Clinical Variability of Charcot-Marie-Tooth Disease and its Association with Neurofilament and Genetic Type of Disease

Summary of the Doctoral Thesis for obtaining the scientific degree “Doctor of Science (PhD)”

Sector Group – Medical and Health Sciences
Sector – Clinical Medicine
Sub-Sector – Neurology

Riga, 2023
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The Doctoral Thesis was developed at Rīga Stradiņš University, Latvia

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Abbreviations used in the Thesis

AFO  ankle foot orthosis
AD   autosomal dominant mode of inheritance
AR   autosomal recessive mode of inheritance
AUC  area under the curve
ADP  adenosine diphosphate
ATP  adenosine triphosphate
CIDP chronic inflammatory demyelinating polyneuropathy
CMAP compound muscle action potential
CMTES CMT Examination Score
CMTNSv2 CMT Neuropathy Score version 2
CNS  central nervous system
Cx32 connexin protein 32
DN4  French *Douleur Neuropathique 4*
EMG  electromyography
GAD-7 General Anxiety Disorder-7 scale
GFAP glial fibrillary acidic protein
GJB1 gap junction protein beta 1
HNPP Hereditary neuropathy with pressure palsies
HMSN hereditary motor and sensory neuropathy
MAM mitochondria-associated endoplasmic reticulum membrane
MFN2 mitofusin 2
miRNS microribonucleic acid
Mit mitochondrial mode of inheritance
MLPA multiplex ligation-dependent probe amplification
MPZ myelin protein zero
MRI magnetic resonance investigation
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA</td>
<td>not applicable</td>
</tr>
<tr>
<td>NCAM</td>
<td>neural cell adhesion molecule</td>
</tr>
<tr>
<td>NCS</td>
<td>nerve conduction study</td>
</tr>
<tr>
<td>NCV</td>
<td>nerve conduction velocity</td>
</tr>
<tr>
<td>NfH</td>
<td>neurofilament heavy chains</td>
</tr>
<tr>
<td>NfL</td>
<td>neurofilament light chains</td>
</tr>
<tr>
<td>NfM</td>
<td>neurofilament medium chains</td>
</tr>
<tr>
<td>OMIM</td>
<td>Online Mendelian Inheritance in Man</td>
</tr>
<tr>
<td>PMP22</td>
<td>peripheral myelin protein 22</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>PRX</td>
<td>periaxin</td>
</tr>
<tr>
<td>ROC</td>
<td>receiver operator curve</td>
</tr>
<tr>
<td>Simoa</td>
<td>single molecule array</td>
</tr>
<tr>
<td>IQR</td>
<td>interquartile range</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SNAP</td>
<td>sensory nerve action potential</td>
</tr>
<tr>
<td>Spo</td>
<td>sporadic</td>
</tr>
<tr>
<td>CMT</td>
<td>Charcot-Marie-Tooth disease</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>TMPRSS5</td>
<td>transmembrane protease serine 5</td>
</tr>
<tr>
<td>VUS</td>
<td>variant of unknown significance</td>
</tr>
<tr>
<td>ES</td>
<td>exome sequencing</td>
</tr>
<tr>
<td>XL</td>
<td>X-linked inheritance</td>
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</table>
Introduction

Charcot-Marie-Tooth disease (CMT) is a hereditary motor sensory polyneuropathy, also referred to as HMSN. It is the most common rare neurological disorder, with a prevalence of 1 in 2500. It is both genetically and clinically highly heterogeneous condition, which can be caused by numerous different gene variants that regulate nerve myelination as well as neuronal viability, function, cytoskeletal structure, and functioning. There are nearly 100 different genes associated with CMT, which can manifest with different phenotypes both within and between its genetic subtypes (1–3).

It is a slowly progressive disease that often leads to functional disability. Unfortunately, no disease-specific treatment is currently available, thus regular rehabilitation activities are essential for patients’ wellbeing. Currently there are several ongoing clinical trials looking for potential treatment options, however the high clinical and genetic variability and the slowly progressive nature of the disease make it challenging to design clinical trials in a way, that allows to assess clinical outcome with the tools currently available, given a relatively short period of time during which a clinical trial is conducted. At present, there are no prognostic biomarkers available, which could be used to predict and assess the rate of disease progression and functional changes in patients with the same genetic variant causing the condition. Identifying such markers would be a key element to accurately assess and predict disease progression, which is also crucial for planning a clinical trial and interpreting the results (3–6).

One of the possible biomarkers currently being investigated is neurofilament light chain (NfL). Neurofilaments are cytoskeletal proteins in a nerve cell, more specifically in its axon, found in the central and peripheral nervous system. These proteins form a structure by binding together the NfL, medium (NfM) and heavy (NfH) chains of the neurofilament. When an axon is damaged, these proteins are released, and can be detected in the extracellular
space, in blood and cerebrospinal fluid. Several studies have demonstrated NfL biomarker properties in some of the CNS diseases such as multiple sclerosis and neurodegenerative diseases, however, there is still no sufficient data regarding the use of NfL as a biomarker in peripheral nervous diseases, including hereditary neuropathies (7–11).

**Aim of the Thesis**

To determine and describe the association between CMT genetic type and clinical variability with plasma NfL levels.

**Objectives of the Thesis**

1. Identify and describe the epidemiological, clinical, and electrophysiological features of the CMT patient group.
2. Identify the CMT disease genetic types and describe their respective clinical presentation.
3. To determine the plasma NfL levels in CMT patients and describe NfL association with disease clinical severity.

**Hypotheses of the Thesis**

1. Clinical variability in CMT patients is affected by its genetic type.
2. Plasma NfL concentration correlates with the severity of clinical presentation and serves as a biomarker in CMT patients.

**Novelty of the Thesis**

CMT clinical variability is one of the factors that make it difficult to ensure a homogeneous population within clinical trials and to interpret the results in large populations. The association between genotype and the severity of the symptoms could explain some of the clinical variability and predict CMT
patient’s prognosis and functional status over time. The existing clinical assessment tools are not sensitive enough, while the NfL has shown promising results as a biomarker which could dynamically assess disease progression and prognosis, since blood cytoskeletal protein concentrations can reflect neuronal damage and thus indicate the severity of neuropathy. The role of neurofilaments in hereditary neuropathies was first reported in 2018 paper by Sandelius et al. (11), which presented promising data, showing that NfL blood levels were increased in patients with CMT and that it correlated with disease severity. In comparison, this work has performed a larger patient group analysis, as well as a more in-depth evaluation of the disease-specific features in all study participants using CMTNSv2 (which includes both clinical and neurophysiological findings) and evaluated for its potential association with serum NfL levels. The results of this work could be used for monitoring the therapy efficacy, explaining the clinical variability and prognosis, and determining the extent of nerve damage.

Up until now, there have been no epidemiological studies on hereditary neuropathies in Latvia, which would include the symptom severity analysis and the factors influencing the disease manifestations. As far as we are concerned, there are not many such studies in the world literature either. The results of this work will hopefully generate new knowledge on biomarkers and their application in clinical practice in CMT patients.
1 Literature review

1.1 CMT disease classification and clinical features

Although there are more than 100 genes and their disease-causing variants known to be associated with CMT, a classification based solely on the genetics is neither feasible nor practical. The most common classification of CMT used in clinical practice is based on neurophysiological data, which suggests the predominant type of nerve lesion – demyelinating, axonal, or mixed (1–4).

To assess the function of the peripheral nerve fibres and to clarify the predominant type of lesion nerve conduction study (NCS) is being used to examine large, myelinated nerve fibres. Unfortunately, it does not provide information regarding the function of thinly myelinated or non-myelinated peripheral nerve fibres, which may present with autonomic dysfunction, including sensory disturbances such as pain, altered perception of temperature. If these complaints are more prevalent, a different test should be considered – the quantitative sensory testing (12–14). NCS reflects the integrity and function of both myelin and axon in myelinated motor and sensory fibres. The sensory nerve action potential (SNAP) amplitude provides information regarding the function of the sensory nerve axon and its integrity starting from the distal receptors in the skin and up to the spinal ganglion. Conversely, the compound muscle action potential (CMAP) amplitude reflects the axonal conduction in motor fibres – from the anterior horns of the spinal cord down to the muscles. Axonal damage or its impaired function is reflected by reduced SNAP or CMAP amplitude. The myelin function, in turn, is assessed by the nerve conduction velocity (NCV) and action potential latency. Prolonged latency or NCV is a sign of impaired myelin function or demyelination (12, 14, 15). Thus the NCS provides information about the predominant type of nerve lesion – axonal or demyelinating – as well as the most affected fibres – motor or sensory. There are
many different structures that ensure axon and myelin function, and if any of them are altered or impaired, nerve impulse conduction can be disrupted. Changes in function of Schwann cells (the cells that make up the myelin sheath) or in neuronal axon will vary depending on the affected gene.

Demyelinating forms with autosomal dominant inheritance are classified as CMT1, axonal forms – as CMT2, while the demyelinating forms with autosomal recessive inheritance are referred to as CMT4 type. CMT3 type, used in the previous classification system, referred to an early-onset clinically severe form of inherited neuropathy, also called Dejerine-Sottas syndrome, is sometimes classified as a demyelinating form of CMT (2–4, 16). Each form or neurophysiologic type (demyelinating (CMT1) or axonal (CMT2)) is then followed by a Latin character associated with a specific disease-causing genetic variant, e.g. peripheral myelin protein 22 (PMP22) gene duplication causes CMT1A and a mitofusin 2 (MFN2) gene variation causes CMT2A (3, 16).

In recent years, the increasing number of newly discovered disease-causing gene variants has resulted in a more complex classification. For example, the genes found to be associated with the form of CMT2 already exceed the number of characters available in the Latin alphabet, which the OMIM (Online Mendelian Inheritance in Man) nomenclature is based on. Besides, some forms of CMT are associated with the same gene but have a different inheritance pattern. For these and other reasons, a new classification model has been proposed (17, 18).

The new classification follows a 3-module approach. The first module indicates the inheritance pattern – AD (autosomal dominant), AR (autosomal recessive), XL (X-linked), Mit (mitochondrial), Spo (sporadic). The second one describes the neurophysiological finding – “De” demyelinating, “Ax” axonal or “In” intermediate form. The third module describes the gene variant that causes the subtype. Note that for a significant portion of patients with inherited
neuropathies, the disease-causing gene variant has not been identified, thus it can be classified as “Unknown” (17, 18). According to the new classification model, the most common form of CMT1A associated with PMP22 duplication should be designated as AD-CMTDe-PMP22dup, which explicitly indicates the inheritance pattern (autosomal dominant), the main neurophysiological finding (demyelinating neuropathy) and the disease-causing gene / gene variant (Table 1.1).

Table 1.1

**Comparison of the new and old CMT classification using the CMT types discovered in the work as an example (17, 18)**

<table>
<thead>
<tr>
<th>Previously</th>
<th>Proposed new classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Designation</td>
<td>1st module Inheritance pattern</td>
</tr>
<tr>
<td>CMT1A</td>
<td>AD</td>
</tr>
<tr>
<td>CMT2A</td>
<td>AD</td>
</tr>
<tr>
<td>CMTX1</td>
<td>XL</td>
</tr>
<tr>
<td>CMT1B</td>
<td>AD</td>
</tr>
<tr>
<td>CMT2F</td>
<td>AD</td>
</tr>
<tr>
<td>CMT2Z</td>
<td>AD</td>
</tr>
</tbody>
</table>


Most experts and specialists believe that the existing classification needed changes and that the proposed new classification has a number of significant improvements, while some specialists argue that the existing, traditional classification is still better (17). It should be said that the former classification is
still more widely used in clinical practice and in the scientific literature. In this work we use the existing and more commonly recognized classification model.

