 7669–7681 (2006).
55. Hijikata, A., Tsuji, T., Shionyu, M. & Shirai, T. Decoding disease-causing mechanisms of missense mutations from supramolecular structures. *Scientific Reports* **7**, 8541 (2017).
56. Mueller, N., Sassa, T., Morales-Gonzalez, S., Schneider, J., Salchow, D. J., Seelow, D., Knierim, E., Stenzel, W., Kihara, A. & Schuelke, M. De novo mutation in ELOVL1 causes ichthyosis, acanthosis nigricans, hypomyelination, spastic paraplegia, high frequency deafness and optic atrophy. *Journal of Medical Genetics* jmedgenet-2018-105711 (2018). doi:10.1136/jmedgenet-2018-105711
57. Kutkowska-Kaźmierczak, A., Rydzanicz, M., Chlebowski, A., Kłosowska-Kosicka, K., Mika, A., Gruchota, J., Jurkiewicz, E., Kowalewski, C., Pollak, A., Stradomska, T. J., Kmieć, T., Jakubowski, R., Gasperowicz, P., Walczak, A., Śladowski, D., Jankowska-Steifer, E., Korniszewski, L., Kosińska, J., Obersztyn, E., Nowak, W., Śledziński, T., Dziem-

bowski, A. & Płoski, R. Dominant ELOVL1 mutation causes neurological disorder with ichthyotic keratoderma, spasticity, hypomyelination and dysmorphic features. *J. Med. Genet.* **55**, 408–414 (2018).

58. Sobreira, N., Schiettecatte, F., Valle, D. & Hamosh, A. GeneMatcher: a matching tool for connecting investigators with an interest in the same gene. *Hum. Mutat.* **36**, 928–930 (2015).

4 Eidesstattliche Versicherung / Anteilserklärung

„Ich, Noomi Müller, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Molecular genetic elucidation of a novel neurocutaneous syndrome“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem Betreuer angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass mir die Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis bekannt ist und ich mich zur Einhaltung dieser Satzung verpflichte.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

_____

Berlin, den 16.10.2019 Unterschrift

Ausführliche Anteilserklärung an der erfolgten Publikation

Mueller N, Sassa T, Morales-Gonzalez S, Schneider J, Salchow DJ, Seelow D, Knierim E, Stenzel W, Kihara A, Schuelke M. De novo mutation in *ELOVL1* causes ichthyosis, *acanthosis nigricans*, hypomyelination, spastic paraplegia, high frequency deafness and optic atrophy. *J Med Genet.* 2019 Mar;56(3):164-175

Noomi Müller hatte an der voranstehenden Publikation folgenden Beitrag im Einzelnen:

- Mitwirken an klinischen Untersuchungen der Patienten (Figure 1A-E, Table S1 und S2)
- Auffinden der *ELOVL1*-Variante durch Analyse der Whole-exome sequencing Daten (Figure S1, Figure 3E)
- Validierung der *ELOVL1*-Variante sowie familiäre Segregationsanalyse mittels Sanger-Sequenzierung und spezifischen Restriktionsenzymverdau (Figure 3A, B, C, E)
- Founder Haplotype Analyse (Figure 3D)
- RT-qPCR im Mausgewebe zur Analyse der mRNA-Expression von *Elovl1* in verschiedenem Gewebe (Figure 6E)
- Klonierung der *ELOVL1*-Expressionsvektoren *ELOVL1(wt)*-EGFP, *ELOVL1*(p.S165F)-EGFP sowie *ELOVL1*(p.S165F)-RFP für biochemische Versuche sowie für subzelluläre Lokalisationsexperimente (Figure 6A, Figure 4C):
    - Herstellung des *ELOVL1*-Inserts (Wildtyp und Mutante) durch RNA-Extraktion aus Patientenfibroblasten, reverse Transkription in cDNA, PCR-Reaktion unter Verwendung speziell generierter Primer, welche die Sequenzen für den spezifischen Restriktionsverdau enthielten. Klonierung des *ELOVL1*-Inserts in pGEM®-T Easy Vektor (Ligation, Transformation, Selektion auf antibiotikahaltigem Medium, Minipräp); spezifischer Restriktionsverdau des klonierten Plasmids. Klonierung des *ELOVL1*-Inserts in zuvor dephosphorylierten und linearisierten pEGFP-N3; Herstellung von *ELOVL1(wt)*-EGFP und *ELOVL1*(p.S165F)-EGFP
    - Herstellung des RFP-Inserts durch PCR-Reaktion auf dem pTagRFP-N Vektor unter Verwendung speziell generierter Primer, welche die Sequenzen für den Restriktionsverdau enthielten; Klonierung in pGEM®-T Easy Vektor; Verdau des klonierten Plasmids, Klonierung des RFP-Inserts in ELOVL1(p.S165F)-EGFP; Herstellung von *ELOVL1*(p.S165F)-RFP
- Zellkultur von Fibroblasten (Patienten und Kontrollen) sowie COS-1 Zellen
- Experimente zur subzellulären Lokalisation: Transfektion der klonierten Vektoren mittels

