Department of Clinical Medicine, The Neuromuscular Diseases Research Group

Charcot-Marie-Tooth disease (CMT)

Statistical analysis and revision of molecular genetic diagnostics in a patient population and identification of the disease locus in a large Norwegian family with CMT.

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Rune Østern

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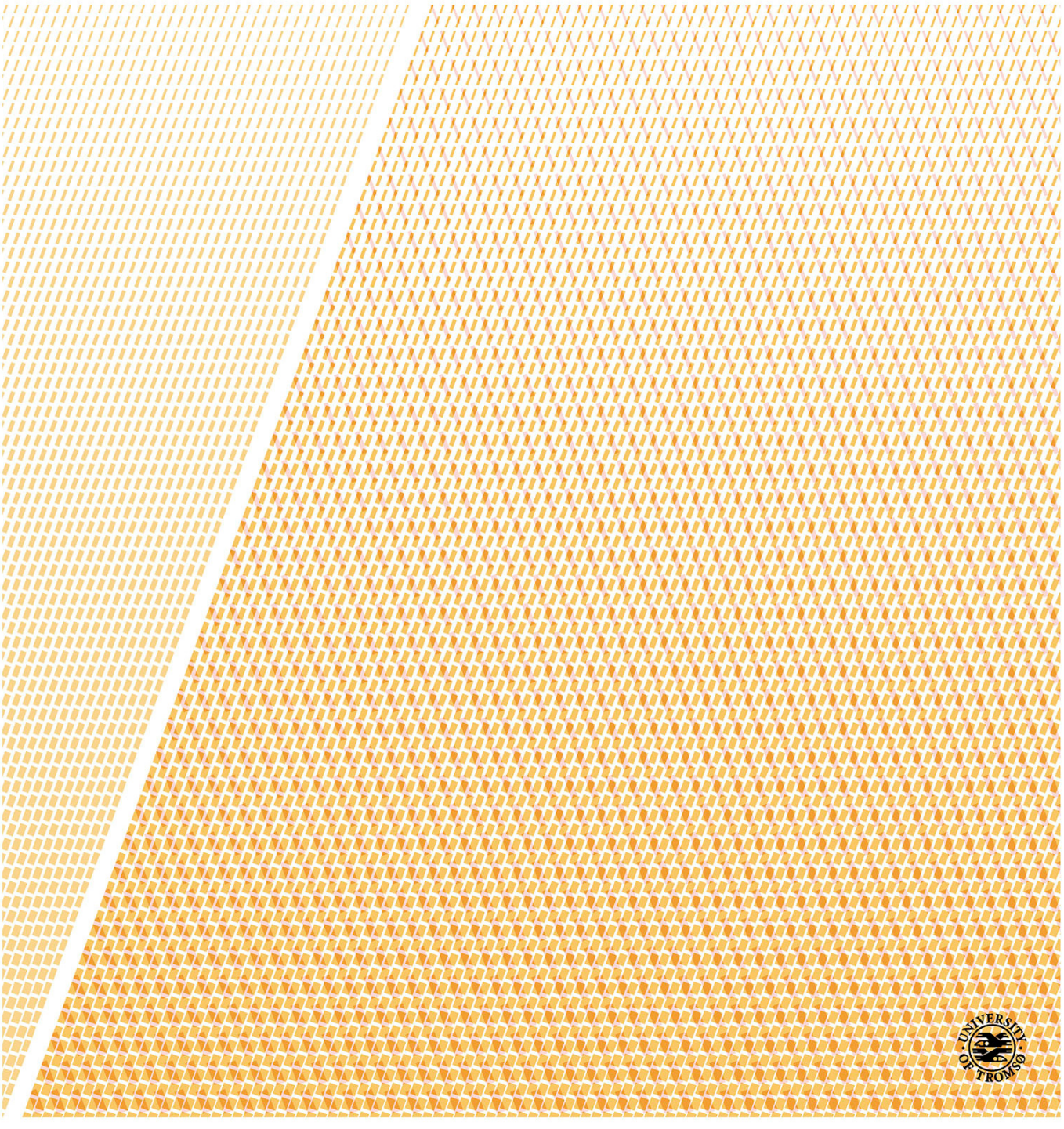

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List of papers

1. **Østern R, Fagerheim T, Hjellnes H, Nygård B, Mellgren SI, Nilssen Ø.**
   Diagnostic laboratory testing for Charcot Marie Tooth disease (CMT): The spectrum of gene defects in Norwegian patients with CMT and its implications for future genetic test strategies
   BMC Med Genet. 2013 Sep 21;14(1):94.

2. **Østern R, Fagerheim T, Hjellnes H, Nygård B, Mellgren SI, Nilssen Ø.**
   Segregation analysis in families with Charcot-Marie-Tooth disease allows reclassification of putative disease causing mutations
   BMC Med Genet. 2014 Jan 21;15(1):12

3. **Østern R, Fagerheim T, Ørstavik K, Holmøy T, Heiberg A, Lund-Petersen T, Strom T, Nilssen Ø, Dahl A.**
   HMN phenotype in a large Norwegian family with a "H46R" substitution in *SOD1*
   Neuromuscul Disord. 2012 Jun;22(6):511-521

Abbreviations

AAS: amino acid sequence

ALS: amyotrophic lateral sclerosis

CDT: cold detection threshold

CHN: congenital hypomyelinating neuropathy

CMT1: Charcot-Marie-Tooth disease type 1

CMT2: Charcot-Marie-Tooth disease type 2

CMT4: Charcot-Marie-Tooth disease type 4

CMTX: Charcot-Marie-Tooth disease type X

CNS: central nervous system

CNV: copy number variation

dHMN: distal hereditary motor neuropathies

DI-CMT: dominant intermediate Charcot-Marie-Tooth disease

dSMA: distal spinal muscular atrophy

DSS: Dejerine Sottas syndrome

EMG: electromyogram

FALS: familial amyotrophic lateral sclerosis

HGVS: Human Genome Variation Society

HMN: hereditary motor neuropathy

HMSN: hereditary motor sensory neuropathy

HNPP: hereditary neuropathy with liability to pressure palsy

HSAN: hereditary sensory and autonomic neuropathy

HSN: hereditary sensory neuropathy

HSP: hereditary spastic paraparesis

LMN: lower motor neurons

MLPA: multiplex ligation dependent amplification

MNCV: motor nerve conduction velocity

MND: motor neuron disease

NCS: nerve conduction studies

NGS: next generation sequencing

PNS: peripheral nervous system

SALS: sporadic amyotrophic lateral sclerosis

SBMA: spinobulbar muscular atrophy

SMA: spinal muscular atrophy

SNP: single nucleotide polymorphism

UMNs: upper motor neurons

WGS: whole genome sequencing

Introduction

1 Polyneuropathy

The peripheral nervous system mediates impulses between the body and the central nervous system (CNS). The peripheral neuropathies may be classified based on the distribution of the affected nerves, the nerve pathology, or on the pathogenesis. The term polyneuropathy describes disorders resulting from diffuse lesions in the peripheral nerves and typically presents as symmetrical sensory loss, pain, and weakness, most often starting distally. Autonomic dysfunction may also occur. Instrumental methods such as nerve conduction studies (NCS) and electromyography (EMG) are crucial to separate primary myogenic and neurogenic conditions, and give important information about the subclasses of peripheral neuropathies.

1.1 Anatomy of the peripheral nervous system

Only a short review of the most relevant elements is presented here, as details are broadly dealt with in numerous textbooks, among others Trepel, 1995 and Lippert, 1996. The somatic part of the peripheral nervous system transfer efferent motor impulses from the motor neurons of the spinal anterior horns to the skeletal muscles via the ventral roots enabling voluntary muscle contractions. Afferent and potentially conscious sensory impulses from the various sensory receptors of the periphery are transferred back to the dorsal horns of the medulla via the peripheral nerves, the pseudounipolar nerve cells of the spinal ganglions, and the dorsal roots.

The autonomous part of the peripheral nervous system sustains important unconscious functions like respiration, and modulates the activity of the heart, intestines, glands and smooth muscles. Overall, the sympathetic nervous system has a mobilizing effect, whereas the parasympathetic nervous system rather triggers rest and digestion. From the lateral horn of the thoracolumbar medulla, nerves of the sympathetic nervous system reach the peripheral ganglia and subsequently the organs. The nerves of the parasympathetic nervous system originate from the brainstem and the lateral horn of the sacral medulla. A part of the nerve fibres serving autonomous functions are however also part of the peripheral nerves and trophic disturbances may therefore be a trait of chronic peripheral nerve dysfunction.

The peripheral nerves are enveloped in a membrane of connective tissue, and contain several sub-compartments that harbour the individual nerve fibres. The peripheral nerves may mediate a single afferent or efferent quality (motor, sensory or visceral), but they usually contain a mixture of nerve fibres supporting all of these functions. Somatic motor and sensory nerve fibres, and fibres involved in muscle reflexes are enveloped in multiple insulating layers of myelin produced by Schwann cells that follow the axons along their paths (Type A fibres). The space between each Schwann cell (nodes of Ranvier) allows the saltatory transmission of nerve signals from gap to gap enabling high nerve conduction velocities. The nerve fibres serving autonomic functions, or transferring the sensation of temperature or dull pain, are wrapped in few, or no layers of myelin (Type A delta/B/C fibres). As a consequence, the nerve conduction velocities are much slower.

A single motor nerve innervates multiple muscle fibres (motor unit). There is extensive variation in the size of the motor units, particularly between muscles involved in fine and gross motor functions. The axons end in the synapse opposite of the specialised motoric endplates of the muscle membrane. The nerve signal triggers the release of acetylcholine that diffuses across the neuromuscular junction, and induces a cascade that generates a depolarisation of the muscle membrane and ultimately muscle contraction.

1.2 Peripheral neuropathies

Peripheral neuropathy, types of neuropathy, causes, and treatments are described in chapters of neurology textbooks (Mellgren, Rasmussen, & Vedeler, 2010). The nerve pathology may be restricted to a single nerve (mononeuropathy), to several individual nerves (multiple mononeuropathy), to a nerve plexus (plexopathy) or the nerve roots (radiculopathy). More commonly the nerve pathology may involve many peripheral nerves in a diffuse manner, usually earlier and more severe in the longest nerve fibres (distal symmetric polyneuropathy, “dying back neuropathy”). If only a single quality is involved, the neuropathy may be designated as a motor, sensory or autonomic neuropathy, whereas the combination of motor and sensory manifestations is called sensorimotor neuropathy.

The polyneuropathy may primarily affect the axon or the myelin sheet. In both cases the lengthiest nerves are affected first resulting in a distal symmetric distribution of symptoms. These may be manifested as positive clinical symptoms such as increased or altered sensations of touch, pain or temperature, or negative clinical symptoms such as reduced

sensation of various qualities, weakened motor reflexes, muscle weakness, hypotonia and atrophy. The involvement of autonomic functions may lead to trophic disturbances, and in some cases central autonomic functions are also implicated.

Finally, the underlying disease causes may be used to categorize the polyneuropathies into the following subgroups: genetic diseases, metabolic and endocrine conditions, malnutrition, polyneuropathy due to toxic substances or medications, infectious causes, connective tissue diseases, cancer, and immunologic diseases. All of these possible causations may be explored in a complete work up of a patient with polyneuropathy. If a disease cause is not found the polyneuropathy is classified as idiopathic, but a substantial proportion of so called idiopathic cases seem to be hereditary.

1.3 Instrumental methods commonly applied in the assessment of polyneuropathies

1.3.1 Nerve conduction studies (NCS)

NCS of the thick, myelinated motor nerves are performed by inducing distal and proximal surface stimulation above the nerve through electrodes and registration of the motor response from multiple individual muscle fibres of the innervated muscle (Sand & Jørum, 2010). The time elapsed between the stimulation and the muscle reaction (distal latency) is measured (ms) as well as the size of the reaction (motor amplitude) (mV). The latter is an expression of the number of recruited nerve cells and axons. The physical distance between the points of proximal and distal stimulation, combined with the difference in distal latency are used to assess the motor nerve conduction velocity (MNCV) (m/s). The nerve impulse produced at the electrode is transferred in orthodromic direction towards the muscle, as well as antidromic towards the cell bodies in the anterior horn. A second, lesser response from the muscle (F-response) is caused by a reflection of the signal back from the anterior horns. As a consequence the NCS may also give information about the proximal part of the peripheral nerves and in particular, the nerve roots. The distal latency, amplitude and conduction velocities can also be assessed for sensory nerves. Nerves commonly evaluated in polyneuropathy cases are the median and ulnar nerve of both arms, and the peroneal, posterior tibial and sensory sural nerve of one of the legs.