Demyelinating forms of CMT have reduced nerve conduction velocity, usually < 35–38 m/s, which reflects damage and dysfunction of Schwann cells (myelin sheath). CMT1 is the most common type of CMT accounting for 50–80% of all CMT cases and has autosomal dominant inheritance pattern. The first symptoms can appear as early as the neonatal period and up to the fourth or fifth decade, but for the most part between the ages of 5 and 25 years, with subsequent slow progression over the course of life. The clinical presentation varies greatly in its severity, and symptoms in patients with CMT1 can range from mild, or even unnoticeable, to marked weakness and severe functional disability. Clinical findings include slowly progressive distal muscle weakness and atrophy in the legs earlier and stronger than in the arms, as well as sensory disturbances and reduced to absent tendon reflexes. The hollow foot (Latin pes cavus) is a common finding, along with the drop foot, which is sometimes bilateral, and hammertoes. As the disease progresses, patients often develop spinal deformities, such as scoliosis. CMT1 does not affect patient survival, however some patients have significant limitations in daily functioning and require assistance and routine long term use of technical aids (2, 3, 19).

The most common form of CMT, which is also the most common form of CMT1 – CMT1A, is caused by PMP22 gene duplication. PMP22 is a glycoprotein located in the compact myelin of Schwann cells and makes up to 5% of the total myelin protein. Together with the other myelin proteins PMP22 maintains a proper myelin structure. Duplication of PMP22 results in overproduction of PMP22, which can cause instability of compact myelin, resulting in demyelination, or remyelination. Repeated demyelination and remyelination can form bulbous nodules – a typical finding in Schwann cell clusters. CMT1A accounts for about 60–70% of all CMT1 patients (1, 16, 19–
In the less common CMT1B type, the myelin protein zero (\textit{MPZ}) gene variant causes the myelin damage and thus plays a central role in the development of the disease. MPZ is a 219 amino acid glycoprotein expressed in Schwann cells, which forms myelin sheath. It forms tetramers and acts as an adhesion molecule in the compact myelin. Half of the myelin of the peripheral nerves is made up of the MPZ protein, but it is not found in the central nervous system (CNS). The protein consists of an extracellular part, which has an immunoglobulin-like structure, an intermembrane part and an intracellular or cytoplasmic part. In CMT1B, the three-part structure of the MPZ is disrupted, leading to inability to form compact myelin, and resulting in its damage and demyelination (22). CMT1B makes up 5–10\% of all CMT cases and has an autosomal dominant inheritance pattern. Some patients may have tonically dilated pupils or Adie pupil, characterized by a reduced pupillary contraction, more pronounced in response to a light stimulus, usually unilateral. This finding is not observed in other CMT types and is only typical in CMT1B patients (4, 19, 23). The typical features of the less common forms can help in the diagnosis, e.g. type CMT1E (\textit{PMP22} point mutation) is associated with sensorineural hearing loss, CMT1C (\textit{LITAF} gene variant) and CMT1F (\textit{NEFL} gene variant) types have an early age of onset, the first symptoms developing as early as 1 year of age, which can manifest as delayed motor development in the infant, child (19).

In CMT2, the lesion lies in the nerve cell itself, more specifically in the axon, which manifests as reduced action potential amplitudes with normal or minimally altered nerve conduction velocity > 45 m/s, indicating relatively preserved myelin integrity and function. CMT2 is not as common as CMT1, accounting for about 1/3 of all CMT patients. The onset of the first symptoms can be highly variable even within the same family, with the first symptoms appearing as early as childhood or as late as the age of 60 or later. Similar to
CMT1, CMT2 patients have gradually progressing distal muscle weakness and atrophy which is more pronounced in the lower limbs than in the upper ones. The sensory disturbances are present, but to a lesser degree. The inheritance pattern can be autosomal dominant or recessive (3, 4, 19).

The most common form of CMT2 is CMT2A, which is caused by the \textit{MFN2} gene variant that encodes the protein mitofusin. This protein embedded in the outer membrane of the mitochondria and extends into its cytoplasm. MFN2 protein has two functions: it regulates mitochondrial fusion and has a central role in forming the mitochondria-associated endoplasmic reticulum membrane (MAM). Thus, several cellular functions are affected in case of MFN2 protein disruption, with the most detrimental impact on cellular transport which ultimately leads to axonal dysfunction (24, 25). Although CMT2A is the most common form of axonal CMT, accounting for more than a third of CMT2 cases, only less than 5\% of all CMT patients have this subtype. CMT2A patients may suffer from optic nerve atrophy, vocal cord paresis and tremor. Up to a quarter of them may have CNS involvement with changes in spinal cord or brain observed radiographically. There have been reports of fatal subacute encephalopathy being associated with the CMT2A form (3, 4, 19, 26). Less common forms of axonal CMT have a wide range of clinical symptoms and phenotypes. Proximal muscle weakness can be quite common, as well as the intercostal muscle and diaphragm weakness, vocal cord paresis, hearing loss, cranial nerve paresis and other less common features of hereditary neuropathy (1, 19).

\textit{HINT1} gene-related hereditary neuropathy with neuromyotonia is caused by changes in histidine triad nucleotide-binding protein 1, or HINT1, which ultimately results in the loss of protein function. Disruption of the HINT1 protein results in changes in the transcription process and signalling pathways, yet its exact role in peripheral nervous system disease remains unknown. The protein
dysfunction causes axonal membrane hyper-excitability, which results in neuromyotonia. *HINT1*-related neuropathy accounts for up to 10% of CMT forms with the recessive inheritance patterns. The first symptoms appear mostly in the first decade of life, although the cases of a later onset (up to the third life decade) has also been reported. The first complaints are usually about the weakness in the distal muscle groups in the legs with subsequent gait changes, as well as the muscle stiffness, muscle twitching, fasciculations and muscle cramps both in the arms and legs. The upper limbs usually get affected later. The typical feature of this CMT form – neuromyotonia – is observed in 70–80% of patients and can be present with or without peripheral neuropathy. As with the other CMT forms, foot deformities or shortening of the Achilles tendon can be observed, and scoliosis is present in about a third of these patients. NCS reveals axonal lesion while electromyography shows neuromyotonic discharges, however these may be absent in around 20–30% of patients or may appear later in the course of the disease (27–30).

Dejerine-Sottas syndrome (DSS) is not usually classified as CMT3, but rather as a part of CMT1 spectre. The genetic finding in these patients revolves around the following disease-causing gene variants: *MPZ, PMP22, PRX* or *EGR2* genes. The roles of MPZ and PMP22 proteins have been described above, while periaxin (PRX) protein plays an important role in myelin structure integrity as well as the signal conduction, whereas EGR2 protein is necessary for peripheral nerve myelination. The gene activation occurs in Schwann cells before the myelination process. When EGR2 function is impaired, Schwann cell differentiation gets compromised. The transcription factor encoded by the *ERG2* gene further regulates the PMP22, PRX, MPZ, connexin 32 proteins, which explains the severe clinical picture in case of EGR2 protein dysfunction. The first DSS symptoms appear in the early infancy, even in neonates, and manifests with delayed motor development and severe clinical course. Patients often develop
distal contractures in their extremities, with occasional kyphoscoliosis and nystagmus. NCS shows significant reduction in nerve conduction speed (< 12 m/s), while the cerebrospinal fluid shows elevated protein levels (19, 21, 31–33).

Intermediate CMT is the form which is characterized by a mixed damage (both demyelination and axonal damage), with nerve conduction velocities of 35–45 m/s. NCS findings can be extremely diverse even within one family, with some family members meeting the criteria for the demyelinating type and others – the axonal type. The diverse NCS findings, that corresponds to the intermediate CMT are also present in the X-linked CMTX1 form, which is the second most common form of CMT, albeit still less common than CMT1A. The disease is caused by gap junction protein beta 1 (GJB1) gene variants, which are located in the X chromosome and encode the connexin 32 protein (Cx32) that makes up the non-compact myelin in the peripheral nervous system, although it is also present in myelin in the CNS. The Cx32 protein has multiple extracellular, intermembrane and intracellular protein parts. Gap junction proteins form connections and transport ions and small molecules between cells. A group of six connexins forms a channel called a connexon. In the case of CMTX1, Cx32 protein expression is reduced, leading to impaired intracellular transport along with impaired ability to form junctions and connexons, or leads to reduced ability to transport molecules between the cells. CMTX1 makes up to 10–20 % of all CMT patients. It manifests with distal muscle weakness and atrophy, hollow foot, sensory impairment in distal extremities, toe walking, Achilles tendon contractures. On rare occasions hearing loss may also be present. The early onset subtype is characterised by motor retardation. CMTX1 is often considered a disease of the central and peripheral nervous system. Patients may experience transient episodes of neurological deficit with monoparesis, paraparesis, sensory disturbances, motor aphasia, dysarthria, dysphagia, cranial nerve deficits, tremor,
and other neurological signs. Magnetic resonance imaging (MRI) of the brain shows hyperintense foci in white matter, corpus callosum in T2 and FLAIR sequences, which disappear over the course of time. This may cause difficulties in establishing the diagnosis, especially if these transient CNS symptoms are the very first manifestation of CMTX1. It should be noted that white matter changes in the CNS of CMTX1 patients may not present with any symptoms (3, 4, 19, 34–38).

A mitochondrial inheritance associated variant of the MT-ATP6 gene may present with clinical and neurophysiological features similar to CMT2. The mitochondrial DNA-encoded adenosine triphosphate (ATP) 6 protein is involved in ATP synthesis, specifically in the mitochondrial membrane ATP synthase function, which produces ATP from adenosine diphosphate (ADP). Impaired ATP6 protein function can lead to severe axonal neuropathy. The disease begins in the first to second decade of life and is marked by high clinical variability, sometimes leading to wheelchair dependence in adolescence. Usually, the symptoms develop gradually until the fifth / sixth decade of life, with a subsequent rapid clinical deterioration, relatively early involvement of proximal muscles of the lower limbs, despite moderate muscle weakness distally. NCS reveals motor or predominantly motor axonal neuropathy. It should be noted that mitochondrial form is characterised by involvement of multiple organ systems in the patient and in their relatives; therefore, collecting a detailed medical history is essential (1, 39).

As already mentioned, CMT disease has a marked clinical variability. A potential involvement of different organ systems or a possibility of an atypical CMT presentation with a less pronounced peripheral polyneuropathy should be considered in patients, which can certainly pose diagnostic challenges.
1.2 CMT clinical diagnosis and genetic testing strategies

Genetic heterogeneity within and between populations, as well as phenotypic variability between families and even within the same families, often make diagnosis challenging. Clinical and neurophysiological findings are essential for correct diagnosis, as well as obtaining an accurate medical and family history. The most accurate diagnosis provides the patient with information about their disease, including its inheritance pattern, which is essential for further family planning (1–3, 19).

The chance of having a hereditary neuropathy is high in patients presenting with symmetrical distal muscle weakness and sensory disturbances that began in adolescence, *pes cavus* foot deformity with hammertoes, reduced nerve conduction velocity on NCS, especially if there is a positive family history. However, the clinical variability in inherited neuropathies is high and patients will not always experience all or the most common symptoms. Diagnosis may be more difficult in case of *de novo* mutations, atypical clinical findings, later onset of symptoms (adult years or older) and/or if NCS shows mostly axonal damage (1–3, 19, 40).