Calcium-Phosphat-Präzipitationstechnik, Fixierung der transfizierten COS-1 Zellen, Mitwirken bei der Immunfluoreszenz-Mikroskopie (Figure 6A)

- Bioinformatische ELOVL1-Proteinstrukturvorhersage (Figure S7)
- Statistische Auswertung der biochemischen Ergebnisse (Figure 4A-C)
- Mitwirken bei der Analyse der Transkriptom-Daten (Table S3)
- Verfassen der Publikationsschrift unter Mitwirkung der Koautoren

_____

Unterschrift, Datum und Stempel des betreuenden Hochschullehrers

_____

Unterschrift der Doktorandin

5 Auszug aus Journal Summary List

Journal Data Filtered By: **Selected JCR Year: 2017** Selected Editions: SCIE,SSCI
Selected Categories: **"GENETICS and HEREDITY"** Selected Category Scheme: WoS

Gesamtanzahl: 171 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NATURE REVIEWS GENETICS	35,680	41.465	0.094300
2	NATURE GENETICS	93,639	27.125	0.234110
3	TRENDS IN ECOLOGY & EVOLUTION	35,124	15.938	0.038820
4	GENOME BIOLOGY	34,697	13.214	0.118500
5	TRENDS IN GENETICS	11,805	10.556	0.023020
6	MOLECULAR BIOLOGY AND EVOLUTION	44,664	10.217	0.101560
7	GENOME RESEARCH	38,842	10.101	0.105060
8	GENETICS IN MEDICINE	8,825	9.937	0.028650
9	Annual Review of Genetics	7,707	9.589	0.013490
10	GENES & DEVELOPMENT	57,469	9.462	0.092720
11	Genome Medicine	4,265	8.898	0.020600
12	AMERICAN JOURNAL OF HUMAN GENETICS	36,546	8.855	0.070550
13	Annual Review of Genomics and Human Genetics	2,729	8.676	0.007160
14	MOLECULAR THERAPY	16,013	7.008	0.029180
15	ONCOGENE	66,411	6.854	0.075960
16	GENOMICS PROTEOMICS & BIOINFORMATICS	1,225	6.615	0.003480
17	Mobile DNA	622	5.891	0.003000
18	Molecular Autism	1,679	5.872	0.006320
19	JOURNAL OF MEDICAL GENETICS	12,239	5.751	0.019280
20	Circulation-Cardiovascular Genetics	3,394	5.664	0.011400
21	Forensic Science International-Genetics	4,168	5.637	0.007560
22	PLoS Genetics	42,988	5.540	0.171040
23	DNA RESEARCH	2,952	5.415	0.005620
24	HUMAN MUTATION	12,927	5.359	0.028300
25	Epigenetics & Chromatin	1,410	5.351	0.007630
26	MUTATION RESEARCH-REVIEWS IN MUTATION RESEARCH	3,440	5.205	0.003220
27	CURRENT OPINION IN GENETICS & DEVELOPMENT	7,791	4.995	0.018550
28	Epigenomics	2,055	4.979	0.006170

6 Publikation

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

http://dx.doi.org/10.1136/jmedgenet-2018-105711

7 Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

8 Komplette Publikationsliste

Wissenschaftliche Originalarbeiten mit Journal Impact Faktor (JIF) laut ISI-WEB 2017

Mueller N, Sassa T, Morales-Gonzalez S, Schneider J, Salchow DJ, Seelow D, Knierim E, Stenzel W, Kihara A, Schuelke M. De novo mutation in *ELOVL1* causes ichthyosis, *acanthosis nigricans*, hypomyelination, spastic paraplegia, high frequency deafness and optic atrophy. *J Med Genet.* 2019 Mar;56(3)