A reduction in the motor nerve conduction velocity in combination with (almost) normal amplitude is indicative of a demyelinating pathologic process. The opposite, a (near) normal motor nerve conduction velocity and small (or absent) motor amplitudes indicates axonal

pathology. As the disease progresses however, signs of both types are usually registered due to a mixture of both demyelinating and axonal pathologic processes.

1.3.2 Electromyography (EMG)

EMG is an invasive method that is based on the insertion of needle electrodes into the muscle under study (Sand & Jørum, 2010). An individual nerve innervates multiple muscle fibres (motor unit) and at voluntary contraction the response from that group of fibres may be assessed as motor unit potentials (MUP), either visually on the computer screen, or acoustically over loud speakers. The width (ms) and height (μV) of a single MUP is registered, as well as the activity (Hz) under resting conditions, and the pattern of recruitment associated with increasing contraction.

In primary myogenic conditions the motor units are preserved, but the muscle pathology leads to small MUPs with short duration. With contraction a high frequency of small MUPs are quickly recruited. No signals are normally registered under resting conditions. Denervation destabilizes the membrane potentials in the respective motor units however, leading to an intermittent period with spontaneous activity seen as fibrillations and positive sharp waves. These are characteristic for acute and sub acute nerve damage. In subacute and chronic conditions signs of reinnervation are also increasingly registered in the form of wider, higher and often polyphasic MUPs. With contraction MUPs are few, but large. In motor neuron diseases such as ALS, nerve conduction studies may show results quite similar to the axonal polyneuropathies. The EMG however, mostly exhibits more denervation activity; fasciculation's and signs of reinnervation.

1.3.3 Quantitative sensory testing

Quantitative sensory testing (Thermotest) evaluates thin fibres mediating cold and heat by defining subjective thresholds for cold and heat sensation (Sand & Jørum, 2010). However, skin biopsy with determination of epidermal nerve fibre density is more objective for demonstration of small fiber neuropathy (Mellgren, Nolano, & Sommer, 2013).

2 Inherited polyneuropathies and related conditions

Inherited polyneuropathies are often an element of a more complex phenotype that is caused by illnesses such as a mitochondrial disease, a genetic syndrome or a metabolic disorder, or the neuropathy may be the only symptom, most often manifesting as a distal symmetric

polyneuropathy (Reilly, 2007). Distal symmetric polyneuropathy is common and can have a number of causes including various underlying conditions, environmental or inheritable factors. The population prevalence is around 2.4 %, in the subgroup aged 55 years or older it is assessed to 8 % (Martyn & Hughes, 1997). In half of the cases the underlying disease is found after neurologic investigation, but the rest remain idiopathic (Dyck, Oviatt, & Lambert, 1981). A large part of the idiopathic cases are genetic (40 %), Charcot Marie Tooth (CMT) is the most frequent entity and makes up about half of this group (Dyck et al., 1981; Ouvrier & Nicholson, 1995).

CMT mainly involves the large and myelinated peripheral motor and sensory nerve fibres, but some small fibre involvement has been reported (Krajewski et al., 2000; Zambelis, 2009). Clinically and neurophysiologically it is distinguished from the distal hereditary motor neuropathies (dHMN) that are associated with motor symptoms without sensory loss, and the hereditary sensory and autonomic neuropathies (HSAN) that are associated with predomination of sensory symptoms. Diseases such as hereditary spastic paraplegia and primary lateral sclerosis mainly implicate the upper motor neurons (UMNs) whereas progressive muscular atrophy and spinal muscular atrophy involve the lower motor neuron (LMN). Amyotrophic lateral sclerosis (ALS) involves both UMNs and LMNs. Some ALS subtypes are also associated with frontotemporal dementia (FTD) (Pan & Chen, 2013; Renton et al., 2011).

The classification of these genetic diseases is, in addition to the clinical signs, based on inheritance patterns and underlying gene defects. Classification systems that are based on clinical symptoms may not correlate well with those based on biology. In the World Muscle Society's gene table (http://www.musclegenetable.fr/), 283 genes were distributed on 16 groups of neuromuscular disorders, 47 associated with variants of CMT and HSAN, 14 with dHMN or dSMA and 15 with subtypes of familial ALS (FALS) (Kaplan, 2011). Many of those genes are linked with multiple disease classes: Three genes are associated with ALS and FTD (*VCP, UBQLN2* and *c9orf72*), two with ALS and HMN (*VAPB, SETX*), one with ALS and CMT (*FIG4*), one with ALS, spastic paraplegia and primary lateral sclerosis (*ALS2*), six with HMN and CMT2 (*DYNC1H1, TRPV4, GARS, AARS, HSPB1* and *HSPB8*), two with HMN and spastic paraplegia (*REEP1* and *BSCL2*), and one with hereditary sensory neuropathy (HSN) and spastic paraplegia (*KIF1A*) (Sivakumar et al., 2005). Several *SOD1* mutations are linked to phenotypes with long survival and absent UMN symptoms, fulfilling the ALS criteria, but with a prognosis different from that normally associated with ALS

(Brooks, Miller, Swash, & Munsat, 2000). Some *SOD1* mutations may also be associated with marked sensory disturbances that contradict a diagnosis of ALS (Rezania et al., 2003).

The large number of genes that may cause a CMT phenotype necessitates extensive testing if one is to examine all possible causations. Moreover, even the exclusion of all CMT associated genes would be insufficient due to the variance in phenotypes associated with single genes. That particularly applies for genes primarily associated with conditions affecting neurons with long axons such as HSAN, HMN, ALS and HSP. Furthermore, all known genes combined only explain a proportion of the heritability, particularly in the case of axonal CMT where many disease associated genes remain unknown.

3 Charcot Marie Tooth disease (CMT)

Based on nerve conduction studies CMT with dominant inheritance is divided into CMT1 and CMT2. CMT1 is associated with demyelination and reduced nerve conduction velocities while CMT2 has (near) normal velocities due to axonal pathology. A MNCV cut-off of 38 m/s in the *N. medianus* is commonly used to separate the two forms (Harding & Thomas, 1980). Recessive CMT is labelled CMT4, and X-linked cases CMTX independent of results on NCS.

The prevalence of the CMT phenotype has been estimated to 10-20:100000 globally (Emery, 1991). Locally, in the population of Western-Norway the prevalence has been reported to be 41:100000 (Skre, 1974), in Eastern-Norway (Akershus) 1:1214 (Braathen, Sand, Lobato, Hoyer, & Russell, 2011), and in Northern-Sweden 20:100000 (Holmberg, 1993). CMT primarily implicates the peripheral nerves, classically occurring earlier and more predominantly in the lower than in the upper limbs. Onset is mostly before 20 years of age, but there is sizable variation in debut as well as severity, particularly in CMT2 cases (Harding & Thomas, 1980). In most cases however, the symptoms are benign and slowly developing (Carter et al., 1995). In the classic phenotype, muscular atrophy and weakness in the distal parts of the legs, absence of Achilles tendon reflexes, *pes cavus* and hammer toes are typical findings.

CMT subtypes are designated by the causative gene defect and the number of subtypes is growing rapidly, particularly in the case of CMT2. A classical presentation of the CMT1 and CMT2 phenotype cannot accurately be divided on the basis of clinical signs (Bienfait et al., 2006). Most CMT2 patients have the classical phenotype and the underlying gene defect can

only be revealed in a subfraction of those cases, usually through the detection of a *MFN2* mutation. Some of the CMT2 cases may present with asymmetric muscle weakness, hypertrophy of the calves, Babinski's sign, hyperreflexia or other manifestations that do not suit the classical CMT phenotype (Bienfait et al., 2007). Especially severe phenotypes with early onset and very slow MNCVs are sometimes designated as Dejerine-Sottas syndrome (DSS, MIM 145900) or congenital hypomyelinating neuropathy (CHN, MIM 605253). Genetically they are heterogeneous and autosomal dominant as well as autosomal recessive transmissions have been described.

3.1 Charcot Marie Tooth Neuropathy Type 1 (CMT1)

CMT1 compose approximately 50 % of the total CMT group and most cases can be identified with molecular genetic testing. There are 6 genetically defined subclasses (Table 1). CMT1A due to a duplication of the *PMP22* region is by far the most common constituting around 70 % of the CMT1 cases (Nelis et al., 1996; Boerkoel et al., 2002). The other subgroups are less frequent.

3.1.1 CMT1A

Patients with a duplication of the *PMP22* region mostly present a classical CMT phenotype, but varying graveness of symptoms is typical, even among members of an individual family. The reported phenotypes spans from congenital onset and severe symptoms to clinically unaffected (Birouk et al., 1997; Baets et al., 2011), but the median MNCV is virtually always < 38 m/s (Birouk et al., 1997; Bienfait et al., 2006; Marques, Jr. et al., 2005; Kim et al., 2012). Hand tremor, hearing loss, scoliosis, calf hypertrophy, pain, diaphragmatic weakness, hypoesthesia and even brain involvement with cognitive impairment are among additional features that have been reported (Bienfait et al., 2006; Marques, Jr. et al., 2005; Chanson et al., 2013).

3.1.2 CMT1B

MPZ mutations may cause demyelination and a severe clinical phenotype with very slow MNCVs (DSS, CHN) or they may generate axonal pathology and a milder CMT1/2 phenotype with intermediate or axonal range MNCVs (Shy et al., 2004). Some mutations are associated with particular phenotypic traits such as pupillary abnormalities, auditory neuropathy and tremor (Shy et al., 2004; Mandich et al., 2009a; Chapon, Latour, Diraison,

Schaeffer, & Vandenberghe, 1999; Starr et al., 2003; Plante-Bordeneuve, Guiochon-Mantel, Lacroix, Lapresle, & Said, 1999).

3.2 Charcot Marie Tooth Neuropathy Type 2 (CMT2)

CMT2 composes about 20 % of the CMT group (Harding & Thomas, 1980). Mutations in the *MFN2* gene is the most frequent and may account for 10-30 % of the classical CMT2 cases in many ethnicities (Zuchner et al., 2004; Lawson, Graham, & Flanigan, 2005; Verhoeven et al., 2006; McCorquodale, III et al., 2011). Disease causing mutations in other CMT2 associated genes are individually rare. These consist of 14 genes and 1 locus in which the involved gene has not been identified (Table 1).

3.2.1 CMT2A

MFN2 mutations are usually associated with a classical CMT2 phenotype. Postural hand tremor, sensorineural hearing loss, pain and signs of UMN involvement like Babinski's sign or hyperrefexia are among additional features that have been reported (Chung et al., 2006; Chung et al., 2010; Zhu et al., 2005). Some however, experience a very severe phenotype that sometimes includes optic atrophy and they frequently have an early onset (< 10 years) (Zuchner et al., 2006; Chung et al., 2006). The severe phenotype may also include scoliosis, knee contractures or brain involvement. In contrast, mutation carriers that are mildly affected or asymptomatic may also be observed in some families (Lawson et al., 2005). A recessive pattern of inheritance has been reported (Polke et al., 2011).

3.3 Dominant intermediate Charcot Marie Tooth Neuropathy (DI-CMT)

A dominant intermediate phenotype also exists. The intermediate range MNCVs are between 25 and 45 m/s and different members of DI-CMT families exhibit MNCVs within both axonal and demyelinating ranges (Nicholson & Myers, 2006). Some genes (*DNM2*, *YARS*) are primarily associated with this type. The *MPZ* gene is associated with DI-type D in addition to CMT1B, 2I/J and 4E. A forth gene associated with DI-CMT (*GNB4*-related I-CMT) was recently identified. DI-CMT type A has been mapped to10q24-25, but the gene in question remains to be identified (Table 1). Mutations in many of the genes traditionally associated with other groups may also cause MNCVs in the intermediate range, this particularly holds true for CMTX1 (*GJB1*).

3.4 Charcot Marie Tooth Neuropathy Type X (CMTX)

Genes involved in CMTX are located on the X-chromosome. Two genes involved in X-linked dominant CMT (*GJB1*, *PDK3*), as well as two genes (*PRPS1*, *AIFM1*) and two loci associated with X-linked recessive CMT have been identified (Table 1).