In case of suspected hereditary neuropathy, a family history of at least 3 generations is recommended. Closer attention should be paid to any cases of early deaths among family members, close marriages and family members with mobility or gait disorders with an uncertain diagnosis. Most of the CMT cases are inherited in an autosomal dominant manner. CMTX1, the second most common CMT form, has the X-linked inheritance pattern, and thus the clinical findings are more pronounced and severe in males than in females. *De novo* mutations can pose a diagnostic challenge; however, a false-negative family history may be reported due to the marked clinical variability of the disease. Onset after the age of 40 years is uncommon, more often observed in CMT2, and
may raise suspicion of an acquired rather than inherited polyneuropathy (1, 2, 40, 41).

NCS is the main investigation to confirm and describe neuropathy – its severity, the nerve fibres affected and the type of lesion – and possibly suggest an inherited aetiology in patients with unspecified polyneuropathy (2, 42). Electromyography (EMG) can also contribute to the diagnosis, especially in the case of less common forms of CMT, such as HINT1 gene-related neuropathy or CMT2Z (MORC2 gene variant), in which neuromyotonic or myokymic discharges can be recorded. Unfortunately, up to 20–30% of HINT1-related neuropathy patients do not present with these specific EMG findings, which complicates the differential diagnosis, although these features may still appear later in the course of the disease (27, 28, 43). NCS can be an informative tool to differentiate hereditary form from the acquired neuropathy. In later, neurophysiological findings are less likely to show such marked and severe damage to the peripheral nervous system with a relatively same or milder clinical features (15, 44–46).

A genetically confirmed diagnosis is achieved in about 60% of CMT patients with identification of disease-causing gene variant. The analysis of genetic testing algorithms reveals that up to 90% of genetically confirmed cases are associated with the following four disease-causing gene variants: PMP22, MPZ, GJB1, MFN2. The addition of the other four genes (GDAP1, HINT1, SH3CT2 and SORD) to the multi-gene panel for analysis would increase the number of patients with a genetically accurate diagnosis. The patients with the demyelinating form of the disease (CMT1) are more likely to have a genetically confirmed diagnosis (> 85%) compared to the CMT2 patients (25–35%). Using next-generation sequencing panels, that include up to 50–60 genes, the genetical diagnosis can be confirmed in 50–90% of CMT1 patients, and in about 15–30% of CMT2 patients (1–3, 47–50).
As a general rule, the first genetic analysis tests for the most common form, CMT1A, which accounts for more than 60% of the genetically confirmed CMT population (1, 3, 21, 47). The same approach is used in Latvia. If the PMP22 copy number does not confirm the diagnosis, further testing tactics may differ.

In some cases, certain genes will be targeted for further analysis depending on the clinical and neurophysiological findings as well as the family history. Alternatively, testing may proceed directly with next-generation sequencing using the neuropathy-related gene panel, which includes 60–200 genes. Utilizing the CMT gene panel the genetic diagnosis accuracy reaches 18–31% (1, 3, 47, 51).

If the genetic testing described so far has not identified the disease-causing gene variant, further testing may require exome sequencing (ES) as a diagnostic option and, in rare cases, whole genome sequencing. Exome sequencing involves the analysis of the coding part of the genome, which can help to detect protein-altering gene variants. For this purpose, a clinical exome, containing about 5000 genes, is commonly used. For patients who have previously tested negative, ES can confirm the genetic diagnosis in 19–45% of cases (1, 3).

1.3 CMT prognostic and diagnostic biomarkers

CMT disease severity and clinical variability is assessed via physical examination using different scales, including those specific to CMT, as well as through neurophysiological examination. Unfortunately, due to the slowly progressive nature of the disease, these methods do not allow to reliably estimate the disease progression over a short period of time (6, 52).
Currently, there is no specific biomarker that would provide information on the prognosis and/or severity of CMT, however the search for such a biomarker has been ongoing for several years. There is more focus on the link between disease severity and its change over time. Given the slow progression of the disease and its symptoms, a biomarker which can reflect short-term changes would be of a greater use. Most of the research up until now has been focused on CMT1A subtype, since it is one of the most common CMT subtype (6, 21, 52–54).

One of the potential biomarkers of disease progression currently being studied, is muscle fat fraction, which can be determined using MRI. So far it has been studied in populations of CMT1A and type 1 hereditary sensory neuropathy. It has been criticized over the lack of clinical relevance, however there is a certain correlation found between the muscle fat fraction and CMTNSv2 scores. Although this potential biomarker has proved to be sensitive enough, and the measurement is technically reproducible, its routine application in clinical practice is not feasible. MRI is an expensive and time-consuming procedure, which may also require sedation in children. It can also vary in its accuracy depending on the different technical parameters or technician’s skills (6, 55–58).

Blood tests, in turn, are widely available, rapid, convenient, and easy to obtain under various conditions and at multiple time points over the course of the disease, making them a good biomarker material. Potential biomarkers that could indicate myelin damage include transmembrane protease serine 5 (TMPRSS5), which is a Schwann cell-specific protein, and neural cell adhesion molecule (NCAM). In chronic neuropathies, axonal damage may be reflected by glia fibrillary acidic protein (GFSP) and neurofilament – a cytoskeletal protein of the nervous system, more specifically neurofilament light chains (NfL). From all the aforementioned potential biomarkers, NfL is the most promising one for the CMT population, since it reflects the axonal damage (6, 11, 59–63).
Neurofilament is a structural protein in the central and peripheral nervous system, and is composed of heavy, medium and light chains. Changes in its levels in both cerebrospinal fluid and blood can indicate the degeneration of a nerve, more specifically an axon. Up until now, the data has shown that NfL have the highest biomarker potential. Initial research on NfL focused on the relationship between CNS diseases and neurofilament levels, but there is now evidence on its role in peripheral nervous system diseases. The aforementioned study by Sandelius et al. published in 2018 examined the changes in plasma NfL levels in a group of 75 CMT patients. The study showed promising results as the data indicated that NfL was significantly higher in the patient group compared to the control group, and plasma NfL levels were associated with the severity of the disease clinical presentation, reflected with CMTES. NfL levels have also been found to be significantly elevated in other neuropathies, such as chronic inflammatory demyelinating polyneuropathy, Guillain-Barré syndrome and vasculitic neuropathy (6, 11, 60, 64–68).

When assessing the markers reflecting myelin damage (NCAM, TMPRSS5, etc.), studies have shown that NCAM levels were higher in demyelinating polyneuropathies (inflammatory and hereditary) compared to controls as well as axonal neuropathies, even though axonal neuropathies also had higher NCAM levels than controls. In the TMPRSS5 study, the data suggested that its levels could be used to distinguish the control group from the CMT1A group, yet no significant differences were observed in the other CMT groups (62, 69).

Promising data has also been reported for microRNAs (miRNAs) as a potential biomarker in the CMT1A group. MiRNAs are small molecules that regulate gene expression on a post-transcriptional level and their expression rates reflect different physiological processes in a cell or damage occurring in a particular tissue. In their recent publication from 2021, Wang et al. have
demonstrated that plasma levels of several miRNAs are elevated in CMT1A patients compared to controls and thus it could be used as a biomarker in clinical trials. Furthermore, miRNAs correlated with plasma NfL concentration as well as with the TMPRSS5 protein levels. In general, the data from the studies suggested utility of miRNAs as a potential CMT biomarker (68, 70).

1.4 CMT pharmacotherapy and rehabilitation

Knowledge and understanding of these disease-causing mechanisms have improved in the recent years, but there is still no disease-specific therapy available to reverse the natural course of the disease. The existing treatments includes symptomatic pharmacotherapy, multidisciplinary rehabilitation and surgical treatment of skeletal deformities (3, 5, 71, 72).

Pain is a common complaint in CMT patients, reported by 23–85 % of those with condition. Patients may have mechanical, nociceptive pain caused by musculoskeletal deformities (spinal or foot deformities) or neuropathic pain. The pain is mostly mild to moderate, nonetheless more than a third of CMT patients are taking painkillers. Fatigue is another common complaint that can have a significant impact on quality of life. This symptom can be addressed by lifestyle changes as medication has little effect and cannot be used as a long-term solution. Muscle cramps are also very common, reported by up to 85 % of patients. According to the current knowledge, pharmacotherapy is not effective in this case either. The efficacy of magnesium supplements, which are frequently prescribed in clinical practice, is also up to debate. Meanwhile, rehabilitation procedures with an emphasis on stretching exercises are able to reduce complaints of pain and muscle cramps (5, 71, 73).

Physiotherapy that focuses on muscle strengthening, aerobic exercise, stretching and exercises to improve posture and balance are the most recommended for CMT patients. There are numerous studies indicating that
rehabilitation positively affects muscle strength, balance, and cardiorespiratory function, and can reduce the time CMT patients spend performing daily activities. The positive effect is achieved not only by working with the functional specialists, but also by patients receiving video material with recommended physiotherapy techniques, which they implement themselves at home (5, 71, 74).

Many CMT patients use insoles or orthopaedic shoes to reduce foot asymmetry, pain caused by foot deformity and ankle foot orthoses (AFOs) to correct foot drop during walking, reduce ankle instability and the subsequent risk of trips and falls. The use of AFOs often has poor compliance and tolerability due to pain and discomfort resulting from physical pressure as well as due to aesthetic reasons, even though it has to be mentioned that the more recent AFO models are lighter and more flexible, which increases their use among patients (71). Around 20 % of the CMT patients have their foot deformities treated surgically. The aim of such surgical intervention is to realign the foot, correcting muscle imbalances and reducing pain. Scoliosis is present in 20–30 % of patients and may require the use of rigid orthosis, physiotherapy and, in more severe cases, surgery (71, 75).

Although there are still no disease-modifying therapies available for any of CMT types, some chemical substances and gene therapy options are currently being evaluated in clinical trials. The most widely studied treatment options is for the most common type of CMT-CMT1A. One of the best-known potential drugs for CMT1A is PXT3003, which uses a combination of drugs already in clinical use – low-dose baclofen, sorbitol, and naltrexone. This drug combination is meant to inhibit the Schwann cell proliferation and reduce PMP22 synthesis. PXT3003 is currently undergoing a Phase III clinical trial, but the results available so far are promising, showing significant improvement in patients’ functioning and a good safety profile (71, 76). At the same time, clinical trials with ascorbic acid, progesterone antagonists or modulators have not yielded any
meaningful results so far (71). Other agents and ways of modifying pathogenic mechanisms underlying the disease are being considered, including gene therapy. Gene therapy with partial gene silencing in case of *PMP22* gene duplication, gene insertion or replacement is being currently investigated, however these options are still in the preclinical research phases (71, 77–80).
2 Materials and methods

2.1 Ethical considerations of the study

The study was carried out in accordance with the World Medical Association Declaration of Taipei, the World Medical Association Declaration of Helsinki, the Convention for the Protection of Human Rights and Dignity of a Human Being with regard to the Application of Biology and Medicine – Convention on Human Rights and Biomedicine (Oviedo Convention) as well as the laws and regulations of the Republic of Latvia.

The study was approved by the Central Medical Ethics Committee of Latvia (No 3/18-03-21), Annex 1. The patients were included in the study only after obtaining their written consent for participation in the study alongside with the written confirmation of having the study explained to them. For those under the age of 18, a written statement from a legal or appointed representative was obtained.

