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EVALUATION OF GENETIC AND PHENOTYPE HETEROGENEITY IN HEREDITARY NEUROPATHIES

SUMMARY OF DOCTORAL DISSERTATION

Biomedical Sciences,
Medicine 06B

VILNIUS 2018

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GENOTIPO IR FENOTIPO HETEROGENIŠKUMO ĮVERTINIMAS PAVELDIMŲ NEUROPATIJŲ GRUPĖJE

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ABBREVIATIONS

AD – autosomal dominant
AR – autosomal recessive
CMT – Charcot-Marie-Tooth disease
CMT1A – Charcot-Marie-Tooth disease type 1A
HNPP – Hereditary neuropathy with susceptibility to pressure palsies
mtDNA – mitochondrial DNA
NCV – nerve conduction velocity
NGS – next-generation sequencing
PCR – polymerase chain reaction
WES – whole exome sequencing

INTRODUCTION

Background

Hereditary neuropathies are a broad clinically and genetically heterogeneous group of neurodegenerative disorders. The clinical phenotypes of all of these diseases overlap. Combinations with the mechanisms of other diseases are also possible.

Hereditary neuropathies, based on their origin, are categorized as neuropathies in which neuropathy is the main symptom of the disease such as Charcot-Marie-Tooth Disease (CMT), hereditary sensory and autonomic neuropathies (HSAN), distal hereditary motor neuropathies (dHMN), etc. and neuropathies in which the clinical manifestation of the neuropathy is part of a widespread neurological or multisystem disorder. Currently, pathogenic variants of hereditary neuropathies and related phenotypes have been clearly described for more than 100 genes, but a large proportion of genetic causes still remain unexplained. Autosomal dominant (AD), autosomal recessive (AR), X-linked and mitochondrial inheritance forms are distinguished. Primary mitochondrial disorders can also manifest as peripheral neuropathies. The use of Next Generation Sequencing (NGS) technology in the study of hereditary neuropathies continually increases the frequency of discovery of genetic causes. Although there is in fact no etiological treatment for hereditary neuropathies, knowing the molecular diagnosis of the disorder is important for many patients as it is not necessary to continue to search for other possible causes of the disease and it is possible to determine the probability of a recurrence risk for children and other family members. An exact molecular diagnosis is important if we want to identify patients for further research, including clinical trials of new therapies.

A lot of effort is being made around the world to investigate the causes of hereditary neuropathies. There were more than 5,000

publications in the PubMed database based on the search criteria "Hereditary Neuropathy" in the period of writing this thesis.

Currently, in different countries, including Lithuania, genetic testing in stages is performed most often, based on the determination of the nerve conduction velocity (NCV) criteria, the pattern of inheritance of the disease in the family, and the prevalence of the disease in the affected population. In Norway, almost 96% of the pathogenic variants identified in patients with CMT were in only four genes (*PMP22* duplication, *GJB1*, *MPZ* and *MFN2*) associated with CMT (Østern R et al., 2013). These genetic findings are consistent with those observed in other countries. In the United Kingdom, more than 90% of the 1607 patients studied had CMT causes in the same four genes (Murphy SM et al., 2012). Meanwhile, in Denmark, where a CMT1A genetic study started in 1990, the results of 1442 genetically tested patients in 1992-2012 showed that the molecular diagnosis of only four major CMT genes confirmed the diagnosis for only 21.6% of the patients (Vaeth S et al., 2017). In Hungary, in 531 patients with CMT, the duplication of *PMP22* was for 40.5%, and the pathogenic variants for the *GJB1* gene were for 9.2% (Milley GM et al., 2018). In Russia, in 174 CMT patients, the duplication of *PMP22* was detected in 59 patients and amounted to 33.9% (Mersiyanova IV et al., 2000). In Lithuania, a CMT1A genomic region study was started in 2000, and for the second most common cause – the *GJB1* gene – only in 2010. Mitochondrial DNA analysis in patients with mitochondrial syndromes have not been previously performed in Lithuania. Usually these patients have multisystem impairment, including peripheral neuropaty. The aim of this study was to identify the most common and novel genetic factors and to evaluate genetic and phenotype relationships in a group of patients with hereditary neuropathies using Sanger sequencing and NGS methods.

The Aim of the Study

To identify the most common and novel genetic defects and to evaluate the genetic and phenotype heterogeneity in a group of patients with hereditary neuropathies.

The Objectives of the Study

1. To determine the distribution of Charcot-Marie-Tooth type 1A disease and analyse the heterogeneity of the phenotype in patients with Charcot-Marie-Tooth disease type 1A and hereditary neuropathy with liability to pressure palsies and to determine the phenotype manifestation dependence on gender in the group of patients with Charcot-Marie-Tooth disease type 1A.
2. To investigate the most common genes associated with Charcot-Marie-Tooth disease (*GJB1*, *MPZ*, *MFN2*), to evaluate the incidence and effect of identified pathogenic variants on the phenotype in patients with Charcot-Marie-Tooth disease.
3. To investigate the genetic region of *MT-ATP6/8* in patients with Charcot-Marie-Tooth type 2 and Charcot-Marie-Tooth intermediate type, to evaluate the incidence and phenotype of the identified pathogenic variants in these groups.
4. To determine and evaluate the incidence of mitochondrial DNA pathogenic variants and the clinical manifestation of neuropathy in the group of patients with classical mitochondrial syndromes.
5. To improve genetic diagnosis using next-generation sequencing technologies and to determine the diagnostic value of the targeted gene panel associated with Charcot-Marie-Tooth disease and related neuropathies in a group of patients with Charcot-Marie-Tooth disease.

Defended Statements

1. When suspecting the mitochondrial syndrome, first of all it is necessary to investigate the most common pathogenic variants of mtDNA, and afterwards, if the molecular diagnosis is not set, perform the complete mtDNA sequencing.
2. The distribution of CMT1A and the manifestation of phenotypes do not differ from the literature data.
3. The pathogenic variants of the *GJB1* gene are the second-most common molecular cause in patients with Charcot-Marie-Tooth disease.
4. Research based on next-generation sequencing technology is an effective tool for rational molecular diagnosis in hereditary neuropathies.