3.4.1 CMTX1

The *GJB1* gene (CMTX1) is a major cause of CMT and responsible for most of the CMTX cases and also about 10 % of the total CMT group (Boerkoel et al., 2002). Males are on average more gravely affected than females who typically exhibit slow or intermediate range MNCVs. Females may be healthy or mildly affected, and usually have intermediate or axonal range MNCVs (Dubourg et al., 2001b; Dubourg et al., 2001a). Upper limb tremor and sensorineural hearing loss are additional symptoms that have been reported with some frequency. Some papers describe rare episodes of CNS disease after provocations such as infections or hyperventilation, but persistent CNS involvement like Babinski's sign or alterations in the corticospinal tract on MRI have also been reported (Srinivasan, Leventer, Kornberg, Dahl, & Ryan, 2008; Marques, Jr., Sweeney, Wood, Wroe, & Marques, 1999; Kassubek, Bretschneider, & Sperfeld, 2005; Siskind, Feely, Bernes, Shy, & Garbern, 2009).

3.5 Charcot Marie Tooth Neuropathy Type 4 (CMT4)

Nine genes have been associated with the recessive CMT4 (Table 1). The phenotype is severe, has an early onset and may include manifestations such as diaphragmatic weakness, scoliosis, hearing loss or glaucoma. In some areas CMT4 may be responsible for as much as 40 % of the CMT group, but in the Northern-European population the recessive forms have been assumed to be rare (Reilly, 2007).

3.5.1 CMT4A

The *GDAP1* gene is causative of one of the most common subclasses of CMT4 (CMT4A), but also the rare dominant CMT2K. The CMT4A cases have an early onset of severe symptoms and some eventually become dependent on a wheelchair. A fraction of the diseased also experiences vocal cord paresis and weakened diaphragm. Axonal range MNCVs is typical, but intermediate or even demyelinating range MNCVs may occur in patients with grave axonal pathology (Cassereau et al., 2011).

3.5.2 CMT4C

The *SH3TC2* gene is linked to the other relatively common subclass of CMT4 (CMT4C). The phenotype is usually severe with early onset and often includes scoliosis (Azzedine et al., 2006). MNCVs are in the demyelinating range. Hypomyelination and extended Ranvier's nodes have been observed on nerve biopsy (Arnaud et al., 2009). In some ethnicities CMT4C is responsible for many cases of demyelinating CMT, but in a recent report from the UK the frequency was estimated to 0.3% (Lassuthova et al., 2011; Murphy et al., 2012)

Table 1. CMT1, CMT2, DI-CMT, CMTX and CMT4 with their subtypes, associated genes or loci and protein products, if known (Bird, 2014).

CMT subgroup	*Gene/Locus*	**Protein**
CMT1A	*PMP22*	Peripheral myelin protein 22
CMT1B	*MPZ*	Myelin P_0 protein
CMT1C	*LITAF*	Lipopolysaccharide-induced tumor necrosis factor-alpha factor
CMT1D	*EGR2*	Early growth response protein 2
CMT1E	*PMP22*	Peripheral myelin protein 22 (sequence changes)
CMT1F/2E	*NEFL*	Neurofilament light polypeptide
CMT2A1	*KIF1B*	Kinesin-like protein KIF1B
CMT2A2	*MFN2*	Mitofusin-2
CMT2B	*RAB7*A	Ras-related protein Rab-7
CMT2B1	*LMNA*	Lamin A/C
CMT2B2	*MED25*	Mediator of RNA polymerase II transcription subunit 25
CMT2C	*TRPV4*	Transient receptor potential cation channel subfamily V member 4
CMT2D	*GARS*	Glycyl-tRNA synthetase
CMT2E/1F	*NEFL*	Neurofilament light polypeptide
CMT2F	*HSPB1*	Heat-shock protein beta-1
CMT2G	12q12-q13	Unknown
CMT2H/2K	*GDAP1*	Ganglioside-induced differentiation-associated protein-1
CMT2I/2J	*MPZ*	Myelin P_0 protein
CMT2L	*HSPB8*	Heat-shock protein beta-8
CMT2N	*AARS*	Alanyl-tRNA synthetase, cytoplasmic
CMT2O	*DYNC1H1*	Cytoplasmic dynein 1 heavy chain 1
CMT2P	*LRSAM1*	E3 ubiquitin-protein ligase LRSAM1
DI-CMTA	10q24.1-q25.1	Unknown
DI-CMTB	*DNM2*	Dynamin 2
DI-CMTC	*YARS*	Tyrosyl-tRNA synthetase
DI-CMTD	*MPZ*	Myelin P_0 protein
GNB4-related I-CMT	*GNB4*	Guanine nucleotide-binding protein subunit beta-4
CMTX1	*GJB1*	Gap junction beta-1 protein (connexin 32)
CMTX2	Xp22.2	Unknown
CMTX3	Xq26	Unknown
CMTX4	*AIFM1*	Apoptosis-inducing factor 1
CMTX5	*PRPS1*	Ribose-phosphate pyrophosphokinase 1
CMTX6	*PDK3*	Pyruvate dehydrogenase kinase isoform 3
CMT4A	*GDAP1*	Ganglioside-induced differentiation-associated protein 1
CMT4B1	*MTMR2*	Myotubularin-related protein 2
CMT4B2	*SBF2*	Myotubularin-related protein 13
CMT4C	*SH3TC2*	SH3 domain and tetratricopeptide repeats-containing protein 2
CMT4D	*NDRG1*	N-myc downstream-regulated gene 1 protein
CMT4E	*EGR2*	Early growth response protein 2
CMT4F	*PRX*	Periaxin
CMT4H	*FGD4*	FYVE, RhoGEF and PH domain-containing protein 4
CMT4J	*FIG4*	Phosphatidylinositol 3, 5 biphosphate

4 Pathomechanisms and therapy

4.1 Pathomechanisms

Some of the genes linked to the CMT phenotype encode proteins that are located in, or are particularly important for specific parts of the peripheral nervous system (PNS) (Figure 1). Examples are for the compact myelin *PMP22* and *MPZ*, non-compact myelin *GJB1*, neuronal cell body *LMNA* and axons *NEFL*. Many of the genes are expressed in several tissues however (*EGR2*, *GARS*, *DNM2*, *RAB7*, *MFN2*, *GDAP1*, *LMNA*, *NEFL*), and it is not known why their defects mainly or solely implicate the PNS. Functional attributes of the nerves are the extraordinary need of energy, the neuronal transmission, and their systems for transportation along the long axons. All of these functions represent possible explanations. The latter attribute in particular fits the length dependence of the polyneuropathy seen in most CMT phenotypes (Juarez & Palau, 2012; Patzko & Shy, 2011; Zuchner & Vance, 2006).

In rare cases the neuropathy is caused by an inborn distortion of the myelination that can be observed on nerve biopsy as hypomyelination without current de- and regeneration (MIM 605253). More commonly there is a secondary myelin involvement that in grave cases is visible on nerve biopsy as hypertrophic nerves with onion bulbs as tokens of current de- and regeneration (MIM 145900). Axonal damage ultimately explains the neuropathy in myelinated fibres seen in CMT2 as well as in CMT1. In CMT2 axons are directly implicated whereas in CMT1 they are involved through intercommunication with the Schwann cells (Krajewski et al., 2000). Nerve biopsy does not currently have a prominent role in the diagnostics of CMT however.

The disease mechanism may be a gene dosage effect such as the over expression observed in CMT1A or the haploinsufficiency causative of hereditary neuropathy with liability to pressure palsy (HNPP). In other cases the disease mechanism is not linked to normal gene function, but rather to a toxic gain of function mechanism. Animal models such as the rat, mouse, fruit fly and others have been helpful in studies of these mechanisms (Sereda & Nave, 2006; Eschenbacher et al., 2012).

Many of the proteins encoded by genes linked to the CMT phenotype have similar functions and some may have underlying disease mechanisms in common. Implicated functions are compaction, preservation and construction of myelin (*PMP22*, *MPZ*), transportation along axons (*KIF1B*, *MFN2*, *NEFL*, *GDAP1*), transcription of genes (*EGR2*), functioning of

mitochondria (*MFN2*, *GDAP1*), synthesis of tRNA (*AARS*, *KARS*, *GARS*, *YARS*), molecular chaperoning (*HSP27*, *HSP22*), trafficking through membranes (*MTMR2*, *MTMR13*), functioning of endosomes (*RAB7*, *SH3TC2*), construction of the cytoskeleton (*NEFL*) and channelling of ions (*GJB1*, *TRPV4*). (Juarez & Palau, 2012; Patzko & Shy, 2011; Zuchner & Vance, 2006). Many of the same metabolic pathways and protein functions have evenly been linked to related inherited phenotypes that also implicate long axons, for example HSAN, HMN, HSP and ALS (Timmerman, Clowes, & Reid, 2013).

In contrast, the phenotype may vary considerably between different mutations in the same gene, which in some cases can be explained by the involvement of different pathomechanisms. Mechanisms implicated in association with various *MPZ* mutations include (partial) loss of function, dominant negative effects and gain of glycosylation (Grandis et al., 2008; Wrabetz et al., 2006; Mandich et al., 2009b; Lee et al., 2010; Prada et al., 2012). The genomic architecture bordering the 1.4 Mb *PMP22* region on chromosome 17p12 predisposes to duplications/deletions due to unequal crossing over (Inoue et al., 2001). In the case of CMT1A that results in a gene dosage effect with over expression of the *PMP22* gene in all CMT1A patients. However, other inborn genetic variants are postulated to play a major regulatory role influencing the level of expression, possibly explaining some of the clinical variation. Some of these regions have been identified (Jones et al., 2012). *GJB1* encodes a gap junction protein (connexion 32) which is not only expressed in Schwann cells, but also in oligodendrocytes, possibly explaining the CNS manifestations exhibited by some of the patients. Most of the sequence variants in *GJB1* lead to protein retention in the endoplasmatic reticulum and loss of function (Sargiannidou et al., 2009). Mitofusin 2 plays a key role in the fusion of outer mitochondrial membranes, compulsory for the admixture of molecules and mtDNA amongst different mitochondria. Defects in mitofusin 2 function may impair the replication of mtDNA, ultimately leading to mtDNA depletion (Vielhaber et al., 2013). Impaired mitochondrial transport can also be an important mechanism in *MFN2* associated disease. It may for example limit the adjustability to local variations in the energy demand along the axons resulting in intermittent local hypoxia and ultimately Wallerian degeneration (Misko, Sasaki, Tuck, Milbrandt, & Baloh, 2012). The potential importance of defective axonal transport in ALS and other neurodegenerative disorders has been reviewed (Morfini et al., 2009). With regard to *SOD1* associated FALS several mechanisms have been proposed, all implicating a dominant negative (toxic) effect. As in the case of *MPZ* associated CMT, different pathomechanisms may apply for different mutations. A peculiarity of the p.H47R

substitution is that it changes one of the four histidines that bind copper in the active site, severely disturbing normal enzyme function (as an antioxidant) (Antonyuk et al., 2005; Winkler et al., 2009; Pan et al., 2012). However, the molecular reason for the benign CMT2-like clinical course associated with p.H47R is not clear.