JIF: **5.751**

Mueller N, Murthy S, Tainter CR, Lee J, Riddell K, Fintelmann FJ, Grabitz SD, Timm FP, Levi B, Kurth T, Eikermann M. Can Sarcopenia Quantified by Ultrasound of the Rectus Femoris Muscle Predict Adverse Outcome of Surgical Intensive Care Unit Patients as well as Frailty? A Prospective, Observational Cohort Study. *Annals of Surgery*. 2016 Dec;264(6)

JIF: **9.203**

Zaremba S*, **Mueller N***, Heisig A*, Shin CH, Jung S, Leffert LR, Bateman BT, Pugsley LJ, Nagasaka Y, Moreno Duarte I, Ecker JL, Eikermann. M (***shared first**). Elevated upper body position improves pregnancy related obstructive sleep apnea without impairing sleep quality or sleep architecture early after delivery. *Chest*. 2015 Oct 1;148(4)

JIF: **7.652**

Fujita N, Grabitz SD, Shin CH, Hess PE, **Mueller N**, Bateman BT, Ecker JL, Takahashi O, Houle TT, Nagasaka Y, Eikermann M. Nocturnal desaturation early after delivery: impact of delivery type and the beneficial effects of Fowler's position. *Br J Anaesth.* 2019 Apr;122(4)

JIF: **6.499**

Shin CH, Grabitz SD, Timm FP, **Mueller N**, Chhangani K, Ladha K, Devine S, Kurth T, Eikermann M. Development and validation of a Score for Preoperative Prediction of Obstructive Sleep Apnea (SPOSA) and its perioperative outcomes. *BMC Anesthesiology*. 2017 May 30;17(1)

JIF: **1.888**

Diaz-Gil D, **Mueller N**, Moreno-Duarte I, Lin H, Ayata C, Cusin C, Cotten JF, Eikermann M. Etomidate and Ketamine: Residual Motor and Adrenal Dysfunction that Persist beyond Recovery from Loss of Righting Reflex in Rats. *Pharmaceuticals* (Basel). 2014 Dec 29;8(1)

JIF: **not available**

D'Angelo OM, Diaz-Gil D, Nunn D, Simons JC, Gianatasio C, **Mueller N**, Meyer MJ, Pierce E, Rosow C, Eikermann M. Anesthesia and increased hypercarbic drive impair the coordination between breathing and swallowing. *Anesthesiology*. 2014 Dec;121(6)

JIF: **6.523**

9 Danksagung

Zum Abschluss möchte ich mich an dieser Stelle bei all denen bedanken, die mich in der Zeit als Doktorandin am NeuroCure Clinical Research Center sowie an der Klinik für Pädiatrie m. S. Neurologie der Charité begleitet und unterstützt haben.

Zuallererst möchte ich Prof. Dr. med. Markus Schülke für die intensive Betreuung meines Promotionsprojektes außerordentlich danken. Er förderte mich großzügig und unterstützte mich mit vollem Einsatz. Seine Freude an der Forschung, sein unermüdliches Engagement sowie breites Fachwissen haben mich in hohem Maße motiviert.

Susanne Morales-Gonzalez gilt mein besonderer Dank für die kompetente Einarbeitung in ein weites Spektrum komplexer Labormethoden. Sie teilte mit mir Hoch- und Tiefmomente der Laborarbeit und stand mir jederzeit zur Seite. PD Dr. med. Ellen Knierim möchte ich für die Betreuung meiner Doktorarbeit, insbesondere für die klinischen Perspektiven auf das Projekt sehr herzlich danken. Der gesamten Arbeitsgruppe um Prof. Schülke möchte ich für die freundliche Aufnahme und Kooperationsbereitschaft danken.

Unseren Kollaborationspartnern Takayuki Sassa, PhD und Prof. Akio Kihara, PhD aus der Hokkaido University, Sapporo danke ich für eine produktive und angenehme Zusammenarbeit.

Weiter gilt mein Dank den beiden Patienten und deren Eltern für die Teilnahme an dieser Studie, für die sie wiederholt aus Polen anreisten.

Für die liebevolle Unterstützung, die ich durch Johannes und meine Familie erhalten habe, kann ich mich nur unzureichend bedanken. Ihr Rückhalt, Verständnis und Vertrauen haben mich während meines gesamten bisherigen Weges bestärkt.