The Scientific Novelty of the Study and Implementation in Clinical Practice

There are no scientific studies in the field of genetic heterogeneity and prevalence of hereditary neuropathies in Lithuania.

The molecular research strategy for patients with hereditary neuropathies in Lithuania is not defined. The aim of this work was to identify the most common causes of CMT in Lithuania and improve the molecular diagnostic strategy of patients with CMT, which would be useful in providing personal health care to patients and would also be useful to their supervising physicians.

MtDNA sequencing for diagnostic purposes has never been performed in Lithuanian clinical genetics practice. The results obtained during the present study allowed to identify molecular causes of mtDNA in a group of patients with mitochondrial syndromes. The obtained results show that molecular research on mtDNA is useful to patients and is important for the differentiation of these phenotypically heterogeneous disorders.

New genetic factors identified during present study have provided more knowledge about the molecular etiology and its impact on the phenotypic spectrum of hereditary neuropathies.

Materials and Methods

Patients Studied

A total of 163 patients were enrolled in the study and composed two groups. Sixteen patients were included in a group based on diagnostic criteria of mitochondrial disorder. Others were attributed to a second group. 122 patients were considered to have Charcot-Marie-Tooth disease based on family history, neurological and neurophysiological examinations after excluding other acquired causes. These patients were classified as demyelinating (CMT1, n = 64), axonal (CMT2, n = 37) or intermediate (CMT-I, n = 21). 22 patients were also included with suspected HNPP or motor neuropathy by means of neurophysiological measurements, i.e. 17 patients with a medical history of transient pareses and/or sensory loss related to typical nerve compression points (pressure palsies), conduction blocks and mild demyelinating neuropathy were considered for a clinical phenotype of hereditary neuropathy with liability to pressure palsies and five patients with motor neuropathy. Three pediatric patients were included in the research study with phenotypicaly unspecified neuromuscular disease with prominent neuropathy features. The present study was approved by the Vilnius Regional Research Ethics Committee.

Retrospective Study

Retrospective study was conducted of 122 unrelated CMT patients who had been referred to a clinical diagnostic laboratory for *PMP22* region analysis from January 2012 to December 2017. 38 (31.14%) patients reached a molecular diagnosis of CMT1A. Clinical data of

37 patients were reviewed by analysing available medical records and compared between female and male patients. In addition, we evaluated phenotypic features of six patients who reached molecular diagnosis of HNPP.

Prospective Study

Most of the genetic studies in the present work have been performed prospectively. The genetic testing shares a common methodological workflow based on the generation of genetic data, analysis and interpretation, and a consecutive validation of the findings. The results were reported back to the physicians and were used in the clinical care of these patients.

Genetic Analysis

Blood samples were collected from all patients and their family members where appropriate. In addition, urine samples were collected from all 16 patients with mitochondrial syndrome. The whole genomic DNA was extracted from peripheral blood and urine sediment cells using the standard phenol-chloroform-isoamyl alcohol extraction method.

Mitochondrial genome analysis. Complete mitochondrial genome (mtDNA) sequencing was performed by Sanger sequencing using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), on automated genetic analyser ABI PRISM 3130xl (Applied Biosystems). The data were analysed and edited, respectively, by software program BioEdit 7.2.6.1. The obtained sequences were compared with the revised Cambridge Reference Sequence (rCRS) from the MITOMAP database (www.mitomap.org). Variants were considered rare when they occurred $\leq$ 10 times on the MITOMAP database with 32,059 mtDNA sequences. Long-range PCR was performed for all patients for the detection of deletions/duplications of mtDNA.

Single gene sequencing. The genetic testing of genes *GJB1*, *MFN2*, *MPZ*, *PMP22* and *MT-ATP6/8* was carried out by Sanger sequencing. It was performed by PCR and the sequencing reactions of each gene, analysing all coding exons and their intronic flanking sequences, except the *GJB1* gene, for which the part of the promoter sequence was also examined. All coding exons of the relevant genes were covered both by forward and reverse strand sequencing using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems) according to the manufacturer's protocol. The obtained sequences were compared with the reference sequences listed in the www.ensembl.org database. *MT-ATP6/8* gene sequences were analysed as mentioned above. Novel sequence variations were analysed with the most used *in silico* prediction programs to predict their functional effects.

Next-generation sequencing (NGS). A custom-designed gene panel was used for sequence detection for 34 subjects. The panel targeted the coding regions (+25 base pairs at exon-intron boundaries) of 150 genes associated with CMT and related hereditary neuropathies. The panel was designed for Ion AmpliSeq™ technology (Ion Torrent, Thermo Fisher Scientific). The DNA libraries were sequenced on an Ion PGM™ Sequencer (Life Technologies). The obtained genetic data were analysed and annotated. FASTQ sequencing files were aligned to human genome assembly GRCh37 on the Ion Torrent Suite™ Server. The average mean read depth was 168.13 ± 61.32 fold coverage in more than 99% of the targeted regions. Variants with population frequency over 1% in the dbSNP v137, Exome Variant Server (EVS; http://evs.gs.washington.edu/EVS/) and 1000 Genome (http://www.1000genomes.org/) databases were filtered out. Only variants predicted to affect the coding regions (including non-synonymous, predicted missense, nonsense, splice acceptor and donor site, and insertions or deletions) were selected for further

analysis. Several *in silico* prediction programs (MutationTaster, PolyPhen-2, SIFT) were used to predict the functional effect as well as the evolutionary conservation score, etc. Variants were correlated with patients' phenotypes and the results of clinical and neurophysiological investigations. Sanger sequencing with specific primers was conducted to confirm the selected variants.

Whole exome sequencing (WES). WES was performed on three of the affected paediatric patients and three adult patients with negative results after targeted NGS in cooperation with partners from Germany and Japan. Exome libraries were captured using hybridization with the Agilent SureSelect V2, and WES performed on an Illumina HiSeq 2000 (Illumina Inc.). High-throughput sequencer data were filtered based on inheritance models, the frequency of the variants in large datasets and the variants' effect.