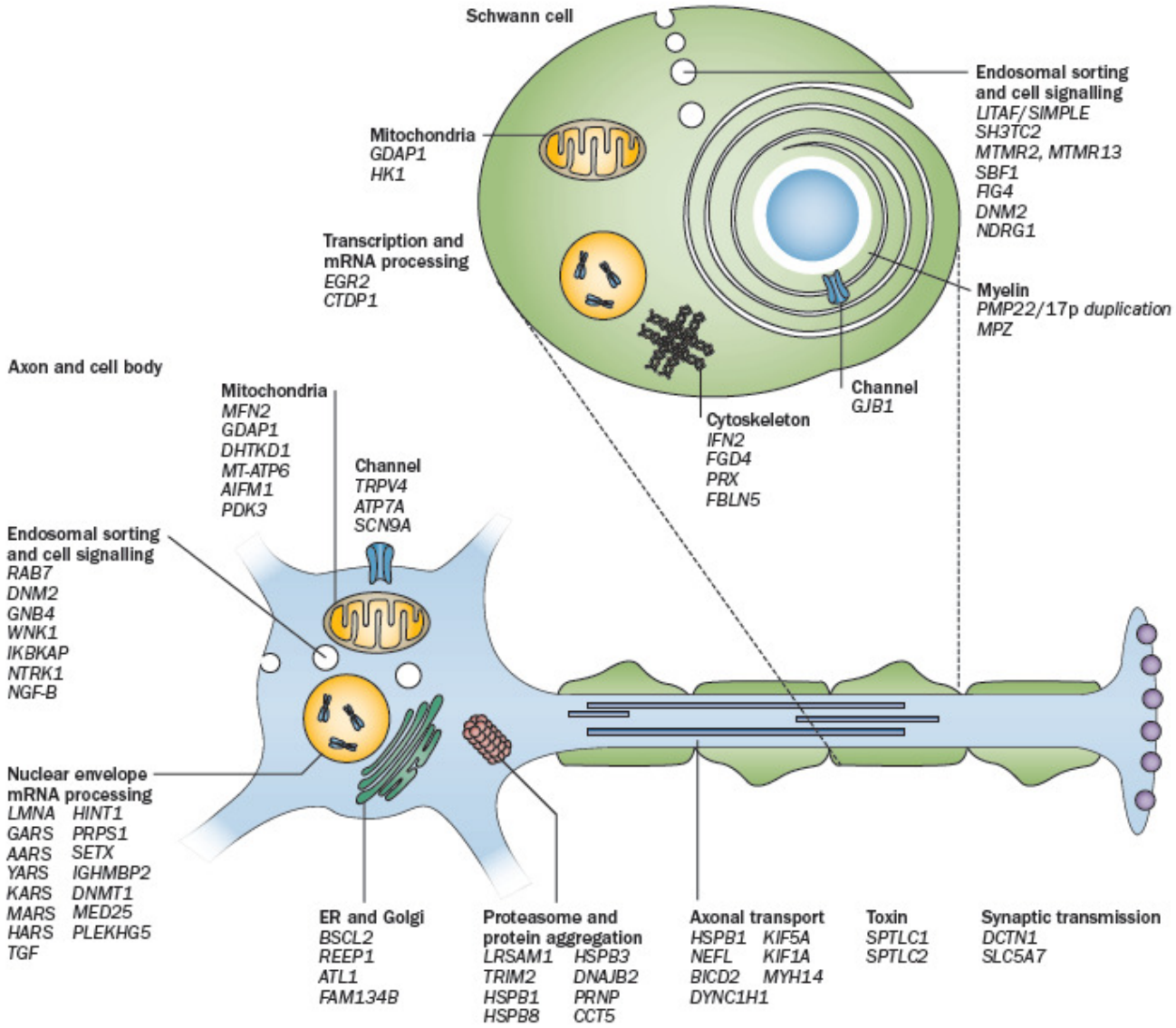

Figure 1: Figure displaying the neuron, axon, Schwann cell and many of the genes involved in CMT and related phenotypes as well as suggested pathomechanisms (Rossor, Polke, Houlden & Reilly, 2013).

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4.2 Therapy

The genetic investigations and the study of relevant pathomechanisms aim at developing causal treatment. That may require complicated procedures that intend to manipulate pathomechanisms directly such as the silencing of toxic gain of function mutations or mutations that cause over expression or oppositely, stimulation of protein production in disorders induced by haploinsufficiency. Other approaches seek to limit neuronal degeneration and activate regeneration independent of the involved pathomechanisms. The role of animal models has recently been reviewed (Bouhy & Timmerman, 2013). One intervention study that has reached clinical trials is the dietary treatment of HSN type 1 with oral L-Serine (ClinicalTrials.gov Identifier: NCT01733407) (Garofalo et al., 2011). Examples of completed studies, regrettably without documented effect, are treatment of patients with the CMT phenotype with coenzyme Q10 (ClinicalTrials.gov Identifier: NCT00541164) and treatment of CMT1A patients with high dose ascorbic acid (ClinicalTrials.gov Identifier: NCT00484510/NCT00271635) (http://clinicaltrials.gov/ct2/home) (Lewis et al., 2013). High throughput tools have been developed that search for agents that can influence regions regulating *PMP22* expression (Jang, Lopez-Anido, MacArthur, Svaren, & Inglese, 2012).The detailed diagnostic studies at neuromuscular centres should aim at having patients ready for relevant clinical trials and treatments as they develop.

5 Challenges in genetic testing of patients with CMT

Diagnostic testing for CMT presents a number of challenges for laboratories because: (i) many different genes are associated with CMT; (ii) mutations in a single gene may cause different clinical pictures, while mutations in different genes linked to the CMT phenotype can cause identical symptoms; (iii) family history might be absent; (iv) the laboratories receive DNA and clinical information from many medical doctors of diverse specialities and clinical backgrounds; (v) important clinical information might be sparse or absent and; (vi) the sensitivity of CMT2 testing is low. Furthermore, a broad scanning of a large number of patients takes a lot of resources and gives a low yield of positive genetic test results.

5.1 Genetic testing of patients with CMT

Recommended procedures for molecular genetic CMT testing presupposes exact clinical details, detailed results from NCS and thoroughly sampled family histories collected under good clinical conditions. They are therefore well suited for inherited polyneuropathy clinics (England et al., 2009; Saporta et al., 2011; Murphy, Laura, & Reilly, 2013). In the everyday

practice for laboratories investigating external samples such favourable circumstances are rarely found and as a consequence, the mutational yield in that group is significantly lower (Murphy et al., 2012). In Norway most patient samples are tested in this setting, and in a recent report from a Neuromuscular Clinic in the UK almost 2/3rds of the tested samples were external (Murphy et al., 2012). In spite of the differences in the context for testing, in depth studies of external samples are lacking. With regards to the guidelines however, the two groups are frequently treated as if they were identical. In paper I we document that > 90% of the mutations are found in *PMP22*, *MPZ*, *MFN2* and *GJB1*, and several other groups have made similar observations (Murphy et al., 2012; Saporta et al., 2011). For screening of external samples, the established algorithms may be too extensive, but for the thorough work-up of patients at neuromuscular centres they may be too limited considering the technology that recently has become available.

5.2 Challenges in the classification of sequence variants

Diagnostic laboratories investigate genes with known association to particular monogenic Mendelian phenotypes. Sequence variants such as nonsense mutations, frame-shifting deletions/insertions and variants affecting canonical splice-sites are predicted to disrupt gene function and they are generally considered pathogenic. This is also the case for missense variants that repeatedly have been identified in patients but not in controls. Sequence variants that have been frequently observed in large control panels are usually considered benign. All other sequence variants are of uncertain clinical significance. This suggests that a classification system with three levels, distinguishing between pathogenic, non-pathogenic and uncertain variants, would be appropriate. For variants of uncertain clinical significance (VUS) different types of documentation may be collected to elucidate their clinical relevance further. Testing of control samples and assessment of the allele frequency in single nucleotide polymorphism (SNP) databases are powerful standard methods to exclude pathogenicity. The documentation of non-segregation in clinically well-defined families, and the documentation of lacking amino acid conservation among species may also help weakening the probability of clinical significance. It is more difficult to obtain support for the clinical relevance of a variant. However, in isolated CMT cases the observation of a *de novo* variant in a known CMT associated gene is substantial evidence for its pathogenicity (Richards et al., 2008; Sunyaev, 2012). In the diagnostic laboratory potential splice site mutations may be investigated with mRNA studies. In some neuromuscular disorders functional studies are currently used in routine diagnostics, for example on muscle biopsies. For CMT associated

genes however, the availability of functional studies is limited (Bell, Bodmer, Sistermans & Ramsden, 2007)

We used Alamut (http://www.interactive-biosoftware.com/) as an initial tool in the assessment of all the genetic variants identified in this study. Alamut works as a web browser with the gene under investigation as the focal point. Evidence related to a particular sequence variant in the gene is collected from multiple sources and subsequently incorporated and presented. These include data from: dbSNP from The National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/), The Human Gene Mutation Database (HGMD Professional) (http://www.hgmd.org/) as well as the Exome Variant Server of the NHLBI Exome Sequencing Project (http://evs.gs.washington.edu/EVS/). Powerful statistical evidence is obtained through investigations of the minor allele frequency (MAF) of a particular variant in the populations included in the databases. Variants that previously have been reported as (likely) pathogenic are highlighted in the reference sequence and individual reports are accessible via links to the HGMD. Extended literature searches are also mediated through PubMed (http://www.ncbi.nlm.nih.gov/pubmed) and Google (http://www.google.com). The proof collected from epidemiological data and earlier reports on affected patients often have the power to justify a primary classification of a missense mutation as (likely) pathogenic/non-pathogenic. In some cases published results on functional studies may also influence the interpretation of a particular sequence variant.

In many cases, however, these sources alone do not provide enough proof to classify a missense mutation as (likely) pathogenic/non-pathogenic. Reports on how to interpret uncertain variants in CMT have been sparse, but the subject has been studied thoroughly in relation to breast cancer. Up to half of the variants in *BRCA1/2* are of uncertain clinical relevance (Gomez Garcia et al., 2009). The International Agency for Research on Cancer (IARC) Unclassified Genetic Variants Working Group favoured a division of interpretations in genes linked to cancer into five; 5 = definitely pathogenic, 4 = likely pathogenic, 3 = uncertain, 2 = likely not pathogenic, 1 = definitely not pathogenic (Plon et al., 2008). In this manner the large group of variants of unspecific significance in the classification system with three levels is divided into three groups (2-4) allowing more specific communication of probability levels, although final proof for or against pathogenicity is lacking.

Alamut integrates several prediction tools that assist in the interpretation of variants of unknown clinical significance. The conservation of a nucleotide or more importantly, of an amino acid, is understood as a sign of functional importance. Conservation scores are estimated from the degree of variation between DNA and amino acid sequences (AAS) from orthologues of growing phylogenetic distance, from human to yeast. DNA and protein annotations as well as structural data, where available, also provide important input into some of the interpretation tools. Grantham scores for individual amino acids are assessed from physiochemical properties such as size, polarity, hydropathy, charge, etc (Ng & Henikoff, 2006). More specifically, align GVGD is a prediction tool supplied by the IARC (http://agvgd.iarc.fr/). It estimates a Grantham score and multiple AAS alignments from orthologues are compared to estimate the degree of variation for a particular amino acid (Grantham Variation). The distance between the Grantham score of the substitute amino acid and the average score of the Grantham Variation is expressed in the Grantham Distance (GD). An increase in the GD is interpreted as an increased likelihood of pathogenicity (Tavtigian et al., 2006; Mathe et al., 2006). SIFT (sorting intolerant from tolerant) is a tool supplied by the J. Craig Venter Institute (http://sift.jcvi.org/). It also evaluates physiochemical properties of amino acids and the degree of conservation in orthologues (Sim et al., 2012). PolyPhen-2 (the prediction of functional effects of human nsSNPs) is supplied by the Harvard Medical School (http://genetics.bwh.harvard.edu/pph2/). A complicated algorithm includes data such as the DNA sequence, amino acid sequence and structural data in humans, as well as comparisons between orthologues (Adzhubei et al., 2010; Adzhubei, Jordan, & Sunyaev, 2013). MutationTaster is provided by the National Genetics Reference Laboratory Manchester (http://www.mutationtaster.org/). It incorporates results from other prediction tools in an interface, and uses Bayesian principles to estimate a common prediction (Schwarz, Rodelsperger, Schuelke, & Seelow, 2010). SwissProt is supplied by the Swiss institute of Bioinformatics (http://web.expasy.org/docs/swiss-prot_guideline.html). It is a validated version of the UniProt Knowledgebase, and provides quality data regarding AAS and protein structure, function, localization and interactions (Bairoch, Boeckmann, Ferro, & Gasteiger, 2004). However, there are still extensive gaps in the coverage provided by SwissProt. Alamut evenly incorporates multiple tools that can assess the potential splice site effect of sequence variants *in silico*. These include SpliceSiteFinder-like, MaxEntScan, NNSPLICE and Human Splicing Finder (http://www.interactive-biosoftware.com/alamut/doc/2.3/splicing.html). The predictions from the different programs vary, and the results are often contradictive. The results must therefore be interpreted with care and the final decision on mutation class must

be computed manually. The reports from the interpretation tools serves as decisional support and in themselves, they do not provide sufficient evidence to alter the interpretation of a sequence variant of unknown pathogenicity.

5.3 Extended diagnostic studies

It is demanding to sort out the patients where a large genetic contribution is credible and the cases where extended testing should be performed. If a positive family history is missing in a polyneuropathy patient, phenotypic characteristics like early onset, sensory symptoms, *pes cavus*, hammertoes and slowly evolving disease may help selecting cases for testing of the prevalent genes (*PMP22dup*, *MPZ*, *GJB1*, *MFN2*) (Reilly, 2007). On the other hand, unusual phenotypes increase the likelihood of association with a rare CMT gene. The recent technological development enables detection of rare, very rare and even new disease causing genes. As such there is a gradate shift from diagnostics to research and vice versa. Quality control measures and the use of registers should accompany this development.