2.2 Inclusion of study participants in the study

The study group consisted of 101 CMT patients aged between five and 81 years (with 18 patients being children) from the geneticist, neurologist and paediatric neurologist clinical practices at the Children’s Clinical University Hospital, Centre for Neuroimmunology and Immunodeficiencies.

The inclusion criteria for the patient group required the patient’s written consent to participate in the study, certified by their signature on the informed consent form. The said form could be signed by the study participant themself or their legal representative. In order to qualify for the patient group, a participant had to meet at least one of the following criteria:

1. Clinical and / or neurophysiological findings consistent with CMT along with the genetic confirmation and / or positive family history.
2. Clinical and neurophysiological features typical of CMT.

3. Genetically confirmed CMT.

The exclusion criteria were a known central or peripheral nervous system comorbidity that could affect the NfL levels and thus the results interpretation, as well as the patients’ refusal to participate in the study.

To compare the neurofilament light chain plasma concentrations, a control group has been included into the study. The control group consisted of 60 individuals (four of them children), aged between five and 62 years. The control group was matched for age and gender distribution. While there were no controls to match the participants over the age of 62, the difference in age between the groups was not statistically significant; the gender distribution between the groups also showed no significant difference. The control group consisted of people available to the research team, such as medical staff, healthy patients’ relatives (only in families with identified disease-causing variant, that was not found in healthy relative), and other people that were physically accessible to the research team without any neurological symptoms. The participants of the said control group did not undergo any additional neurological examination or assessment, but were required to meet both of the following two criteria:

1. Healthy individuals without any known neurological disease and / or neurological symptoms.
2. Consent to participate in the study.

2.3 Characterization and evaluation of study participants

Patients or their legal representatives had to answer a set of socio-demographic questions and undergo clinical and neurophysiological examinations and assessments with the help of standardised tests.
NCS was performed to define their neurophysiological parameters. The investigation was performed by one NCS specialist as per the standardised polyneuropathy protocol. NCS was performed using the Dantec Keypoint Focus EMG / NCS / EP system. According with the polyneuropathy protocol (81), NCS included the motor nerves examination in the lower limbs (n.peroneus, n.tibialis), followed by the sensory nerves (n.suralis, n.peroneus superficialis). After the lower limb nerve examination, the upper limb nerves were assessed. The motor and sensory fibres of the n.ulnaris and the sensory fibres of the n.radialis were examined. For this study, the NCS was performed in order to define the type of disease as well as for the detailed data collection for the disease-specific severity scales. Based on the NCS results, the patients were divided into demyelinating (CMT1), axonal (CMT2) or mixed forms according to the nerve conduction velocity classification for the CMT disease (3).

The clinical assessment included the patient’s symptoms and the objective neurological findings, summarizing prevalence of the most common symptoms that could indicate the diagnosis of hereditary neuropathy (Annex 2). To describe the clinical manifestation, the disease-specific clinical severity rating scales CMTNSv2, CMTES (82) were used, Annex 3. The CMTNSv2 score includes sensory symptoms description – indicating the level of sensory disturbance in the lower limbs, vibration sensation test, needle prick test; motor function description – separately for the upper and lower limbs, which are described by muscle strength according to the Medical Research Council (MRC) scale, the functioning level when performing daily activities. The seven sections of the CMTNSv2 described above form the clinical score section or CMTES, and in addition in the CMTNSv2 scale, there is a section regarding the neurophysiological data. This section presents neurophysiological data from both arm nerves. The first parameter allows to choose between ulnar or median nerve CMAP, the second parameter provides the amplitude range of radial nerve
SAP. The inclusion of these nerve functional parameters can be confusing, as the CMT affects mostly the lower limbs, however, it should be noted that in some cases the neurophysiological data obtained from the leg nerves is already so altered, that there is little to no temporal variability. As a result, the CMTNSv2 produces two separate disease severity indicators – the CMTNSv2 or the complete version, which includes the neurophysiological data, and the CMTES, which reflects only the clinical assessment results and is basically the first part of the CMTNSv2 scale. The result in each section is rated from zero to four, the total score for CMTNSv2 can vary from 0 to 36, while for CMTES from 0 to 28. The higher the score, the more severe the clinical presentation. Sometimes CMTNSv2 results may be grouped depending on the score, for better interpretation: 0–10 mild, 11–20 moderate and 21–36 severe clinical phenotype. In this work, these two results (CMTNSv2 and CMTES) will be reviewed and analysed separately.

To understand the prevalence of neuropathic pain in the CMT group, the Neuropathic pain scale 4 (DN4, French *Douleur Neuropathique 4*) was applied, which consists of four sections (Annex 4). Each section must have at least one positive response for the pain to be classified as neuropathic. The first section inquires whether the pain produces a burning sensation, a painful freezing sensation, an electric shock sensation; the second section asks whether the pain is accompanied by any additional sensations – tingling, pins and needles pricking sensation, numbness and itching; the third section asks whether the localization of the pain corresponds to the objective finding of tactile hypoesthesia, hypoalgesia; the fourth section asks whether the pain is caused / exacerbated by touch. There must be at least four positive answers (out of the possible 10 points), at least one in each of the four sections to confirm the neuropathic nature of the pain.
The Generalized Anxiety Disorder 7 (GAD-7) scale has been used (Annex 5) to examine the possible associations between anxiety and pain as well as reduced daily functioning ability. The scale is widely used in the adult population (83), which makes the biggest part of the participants in our study. The scale includes seven statements regarding the patient’s feelings in the last two weeks, with the patient choosing the frequency that best matches the feeling described. Frequency is expressed as “not at all” (0 points), “several days” (1 point), “more than half of the days” (2 points) and “nearly every day” (3 points). After completing the scale, the total number of points is calculated. The patients who score more than 5 points have at least a mild level of anxiety, more than 10 points – a moderate level, and more than 15 points have a severe anxiety level.

For objective assessment of memory and cognitive abilities, a computerized neurocognitive assessment tool “CNS Vital Signs” (CNVS) was used, which is freely available at www.cnvs.com. The program is validated (84) and allows testing in both Latvian and Russian languages and provides an opportunity to compare the participant’s results with the age-appropriate standardized score ranges (above average or > 74th percentile, average or 25–74th percentile, lower average or 9–24th percentile, low or 2–8th percentile, very low or < 2nd percentile) for both verbal and visual memory domains.

In addition to the above, information on patients’ involvement and participation in regular rehabilitation activities was collected, as well as the regular use of technical aids, such as orthoses, in daily activities.

2.4 Blood sample collection and testing procedures

The collection and storage of blood samples followed a strict protocol. Certified medical personnel collected two blood tubes with EDTA preservative from the patients and one blood tube from those in the control group after
an outpatient visit. The blood samples were processed within one hour. To detect the NfL, one of the EDTA-containing blood tubes was subjected to centrifugation at ambient temperature for 10 min at 3500 rpm. The plasma was then divided into aliquots and stored at −20 °C. The other was stored at a temperature of +4 °C and transported to the Rīga Stradiņš University (RSU) Scientific Laboratory of Molecular Genetics (SLMG) for DNA isolation within one-week period.

2.4.1 Genetic testing

Two different methods were used to isolate the DNA. Both the commercially available method (Analytic Jena, Germany) and the customized phenol-chloroform method were used to isolate DNA from peripheral blood samples collected from the patient group (85). The quality of the DNA was quantified using a Nanodrop UV/VIS spectrophotometer (ThermoFisher Scientific, USA) whereas the DNA concentration was measured using a Qubit fluorometer (ThermoFisher Scientific, USA), then sent for exome sequencing. The first step in genetic testing was to quantify the PMP22 copy number using the multiplex ligation-dependent probe amplification (MLPA) kit P405 (MRC Holland, The Netherlands) as per the manufacturer’s protocol, using Coffalyser.Net software. For patients with clinical signs or family history of possible CMTX1, and patients with normal PMP22 copy number, exons and exon/intron junctions of the GJB1 gene were analysed using bidirectional Sanger sequencing with the help of the BigDye Terminator 3.1 kit (ThermoFisher Scientific, USA) as per an adapted manufacturer’s protocol, using the primers as described earlier (36). Patients with negative results had their exome sequenced in an ISO:15189 accredited Medical laboratory, CeGaT (Germany) using Twist Bioscience reagents (Twist Bioscience, USA). Biological data computation and analysis was performed at RSU SLMG using a laboratory-developed bioinformatic pipeline that allows the analysis of single nucleotide variation,
small insertions/deletions and copy number changes. The bioinformatics algorithm was validated on samples with known genotypes, both with microsatellite repeats and exon duplications/deletions. Genetic variants were annotated using the *Illumina Variant Interpreter* platform (Illumina, USA). Exome sequencing only targeted genes associated with neuropathy (Annex 6). The list of genes included for analysis was constructed by selecting genes using publicly available information from *Panelapp* (http://panelapp.genomicsengland.co.uk/#!), *Blueprint* laboratory, publications.

The genetic variants identified in the selected genes were classified according to the criteria recommended by the American College of Medical Genetics (ACMG) into the following groups: benign, likely benign, variants of unknown significance (VUS), likely pathogenic, pathogenic variants (86). Identified pathogenic and probable pathogenic, as well as some VUS variants, were confirmed using bidirectional Sanger sequencing in both the patient and family members if they agreed to participate in the study.

The following work uses pathogenic and likely pathogenic gene variants as a diagnostic confirmatory, listed together with the gene in which the variant was found. In addition, a group of patients with VUS has been identified, it should be noted that this group includes the patients with a gene variant identified as more likely to be disease causing according to the guidelines of the ACMG (86). The patients with a likely benign VUS were classified as ES-negative. For patients with VUS, healthy and/or symptomatic relatives were invited to undergo the test in order to clarify the clinical significance of VUS. However, obtaining a blood sample from a relative with a subsequent genetic analysis was not always possible.
2.4.2 Neurofilament light chain concentration measurement

The NfL tests were carried out at the Institute of Neuroscience and Physiology, Sahlgrenska Academy, University of Gothenburg. The time from blood sample collection to NfL analysis was 3–4 months, until then the plasma samples were stored at the temperature −20 °C. Plasma NfL concentration was measured using the single molecule array (Simoa) NfL assay (Quanterix, USA). The samples were analysed in a randomized order without knowing the patient and the control group sample distribution. The samples were diluted 4 times and processed separately; the result interpretation was adjusted in accordance with the dilution degree. The dynamic range for NfL levels was 1.9–1800 pg/ml.

2.5 Statistical analysis

All the calculations were performed in R v3.6.0 software (87), as well as IBM SPSS Statistics, version 27.0. The normal distribution of the continuous data was assessed by histograms, Q-Q plots and the Shapiro-Wilk test. T-test was performed on the normally distributed data to compare means between groups, while a Mann-Whitney U test was used for data that did not follow a normal distribution. Discrete data were compared using Pearson’s chi-square test. Correlation between the continuous data was analysed using Spearman’s correlation coefficient. The difference between the correlation coefficients was assessed using the Fisher’s Z test, with the use of the Cockor package in R software (88). To assess the utility of NfL in distinguishing between the controls and CMT patients, a receiver operator curve (ROC) was generated and analysed using the pROC package in R (89). After that the best NfL threshold was determined using the Juden index, while the area under the curve (AUC) was calculated using pROC with 95 % confidence intervals (95 % CI).
One patient with an NfL level below the detection threshold (NfL result < 1.9 pg/ml) was assigned an NfL value corresponding to half of the NfL detection range, or 0.95 pg/ml, as per recommendations (90, 91).
3 Results

3.1 Characteristics of the CMT group and genetic testing results

The study involved 101 hereditary neuropathy patients from 72 families. The mean age was 37.9 ± 18.4 years; sex distribution: 46 men and 55 women. Study included group of children (n = 18), the mean age in this subgroup was 12.6 ± 3.7 years, with nine boys and nine girls.