Statistical analysis. Free software environment R, version 3.4.3, and Excel (Microsoft) 2016, were used to generate descriptive statistics. Continuous quantitative variables are presented as means ± Standard Deviation (SD). Categorical variables are presented as numbers and percentages. The Shapiro-Wilk test was used to determine the normal distribution of the data. If the data was not normally distributed, the non-parametric Mann-Whitney rank-sum test was performed. Group differences were analysed by t-test (2-tailed, unpaired). Probability values of $p < 0.05$ were considered statistically significant.

RESULTS

Results of Retrospective Studies

Evalutation of CMT1A Cohort Phenotypes

A total of 38 (31.14%, 38/122) unrelated CMT1A patients were diagnosed between 2012 and 2017. Clinical data were available in 37 patients. The mean age at the time of onset was 9.08 ± 8.37 years,

and the mean age at molecularly confirmed diagnosis of CMT1A was 28.10 ± 17.82 years. The mean age at the time of onset was 9.80 ± 9.27 in females, and 8.23 ± 7.36 in males. The mean age at molecularly confirmed diagnosis was 34.25 ± 19.60 in females, and 20.88 ± 12.47 in males (Fig. 1).

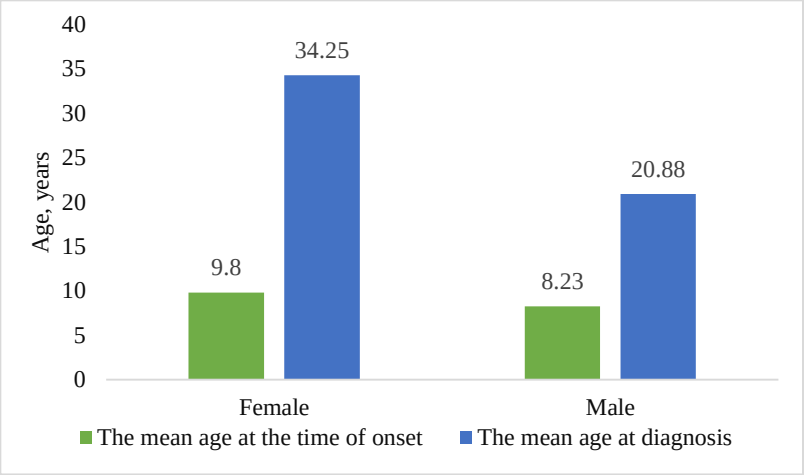

Figure 1. The age distribution between genders among the CMT1A patients.

The most common presented complaints were difficulties in walking, running and jumping. Atypical presentation in our cohort observed one male patient with predominantly upper limb involvement (postural hand tremor and difficulty writing). In female patients, sensory impairment in the lower limbs and absent tendon reflexes were significantly frequent, $p < 0.05$. Female patients received a rehabilitation treatment significantly more periodically, $p < 0.05$. An occasional finding was scoliosis. Auditory disturbances were not observed in our cohort. The clinical characteristics recorded in the mutation-positive patients with CMT1A are summarized in Table 1.

Table 1. Clinical characteristics of CMT1A patients analyzed in this study

Items	Female, n=20	Male, n=17	Total, n=37	p
Mean age at onset, years	9.80 ± 9.27	8.23 ± 7.36	–	0.713
Mean age at diagnosis, years	34.25 ± 19.60	20.88 ± 12.47	–	0.067
Initial symptoms				
Lower limb, %	20 (100 %)	16 (94.12 %)	36 (97.30 %)	0.460
Upper limb, %	–	1 (5.88 %)	1 (2.70 %)	0.460
Muscle weakness				
Lower limb, %	17 (85.00 %)	12 (70.59 %)	29 (78.38 %)	0.428
Upper limb, %	8 (40.00 %)	3 (17.65 %)	11 (29.73 %)	0.138
Muscle atrophy	15 (75.00 %)	9 (52.94 %)	24 (64.86 %)	0.161
Sensory impairment				
Lower limb, %	16 (80.00 %)	7 (41.18 %)	23 (62.16 %)	0.015*
Upper limb, %	7 (35.00 %)	2 (11.76 %)	9 (24.32 %)	0.137
Absent tendon reflexes				
Lower limb, %	20 (100 %)	11 (64.71 %)	31 (83.78 %)	0.005*
Upper limb, %	6 (30.00 %)	1 (5.88 %)	7 (18.92 %)	0.097
Gait disturbance, %	12 (60.00 %)	7 (41.18 %)	19 (51.35 %)	0.254
Gait disturbance on heels, %	19 (95.00 %)	15 (88.24 %)	34 (91.89 %)	0.584
Foot deformation, %	19 (95.00 %)	15 (88.24 %)	34 (91.89 %)	0.584
Hand tremor, %	6 (30.00 %)	4 (23.53 %)	10 (27.03 %)	0.725
Medication for pain in legs, %	6 (30.00 %)	2 (11.76 %)	8 (21.62 %)	0.246
Surgery, %	3 (15.00 %)	4 (23.53 %)	7 (18.92 %)	0.680
Rehabilitation, %	14 (70.00 %)	4 (23.53 %)	18 (48.65 %)	0.005*

*: statistically significant p value.

Phenotypic Findings of Patients with Hereditary Neuropathy with Liability to Pressure Palsies

In the retrospective study of this cohort, we analyzed the phenotypic data of six patients with a deletion of CMT1A genetic domain that determined HNPP. The mean age of the initial

symptoms was 24.83 ± 8.95 years, and the mean age at diagnosis was 35.33 ± 12.58 years. The genealogy data of three patients showed AD inheritance. Neuropathic pain and provocative factors have not been reported by any patient. The classic HNPP phenotype was found in 50% of patients. The phenotype of CMT-like disease was present in 33.33%, and chronic sensory neuropathy in 16.67% of patients. In all patients, the initial symptoms were leg insensitivity and numbness. Foot deformity (*pes cavus*) was observed in only one patient.