5.3.1 Targeted testing

Specific handles such as ethnicity or particular clinical clues may help pinpointing cases where a major genetic contribution is likely and may also enable targeted testing of a limited number of rare candidate genes. Examples are Dejerine Sottas Syndrome (*MPZ, EGR2, PMP22, PRX*), congenital hypomyelinating neuropathy (*MPZ, EGR2*), axonal CMT with Argyl Robertson pupil (*MPZ*), upper limb predominance of weakness with or without sensory findings (*GARS, BSCL2*) – or with spasticity (*BSCL2*), pronounced sensory loss and insensitivity to pain (*RAB7*), vocal cord paresis (*GDAP1*, *TRPV4*), or intermediate range MNCVs and glomerulopathy (*IFN2*) (http://www.musclegenetable.fr/) (Boyer et al., 2011). Only a small fraction of the patients seen in inherited polyneuropathy clinics belong to that group however.

5.3.2 Linkage studies and massive parallel sequencing

Parallel sequencing of all the protein coding regions of the genome (exome sequencing) was presented as a way to uncover mutations in rare monogenic disorders in 2009, revealing the mutations in four individuals with Miller syndrome in 2010 (Ng et al., 2009; Ng et al., 2010). These next generation sequencing methods (NGS) are especially advantageous in the diagnostics of multifarious phenotypes like CMT and other heterogeneous neuromuscular disorders. This was first illustrated in a family with a *GJB1* mutation, and subsequently in

many other research publications (Montenegro et al., 2011; Choi et al., 2012; Landoure et al., 2012; Weedon et al., 2011; Weterman et al., 2012; Kennerson et al., 2013). At present NGS is increasingly incorporated into the repertoires of regular diagnostic laboratories and practical approaches have already been discussed extensively in the literature (Vasli et al., 2012; Vasli & Laporte, 2013). In large dominant, X-linked or consanguineous families, traditional linkage studies may help identifying candidate regions for further analysis with NGS. It is not necessary to obtain the high significance levels traditionally used (LOD > 3) for that purpose. Similarly, analysis of copy number variation and the identification of homozygous regions may help prioritizing candidate genes in recessively inherited cases.

5.4 Family investigations

Genetic diagnostic evaluations of index patients with the CMT phenotype have been comprehensively reported. The subsequent molecular genetic family investigations however, have received less focus. Diagnostic, presymptomatic and prenatal testing is feasible for variants with definite or likely pathogenicity, in some cases also preimplantation diagnostic testing (PGD). Predictive and prenatal testing of the common CMT linked genes are affiliated with psychological and ethical issues. Medically, the broad clinical variation, even amongst family members might create uncertainty. Furthermore, the medically defined gravity of the phenotype is often of limited use in the context of genetic counselling and testing (Wertz & Knoppers, 2002). Quality of life studies have only been published for the major subtypes and in those, the wellbeing is reduced in afflicted children as well as adults. Physical limitations related to the core phenotype is one factor, but treatable manifestations like cramps, tremor and fatigue are also major contributors. Social stigma may also lower the individual sense of well being (Burns, Ramchandren, Ryan, Shy, & Ouvrier, 2010; Pfeiffer, Wicklein, Ratusinski, Schmitt, & Kunze, 2001; Boentert et al., 2010). Private class 5 and class 4 mutations in rare CMT genes, for which no natural history studies exist, cause even greater ambiguities regarding prognosis. The interpretation of class 3 variants (VUS) often remains indeterminate after family investigations. These obscurities may be difficult to convey for the laboratory, and difficult to understand for the physicians and their patients.

6 Summary

The CMT phenotype is the most common inherited neurologic disorder, but it is also genetically one of the most multifarious, and with only four exceptions (*PMP22dup*, *MPZ*, *MFN2* and *GJB1*) the CMT subtypes are individually extremely rare. Furthermore, there are

phenotypic overlaps with related and almost equally genetically diverse conditions like HSAN, HMN, ALS and HSP. The pathomechanisms of these conditions involve many of the same metabolic pathways and some of the same disease associated genes. Diagnostic testing of index patients for CMT therefore presents a number of challenges, both for the screening of the main bulk of the patient samples, and also for the group prioritized for extended studies. Genetic counselling of families with mutations in *PMP22dup* and other prevalent CMT genes are complicated by the large clinical variability. Genetic variants in rare disease associated genes, and variants of uncertain clinical significance are affiliated with uncertainties that are difficult to convey and difficult to comprehend.

Aims of these studies

The Department of Medical Genetics at the University Hospital of North-Norway serves as part of the National Neuromuscular Centre and receives samples for genetic testing of neuromuscular disorders from all parts of Norway among which CMT testing makes up the largest group. This study is based on 559 samples received for CMT testing during the seven years from 2004-2010. The overall aim was to improve the diagnostics of inherited polyneuropathies. As a part of this effort we defined sub goals as outlined below:

1 Statistical analysis and revision of molecular genetic diagnostics in a population of Norwegian patients with CMT.

1.1 Diagnostic testing of index patients

- Describe the spectrum of genes and mutations involved in Norwegian patients with CMT.
- Based on the referral forms following the patients; identify factors that are associated with positive and negative genetic findings.
- Analyse and, if necessary, revise the test protocols and formulate criteria for mandatory information to be obtained before genetic testing for CMT.

1.2 Molecular genetic family investigations

- In families of patients with definite or likely pathogenic genetic variants (class 4-5).
    - Give a quantitative estimate of the number of relatives tested, as well as the relative proportions of the indications for testing.
    - Describe the characteristics of the cases within each indication group.
- In families of patients with genetic variants of unknown clinical significance (class 3).
    - Assess the impact of segregation analyses.

2 Extended studies - identification of the disease gene in a large Norwegian family with a CMT2-like disorder.

- Describe the clinical and neurophysiologic phenotype in affected patients.
- Identify the chromosomal region where the disease associated gene is localized.
- Identify and characterize the gene and the mutation that are associated with the disease.

In part 1.1 we aim at improving the strategy for molecular genetic testing of CMT in diagnostic laboratories. It should also be of help in delineating the patient group that could

benefit from extended testing at an interdisciplinary neuromuscular clinic. This may be of advantage for health services, patients and users of the laboratory as we expect an increase in conclusive test results, a decrease in false positive results and a more efficient use of resources. Part 1.2 will aid in the assessment and planning of resources needed for family studies. In part 2 the purpose was to identify and characterize a new common cause of CMT2 in Norway. This will allow us to create a new diagnostic test which will benefit patients and the results will shed new light and knowledge on the molecular aetiology of CMT2 and related inherited disorders.

Strategy

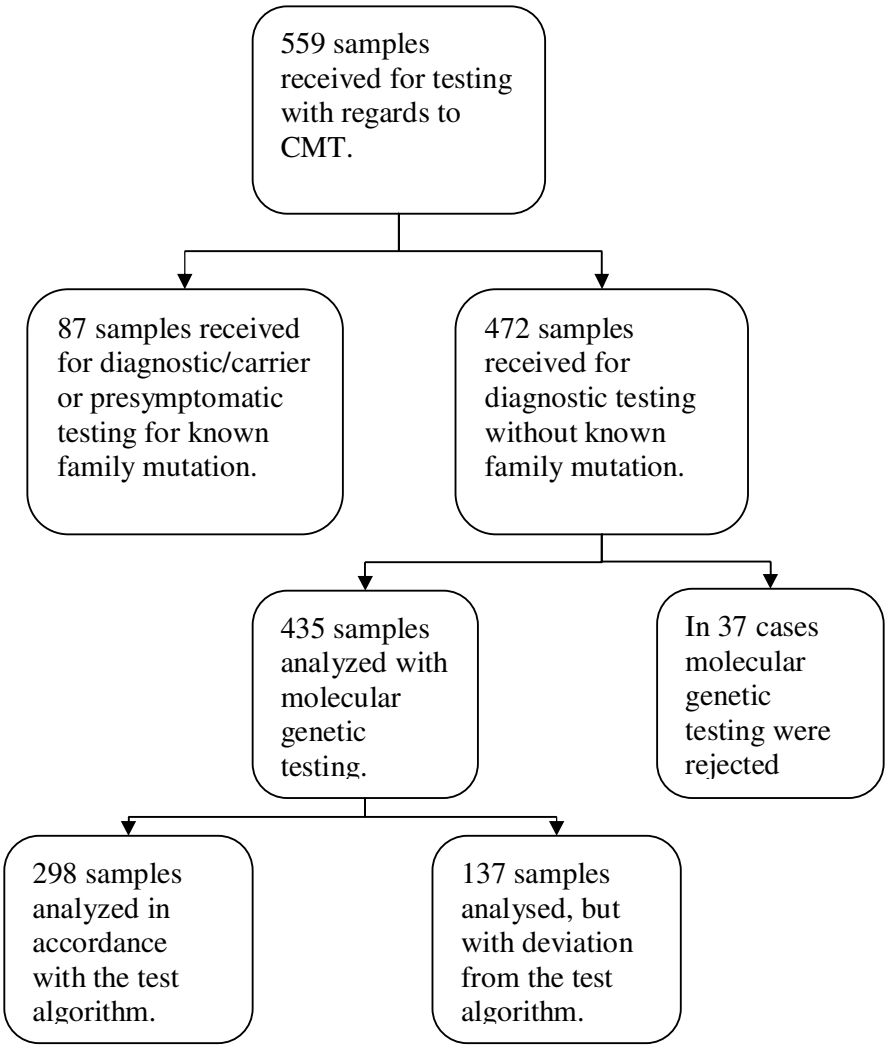
Part I - screening of index patients (1.1) and subsequent molecular genetic family investigations (1.2)

Clinical investigations, data collection, statistics and endpoint measures

During the seven years from 2004 to 2010 we received 559 samples with the request to carry out molecular genetic testing for CMT (Figure 2). In 472 cases diagnostic testing of index patients was called for, but in 37 cases the request was cancelled by the laboratory. In the remaining 435 cases tests were performed in compliance with the protocol (see below; Molecular investigations) in 298 cases. In 137 cases there were discrepancies from test protocol. In that group 2.7 genes were investigated pr. patient on average. We identified a sequence variant in 72 index cases. Following family studies for diagnostic, carrier or presymptomatic testing were requisitioned for 75 patients belonging to 31 families (43%). We also received samples from 12 family members of 10 index patients diagnosed at other laboratories. In total, testing of 87 family members from 41 families was requisitioned.

From the laboratory request forms important data were extracted and systematized to examine to which extent they modified the outcome of genetic diagnostics. Among others, these encompassed the test indication, clinical information, NCS results, age at onset and at testing, speciality of the referring physician, family history with regards to mutation status and symptoms. The group of index cases that underwent diagnostic testing and the group of affected and healthy relatives tested for known family mutations were divided and analyzed separately. In part 1.2 the separate variables were assessed and data from the mutation positive and the mutation negative patient samples were compared. The Statistical Package for the Social Sciences (SPSS) version 20.0 was used for the statistical analyses.

Figure 2. Flowchart of the investigations performed on 559 samples received for CMT mutation analysis.



Molecular investigations

Index patients with demyelinating or mixed (axonal and demyelinating) polyneuropathy underwent testing with a CMT1 panel containing Multiplex Ligation dependent Probe Amplification (MLPA) of the *PMP22* region as well as DNA sequencing of the *MPZ*, *EGR2*, *LITAF*, *NEFL*, *PMP22* and *GJB1* including all coding exons and the adjacent intron sequences (Table 2). Requisitions indicating axonal polyneuropathy and those with normal NCS results were investigated with a CMT2 panel containing DNA sequencing of the *MFN2*, *MPZ*, *NEFL* and *GJB1* genes including all coding exons and the adjacent intron sequences. Requisitions, in which the type of polyneuropathy was non-specifiable, were categorized as

"deviation from test protocol" if they were not studied with both the CMT1 and CMT2 test batteries. Clinical and family information determined if the *GDAP1* gene should be sequenced in individual cases. In total 58/435 patient samples from all polyneuropathy groups were analyzed. MLPA of *MPZ/MFN2* was carried out on 229/435 samples. Relatives were tested for the particular sequence variant already identified in the index patient. Genetic variants were assessed with the Alamut software (Interactive Biosoftware, San Diego, CA, USA), which incorporates data from multiple internet sources, and additional manual interpretation. The sequence variants were categorized into five groups that communicate the likelihood of their pathogenicity in the range from definitely pathogenic (5) to definitely not pathogenic (1). Variants that were determined to be non-pathogenic or likely non-pathogenic (class 1 and 2) were designated as negative findings. For variants of uncertain clinical significance (class 3) segregation studies of relatives were carried out whenever possible.

Table 2. CMT1, CMT2, CMT4, CMTX and DI-CMT subtypes with gene symbols or loci (Bird, 2014).