The results of the genetic analyses are described separately for the study participants, as well as for the index group. The index patient is the first identified case within the same family. This allowed a subsequent follow up of the other family members enrolled in the study if they met the inclusion criteria. After performing genetic testing, 44 patients (index patients n = 33) were found to have a PMP22 gene duplication (CMT1A) – Figure 3.1. The second biggest group of CMT patients (n = 13; index patients n = 6) had a disease-causing variant of the GJB1 gene (CMTX1), followed by disease-causing variants of the HINT1 gene in 6 patients (index patients n = 3), that presents as axonal neuropathy with neuromyotonia. Several patients had a PMP22 deletion causing HNPP (n = 3; index patients n = 2), as well as disease-causing variants of MFN2 (n = 2; index patients n = 1; cause CMT2A), HSPB1 (n = 2; causes distal hereditary motor neuropathy), MPZ (n = 1; causes CMT1B), BSCL2 (n = 1; causes distal hereditary motor neuropathy) and MORC2 (n = 1; causes CMT2Z) genes. VUS were found in some patients (n = 7; index patients n = 5). VUS were found in the following genes: PMP22 (n = 1), MFN2 (n = 3, index patients n = 1), AARS1 (n = 2, index patients n = 2) and BICD2 (n = 1). Additionally, 1 HINT1 gene VUS was identified in a family with a HINT1 gene disease-causing variant (n = 3, index patient n = 1). Furthermore, 21 patients (index patients n = 17) received ES negative finding (Figure 3.1).
Figure 3.1 Genetic profile of study participants (A; n = 101) and index patients (B; n = 72) depending on the disease-causing gene

VUS – variant of unknown significance, ES – exome sequencing, del – heterozygous deletion, dup – heterozygous duplication

The genetic testing strategies evaluation used in the analysis were based on the genetic testing results of the index patients (n = 72), so that the family size and involvement of other relatives in the study did not influence the interpretation of the said results. The data showed that the diagnosis was confirmed in 48.6% of cases using PMP22 copy number analysis, thus supporting the diagnoses of CMT1A (n = 33) and HNPP (n = 2). Additionally, analysis of the other most common disease-related gene, GJB1, confirmed the diagnosis of CMTX1 in 6 patients, which is 16.2% of the previously genetically undefined cases. Further genetic testing consisted of ED of the remaining patient group (n = 31), this confirmed the diagnosis in 14 patients or 45.2% – pathogenic, probable pathogenic or VUS more likely to cause disease according to ACMG guidelines.
Overall, DNA testing was able to identify the disease-causing gene variant (including the VUS) in 76.4% of patients. In the paediatric population, PMP22 copy number measurements improved the diagnosis in 37.5% of cases, GJB1 gene testing in 20.0% of cases, whereas the ES – in 50.0% of the cases. The applied DNA testing strategy was able to improve the diagnosis in 75.0% of the children. Comparing genetic diagnosis accuracy in CMT1 and CMT2, the majority of CMT2 patients remained genetically undefined after the ES; 31.3% (n = 5) of CMT2 patients versus 19.2% (n = 15) of CMT1 patients.

For genetic type comparison, the most common CMT types were identified, while the less frequent and genetically unspecified forms of CMT were grouped together under the “Other CMT”. This was done due to a small number of patients with the less common forms. Note that this group may not be representative of all the patients with a particular disease-causing gene variant.

NCS has been used to classify the CMT group based on the myelin function or nerve conduction speed results. Most of the patients (n = 78) were found to have nerve conduction velocities below 35 m/s, indicating myelin damage and meeting the criteria for demyelinating hereditary neuropathy (CMT1). There were 16 patients with a relatively preserved nerve conduction velocity (> 45 m/s), however, most of the NCS findings showed prolonged latencies and reduced action potential amplitudes, suggesting axonal damage with relatively preserved myelin function, indicating hereditary axonal neuropathy or CMT2 type. Six patients had mixed NCS findings with demyelinating and axonal lesions, showing slightly reduced nerve conduction velocities between 35 and 45 m/s – a pattern consistent with an intermediate CMT. One participant was found to have no evidence of neuropathy in NCS. The patient met the inclusion criterion for having a genetically confirmed disease. The woman was 33 years old, a family member of CMT1A patient with molecularly confirmed PMP22 duplication, had her NCS parameters within the
reference range. Clinical assessment of the patient revealed no abnormalities in the CMTNSv2, CMTES scales.

3.2 Clinical variability and differences between genetic types

A detailed clinical assessment has been performed for the CMT study group (Figure 3.2). Typical symptoms of hereditary polyneuropathy, such as hollow feet, hammer toes and gait disturbances, were observed in most of the patients. A common clinical finding was a hollow foot or pes cavus (79.2 %), followed by decreased deep tendon reflexes (76.2 %) and difficulty running (74.3 %). The same most common clinical manifestations were observed in the paediatric subgroup. Only a few patients reported difficulties with manual manipulation (33.7 %) and acrocyanosis (18.8 %).
Figure 3.2 CMT clinical variability in relation to the disease-causing gene

TR – tendon reflexes.

The disease severity was assessed using the CMTNSv2 and CMTES rating scales (Table 3.1). A significant correlation was found between patient age and severity of symptoms (CMTNSv2, CMTES) (p < 0.05).
According to CMTNSv2 and CMTES scores, the most severe clinical picture was in the GJB1 (CMTX1) group; however, the differences between the groups were not statistically significant (p > 0.05). When assessing differences between sexes in the GJB1 group, male patients (n = 6) had higher scores in severity of neuropathy (CMTNSv2 18.2 ± 9.9; CMTES 12.7 ± 6.8) compared to females (n = 7; CMTNSv2 12.7 ± 9.8; CMTES 7.6), the difference was not statistically significant (p > 0.05). Interestingly, 13 patients did not score any points on the CMTES and would have been considered asymptomatic according to this scale. In the patient group (n = 13) that did not score any points on the CMTES, the age range was eight to 52 years (mean 25.8 ± 15.2 years), the gender distribution was equal (7 males, 6 females), and the majority had an ES negative finding (n = 5) or PMP22 duplication – CMT1A (n = 5), the rest (n = 3) were HNPP patients.

Table 3.1

Disease severity characteristics in relation to disease genetic type

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Total n = 101</th>
<th>PMP22 dup (CMT1A) n = 44</th>
<th>GJB1 (CMTX1) n = 13</th>
<th>HINT1 n = 6</th>
<th>Other CMT n = 38</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMTNSv2 (SD), range (0–36)</td>
<td>10.7 (7.6), 0–33</td>
<td>11.9 (6.5), 0–29</td>
<td>15.2 (9.9), 2–30</td>
<td>10.2 (5.1), 2–15</td>
<td>7.9 (7.5), 0–33</td>
</tr>
<tr>
<td>CMTES (SD), range (0–28)</td>
<td>7.2 (5.7), 0–25</td>
<td>7.0 (5.2), 0–22</td>
<td>10.8 (7.2), 2–24</td>
<td>8.2 (4.2), 2–12</td>
<td>6.1 (5.6), 0–25</td>
</tr>
</tbody>
</table>

SD – standard deviation; CMTNSv2 – CMT Neuropathy Score, second version; CMTES – CMT Examination Score.

More than a third (41.0 %) of adult patients reported musculoskeletal pain (Table 3.2). The DN4 rating scale revealed that 27.7 % of patients had neuropathic pain – about one in four PMP22dup (CMT1A) patients and one in two GJB1 (CMTX1) patients. Patients with neuropathic pain had higher neuropathy severity scores than patients in the same genetic group without
neuropathic pain – *PMP22dup* group: CMTNSv2 14.0 ± 7.4 vs 11.4 ± 6.5 and CMTES 9.7 ± 5.2 vs 6.5 ± 4.9; *GJB1* group: CMTNSv2 20.0 ± 8.3 vs. 15.2 ± 9.9 and CMTES 14.2 ± 7.2 vs. 10.5 ± 6.9 – yet the difference was not statistically significant (p > 0.05), and no significant differences were observed between genders.

Table 3.2

Types of pain in the adult patient group in relation to disease genetic type

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Total n = 83</th>
<th>PMP22 dup (CMT1A) n = 37</th>
<th>GJB1 (CMTX1) n = 11</th>
<th>HINT1 n = 4</th>
<th>Other CMT n = 31</th>
</tr>
</thead>
<tbody>
<tr>
<td>Musculoskeletal pain</td>
<td>34 (41.0 %)</td>
<td>15 (40.5 %)</td>
<td>7 (63.6 %)</td>
<td>1 (25.0 %)</td>
<td>11 (35.5 %)</td>
</tr>
<tr>
<td>Neuropathic pain (DN4)</td>
<td>23 (27.7 %)</td>
<td>9 (24.3 %)</td>
<td>5 (45.5 %)</td>
<td>0 (NA)</td>
<td>9 (29.0 %)</td>
</tr>
</tbody>
</table>

DN4 – *Douleur Neuropathique* 4 scale, NA – not applicable.

The GAD-7 scale was used to measure the presence and levels of anxiety in the adult patients’ group (n = 82; n = 1 missing data) (Table 3.3). The GAD-7 scores of 5, 10 and 15 were used as the reference points for mild, moderate and severe anxiety, respectively. Mild anxiety was present in at least 20.7 % of all adult patients and was even more common in the *GJB1* group (36.4 %) and the *PMP22dup* group (25.0 %). Moderate and severe anxiety was present in 13.4 % of the adult patients, more often in the *GJB1* group (27.3 %). No significant differences between genders were observed. Patients presenting with at least mild anxiety had higher CMTNSv2 (15.7 ± 7.6 vs. 10.7 ± 7.4) and CMTES (10.8 ± 6.1 vs. 7.4 ± 5.3) disease severity scores than patients without high anxiety scores; however, the difference was not statistically significant (p > 0.05). Furthermore, patients with elevated GAD-7 scores had a significantly higher prevalence of musculoskeletal pain (70.6 % vs. 33.8 %, p < 0.05) and a higher prevalence of neuropathic pain, but this trend also did not reach
statistical significance (35.3 % vs. 26.2 %, p > 0.05). Gait disturbances such as stumbling (76.5 % vs. 63.1 %, p > 0.05) and difficulty walking (64.7 % vs. 61.5 %, p > 0.05) were more common in patients with elevated anxiety levels.

Table 3.3

Anxiety scores in the adult patients in relation to the disease-causing gene

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Total n = 82</th>
<th>PMP22 dup (CMT1A) n = 36</th>
<th>GJB1 (CMTX1) n = 11</th>
<th>HINT1 n = 4</th>
<th>Other CMT n = 31</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAD-7 score ≥5</td>
<td>17 (20.7 %)</td>
<td>9 (25.0 %)</td>
<td>4 (36.4 %)</td>
<td>0 (NA)</td>
<td>4 (12.9 %)</td>
</tr>
<tr>
<td>GAD-7 score ≥10</td>
<td>11 (13.4 %)</td>
<td>5 (13.9 %)</td>
<td>3 (27.3 %)</td>
<td>0 (NA)</td>
<td>3 (9.7 %)</td>
</tr>
<tr>
<td>GAD-7 score ≥15</td>
<td>3 (3.7 %)</td>
<td>2 (5.4 %)</td>
<td>0 (NA)</td>
<td>0 (NA)</td>
<td>1 (3.2 %)</td>
</tr>
</tbody>
</table>

GAD-7 – General Anxiety Disorder-7 questionnaire, NA – not applicable.