Table 2. Demographic and phenotypic data of the HNPP patients

Gender	Age at diagnosis, y	Age at onset, y	Classic phenotype		Atypic phenotype		Positive genealogy	NCV (*N.medianus*)	
			Reccurent palsies	Isolated palsy	CMT-like disease	Chronic sensory neuropathy		< 38 m/s	> 38 m/s
F	56	20	-	-	+	-	+	+	-
M	38	36	-	-	-	+	+	-	+
M	31	22	+	-	-	-	-	+	-
M	17	15	-	-	+	-	-	+	-
M	34	20	+	-	-	-	+	-	+
F	36	36	+	-	-	-	-	-	+

F: female; M: male; +: yes, -: no.

The results of the NCV study showed an increase in the rate of conduction of the middle nerve. In three patients, the results of the NCV showed a motor and sensory demyelinating type of neuropathy after detecting a slow pulse rate at extended distal latent periods. Patients' demographic and phenotypic data are summarized in Table 2.

Mitochondrial Genome Findings and Phenotypes of Patients with Primary Mitochondrial Syndromes

We ascertained 16 patients with suspicion of mitochondrial syndrome (between 2015 and 2017) on the basis of clinical criteria for mitochondrial disorders (*Leigh* syndrome n = 3; Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) n = 4; Neuropathy, ataxia and retinitis pigmentosa (NARP) n = 1; Progressive External Ophthalmoplegia n = 4; *Kearns-Sayre* syndrome (KSS) n = 1; Leber hereditary optic neuropathy (LHON) n = 3). Objective signs of neuropathy were observed in all patients. Other clinical findings classically showed a multisystem involvement and the findings differed amongst all patients. Seven patients showed pathogenic variants in the mitochondrial genome. Maternally inherited *Leigh* syndrome was confirmed for two children (patient 1 and patient 2) with pathogenic variants NC_012920.1: m.9185T>C (p.(Leu220Pro)) and NC_012920.1:m.8993T>C (p.Leu156Pro)) in the *MT-ATP6* gene, respectively. Brain MRI abnormalities in these *Leigh* syndrome patients included symmetric alterations to the basal ganglia or brain stem. Other phenotypic findings are summarised in Table 3.

Three adult patients (patients 3, 4 and 5) were identified as harbouring the pathogenic variant NC_012920.1:m.3243A>G (p.(Ala3243Gly)) in the *MT-TL1* gene corresponding with MELAS, and in one patient (patient 6) the MERRF-like syndrome was concluded by detecting the new variant NC_012920.1:m.8353T>C *tRNA (Lys)* gene. Phenotypically, all three patients with m.3243A>G had short stature, ptosis, hearing impairment, dysarthria, dysphonia, feeding difficulties, exercise intolerance and muscle weakness as well gastrointestinal dysmotility, diarrhoea, constipation and abdominal distension (Table 4).

Table 3. Phenotypic findings of patients with *Leigh* syndrome

Organ systems	**Pt 1**	**Pt 2**
Age of onset	By 6 months	By 6 months
Motor delay	+	+
Ocular signs (nystagmus, strabismus)	+	+
Dysphagy	+	-
Epilepsy	-	-
Failure to thrive	+	-
Hearing impairment	-	-
Breathing disorders	-	-
Cardiologic dysfunction	-	-
Liver dysfunction	-	-
Renal dysfunction	-	-
Haematologic disorders	-	-
Neuropathy	+	+
Growth delay	-	-
Microcephaly	-	-
Intellectual dissability	-	-

Pt: patient; +: yes; -: no.

Table 4. Phenotypic characterization of the three female patients with m.3243A>G included in this study

Organ systems	**Features**	**Pt 3**	**Pt 4**	**Pt 5**
	Onset	2rd-3rd decade	1st-2nd decade	2rd-3rd decade
	Age at last examination, years	36	30	55
	Height, cm	157	162	162
	Weight, kg	33	58	45
	BMI	13.4	22.1	17.1
CNS	Stroke/stroke-like episodes	-	-	-
	MRI findings	Brain atrophy, cerebellar atrophy	Subcorti-cal lesions in the occipital lobes	Subcor-tical lesions
	Encephalopathy	-	-	-

Organ systems	Features	Pt 3	Pt 4	Pt 5
	Cognitive dysfunction	-	-	+
	Ataxia	+	-	-
	Seizures	-	-	-
	Migraine	-	-	-
PNS	Neuropathy/hyporeflexia	+	+	+
Muscular	Myopathy/weakness	+	+	+
	Exercise intolerance	+	+	+
	Myopatic EMG	-	+	-
Ear	Hearing loss	+	+	+
Eye	Ptosis/ophthalmoplegia	+	+	+
	Pigmentary retinopathy	+	-	-
Gastro-intestinal	GI dysmotility	+	+	+
	Feeding difficulties	+	+	+
Heart	Cardiomyopathy	-	-	-
	ECG abnormalities	+	-	-
Liver	Hepatic dysfunction	-	-	+
Endocrine	DM type I	+	-	-
	DM type II	-	-	+
Metabolic	Lactic acidosis	-	+	-

Pt: patient; BMI: body mass index; DM: diabetes mellitus; ECG: electro-cardiography; EMG: electromyography; MRI: magnetic resonance imaging; +: yes; -: no.

One patient (patient 7) of the 16 patients studied for mtDNA deletions and duplications was identified as carrying the novel 5888 bp mtDNA deletion NC_012920.1:m.6069_11956del. This patient exhibited a classical *Kearns-Sayre* syndrome phenotype.

Results of Single Gene Analysis

GJB1 gene

We identified 5 pathogenic and/or likely pathogenic *GJB1* variants in 6 patients, and 2 of them were novel: the missense hemizygous variant NM_001097642.2:c.290A>G (p.(His97Arg)) and heterozygous

frameshift deletion NM_000166.5:c.476delG (p.(Gly159Alafs)). The phenotypes and genotypes of patients with *GJB1* variants in this study are summarized in Tables 5 and 7, respectively.