X linked	**Autosomal dominant**		**Recessive**
CMTX; demyelinating, or axonal	CMT1; demyelinating	CMT2; axonal	CMT4; demyelinating or axonal
CMTX1: *GJB1**	CMT1A: Dup 17p*	CMT2A1: *KIF1B*	CMT4A: *GDAP1**
CMTX2: Xp22.2	CMT1B: *MPZ**	CMT2A2: *MFN2**	CMT4B1: *MTMR2*
CMTX3: Xq26	CMT1C: *LITAF**	CMT2B: *RAB7*	CMT4B2: *SBF2*
CMTX4: *AIFM4*	CMT1D: *EGR2**	CMT2B1: *LMNA*	CMT4C: *SH3TC2*
CMTX5: *PRPS1*	CMT1E: *PMP22**	CMT2B2: *MED25*	CMT4D: *NDRG1*
CMTX6: *PDK3*	CMT1F: *NEFL**	CMT2C: *TRPV4*	CMT4E: *EGR2*
		CMT2D: *GARS*	CMT4F: *PRX*
	DI-CMT: mixed**	CMT2E: *NEFL**	CMT4H: *FGD4*
	DI-CMTA: 10q24-25	CMT2F: *HSPB1*	CMT4J: *FIG4*
	DI-CMTB: *DNM2*	CMT2G: 12q12-q13	
	DI-CMTC: *YARS*	CMT2K: *GDAP1*	
	DI-CMTD: *MPZ**	CMT2I/J: *MPZ**	
	GNB4 related I-CMT: *GNB4*	CMT2L: *HSPB8*	
		CMT2N: *AARS*	
		CMT2O: *DYNC1H1*	
		CMT2P: *LRSAM1*	

*genes analyzed in this study. ** mixed axonal and demyelinating neuropathy

Part II – extended studies - identification of the disease locus in a large Norwegian family with a CMT2-like disorder.

Patients, clinical investigations and neurophysiologic evaluations

The subjects of the investigations in part II belonged to an extensive Norwegian "CMT2" kindred. The earliest obligate mutation carrier in the part of the family that was described in paper 3 was born in 1763. That part of the family spanned over 7 generations. We evaluated 10 departed and 12 living relatives. Before the project started a meeting with the family was arranged, initiated by the family members, The Norwegian Association for Patients with Muscle Diseases, The Centre for Rare Disorders at Oslo University Hospital and The Department of Medical Genetics, University Hospital of North Norway. During the project the family members were updated at re-examination, and through written information. A follow up meeting was held at the end of the project and individual results were given upon personal request at counselling. The family was thereafter transferred to The Centre for Rare Disorders at Oslo University Hospital.

The phenotype in the family was originally categorized as CMT2. In order to obtain a precise description of the phenotype 12 family members were examined anew clinically. Nine of them also underwent neurophysiologic investigations. NCS of the median and ulnar nerves was performed in one arm as well as of the superficial peroneal, tibial, and sural nerves of both legs. Needle-EMG was registered in the opponens pollicis, extensor digitorum communis and deltoideus posterior muscles in one arm, as well as in the anterior tibial, medial gastrocnemius in both legs and lateral vastus in the right leg. Threshold temperatures for sensations of cold, warmth, cold-pain and heat-pain were measured at the base of the thumb, lateral at the left thigh, at the lower leg and at the back of the foot bilaterally (Thermotest, ® Somedic AB, Sweden). Allodynia was estimated by brushing of all 4 limbs. In addition, indications of hyperalgesia to punctate stimuli were assessed by an 83.7 mN von Frey filament.

Molecular investigations

Genome-wide genotyping was executed with Human CNV370 chips (Illumina) with the presumption of autosomal dominant inheritance, a penetrance of 95%, a frequency of the disease associated allele of 0.0001, and a phenocopy rate of 0.001. Multipoint linkage analysis was completed implementing MERLIN (Abecasis, Cherny, Cookson, & Cardon, 2002).

About 42,500 markers with a minor allele frequency of >= 0.15 were picked for the study. Bidirectional sequencing was executed with BigDye version 3.1 and an ABI 3130xl (Applied Biosystems). Analysis of the most likely disease associated gene in the linkage region was performed by sequencing of all coding exons in the *SOD1* gene in the index patient and consecutive segregation studies of the family. PCR amplification and partial sequencing of exon 4 of the *CHGB* gene was also done.

Summary of papers

Paper I: Diagnostic laboratory testing for Charcot Marie Tooth disease (CMT): The spectrum of gene defects in Norwegian patients with CMT and its implications for future genetic test strategies.

We assessed the spectrum of gene defects documented in CMT patients analyzed at The Department of Medical Genetics at the University Hospital of North-Norway during the course of seven years (2004 – 2010). The information given in 435 requests for diagnostic investigations of index patients were assessed retrospectively. Testing was executed according to polyneuropathy type; demyelinating/mixed: *PMP22* duplication, *MPZ*, *EGR2*, *LITAF*, *NEFL*, *PMP22*, *GJB1*, axonal: *MFN2*, *MPZ*, *NEFL*, and *GJB1*. Clinical details such as family history, age at first symptoms and age at testing, clinical details and results on nerve conduction studies (NCS) were registered. The group consisting of cases with a positive finding and the group with mutation negative cases were compared utilizing a statistical tool (SPSS 20.0). Seventy-two (16.6%) genetic variants of uncertain (12), likely (12) or certain (48) pathogenicity were detected. Most (94.6 %) of the mutation positive index patients experienced disease onset prior to 50 years of age. In total 31.2 % of the samples received by the laboratory belonged to index patients with onset after 50 years of age however. Few positive findings were made in that group. *PMP22* duplications and sequence variants in *MPZ, GJB1* and *MFN2* composed 95.8 % of the positive findings. The mutation detection rates within the polyneuropathy types were; demyelinating 33.8 %, mixed (both demyelinating and axonal) 29.0 %, axonal 8.8 %, and unspecified 16.5 %. A classical CMT phenotype was indicated in all cases with a positive finding and an acceptable detection rate was also achieved in requests with little complementary information. In contrast no findings were made in cases where the requests indicated atypical CMT, a more complex phenotype or a primary assumption of another condition. The inheritance patterns estimated from the requests, and the inheritance patterns deducted from the detected gene defects did not correlate well. The study revealed 11 novel sequence variants that had not been described in the HGMDp database version 2013.2. These were detected in the *MPZ* gene (c.679A>T, c.368G>T, c.410G>A), in *NEFL* (c.1027_1029del), *GJB1* (c.775del), and in the *MFN2* gene (c.2146_2148dup, c.250A>G, c.612T>A, c.653T>C, c.692C>T and c.1921T>C). The high prevalence of *PMP22*dup, and sequence variants in *MPZ, GJB1* and *MFN2* indicate that the first screening of external samples may be limited to the four most frequent genes. The

decision on which genes to test should primarily be founded upon NCS results. These results also support a more strict practice for testing of index patients with onset after 50 years of age with this gene panel. The same applies for patients with a more complex phenotype, atypical CMT, or a primary assumption of a different diagnosis. These latter groups can be met with higher demands regarding family history and clinical details, but also with a potential referral to extended investigations. This also applies for patients with a negative result for the four most prevalent genes. The extended investigations should involve an evaluation at a neuromuscular clinic that is capable of targeted molecular genetic analysis of "rare" CMT genes as well as linkage studies and diagnostics utilizing next generation sequencing technology

Paper II: Segregation analysis in families with Charcot-Marie-Tooth disease allows reclassification of putative disease causing mutations.

During the years from 2004-2010 we identified 72 genetic variants of definite (48), likely (12) or uncertain (12) clinical significance in index cases with CMT. Our intention was to evaluate the follow up investigations with family studies, including the relative proportions of the various test indications as well as the test results and reported clinical details. The main objective however, was to assess the family investigations executed as part of the evaluation of variants of unknown pathogenicity. We analyzed 87 requests for testing of relatives akin to 41 index patients, including 12 family members of 10 index patients diagnosed at other institutions. The mean number of relatives investigated was 2.1 per index patient. Diagnostic investigations of afflicted relatives for genetic variants of definite/likely pathogenicity were completed in 41 relatives from 29 families. Mean age at investigation was 27.6 years (range 2 months – 78 years). Six family members (14.6%) tested negative whereas 35 relatives from 26 families tested positive. NCS results were reported only in 28.6 % of the afflicted family members and in the greater part (65.7 %) only limited clinical information was given. The unexpectedly high rate of normal results on diagnostic testing of afflicted relatives underscores the need for an accurate clinical diagnosis, even in that group. Presymptomatic testing of healthy relatives for genetic variants of definite/likely pathogenicity was executed in 20 relatives from 12 families. Mean age at investigation was 37.0 years (range 2 – 92 years). The low detection rate in the presymptomatic testing group (21 %) is related to the high average age at testing, *de novo* mutations, and investigations of family members who were not first degree relatives. The *de novo* mutations comprised a c.490C>T missense substitution in *GJB1*. Prenatal testing was requested by one of nine females at fertile age (21-

42 years) carrying a genetic variant of certain pathogenicity. Twenty-two relatives (9 clinically affected) from 8 families were included in the family investigations executed as part of the evaluation of VUS. Mean age at investigation was 44.7 years (range 3 – 66 years). All except two cases were more than 30 years of age. Three instances of *de novo MFN2* sequence variants were revealed in two patients with a classic CMT2 phenotype (c.2146_2148 dup and c.692C>T) and in one patient with a severe phenotype (c.250A>G). That led to the upgrading of the classification from uncertain to likely for two of the sequence variants. Moreover, following segregation studies, one *MFN2* substitution (c.1709A>G) was downgraded from uncertain to unlikely pathogenic, and one *MPZ* substitution (c.103G>A) was upgraded from unknown to likely pathogenic.

Paper III: Hereditary motor neuron disease in a large Norwegian family with a "H46R" substitution in the superoxide dismutase 1 gene.

The study of an extensive "CMT2" kindred with whole genome linkage analyzes and haplotype investigations pinpointed a 1.9Mb candidate region on chromosome 21. *SOD1* was the most likely candidate gene and follow up Sanger sequencing showed a c.140A>G (p.His47Arg, alias "H46R") substitution. The disease was transferred in an autosomal dominant way in the family and we investigated 10 departed and 12 living relatives. Amongst the departed one case of likely non-penetrance in an obligate mutation carrier was observed. The 12 living relatives were reassessed clinically and 9 individuals were studied with NCS, EMG, thermotest and evaluations of allodynia and hyperalgesia. The mean age at disease onset was 42.5 years (22-65) and the initial symptom was distal weakening of a leg in all cases that had specific information. The impairments progressed to involve the hands years later. Seven of the 12 individuals developed wheelchair dependency after a mean duration of 9.7 years. Sensory investigations were normal with the exception of one individual who had increased vibratory thresholds beneath the knees. Some manifestations indicating an implication of upper motor neurons were observed in 3 patients demonstrating a certain, and 2 patients a possible Babinski's sign. Spasticity or increased reflexes were not observed however, and the upper motor neuron involvement was not more pronounced than what has been described in connection with dHMN. Cognitive decay, respiratory failure or bulbar symptoms were not detected. Bone fractures were registered in 6 patients, many in association with minor trauma, perhaps caused by osteoporosis due to inactivity. Average period from onset of the first symptoms to passing was 29 years (11-51). NCS exhibited reduced

amplitude of motor nerves in the lower extremities indicating axonal motor involvement. Indication of sensory involvement of the upper limbs was not registered. Some patients exhibited signs of axonal involvement of the sensory nerve fibres in the lower limbs however. On EMG extensive neurogenic alterations with denervation activity, pathological motor unit potentials and complex repetitive discharges in numerous muscles were registered, much in compliance with observations known from motor neuron diseases. Only few muscles demonstrated fasciculation potentials however. Six patients had heightened thresholds for cold detection implying a small fiber involvement. We also tested all family members for the chromogranin B (*CHGB*) variant c.1238C>T (p.Pro413Leu) to study the possible association with disease onset, none of the relatives was carriers however. The diagnosis was altered to dHMN after the clinical reassessment due to the scarcity of sensory symptoms and findings. Later on the involvement of proximal muscles made the clinical picture more consistent with the ALS phenotype. Moreover, the one-sided onset with weakening of a calf muscle is not to be anticipated in a dHMN/dSMA. The p.His47Arg substitution has mainly been published in Japanese patients, but the description of this sizable kindred demonstrated that it has been underrated as a cause of HMN in Norway, and possibly also in other populations. The kindred exemplify the variety of non-demyelinating inborn neuropathy phenotypes that have been consolidated under the CMT2 umbrella. We therefore propose that patients with a phenotype starting with adult one-sided weakness in a calf muscle advancing to a distal hereditary motor neuropathy-like clinical picture, ought to be investigated for the c.140A>G (p.His47Arg) substitution.