Some patients had their memory / cognitive abilities assessed with memory tests using the CNSVS software (www.cnsvs.com). This pilot study group was comprised of 21 patients from all the genetic groups – nine PMP22dup patients, five GJB1 patients and seven other CMT patients. All patients were over 18 years old, mean age of the group was 37.3 ± 12.5 years. No abnormalities were found for CNSVS memory domain scores in verbal and visual memory. All patients’ scores were within the mean reference interval according to the software guidelines and no differences were observed between the different genetic groups.

Most of our patients reported that they do not engage in regular rehabilitation activities (Table 3.4). Only 12.9 % (n = 13) reported having regular rehabilitation activities such as physiotherapy. Moreover, only 6.9 % of the patients (i.e. 7 out of 13 who engaged in regular rehabilitation) used orthotics, despite most patients (65.3 %) having a drop foot. Comparing clinical
presentation and symptom severity in those who attend rehabilitation and those who do not, the data revealed that patients who engage in regular rehabilitation activities had higher CMTNSv2 and CMTES severity scores and experienced more difficulties with daily life activities. The prevalence of musculoskeletal and neuropathic pain was similar in both groups (p > 0.05).

Table 3.4

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CMTNSv2 (SD)</th>
<th>CMTES (SD)</th>
<th>Musculoskeletal pain</th>
<th>Neuropathic pain</th>
<th>Difficulty walking</th>
</tr>
</thead>
<tbody>
<tr>
<td>Receive rehabilitation</td>
<td>12.8 (7.8)</td>
<td>9.4 (6.7)</td>
<td>5/13 (38.5 %)</td>
<td>3/13 (23.1 %)</td>
<td>9/13 (69.2 %)</td>
</tr>
<tr>
<td>Do not receive rehabilitation</td>
<td>10.4 (7.6)</td>
<td>6.9 (5.5)</td>
<td>32/88 (36.4 %)</td>
<td>23/88 (26.1 %)</td>
<td>47/88 (53.4 %)</td>
</tr>
</tbody>
</table>

SD – standard deviation, CMTNSv2 – CMT Neuropathy Score, second version, CMTES – CMT Examination Score.

3.3 Significance of neurofilament light chain concentration

This part of the study involved CMT patients (n = 96) and a control group (n = 60) (Table 3.5).
Table 3.5

Disease severity and plasma NfL levels in study participants

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Total</th>
<th>PMP22 dup (CMT1A)</th>
<th>GJB1 (CMTX1)</th>
<th>Other CMT</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total (females / males)</td>
<td>96 (51/45)</td>
<td>43 (24/19)</td>
<td>10 (5/5)</td>
<td>43 (22/21)</td>
<td>60 (41/19)</td>
</tr>
<tr>
<td>Mean age (SD)</td>
<td>38.6 (18.4)</td>
<td>36.7 (16.3)</td>
<td>35.5 (17.7)</td>
<td>41.2 (20.5)</td>
<td>35.7 (11.8)</td>
</tr>
<tr>
<td>Mean NfL, pg/mL (IQR)</td>
<td>12.5 (7.9)</td>
<td>12.5 (5.9)</td>
<td>16.0 (5.8)</td>
<td>11.8 (9.2)</td>
<td>5.2 (2.8)</td>
</tr>
<tr>
<td>Mean CMTNSv2 (IQR)</td>
<td>10 (10.0)</td>
<td>12 (7.0)</td>
<td>10 (16.8)</td>
<td>9 (10.5)</td>
<td>NA</td>
</tr>
<tr>
<td>Mean CMTES (IQR)</td>
<td>7 (6.5)</td>
<td>6 (5.0)</td>
<td>7.5 (11.2)</td>
<td>7 (9.0)</td>
<td>NA</td>
</tr>
</tbody>
</table>


Although the age distribution in the patient group ranged from five to 81 years and from five to 62 years in the control group, it is important to note that there was no significant difference in age or sex between the patient and control groups (p = 0.238 and p = 0.087, respectively). No differences in plasma NfL levels were observed between sexes in the patient and control groups (p = 1.00 and p = 0.14), although NfL levels were moderately correlated with age in both the control group and the CMT patient group (rs = 0.42, p = 0.001 and rs = 0.31, p = 0.002, respectively) (Figure 3.3). Even though the control group showed a stronger correlation between NfL levels and age compared to the CMT group, there was no significant difference in correlation levels (z = −0.81, p = 0.42).
Figure 3.3 **Correlation between NfL concentration and age in the CMT patient and control groups**

Plasma NfL concentration were significantly higher in the CMT patient group than in the control group (p < 0.001). The NfL concentration in the study groups are illustrated in Figure 3.4 and the mean NfL levels with interquartile range (IQR) are shown in Table 3.5. There was one outlier observed in the patient group with a significantly high NfL level (NfL = 84.4 vs. mean 12.5 pg/ml), who was also included in the further analysis.
When comparing NfL levels between the different CMT genotypes, NfL was higher in the CMTX1 group than in the other two CMT groups ($p = 0.0498$) (Table 3.5 and Figure 3.4), although the CMTX1 subgroup did not have the highest disease severity scores according to CMTNSv2 in this part of the study. While CMTX1 predicts a more severe clinical picture in men, our cohort data (five females, five males) revealed no significant difference in CMTNSv2 scores between sexes ($p = 0.222$), including the plasma NfL levels ($p = 0.841$). It should be taken into consideration that the size of the CMTX1 group was relatively small, and that statistically significant trends could
potentially be observed with a larger number of subjects. In the context of current results when $p = 0.05$, even one patient could affect the scores in the CMTX1 group with the subsequent statistically significant confidence scores shifting for or against significant differences between the groups.

To determine the correlation between plasma NfL levels and the severity of the disease, we have investigated the association with the total CMTNSv2 score, (includes NCS findings and the clinical features), and the CMTES (focuses only on the clinical assessment). The NfL levels showed a significant but weak correlation with CMTNSv2 score ($r_s = 0.25$, $p = 0.012$) (Figure 3.5). The mean values of CMTNSv2 and IQR for the different groups are shown in Table 3.5. Since the CMTES correlated with CMTNSv2 ($r_s = 0.92$, $p < 0.001$), CMTES also had a weak correlation with plasma NfL levels ($r_s = 0.24$, $p = 0.016$).
Although the duration of patient-reported symptoms had a moderate correlation with age ($r = 0.65, \ p < 0.001$), it had a weak correlation with CMTNSv2 scores ($r_s = 0.28, \ p = 0.006$) and no association with plasma NfL levels ($r_s = 0.15, \ p = 0.16$). ROC analysis shows that the NfL levels can be used to distinguish between patients with CMT disease and the control group with an AUC of 0.881 (95% CI: 0.83–0.93) (Figure 3.6).
Figure 3.6 Specificity and sensitivity of plasma NfL concentration as a CMT biomarker

AUC – area under the curve.
4 Discussion

This study describes the clinical, genetic and diagnostic characteristics in a large cohort of hereditary peripheral polyneuropathy patients in Latvia and evaluates plasma NfL levels as a potential biomarker in CMT patients.

4.1 CMT clinical variability and association with genetic type

According to the genetic testing results, part of the study participants confirmed their genetic diagnosis with the help of PMP22 copy number analysis followed by GJB1 gene analysis. The other participants were further analysed with ES, which also increased the number of patients with known disease-causing gene variant. Almost half of the study participants had the CMT1A subtype, with the next largest group being CMTX1, which corresponds to the previously published information regarding the genotype prevalence tendencies (3, 92, 93). After using the ES, 76.4 % of patients had a disease-causing gene variant (pathogenic, likely pathogenic or VUS) identified, while others had a negative result, which is a relatively high rate of diagnostic yield. Global reports suggest that ES yields better results in paediatric patients (78 %) and in patients with predominantly neurological symptoms (65 %) (94).

One molecularly confirmed CMT1A patient showed no abnormalities indicative of hereditary neuropathy on the NCS investigation. The patient was not an index patient and was included in the study based on genetic confirmation of the disease in other family members. Asymptomatic CMT1A disease has been previously described in the literature, when a worsening of the clinical picture occurs, induced, for example, by neurotoxic medication (71, 95–97). In addition, it is still unclear what proportion of CMT1A patients remain undiagnosed due to a mild or atypical clinical findings, with studies suggesting that the proportion could exceed 40 % in the neuropathy group with unspecified aetiology (96, 98).
There is not sufficient data that would show a CMT1A population without clinical symptoms and neurophysiological abnormalities. Identifying such patients is likely to be challenging in regard to patient cohort selection as well as ethical considerations related to the need for genetic testing.

The neuropathy severity rates in the CMT1A study group were lower than those in the Inherited Neuropathy Consortium study, which cross-analysed 1652 CMT patients from 13 international centres (50), while the CMTX1 group showed similar results as suggested by our study group analysis. We found that, irrespective of gender, the CMTX1 group tended to have higher neuropathy severity scores than other CMT types, which is consistent with the previous reports (50). It was more pronounced in the male subgroup, which is typical for X-linked disease, but the difference between the genders was not statistically significant. Also, note the relatively small size of the CMTX1 group, which may have affected the results.

The disease severity in the study group as a whole and in its genetic subgroups showed wide clinical variability and was related to the patient’s age. The disease severity ranged from no symptoms and no neurophysiological changes on the CMTNSv2 scale to severe disability, even within the same genetic group. It should be noted that the relatively low CMTNSv2 scores in some patients may not reflect those CMT symptoms, that are not included in the assessment scale, yet cause functional impairment and disability. The CMTNSv2 with the corresponding CMTES does not reflect all possible symptoms and neurophysiological findings of hereditary neuropathy. Such results only highlight the need for more specific and sensitive clinical assessment tools. Clinical variability is pronounced and widespread, but is still not fully understood in the CMT population; several contributing factors could be involved, both of environmental and genetic nature (99, 100).
A significant number of patients reported complaints of pain, with more than one third of them complaining of musculoskeletal pain. Evidence suggests that a significant portion of CMT patients (sometimes more than a half) may suffer from chronic pain, with up to 50% of patients also suffering from neuropathic pain (101, 102). Some patients had neuropathic pain according to DN4, indicating possible damage to fine nerve fibres. The patient group with higher levels of anxiety (as per GAD-7) had a significantly higher prevalence of musculoskeletal pain. Anxiety is a common psychological condition in patients with chronic pain. Anxiety in patients can aggravate long-term functional disability and hinder the process of personalized physiotherapy rehabilitation (103–105).

In a pilot project with a small number of CMT patients, we assessed their memory and cognitive abilities. According to the CNSVS results no memory impairment was observed in any of the CMT genetic subgroups. Other studies that assessed CNS involvement in CMT patients have produced conflicting data. For example, one prospective study with 30 patients reported that 70% of patients with CMT1A and HNPP had cognitive impairment and lower white matter volumes in the brain (20, 106). At this point, it is unclear whether these observations are random or due to a common underlying cause these two processes share. Therefore, imaging studies using MRI are required in larger patient groups to examine the CNS and cognitive involvement in CMT patients.