Table 5. Phenotypic features of patients carrying *GJB1* variants

Items	**Pt 1**	**Pt 2**	**Pt 3**	**Pt 4**	**Pt 5**	**Pt 6**
Gender	F	M	M	M	F	M
Variant	c.34G>A	c.43C>T	c.290A>G	c.290A>G	c.476delG	c.547C>T
CMT type*	CMT2	CMT-T	CMT-T	CMT2	CMT1	CMT-T
Age at onset, years	15	8	16	15	35	12
Age at diagnosis, years	21	28	29	19	36	12
Initial symptoms	Distal limb numbness	Steppage gait	Steppage gait	Steppage gait, distal muscle weakness	Right foot drop and numbness	Acute transient CNS manifesta-tions
Muscle weakness (UL/LL)	- /+	++/++	+/++	++/++	-/++	-/-
Muscle atrophy	Mild	Severe	Moderate	Moderate	Mild	-
Sensory disturbance	-	V	P>V	V	-	-
Tendon reflex (knee/ankles)	B/B	A/A	A/A	B/A	B/A	B/A
Foot deformity	+	+	+	+	+	+
Other symptoms	Tremor, cognitive impairment	-	Tremor	Scoliosis	-	-

Pt: patient; UL: Upper limb; LL: Lower limb; Muscle weakness: -: Normal; +: Distal weakness ≥4/5 on MRC; + +: Distal weakness <4/5 on MRC scale. P: Pinprick; T: Touch; V: Vibratory; Tendon reflex: B: brisk; A: absent; Foot deformity: +: *pes cavus*; -: not observed; CMT: Charcot-Marie-Tooth; * based on nerve conduction velocity findings.

MPZ gene

The sequencing of the *MPZ* gene was performed for 76 patients. The pathogenic variants of the *MPZ* gene were detected in 2 (2.63%) patients. PN occurring during infancy associated with pathogenic variants of the *MPZ* gene is characterized by delayed self-paced walking when children begin to walk from at least 15 months of age (on average 18-48 months) and symptoms up to 5 years of age. Patient 1 is a 4-year-old girl who presented a generalized hypotonia at 2 months of age with a history of gradual weakness, more prominent in the upper limbs proximally. Respiratory and bulbar difficulties led to tracheostomy tube and gastrostomy tube at 4 and 8 months, respectively. Deep tendon reflexes were absent. Needle EMG/NCVs indicated very severe sensorimotor demyelinating-axonal polyneuropathy with predominant severe demyelination. WES identified a novel *de novo* heterozygous frameshift deletion NM_000530.7:c.558delG (p.(Arg196Serfs*)) in the *MPZ* gene.

Patient 2 is a 7-year-old girl who presented generalized hypotonia and delayed gross motor skills. She was able to walk independently at the age of 2 years and 4 months. Her neurological examination revealed an inability to walk on heels or toes, proximal and distal weakness. Deep tendon reflexes were preserved. Needle EMG/NCVs demonstrated borderline CMAP amplitude with neurogenic changes on needle examination, asymmetric, length-dependent, sensory-predominant sensorimotor, primarily demyelinating polyneuropathy. She harboured a novel *de novo* heterozygous missense mutation of NM_000530.7:c.263A>G (p.(Tyr88Cys)) in the *MPZ* gene. Characterization of the novel *MPZ* variants are presented in Table 7.

MFN2 gene

We performed a *MFN2* gene screening in a clinically well-characterized group of 54 unrelated index patients with CMT2 (n=34) and CMT-I (n=18). We detected a pathogenic variant in one patient (1.85%) with CMT2. The patient carried a heterozygous

mutation NM_014874.3:c.281G>A (p.(Arg94Gln)) in close proximity of the GTPase domain. At the onset of the disease at the age of 12, foot instability, *pes cavus* and painful involuntary muscle contractions occurred. These symptoms have slowly progressed over time. At the age of 32, the patient had mild sensory impairment in the hands, distal sensory loss in her legs, distal muscle weakness and atrophy. ENG revealed a reduced amplitude of the compound motor nerve action potentials of the peroneal and tibial nerves and normal nerve conduction velocities (Table 6).

Table 6. Motor and sensory nerve conduction velocity results

	Nerve	Amplitude (uV) (ref.)	Latency (mS) (ref.)	Conduction velocity (m/s) (ref.)
Motor nerve	Median	8.75 (3.9)	3.96 (3.9)	54.3 (48.4)
	Ulnar	7.35 (4.8)	2.74 (3.7)	50.0 (49.9)
	Right nervi tibialis	0.66 (3.0)	3.80 (3.9)	37.4 (40.3)
	Left nervi tibialis	2.97 (3.0)	4.30 (3.9)	41.1 (40.3)
	Right peroneal nerve	9.92 (3.8)	3.30 (4.3)	41.1 (40.0)
	Left peroneal nerve	9.51 (3.8)	2.90 (4.3)	37.2 (40.0)
Sensory nerve	Median	10.7 (15.1)	4.60 (3.7)	38.0 (48.1)
	Ulnar	Not elicited		
	Suralis	Not elicited		

PMP22 gene

The sequencing of the *PMP22* gene was performed for 31 patients (CMT1, n = 15, and HNPP, n = 16). Only the previously reported pathogenic variant NM_000304.3:c.319+1G>A (p.?) was identified in intron 4 of the *PMP22* gene in a patient with CMT1. The patient developed gait abnormalities from the age of 16. Neurological examinations at age 20 demonstrated diffusely diminished deep tendon reflexes in the legs. NCVs showed generalized prolonged distal latencies and F-wave latencies of the motor or sensory nerves

and markedly decreased motor and sensory nerve conduction velocities in the hands.

Table 7. Pathogenic/likely pathogenic and novel variants identified by the use of single gene analysis.