Results and discussion

1 The impact of clinical, nerve conduction studies, and family information on genetic diagnostic testing of index patients with CMT

The conventional algorithms for molecular genetic CMT testing presuppose accurate and complete inputs concerning clinical symptoms, NCS and family history. For external samples however, such precision and fullness of the input may not always be assumed. In spite of obvious differences between the carefully selected patient cohort ascertained at neuromuscular clinics, and the heterogeneous cohort of external samples analyzed at diagnostic laboratories, they have traditionally been handled as if they were the same. Thorough descriptions of the external sample cohort have been absent, although it quantitatively represents the largest group. Our intention was to do a complete evaluation of the diagnostic testing performed on our CMT samples, which are requested by various external medical doctors.

Firstly, based on the requisitions, we aimed at recognizing elements that were associated with positive and negative test results. In paper I we included 435 afflicted index patients genetically tested for CMT and as a result 72 positive genetic findings were documented. Close to half of the positive results were obtained amid requests indicating a clear suspicion of CMT, but with little specified supporting information. This group of requests was numerically the largest. The yield of positive findings was clearly higher amongst requests giving a more thorough description of a classical CMT phenotype, particularly when additional features known to be associated with the most prevalent CMT genes were specified. The requests specifying a particularly severe phenotype showed the lowest detection yield. Seventy-three requests stated symptoms indicating atypical CMT, a more complex phenotype, or a primary suspicion of a differential diagnosis. In that group no positive findings were made with the gene panel applied in this study.

These results support an approach of limited testing (*PMP22, MPZ, GJB1, MFN2*) of external patients with a clearly stated suspicion of CMT, but with few clinical details supporting the diagnosis, The evaluation of *PMP22dup* and sequence variants in *GJB1* are also straightforward in most cases. A subsequent summoning of complementary information or the initiation of segregation studies is therefore mostly not necessary. The low yield of positive

findings associated with requests that indicate a primary suspicion of other diagnoses supports a restrictive policy for the gene tests applied in this study. Some of these patients should rather be examined clinically and neurophysiologically at a neuromuscular clinic, before extended molecular genetic testing. The group of index patients with severe symptoms reported in paper I was small (14 patients) and only one positive finding was documented (a patient homozygous for the *PMP22* duplication). No positive results were obtained upon testing of *EGR2* or amongst 58 patients selected for *GDAP1* testing. This would suggest that the causes for recessive CMT in Norway are still elusive. However, after the completion of this study several cases associated with mutations the *SH3TC2* gene (CMT4C), were identified. From the cohort of CMT patients received by our Department before 2013, thirty-two affected index patients were selected for *SH3TC2* testing. The selection was based on individual assessment of the cases, but a severe phenotype with early disease onset, scoliosis, foot deformities, demyelinating polyneuropathy and a family history compatible with autosomal recessive inheritance were important criteria. Twelve patients (37.5 %) were diagnosed with CMT4C. Eight were homozygous for the common nonsense variant c.2860C>T, p.Arg954*, four were compound heterozygous for the c.2860 C>T variant and a missense variant (c.798T>C, c.3511C>T, c.956G>T) (unpublished results). This indicates that CMT4C may be a common cause of recessive CMT in Norway. Dominant cases with mutations in *MPZ* and *MFN2* would have been expected in the severely affected group if the cohort had been larger. In fact, one severe case in an infant from a DSS family with a *MPZ* substitution, as well as a severe case with a *de novo* substitution in *MFN2* was described amongst the relatives reported in paper II.

The results in paper I clearly confirmed an association between a positive family history and a positive genetic test result. In the prevailing test algorithms however, assumptions about the inheritance patterns are often used to select genes for testing (England et al., 2009; Saporta et al. 2011). The quality and amount of specifications regarding family history in the requisition forms varies greatly, they are often insufficient, or more concerning, inaccurate. We demonstrated a poor correlation between the inheritance patterns assumed from the requisitions, and the correct transmission form documented by the identified gene defect. Testing of *GJB1* is superfluous in families with unambiguous male to male transmission however. Family histories compatible with autosomal recessive inheritance should create awareness that the causative mutation may well be found outside the four most prevalent

CMT genes. Amongst ethnic Norwegians *SH3TC2* is the most likely candidate gene in such cases.

A clear association between the various types of polyneuropathy indicated by NCS, and the likelihood of obtaining a positive test result was also confirmed in paper I. The highest detection rate was observed in the demyelinating and mixed (demyelinating and axonal) polyneuropathy groups (33.8 %; 29.3 %). The lowest proportion of positive findings was found in the axonal polyneuropathy group (8.8 %). In retrospect, the observed gene defect was compatible with the polyneuropathy type indicated on the requisition form in all cases. The prevailing test algorithms are predominantly based on the CMT classification which relies on assumptions about the inheritance pattern as well as the NCS result. In paper I we demonstrated that the majority (63.9 %) of our external samples could not be categorized according to those criteria, primarily due to insufficient family details, more scarcely due to the absence of NCS results. The practicality of the CMT classification was diminished even further by the low correlation between the transmission modes deduced from the requisitions and the inheritance patterns documented by the identified gene defects. We concluded that the NCS results alone are far more robust and precise in the prioritization of genes to be analysed in external samples. Of note, index patients with requisitions lacking NCS results obtained a positive result in 16.5 % of the cases, equal to the proportion detected in the cohort of index patients as a whole (16.6 %). We did however spend more resources on that group, because more genes had to be analyzed according to protocol.

During the years we have noticed that a major part of the requisitions for CMT testing received by our laboratory are associated with individuals that are of some age. In paper I we documented that 53 % of the requisitions belonged to patients that were > 50 years when the blood was sampled. However, 23 positive findings were observed in this group, presumably due to a delay from age at onset to age at testing. When we evaluated the group with known age at onset in isolation (52.6 %), we could show that virtually all positive results were documented in indexes with onset < 50 years of age (95.8 %). Of note, in the selected group with known age at onset (231/435), approximately one third (31.2%) of the requests concerned indexes with onset > 50 years of age. Only one certain finding (*PMP22dup*) was found amongst those 72 patients (and one VUS in *MPZ*). Most of the patients with onset > 50 years of age had either an axonal polyneuropathy (59.7 %) or no NCS results at all (21 %), many combined with the absence of a specified family history. Hence phenocopies most

likely constitute a major fraction of this group. All in all these data support a more restrictive practice for the gene tests applied in this study for patients with onset > 50 years of age. Requisitions that are well founded based on thorough clinical information, specified family details and NCS should be tested regardless of age at onset however.

The requisition forms of relatives of index patients (paper II) mostly contained only little clinical information. To some surprise, 6/41 affected family members (14.6 %) tested negative for the family mutation. In those cases clinical details were limited and NCS results were not reported. Amongst the relatives with a positive test result, a female with a c.490C>T (p.Arg164Trp) substitution in the *GJB1* gene had tremor from early childhood, particularly of the head, muscular cramps and fasciculation's in addition to the polyneuropathy. Dejerine-Sottas syndrome (DSS) was reported in a family with a c.368C>A (p.Gly123Val) substitution in the *MPZ* gene. Requisition forms of affected relatives that were supplied with detailed clinical information (10/41); all described a classic phenotype concordant with the index patient group. The investigation of relatives could potentially reveal cases with uncommon phenotypes due to a lower threshold for testing, but this was not the case in our cohort.

2 The relative distribution of gene defects among mutation positive index patients in our population

Secondly, we wanted to characterize the spectrum of sequence variants and genes implicated in CMT in Norway. The mutation detection rate amongst index patients with available NCS results were 33.8 % for the demyelinating, 29.3 % for the mixed (axonal and demyelinating), and 8.8 % for the axonal polyneuropathies, as compared to 16.6 % for the total group. Almost all positive findings were made up by *PMP22dup* and sequence variants in *MPZ*, *GJB1* or *MFN2*. None of the index patients reported in paper I carried deletions/duplications of the *MPZ* gene. Moreover, sequencing of *LITAF/SIMPLE, EGR2, PMP22* and *GDAP1* showed negative results in all tested cases, implying that sequence variants in these genes are rare causes of CMT in Norway. All in all we found 11 new sequence variants not described in the HGMDp database version 2013.2. These were discovered in *MPZ* (c.679A>T, c.368G>T, c.410G>A), *NEFL* (c.1027_1029del), *GJB1* (c.775del), and *MFN2* (c.2146_2148dup, c.250A>G, c.612T>A, c.653T>C, c.692C>T and c.1921T>C).

The reported frequency and spectrum of sequence variants and genes implicated in CMT varies considerably across different studies and populations. The proportion of gene defects in

CMT1 spans from 23.3% to 60.7% for *PMP22dup* (average 41.8%), from 2.4% to 13.0% for *MPZ* (average 5.0%), and from 5.5% to 25.8% for *GJB1* (average 8.8%) (Bort et al., 1997; Choi et al., 2004; Mostacciuolo et al., 2001; Nicholson, 1999; Silander et al., 1998; Abe et al., 2011; Mersiyanova et al., 2000; McCorquodale, III et al., 2011). This might mirror unequal frequencies of particular CMT associated gene defects in various populations, but perhaps even more so, differences in sampling methods and strategies for testing and reporting of the results. Notably, the ways the patients are ascertained appear to influence the detection frequency to a large extent. The patient cohort sampled at neuromuscular clinics have a significantly higher fraction of mutation positive cases than the heterogeneous patient group tested as external samples (Saporta et al., 2011; Murphy et al., 2012). The comparatively low detection frequencies in our cohort ought to be interpreted bearing this in mind. However, the *PMP22dup* frequency (18.7%) is even lesser than anticipated relative to the prevalence of *MPZ* mutations (6.0%) and *GJB1* mutations (6.7%). A comparable prevalence of *PMP22* duplications (13.6 %) was reported in families with CMT amongst the inhabitants of Akershus County, Norway (Braathen, 2012). The results may imply that we obtain fewer CMT1A patients at our laboratory, relative to the other gene defects. In comparison, the requested family investigations exhibited a tendency in the same direction; when the index patient had a *PMP22dup* subsequent testing of relatives was requested in 23 %, as compared to 60 % for *GJB1*, 64 % for *MPZ* and 42 % for *MFN2*.

3 Revision of the test protocol and criteria for the implementation of genetic testing of index patients – summary

Finally and based on the project results, we aimed at revising our CMT test protocol and formulate criteria for the information compulsory for genetic testing. In the meantime however (the project started in 2010) a radical technological transformation occurred within the field of medical genetics with the introduction of massive parallel sequencing technologies. At first it was introduced as a new and potent research tool, but it was also soon applied by diagnostic laboratories as well. Extensive panels of known disease associated genes bypass some of the technological and ethical challenges that still apply, and are therefore most widely used at present. With time and with increasing experience though, these challenges will be overcome and exome/whole genome sequencing will become standard, also in the diagnostic setting. That particularly applies for heterogeneous conditions such as CMT.

At this point in time, and in our experience as a small/medium size diagnostic laboratory primarily testing external samples, it is still appropriate to filter the incoming requests and to investigate them in a stepwise manner. The combination of scarce clinical information (such as "polyneuropathy") and a certain genetic finding (such as *PMP22dup* or mutations in *GJB1*) is manageable for diagnostic laboratories. Extensive genetic testing of uncertain phenotypes (clinical group 1 in our study), however, would inevitably produce a large group with the combination of an uncertain phenotype and an uncertain genetic finding (class 3 variants). A professional handling of these cases requires a posterior summoning of additional clinical information and in many cases also the initiation of segregation analyses. In paper 1 we documented the poor compliance from referring physicians that initially had sent a request form falling within clinical category 1. Certain findings in rare disease associated genes and/or in atypical phenotypes should also be documented well. The principle question is if this information should be sampled before or after the investigation with NGS technologies (Vasli et al., 2012; Vasli & Laporte, 2013).

On this backdrop we propose a two-tier strategy, the first tier is intended for referral centres similar to ours, receiving blood or DNA, not patients. The choice of genetic tests in this context should chiefly be based on NCS. Index cases with demyelinating or mixed (axonal and demyelinating) polyneuropathy and patients where NCS is not specified, ought to be investigated with *PMP22* MLPA and sequencing of *MPZ* and *GJB1*. Index cases with axonal polyneuropathy should be investigated with sequencing of *MFN2*, *MPZ* and *GJB1*. This strategy would reduce labour substantially and still only miss about 4 % of the positive findings in our cohort. An analogous proposal was forwarded by a UK group (Murphy et al., 2012). The results of our studies advocate a stricter routine for genetic investigations of cases with onset > 50 years, cases with an atypical or more complex phenotype, or requisitions primarily indicating the suspicion of a differential diagnosis (at least for the genes tested in this study). These particular cases can be met with higher demands regarding the family history and clinical details, but also by a potential transferral to the second tier of the investigations. The second tier should include an interdisciplinary evaluation at a neuromuscular centre that is able to perform detailed clinical and neurophysiologic investigations, targeted molecular genetic testing of rare disease associated genes, NGS and (for some cases) linkage analyses or analyses of CNV (copy number variation).