Physiotherapy, occupational therapy and application of various technical aids (especially orthotics) are essential for CMT patients to maintain daily activities, improve functioning, independence and overall quality of life (5, 71, 72, 107). Sadly, only 12.9% of our patients reported engaging in rehabilitation activities on a regular basis, and only about half of this group used orthotics. The study has found that patients who had regular rehabilitation had higher disease severity scores. These results should be interpreted with caution,
however, as it does not necessarily indicate that rehabilitation is ineffective. First, there were few patients engaging in rehabilitation \((n = 13)\); second, rehabilitation can be helpful and effective even in patients with mild to moderate CMT, slowing down its progression and helping to maintain functional capacity for as long as possible. Rehabilitation plays an important role in maintaining or prolonging patient’s independent functioning and is currently the main treatment option for patients with CMT \((3, 5, 71, 72)\). This should be emphasised not only when educating the patient themselves, but also when consulting their family, relatives.

To properly evaluate the effectiveness of rehabilitation in CMT patients, it would be essential to have a larger study group, comprised of patients presenting with different levels of disease severity, and assessing them dynamically. It should be mentioned that patient’s own cooperation and involvement also plays a role in the effectiveness of rehabilitation.

4.2 CMT clinical variability association with neurofilament light chain concentration

The study involves a large group of CMT patients with a detailed phenotype and a control group to assess the potential use of NfL in clinical practice and in future studies. The data support plasma NfL levels as a potential biomarker of CMT severity.

In Sandelius et al. research \((11)\) it was reported, that plasma NfL concentration is associated with disease severity in CMT patients, suggesting that it could be used to monitor disease severity and progression. However, so far these findings have not been reproduced in larger studies. To minimise any discrepancies with Sandelius et al. study, the patients and the control group were evaluated under the similar conditions, using the same laboratory and method to determine the NfL levels, as per the aforementioned protocol \((11)\). Compared with the Sandelius et al. research, this study recruited more patients, performed
a more detailed phenotype characterisation, and subjected a significantly larger number of patients to CMTNS analysis (11) – the number of patients with CMTNS evaluation / number of patients in total: Sandelius et al. 30/75, within this work 96/96. It is important to note, that NfL, being a cytoskeletal protein of a nerve cell, is more likely to reflect axonal damage, which is already indirectly included in the CMTNS, in the neurophysiological data section, in the form of the CMAP and SAP amplitudes for the upper limb nerves (108).

The results we obtained confirm that plasma NfL concentrations are significantly higher in CMT patients than in the control group. Just as previously reported (11), the data shows that the CMT patients can be distinguished from the control group using plasma NfL. To discriminate between the patients with CMT and the control group, the AUC value was 0.881, while a significantly lower AUC of 0.755 has been reported until now (11). However, despite the high AUC, there is still a certain overlap between the CMT patient and the control group.

In the study by Sandelius et al. (11) a significant correlation was reported between plasma NfL concentrations and the disease severity as per CMTES and CMTNS measurements, with a stronger correlation observed with CMTES ($r = 0.46, p < 0.0001$) than with CMTNS ($r = 0.37, p = 0.044$). The results of this study confirm the association between the plasma NfL levels and the severity of CMT, however the association is relatively weak (CMTNS: $r_s = 0.25; p = 0.012$ and CMTES: $r_s = 0.24; p = 0.016$). As the two measures were highly correlated, no significant difference was observed between the correlation of NfL with CMTES or CMTNS, which was reported in Sandelius et al. research work (11). Although both studies confirm a correlation between the severity of CMT (as assessed by CMTNS or CMTES) and plasma NfL concentration, the correlation ranges from weak to moderate. More data is necessary to determine whether plasma NfL concentration can indeed be used to assess the disease progression.
Plasma NfL concentration had a moderate correlation with age of CMT patients, but the same was observed in the control group. This, however, is not completely unexpected, as age-related rise of NfL has been repeatedly reported in both healthy individuals and those with different health conditions (109). Presumably, the age-related increase in plasma NfL levels could suggest a certain neuronal damage occurring in elderly people, especially in case of neurological diseases where increase in NfL is even more pronounced, but it may also distinguish the elderly population from the control group (11, 110, 111). It should be noted that NfL is not a disease-specific marker and its release and subsequent elevation of plasma concentration can also be caused by certain physiological processes (10, 64, 112, 113). Similarly to Sandelius et al. (11), our study found no difference in plasma NfL concentration between sexes in the CMT patients and in the control group.

Self-reported duration of symptom was moderately correlated with age, weakly correlated with disease severity and had no correlation with plasma NfL levels. In hereditary conditions, it is the patient’s age, and not the onset of symptoms, which better suggests the potential duration of the disease, which may explain such findings. Additionally, there are several confounding variables that can affect the estimated duration of patient-reported symptoms: patients’ perception and understanding of the onset of clinical symptoms may differ; mild symptoms may go unnoticed, or even in case of more severe symptoms the patient may gradually adapt and grow used to their functional impairment. It may also be possible that a patient may not remember the exact onset of their first symptoms, and in case with underaged patients, their caregivers’ opinion may influence their perception and knowledge about the timing of their first symptoms. Considering all the aforementioned factors, the patient’s age should be seen as a more reliable independent indicator than self-reported symptom duration.
The study showed that CMTX1 patients had higher plasma NfL levels than the other CMT subgroups. This observed difference was not found to be related to patients’ gender or the disease severity. This elevation could be due to nerve cell degeneration and symptomatic / asymptomatic CNS manifestations previously reported in CMTX1 patients (37), as well as described in the literature section. Although the medical history and examination of the CMTX1 patients did not reveal any CNS involvement, the neuronal damage could be subclinical and asymptomatic. It also should be noted that this could be a false positive finding, since the size of the CMTX1 group was very small (n = 10), comprised of five females and five males with a mean age of 35.5 years, while the entire CMT group (n = 96) had a slightly higher mean age of 38.6 years, although this difference was not statistically significant. It should be noted that the p value p = 0.05, which shows the differences between the groups, can be easily influenced by increasing the group even by one patient, which could result in no significant differences or the opposite. In the study by Sandelius et al. (11) with a similar CMTX1 group size (n = 11) no such association could be found. This could be explained by the sex distribution in the CMTX1 group (nine females and two males), with the mean age of 43.3 years in the CMTX1 group compared with the overall group (n = 75), where the mean age was 46.2 years. The absence of the difference could be due to a relatively small number of male patients in the CMTX1 group. One would expect that men in the CMTX1 group would have a more severe clinical picture and therefore higher plasma NfL levels compared to women, however this has not been confirmed. Further studies using a larger group of age dispersed CMTX1 patients would be required to check the assumption regarding the plasma NfL concentration in different genetic types of the disease.
The absolute NfL levels in the study were lower compared to the data found in the other publications (11, 110). In a study of 335 healthy subjects aged between 38.5 and 85.6 years, the mean plasma NfL concentration was 32.30 pg/ml (SKA: 23.15–43.95) (110). In another study, the mean plasma NfL levels in healthy controls (n = 59) was 17.8 ± 6.4 pg/ml (114). A Danish study proposed NfL reference ranges for healthy controls in different age groups; for the age groups 18–40, 41–65 and > 65 years 2.8–9.7 ng/l, 4.6–21.4 ng/l and 7.5–53.8 ng/l, respectively (115). In our study, the mean plasma NfL concentration in the control group, determined using the same method, was 5.2 pg/ml, which fits the recommended reference ranges. The differences observed may be due to the fact that the measurements used in research studies are coming from different laboratories and are not standardised. No validated reference intervals and / or standardisation methods are available as of now. This highlights the need for both test and NfL reference value standardisation between laboratories for safe and convenient use in clinical practice, as well as the need for dynamic evaluation of figures within the same laboratory. Having a dynamic assessment tool within the same laboratory would also be of a great use.

One CMT1A patient (61-year-old male) had markedly elevated plasma NfL concentration (84.4 pg/ml) and a moderate clinical phenotype (CMTNSv2 18). This patient had a comorbidity suspected – chronic inflammatory demyelinating polyneuropathy (CIDP). Co-occurrence of acquired neuropathy (e.g. CIDP) with hereditary neuropathy has been reported a number of times (116–118). This co-existence and overlap of symptoms make it difficult to properly diagnose such cases and often leave patients with an uncertain diagnosis for years. One isolated case of marked NfL elevation among other CMT patients was also reported in the study by Sandelius et al. (11), however, unlike in our study, the reason for such elevation has not been identified.
Overall, the study results indicate that the plasma NfL concentration is a promising biomarker in CMT patients. However, for its further use as biomarker, either alone or in combination with others, several issues should be addressed: the NfL level overlap, observed between the CMT patients and the control group; the lack of standardisation, reference and value ranges, which is an important aspect to address for it to be used in the clinical settings; the lack of specificity, since the elevated NfL levels can be observed in various neurological conditions; as well as generally not so strong correlation with the disease severity.

Besides, it is currently unknown whether the changes in plasma NfL levels over time can be used to assess the disease activity and progression in a short and/or long term. A longitudinal study with NfL is required to determine its use in assessing the disease progression.
Conclusions

1. CMT disease occurs equally often in men and women, the first manifestations begin in adolescence, the clinical picture is highly variable with the main signs and symptoms of peripheral polyneuropathy, the neurophysiological findings can be divided into predominantly axonal or demyelinating lesions, in some cases – mixed.

2. In the group of CMT patients, the most frequently confirmed genetic types were CMT1A and CMTX1. The association of the genetic type with the clinical findings was not identified, but CMTX1 patients, predominantly males, tended to have a more severe clinical picture compared to other CMT patients.

3. The concentration of NfL in plasma is significantly higher in CMT patients than in control group, however the result may overlap between the above-mentioned groups. NfL levels are associated with CMT disease severity, but the association is weak and does not differ between different genetic types.
Publications

Presentation at a scientific conference of international and local importance with a poster report:


Presentation at a scientific conference of international and local importance with an oral report:


Scientific publications included in international databases:


Annexes
Annex 1

Centrālā medicīnas ētikas komiteja

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Rīgā

21.03.2018. Nr.3/18-03-21

Rīgas Stradiņa universitātes
Molekulārās Ģenētikas Zinātņiskajai laboratorijai

Atzinums par pētījumu
„Pārmantoto neiromuskulāro slimību ģenētiskā analīze”

Centrālā medicīnas ētikas komiteja 2017. gada 23. novembrī ir izskatījusi Rīgas Stradiņa Universitātes Molekulārās Ģenētikas Zinātņiskās laboratorijas iesniegto pētījumu „Pārmantoto neiromuskulāro slimību ģenētiskā analīze”.

Pamatojoties uz Centrālās medicīnas ētikas komitejas 2017. gada 23. novembra sēdes protokola Nr.2017-4 punktu Nr.1 un iesniegtajiem labojumiem, tiek iesniegts atzinums, ka Rīgas Stradiņa Universitātes Molekulārās Ģenētikas Zinātņiskās laboratorijas pētījums „Pārmantoto neiromuskulāro slimību ģenētiskā analīze” nav pretrunā ar bioētikas normām.