Variant	**Geno - type**	**Data from large databases[1]: 1000G; ESP; ExAC**	***In silico* prediction[2]: GERP; SIFT; PP2; CADD**
NM_000166.5(*GJB1*):c.34G>A (p.(Gly12Ser))	htz	-	5.63; 0*; 1*; 34*
NM_000166.5(*GJB1*):c.43C>T (p.(Arg15Trp))	hmi	-	4.36; 0*; 0.99*; 31*
NM_000166.5(*GJB1*):c.290A>G (p.(His97Arg))	hmi	-	4.67; 0*; 0.79*; 19.98*
NM_000166.5(*GJB1*):c.476delG (p.(Gly159Alafs))	htz	-	4.81; -; -; -; -
NM_000166.5(*GJB1*):c.547C>T (p.(Arg183Cys))	hmi	-	4.99; 0*; 1*; 29.2*
NM_000530.7(*MPZ*):c.263A>G (p.(Tyr88Cys))	htz	-	4.69; 0*; 0.99*; 25.1*
NM_000530.7(*MPZ*):c.558delG (p.(Arg186Serfs))	htz	-	4.7 ; -; 0.97*; -
NM_014874.3(*MFN2*):c.281G>A (p.(Arg94Gln))	htz	-	5.4; 0*; 0.99*; 30*
NM_000304.3(*PMP22*):c.319+ 1G>A (p.?)	htz	-	5.76; -; -; -

Genotype: hmi: hemizygous; htz: heterozygous;
[1] 1000G: 1000 Genome project (http://www.1000genomes.org); ESP: Exome Sequencing Project (http://evs.gs.washington.edu/EVS/); ExAC: Exome Aggregation Consortium (http://exac.broadinstitute.org/);
[2] GERP: Genomic Evolutionary Rate Profiling; PP2: PolyPhen2 (http://genetics.bwh.harvardPedu/pph2/); SIFT: (http://sift.jcvi.org/); CADD: (https://cadd.gs.washington.edu/);
* predicts pathogenicity.

MT-ATP6/8 gene

A standard PCR reaction was performed to amplify overlapping genes *MT-ATP6/8* in 49 CMT patients (CMT2, n = 34, and CMT-I, n = 15). All patients showed negative results after CMT1A genetic locus analysis and single genes, the *GJB1*, *MPZ* and *MFN2*, sequencing. Patients whose genealogy data showed a clear male-to-male inheritance were not included in the study. No pathogenic variants of *MT-ATP6/8* genes have been identified in this group. Since the mitochondrial genome is characterized by significant variability, all non-pathogenic changes identified in this study are presented in Table 8.

Table 8. mtDNA variants identified in this study

Variant	Number of patients	State
CO2:m.8167T>C (p.(Gly194=))	1	homoplasmic
CO2:m.8251G>A (p.(Gly222=))	2	homoplasmic
NC7:m.8270_8278delCACCCCCTC	1	homoplasmic
NC7:m.8289_8290insCCCCCTCTA	2	homoplasmic
ATP8:m.8395C>T (p.(Pro10=))	1	homoplasmic
ATP8:m.8410C>T (p.(Pro15=))	1	homoplasmic
ATP8:m.8422A>G (p.(Thr19=))	1	homoplasmic
ATP8:m.8464C>T (p.(Tyr33=))	1	homoplasmic
ATP8:m.8472C>T (p.(Pro36Leu))	1	homoplasmic
ATP8:m.8473T>C (p.(Pro36=))	1	homoplasmic
ATP8:m.8512A>G (p.(Lys49=))	1	homoplasmic
ATP6:m.8610T>C (p.(Pro28=))	2	homoplasmic
ATP6:m.8697G>A (p.(Met57=))	5	homoplasmic
ATP6:m.8715T>C (p.(Thr63=))	1	homoplasmic
ATP6:m.8818C>T (p.(Leu98=))	1	homoplasmic
ATP6:m.8825T>C (p.(Met100Thr))	1	homoplasmic
ATP6:m.8836A>G (p.(Met104Val))	1	homoplasmic
ATP6:m.8860A>G (p.(Thr112Ala))	49	homoplasmic
ATP6:m.8994G>A (p.(Leu156=))	2	homoplasmic
ATP6:m.9055G>A (p.(Ala177Thr))	2	homoplasmic
ATP6:m.9123G>A (p.(Leu199=))	1	homoplasmic
ATP6:m.9614T>C (p.(Leu30=))	2	homoplasmic

Efficacy of targeted next-generation sequencing

In this study we analyzed 34 patients with CMT and evaluated the effectiveness of the targeted sequencing of a CMT gene panel using NGS. The studied group consisted of 34 patients with no evidence of duplication in the genetic locus comprising the *PMP22* gene, and no pathogenic variants of the *GJB1*, *MPZ* and *MFN2* genes using the Sanger sequencing method. Patient selection was based on an individual assessment, which included genealogy data, clinical neurological findings and NCV results. In suspicion of AR inheritance, we selected all patients with early onset and severe phenotype, scoliosis, foot deformities and other accompanying symptoms. For other patients, an AD inheritance pattern was suspected.

In the NGS group, 25 (73.53%, 25/34) of the patients were selected with CMT2 type diagnosed by clinical evaluation, because after genetic testing of the most common CMT genes, the genetic defect rate was the lowest in this group. Five (14.71%, 5/34) and four (11.76%, 4/34) patients were assigned for CMT-I and CMT1 types, respectively. The ratio of women to men was 0.89. The mean age at the time of the CMT diagnosis was 26.79 ± 17.46 years. The mean age at the time of the first symptoms was 20.26 ± 17.63 years. The predominant first symptoms were gait disturbances and foot deformities. Analysis of the targeted gene panel NGS results revealed that pathogenic or likely pathogenic variants were detected at 29.41% (10/34), novel pathogenic variants were identified 17.65% (6/34), and variants of unknown clinical significance in 17.65% (6/34) of patients. Characteristics of pathogenic, novel pathogenic and variants of unknown clinical significance are presented in Table 9.

Table 9. Characteristics of variants identified by the targeted NGS panel.

Variant	Genotype	Data from large databases[1]: 1000G; ESP; ExAC	*In silico* prediction[2]: GERP; SIFT; PP2; CADD
Pathogenic/likely pathogenic variant			
NM_001122955.3(*BSCL2*):c.455A>G (p.(Asn152Ser))	htz	-	5.63; 0*; 1*; 34*
NM_001005361(*DNM2*): c.1609G>A (p.(Gly537Ser))	htz	-	5.36; 0.06*; 0.99*; 27.4*
NM_018972.2(*GDAP1*): c.844C>T (p.(Arg282Cys))	hmz	-	4.99; 0*; 1*; 35*
NM_000166.5(*GJB1*): c.34G>A (p.(Gly12Ser))	htz	-	4.36; 0*; 0.99*; 25.6*
NM_005340.6(*HINT1*): c.110G>C (p.(Arg37Pro))	hmz; htz	0.0002; 0.0001; 0.0001	-0.93; 0.02*; 0.115; 23.3*
NM_000304.3(*PMP22*): c.353C>T (p.(Thr118Met))	htz	0.0008; 0.0053; 0.0029	5.15; 0*; 1*; 28.8*
NM_021625.4(*TRPV4*): c.68G>A (p.(Ser23Asn))	htz	-	3.62; 0.01*; 0.12; 17.05
Novel pathogenic variant			
NM_018188.3(*ATAD3A*): c.556C>T (p.(Arg186Trp))	htz	-; 0.0001; 0.00004	3.87; 0*; 1*; 34*
NM_001605.2(*AARS*): c.1643T>C (p.(Leu548Pro))	htz	-	6.15; 0*; 0.93*; 31*
NM_004082(*DCTN1*): c.1295C>G (p.(Ala432Gly))	htz	-	5.65; 0.01*; 1*; 27.9*
NM_021629(*GBN4*): c.847C>T (p.(Arg283Cys))	htz	-	4.51; 0.01*; 0,99*; 35*
NM_004990.3(*MARS*):c.1340G>A (p.(Ser447Asn))	htz	-	3.88; 0*; 0.948*; 23.7*
NM_001136472.1(*LITAF*):c.418G>A (p.(Ala140Thr))	htz	-; -; 0.00001	5.01; 0.03*; 0.64; 27.1*

Variant	Genotype	Data from large databases[1]: 1000G; ESP; ExAC	*In silico* prediction[2]: GERP; SIFT; PP2; CADD
Variants of unknown clinical significance			
NM_014629.3 (*ARHGEF10*):c.2197C>T (p.(His733Tyr))	htz	0.001; 0.0016; 0.001	3.95; 0.03*; 0.76*; 23.1*
NM_014629.2(*ARHGEF10*): c.2456T>C (p.(Val819Ala))	htz	0.0002; -; 0.0001	-0.02; 0.02*; 0.12; 12.9
NM_018188.4(*ATAD3A*): c.1847A>T (p.(Lys616Met))	htz	0.0017; -; 0.0034	2.75; 0.02*; 0.99*; 23.7*
NM_000902.3(*MME*): c.157G>A (p.(Asp53Asn))	htz	-; 0.0001; 0.0002	5.24; 0.43; 0.01; 18.72
NM_005340.6(*HINT1*): c.299A>G (p.(Glu100Gly))	htz	-	3.52; 0.04*; 0.19; 24.9*
NM_021625.4(*TRPV4*): c.2518G>A (p.(Glu840Lys))	htz	0.0024; 0.0095; 0.0053	5.39; 0.01*; 0.11; 26.8*

Genotype: htz: heterozygous; hmz: homozygous;

[1] 1000G: 1000 Genome project (http://www.1000genomes.org); ESP: Exome Sequencing Project (http://evs.gs.washington.edu/EVS/); ExAC: Exome Aggregation Consortium (http://exac.broadinstitute.org/);

[2] GERP: Genomic Evolutionary Rate Profiling; PP2: PolyPhen2 (http://genetics.bwh.harvardPedu/pph2/); SIFT: (http://sift.jcvi.org/); CADD: (https://cadd.gs.washington.edu/);

* predicts pathogenicity.

The diagnostic yield of the targeted 150 gene panel NGS was 44.12% (15/34) in the studied cohort of CMT patients. However, slightly more than half of the patients remained with unidentified molecular CMT diagnosis. *GJB1* gene pathogenic variant NM_001097642.2:c.34G>A (p.(Gly12Ser)) was not identified with the Sanger sequencing method. *HINT1* gene pathogenic variants

were identified in three patients with neuromyotonia and axonal neuropathy. Digenic inheritance was identified in one patient.

Results of Whole Exome Sequencing

Six patients (three children with unspecified neuromuscular disease and three adults with pure CMT2) were included in this study. In the three adult patients we did not identify any pathogenic/likely pathogenic or variants of unknown significance that segregate with the CMT phenotype. Molecular genetic diagnoses were attributed to all three paediatric patients.

The heterozygous *de novo* deletion NM_000530.7:c.558delG (p.(Arg186Serfs)) in the *MPZ* gene was identified in a 7-year-old girl (described in the section *MPZ* gene). The second patient harboured a rare heterozygous missense variant NM_001376.4:c.1792C>T (p.(Arg598Cys)) in the *DYNC1H1* gene. This girl presented motor developmental delay with onset of independent walking at 17 months of age. Her gait was waddling and she was not able to crouch or climb stairs unaided. On examination at 5 years of age, a symmetric distal muscle weakness and hypotrophy of the lower limbs were observed. The muscle hypotrophy and weakness were mainly restricted to the distal lower limbs along with absent reflexes in the lower extremities. The symptoms were predominantly motor. NCVs were not performed due to their unavailability. *DYNC1H1* gene pathogenic variants are linked to two distinct phenotypes. Detailed phenotypic information is required for correlating potential disease-causing variants to the clinical phenotype of the patients. The identified pathogenic variant was *de novo* origin. A third patient was found to carry a compound heterozygous variants in the *SLC52A2* gene. This patient presented motor delay from early childhood, hypotonia, bulbar paralysis and, later, signs of involvement to the lower motor neuron. Two variants were identified by WES in the *SLC52A2* gene. The rare pathogenic missense variant NM_024531.4:c.1016T>C (p.(Leu339Pro)) was

inherited from the father. The paternally inherited variant (c.1016T>C) had previously been reported for many patients. In vitro studies have shown that such an altered protein does not connect ^{3}H-riboflavin (Foley AR et al., 2014). Another novel missense variant NM_024531.4:c.377G>A (p.(Cys126Tyr)) was inherited from the mother. The maternally inherited missense variant (minor allele frequency < 0.01) had not been previously reported. The variant is evaluated as likely pathogenic based on DANN (0.99), GERP (3.65), LRT (0.84), MutationAssessor (0.928), MutationTaster (1), SIFT (0), PolyPhen2 (0.99) and PROVEAN (-10.88) scores. Based on a phylogenetic point of view, the novel variant located in the moderately conservative reading frame results in a loss of the protein function and is a possible pathogenic mechanism that corresponds to previous pathogenicity observations.