Centrālās medicīnas ētikas komitejas priekšsēdētājs

V. Silis

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Annex 2

A compilation of the most frequent symptoms of inherited neuropathy created by the authors of the study

<table>
<thead>
<tr>
<th>Symptom / complaint</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pes cavus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hammer toes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Difficulty in running</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tripping</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Difficulty in walking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Foot drop</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steppage gait</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wasting</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Difficulties in hand manipulation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reduced or absent deep tendon reflexes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hand tremor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle cramps</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cold feet</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acrocyanosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Musculoskeletal pain</td>
<td></td>
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</tr>
</tbody>
</table>
## CMT Neuropathy Score, version 2 (108)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensory symptoms *</td>
<td>None</td>
<td>Symptoms below or at ankle bones</td>
<td>Symptoms up to the distal half of the calf</td>
<td>Symptoms up to the proximal half of the calf, including knee</td>
<td>Symptoms above knee (above the top of the patella)</td>
</tr>
<tr>
<td>Motor symptoms (legs) †</td>
<td>None</td>
<td>Trips, catches toes, slaps feet, shoe inserts</td>
<td>Ankle support or stabilization (AFOs) Foot surgery †</td>
<td>Walking aids (cane, walker)</td>
<td>Wheelchair</td>
</tr>
<tr>
<td>Motor symptoms (arms)</td>
<td>None</td>
<td>Mild difficulty with buttons</td>
<td>Severe difficulty or unable to do buttons</td>
<td>Unable to cut most foods</td>
<td>Proximal weakness (affect movements involving the elbow and above)</td>
</tr>
<tr>
<td>Pinprick sensibility ‡</td>
<td>Normal</td>
<td>Decreased below or at ankle bones</td>
<td>Decreased up to the distal half of the calf</td>
<td>Decreased up to the proximal half of the calf, including knee</td>
<td>Decreased above knee (above the top of the patella)</td>
</tr>
<tr>
<td>Vibration</td>
<td>Normal</td>
<td>Reduced at great toe</td>
<td>Reduced at ankle</td>
<td>Reduced at knee (tibial tuberosity)</td>
<td>Absent at knee and ankle</td>
</tr>
<tr>
<td>Strength (legs) †</td>
<td>Normal</td>
<td>4+, 4, or 4− on foot dorsiflexion or plantar flexion</td>
<td>≤3 on foot dorsiflexion or ≤3 on foot plantar flexion</td>
<td>≤3 on foot dorsiflexion and ≤3 on plantar flexion</td>
<td>Proximal weakness</td>
</tr>
<tr>
<td>Strength (arms) †</td>
<td>Normal</td>
<td>4+, 4, or 4− on intrinsic hand muscles ‡</td>
<td>≤3 on intrinsic Hand muscles ‡</td>
<td>≤5 on wrist extensors</td>
<td>Weak above elbow</td>
</tr>
<tr>
<td>Ulnar CMAP (median)</td>
<td>≥6 mV</td>
<td>4–5.9 mV</td>
<td>2–3.9 mV</td>
<td>0.1–1.9 mV</td>
<td>Absent</td>
</tr>
<tr>
<td>Radial SAP amplitude, antidromic testing</td>
<td>≥15 μV</td>
<td>10–14.9 μV</td>
<td>5–9.9 μV</td>
<td>1–4.9 μV</td>
<td>&lt;1 μV</td>
</tr>
</tbody>
</table>

AFO – ankle foot orthoses; CMAP – compound muscle action potential; SAP – sensory action potential.
1 Use the picture below to discriminate the level of symptoms.

2 Uses aid most of the time. The patient was prescribed to wear / use or should be wearing the aid in the examiner’s opinion.

3 See indications for eligible foot surgery.

4 Abnormal if patient says it is definitely decreased compared to a normal reference point.

5 Use Rydel-Seiffer tuning fork. Defined normal: ≥ 5.

6 Limb scores refer to MRC grade.

7 Intrinsic hand muscles strength assessment: test only abductor pollicis brevis (APB) and first dorsal interosseous (FDI), then choose the strongest.
### DN4 scale (119)

<table>
<thead>
<tr>
<th>No.</th>
<th>Questions and answer options</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>Does the pain have one or more of the following characteristics?</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Burning</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Painful cold</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Electric shocks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II.</td>
<td>Is the pain associated with one or more of the following symptoms in the same area?</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tingling</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pins and needles</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Numbness</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Itching</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III.</td>
<td>Is the pain located in an area where the physical examination may reveal one or more of the following characteristics?</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hypoesthesia to touch</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hypoesthesia to pinprick</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV.</td>
<td>In the painful area, can the pain be caused or increased by:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Brushing</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

There must be positive answers in all 4 parts of the questions (at least one positive in each of I-IV), i.e. at least 4-5 points out of a maximum of 10 possible to confirm the neuropathic nature of the pain.
Generalized Anxiety Disorder Scale 7 (GAD-7) (83)

<table>
<thead>
<tr>
<th>Over the last 2 weeks, how often do you have been bothered by the following problems? (Circle the appropriate answer on each line)</th>
<th>Not at all</th>
<th>Several days</th>
<th>More than half the days</th>
<th>Nearly every day</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Feeling nervous, anxious or on edge</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>2. Not being able to stop or control worrying</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>3. Worrying too much about different things</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>4. Trouble relaxing</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>5. Being so restless that it is hard to sit still</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>6. Becoming easily annoyed or irritable</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>7. Feeling afraid as if something awful might happen</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Overall assessment:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Annex 6

**Genes included in exome sequencing**

DCAF8; GLE1; ZFYVE26; TBK1; ZFYVE27; TFG; CLP1; SCN11A; APIS1; MPV17; PRKCG; UNC13A; PDYN; SOX10; SLC5A7; SCN10A; SOC2; MAP1B; HMBS; FDX2; OPTN; GNE; MTMR2; COL13A1; DHH; XPA; PRDM12; AAAS; PLA2G6; SLC5A2; SLC5A3; GJC2; TTPA; PIP5K1C; ABCA1; DNAH10; HACE1; JAG1; PPM2; ERLIN1; TRIP4; ERLIN2; LICAM; SETX; FA2H; GJB1; LAS1L; GJB3; TDP1; PAH; CDK16; LRSAM1; HEBX; HEXA; LITAF; LYST; FARS2; AIFM1; TRIM2; RARS; FVLCR1; NEFL; TK2; NEFH; SLC18A3; MYBPC1; ELOVL4; ELOVL5; ARG1; KCND3; NIPA1; TIMM22; TMEM173; FAM126A; HADHB; HADHA; MED25; INF2; MYOT; PTRH2; VAMP1; PMP2; GARS; L2HGDH; ASAHI1; GRN; CTDPI; AP5Z1; ATL3; WDR45B; ATL1; ATLA2; KCNA2; PRUN1; C1orf194; HOXD10; FGD4; CHRNND; CHRNG; TMEM65; CHRNE; MFN2; ZNF106; HARS; GSN; KIDINS220; ELPL1; TUBB4A; FIG4; PLP1; UBAP1; COQ8A; AGRN; RPH3A; ABHD12; NAGLU; TUBB3; CHPI; NEK1; SLC25A42; SLC16A2; MTPAP; SLC25A46; NKX6-2; DGAT2; PCYT2; AGTPBP1; GPT2; VRK1; TUBA4A; BSCL2; MAG; STIM1; SQSTM1; RNASEH2B; DNMT1; ITPR3; ATXN2; KLC2; BAG3; PSAP; CLTCL1; RNF170; AP4S1; SMN1; TIA1; FUS; WFS1; FBXO38; GFPT1; REEP1; DMXL2; REEP2; SLC25A1; SNAP25; CNTNAP1; PTEN; BICD2; AP4M1; ATP7B; OPA1; SPR; OPA3; EMILIN1; SERAC1; ATP7A; SPG11; SNAP29; COA7; DST; PLEKHG5; SIGMA1; VPS13A; SORD; APOA1; VPS37A; NGF; COASY; DNAJC3; SLC25A15; NEMF; RRM2B; SLC25A19; ANG; AGXT; SLC25A4; FXN; C9orf72; PRPS1; LRP4; DNAJB2; TTR; MSTO1; DNAJB5; SH3BP4; IARS2; HNRNPA1; UBQLN2; NTRK1; MGME1; KCNJ10; TRPA1; ASCC1; AHNAK2; DDHD2; NAGA; PTPN11; DDHD1; MCM3AP; SUCLA2; POLR3A; VAPB; MYO1A; STUB1; MMACHC; SPG21; ARL6IP1; PRF1; NUDT2; GBA2; EDNRB; FLAD1; PIEZO2; PDK3; SLC12A6; SIL1; C12orf65; ARSA; ENTPD1; MUSK; ALG6; IFRD1; ALG2; CPT1C; CYP27A1; AFG3L2; BTD; CYP2U1; KARS; SUCLG1; DNA2; PFN1; SPTBN4; MTTP; PIK3R5; DGUOK; SGPL1; SPAST; MICAL1; B4GALNT1; SURF1; EGR2; XRCC4; SLC33A1; XRCC1; PRG4; NOTCH2NL; SPART; AIMP1; TH; WNK1; NDUFAF5; ATM; ALDH18A1; RETREG1; TECPR2; FBXL4; AARS; FASTKD2; GBE1; AP4E1; SIPA1L2; UCHL1; HINT1; SPTLC1; SPTLC2; SPTLC3; PRX; KIF1C; KIF1B; KIF1A; SPTAN1; WARS; MME; MRPS25; TOP3A; AMPD2; CYP7B1; GAN; TNNT2; MAPT; RAPSN;
Annex 6 continued

RAB7A; IGHMBP2; HSD17B4; IBA57; TYMP; DRP2; LMNA; EXOSC8; CD59; EXOSC3; CCT5; DYNC1H1; GCH1; GBF1; PEX10; PEX12; FAH; C19orf12; MYO9A; APTX; COLQ; XK; KIF26B; MARS; CTNNB1; PNPLA6; TARDBP; SCARB2; DPAGT1; WASHC5; CPOX; COX6A1; COX6A2; YARS2; MPZ; SCN9A; KIF5A; SACS; CAPN1; SBF1; SBF2; CHRN; PHYH; PUS1; ALG14; ANXA11; ATAD3A; ALDH3A2; PREPL; POLG2; SCN8A; DOK7; TBC1D24; MATR3; CHRNA1; RTN2; VCP; UBA5; CHAT; SLC1A4; KLHL13; NDRG1; TOR1AIP1; FBLN5; CACNA1G; RNASEH1; MYH14; MRC2; COX10; PRPH; YARS; PRNP; DARS; SMAD3; PDHA1; SYT2; ZFHX2; SLC52A2; SLC52A3; CHCHD10; COQ9; AP4B1; COQ7; PHOX2B; COQ6; COQ4; COQ2; GCLC; AMACR; TRPV4; CHMP2B; TBCE; GNB4; UBA1; DEGS1; COX20; KDM5C; HSPB8; NGLY1; SLC2A1; HSPB1; ADAR; LDB3; HSPB3; GRM1; DHTKD1; HK1; IRF2BPL; GMPPB; SEPT9; GMPPA; RBM7; ABCD1; SCYL1; POLG; ARHGEF10; MARS2; BCKDHB; TWNK; DARS2; PEX1; DNM2; PEX7; ALS2; ACOX1; SCN4A; PLEC; LAMA5; DCTN2; DCTN1; PNKP; ETFDH; SPG7; ATP1A1; PPOX; NT5C2; CLCN2; HSPD1; ERBB3; SH3TC2; ERBB4; LRIG3; LAMB2; GDAP1; SOD1; RPIA; GALC; PMP22; ERCC8; ATP13A2; ERCC6; GLA.