DISCUSSION

The overall aim of the present study was to elucidate novel genetic causes for hereditary neuropathies and to evaluate genotype-phenotype correlations in patients with hereditary neuropathies.

In the study of CMT1A and HNPP patients we do not observed any differences with literature data. We observed gender differences between CMT1A patients with regard of data of sensory impairment, absent tendon reflexes and rehabilitation treatment with higer deterioration in women. It could be explained by the older age at diagnosis and that female patients have finer perception of symptoms than men (Colomban C et al., 2014).

Phenotype-genotype correlations in 6 patients with the *GJB1* pathogenic variants indicate that, based on NCV criteria, these patients could be classified as CMT1, CMT-I or CMT2. The novel *GJB1* variant NM_001097642.2:c.290A>G (p.(His97Arg)) is associated with a clinically distinct phenotype characterized by

classical onset, distal muscle weakness and marked sensory abnormalities. NCVs of the motor median nerve vary from marked axonal demage to intermediate values in these patients. A rare presentation was found in a patient harbouring the NM_001097642.2:c.547C>T (p.(Arg183Cys)) variant and these findings are in line with the literature data (Scherer SS and Kleopa KA, 2012). In the process of analyzing the most common CMT-related genes, two novel pathogenic variants of the *MPZ* gene were identified, one of which was identified by WES.

Mitochondrial DNA analysis in patients with mitochondrial syndromes have not previously been performed in Lithuania. In this study, seven patients were diagnosed using combined mtDNA molecular testing. Three of these patients had the most common variant of the *MT-TL1* gene m.3243A>G and two were confirmed to have maternally inherited *Leigh* syndrome. One patient harboured a novel deletion of mtDNA that is responsible for *Kearns-Sayre* syndrome.

A targeted next-generation sequencing study of 150 hereditary neuropathy-related genes was performed on 34 selected patients. We identified a diagnostic efficiency of the targeted gene panel NGS for 44.12% (15/34) of the patients. Thus, we identified pathogenic variants that confirmed the molecular CMT diagnosis in less common CMT-associated genes, except for one patient who had not been diagnosed with the *GJB1* gene variant NM_000166.5:c.34G>A (p.Gly12Ser) by the Sanger sequencing method. Three patients were identified with pathogenic variants of the *HINT1* gene. The pathogenic variant NM_005340.6:c.110G>C (p.(Arg37Pro)) was detected in a homozygous condition for two patients, and in one heterozygote in combination with a novel variant of NM_005340.6:c.299A>G (p.(Glu100Gly)). Milovidova TB et al. tested 700 CMT patients in Russia and identified the *HINT1* gene homozygous variant NM_005340.6:c.110G>C (p.(Arg37Pro)) for 30 patients (http://www.abstractsonline.com/pp8/#!/4652/presentation/2285). In

the Czech Republic, variants of the *HINT1* gene are also the most common cause of neuromyotonia and axonal neuropathy (Lassuthova P et al. 2015), but they are rare in Spain and the United Kingdom (Horga A et al., 2015). Based on these results, it is rational for patients with neuromyotonia and axonal neuropathy to first choose the *HINT1* gene NM_005340.6:c.110G>C (p.(Arg37Pro)) variant testing. The digenic inheritance CMT-I variant was identified for one patient by identifying variant NM_001005361:c.1609G>A (p.(Gly537Ser)) of the *DNM2* gene and a novel variant NM_021629:c.847C>T (p.(Arg283Cys)) of the *GNB4* gene. The digenic effect of two pathogenic variants in different genes may modulate the phenotype, since both gene products are involved in endosomal sorting and cell signaling, possibly interacting in similar pathways.

Targeted NGS efficiency in this study almost matched the expected frequency of molecular diagnosis, as phenotypes of the patients were clearly defined, and according to recent literature, molecular diagnosis identified with the NGS method is on average half of well-defined patients. WES was performed for six patients, of which three were identified with molecular disease causes. A patient who was confirmed with a type 2 riboflavin transporter deficiency has promptly obtained the results of the study and specific treatment was immediately introduced. This case highlights the clinical benefit of WES where a molecular diagnosis was established without delay and the patient started to be properly treated, avoiding alternative treatments that not only do not help but could even be harmful during intercurrent illnesses.

In conclusion, we described novel pathogenic variants that were identified by Sanger sequencing and NGS methods. NGS is an effective tool for determining molecular diagnosis in patients, particularly with rare and heterogeneous diseases, whose genetic causes of the single genes analysis are not determined by conventional molecular genetic methods. A defined molecular diagnosis is vital to guide patients' clinical care and informed genetic counselling and is often required for the inclusion of patients in clinical trials.

CONCLUSIONS

1. Charcot-Marie-Tooth disease type 1A is the most common form of CMT. It comprises two-thirds of CMT1 unrelated patients and one third of the entire CMT group during the period 2012 to 2017. No statistically significant gender differences were observed in patients with Charcot-Marie-Tooth disease type 1A ($p = 0.592$).
2. The pathogenic variants of the *GJB1* gene are the second most common genetic factors of Charcot-Marie-Tooth disease. Concurrently, the pathogenic variants of genes *MPZ* and *MFN2* are rarely determined in the analyzed CMT group.
3. The pathogenic variants of the genes *MT-ATP6/8* in this study are not identified.
4. Nearly half of the patients phenotypically diagnosed with classical mitochondrial syndrome have identified pathogenic variants of the mitochondrial DNA. These patients were observed with normal to mild appearance of axonal-type neuropathy according to the electrophysiological pattern.
5. The next-generation sequencing approach for a targeted gene setup is a valuable and an effective diagnostic tool for the molecular diagnosis of patients with comprehensively characterised Charcot-Marie-Tooth disease. However, substantial genetic and phenotype heterogeneity of CMT makes it challenging for molecular diagnosis by single gene and gene panel testing. Digenic inheritance may not be established if only single genetic studies are performed.

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