4 The application of presymptomatic and prenatal testing for genetic variants of definite or likely clinical significance

The intention of the study leading up to paper II was, broadly defined, to evaluate the molecular genetic family investigations following a diagnostic finding in an index patient. We wanted to describe the relative proportions of the different indications for testing as well as the results, and the reported clinical information.

In total, presymptomatic testing was requested for 20 healthy relatives of afflicted index patients harbouring sequence variants of known or likely pathogenicity. A positive finding was detected in 21%. The low rate of positive findings is likely due to the fact that; several of the tested family members were adults (average age 37 years), three second degree relatives were included and two patients turned out to harbour *de novo* mutations (a *PMP22* duplication and a c.490C>T missense mutation in *GJB1*). *De novo* duplications of the *PMP22* region are common, but in *GJB1 de novo* sequence variants are allegedly rare (Meggouh et al., 1998). Six of the requests for presymptomatic testing concerned minors aged 15 years or younger. This seems to contradict recommendations for genetic testing of benign conditions with limited available treatment measures. Four of the cases were requested by different departments of medical genetics, two were ordered by our own department. Data that potentially could elucidate the backgrounds for these decisions were not available to us, but they are all founded on individual considerations by various expert professionals, and in that way they represent common practice.

Prenatal diagnostic testing is available in childbearing carriers of genetic variants of certain pathogenicity. Only 1/9 healthy female relatives at fertile age wished prenatal testing however (paper II). Amongst 72 affected index cases (paper I), 15 women aged 20 - 46 years harboured class 4/5 variants. None of these desired prenatal testing. Thus, in our experience, prenatal diagnostics is rarely applied for CMT in Norway.

5 Evaluation of the molecular genetic family investigations initiated after the identification of genetic variants of unknown clinical significance (class 3)

The main objective of paper II was to evaluate the family studies executed in order to assess sequence variants of uncertain pathogenicity (VUS). We included 22 relatives (9 afflicted) from 8 families. The mean age was 44.7 years. In 4 of the 8 families classifications were adjusted, much due to the documentation of multiple *de novo* mutations in *MFN2*. A comparatively high occurrence of *de novo* sequence variants in *MFN2* has been observed in

other patient populations, mainly with severe symptoms. The *de novo* occurrence of c.280C>T has been reported multiple times (Zuchner et al., 2004; Verhoeven et al., 2006; Chung et al., 2006; Cho, Sung, Kim, & Ki, 2007). Two of our patients with spontaneous *MFN2* mutations (c.2146_2148dup and c.692C>T) and a disease onset before the age of 10 years showed a classical CMT2 phenotype however. This verifies that *de novo MFN2* sequence variants are also recurrently documented in patients with a milder phenotype (Braathen, Sand, Lobato, Hoyer, & Russell, 2010). The third *de novo* case (c.250A>G) and a disease onset before the age of 10 years exhibited a severe phenotype more in compliance with the early onset group described elsewhere (Verhoeven et al., 2006; Chung et al., 2006; Chung et al., 2010). Posterior to segregation studies one *MFN2* mutation (c.1709A>G) was reclassified from uncertain to unlikely pathogenic and one *MPZ* mutation (c.103G>A) was upgraded from uncertain to likely pathogenic.

Segregation analysis is only one of several tools used in the evaluation of variants of uncertain pathogenicity. The assessments include the sampling of documentation from several sources in an attempt to build up evidence for or against pathogenicity. The effort to quantify uncertainty is neither standardized nor unproblematic, and final proof for or against the clinical significance of a variant is not obtained (Gomez Garcia et al., 2009). Cancer is the disease group where most work has been done in this regard. One of the main outcomes there is to decide whether possibly life-saving prophylactic treatment shall be initiated. A larger risk of over-interpretation may be tolerable in that context. Cancer susceptibility genes such as *BRCA1/2* are also amongst the most extensively studied. As a consequence, a massive amount of data has been accumulated that can be incorporated into the evaluation of new sequence variants (Eggington et al., 2013). In contrast, the main outcome in benign diseases without causal treatment options like CMT is a precise diagnosis, accurate genetic counselling and potentially prenatal and presymptomatic testing. Most of the CMT subtypes are rare or extremely rare, and associated with genes that are poorly studied compared to the common cancer susceptibility genes. Less evidence is therefore available for the evaluation of new variants complicating the application of Bayesian approaches.

Although the introduction of 5 interpretation classes may seem aspiring, we have chosen to implement them knowing that many of the *a priori* class 3 variants will and should remain in the VUS group, even after follow up investigations (Plon et al., 2008). This is in compliance with the recommendations from The Norwegian Society of Human Genetics. The ACMG

Laboratory Quality Assurance Committee proposed an analogous classification that additionally included a sixth category for low penetrant alleles (Richards et al., 2008). Guidelines approved by the UK Clinical Molecular Genetics Society proposed 4 interpretation classes in the range; not, unlikely, likely and certainly pathogenic (Bell, 2007). The advisory groups assent with one another that uncertainties associated with the genetic variants must be disclosed to the clinicians and their patients. Furthermore, it is essential that class 3-5 sequence variants are reported, particularly in rare disorders like CMT.

6 The clinical and neurophysiologic phenotype in patients with CMT2-like-disease

In paper III we studied a large Norwegian kindred with a phenotype that for decades had been labelled as CMT2. Extensive clinical and neurophysiologic examinations of afflicted relatives were executed in order to achieve an accurate description of their phenotype.

At the age of 42.5 years on average (range 22-65) the mutation positive family members developed weakness distally in a leg. In relatives with sufficient clinical data the initial weakness was situated in one of the calf muscles in all cases. Dependency on a wheelchair occurred after 9.7 years (range 4-20), and weakness in the hands after 10.3 years (range 1-20) on average. The stretch muscles of the wrists and digits were (relatively) preserved, even late in the disease course. Bone fractures (six cases) and facial weakness (one case) were additional traits registered. The manifestation of bulbar symptoms however, was not found and none of the relatives were in need of ventilation support. The average time from the initial symptoms until departure was 29 years (range 11-51 years).

The NCS first and foremost displayed signs implying axonal motor nerve involvement, but also demonstrated the involvement of axonal sensory nerves in the legs. The EMG recorded signs indicating subacute and chronic neurogenic pathologic processes compatible with motor neuron disease, but hardly any fasciculation potentials as opposed to what is usually registered in ALS (Wijesekera et al., 2009; Douglass, Kandler, Shaw, & McDermott, 2010). Some signs indicating sensory as well as small fibre (mainly cold) involvement was registered with Thermotest. Earlier reports have observed the same in up to one third of the sporadic cases with ALS. These findings might be more evident in patients with *SOD1* associated disease (Hammad, Silva, Glass, Sladky, & Benatar, 2007; Rezania et al., 2003).

The kindred reported in paper III was presented to, and considered by our neuromuscular clinic as a CMT2 family as the signs were distal onset with (relatively) slow progression, discrete UMN signs and the nonappearance of bulbar symptoms. On re-investigation, the registration of few (or none) sensory symptoms did not comply with CMT and the diagnosis was altered to dHMN. The diagnosis was still in compliance with the long survival that all members of this family experienced. The EMG however, registered findings compatible with motor neuron disease, and cases with advanced disease exhibited prominent proximal involvement in agreement with ALS. The asymmetry of the initial symptom with paresis in a calf muscle also separated the phenotype from dHMN/dSMA or CMT2. The phenotypic divergence of *SOD1* associated disease that this family demonstrated has also been linked to multiple other genes involved in diseases involving neurons with long axons. The phenotypes associated with the p.Pro56Ser substitution in *VAPB* for instance, range from ALS, over atypical ALS to late onset SMA (Nishimura et al., 2004; Millecamps et al., 2010). *SETX* mutations have been revealed in cases with benignant autosomal dominant juvenile ALS (ALS4), dHMN type II and sporadic ALS (Chen et al., 2004; Zhao et al., 2009). Of note, the absence of UMN involvement as a trait does not necessarily correlate with a benign disease course in *SOD1* associated disorders (Cudkowicz, McKenna-Yasek, Chen, Hedley-Whyte, & Brown, Jr., 1998).

7 Characterization and discussion of the mutation that is associated with the disease in the "CMT2" family

In paper III we used extended studies, including linkage analysis; to identify the disease associated locus and the causative gene in a kindred with a CMT2-like phenotype, but without carriers of sequence variants in the most prevalent CMT2 genes.

Genome-wide linkage testing with SNP array genotyping and consecutive haplotype analysis pointed to a 1.9 Mb area on chromosome 21 harbouring only 7 genes. *SOD1* was the most conspicuous candidate gene and we therefore progressed directly to Sanger sequencing which revealed a c.140A>G substitution resulting in p.His47Arg. The mutation segregated with the trait in the family. Moreover, the c.140A>G substitution has previously been defined as disease associated in multiple Japanese families with a resembling phenotype (Aoki et al., 1994; Ohi et al., 2002), thus the substitution was clearly the cause of the disease in this kindred. However, as opposed to the publications from Japan none of the Norwegian patients exhibited bulbar symptoms.

The count of published sequence variants in *SOD1* surpasses 170, many are of unknown pathogenicity (Felbecker et al., 2010) (www.alsod.iop.kcl.ac.uk). Some are coupled with rapidly developing disease (survival < 2 years), others with a more classic type or a comparably benevolent disease course (survival > 5 years). The p.Asp91Ala substitution for instance is frequent in some areas of Scandinavia and leads to a relatively benign phenotype in homozygous cases (Andersen et al., 1996). As far as we know, the affected relatives presented in this work have the lengthiest average duration from onset to departure published in connection with any *SOD1* mutation (paper III).

Probable non-penetrance in a female obligate mutation carrier was documented as several family members described her as unaffected until her passing at the age of 79. That, together with the outspread time of onset amongst the afflicted relatives clearly suggested an implication of modifying factors, as suspected in other cases of *SOD1* associated disease (Parton et al., 2002). The p.Pro413Leu substitution (c.1238C>T) in the Chromogranin B gene (*CHGB*) has been linked to an earlier disease onset in patients afflicted with sporadic amyotrophic lateral sclerosis (Gros-Louis et al., 2009). Efforts to replicate the association have however failed (Blasco et al., 2011; van Vught, Veldink, & van den Berg, 2010). The extensive intrafamilial variation in age at manifestation of the first symptoms invited an evaluation of the potential modifying role of the p.Pro413Leu substitution in this kindred. None of the relatives turned out to be carriers however and we could therefore not contribute to a further clarification of the modifying role of the p.Pro413Leu substitution.

Even if the greater number of reports on the p.His47Arg substitution describes patients of Japanese ancestry, single patients of Pakistani, German and North American descent have also been published (Holmoy, Bjorgo, & Roos, 2007; Rabe et al., 2010; Radunovic & Leigh, 1996). It remains to be shown if this is due to recurrent mutations or to (an) ancient common founder(s). It is possible that the c.140A>G allele (p.His47Arg) is underrated as agent of a CMT2/HMN/SMA-like phenotype in Norway, and this may also apply for other populations. Therefore individuals with adult manifestation of one-sided weakness in a calf muscle advancing to a distal hereditary motor neuropathy-like phenotype, ought to be considered for c.140A>G (p.His47Arg) testing.

8 Future challenges

Diagnostic testing of index patients for CMT in it selves presents multiple challenges with regards to the screening of the main bulk of the patient samples, as well as for the group prioritized for extended studies. The patient group is heterogeneous and the cohort investigated at neuromuscular clinics, and the external samples investigated by diagnostic laboratories differ significantly. Extended studies now have the capacity to detect rare, extremely rare and even new disease associated genes; the latter group may be categorized as research. This development should be accompanied by the evolvement and strengthening of national and international registers and quality control. The new NGS technology will provide new insights into the disease mechanisms that lead to CMT and other conditions implementing neurons with long axons, and eventually also effective treatment measures. Neuromuscular centres should aim at having patients ready for relevant clinical trials and treatments as they develop. Broad diagnostic screening with new technology will more often identify disease associated genes that primarily are associated with other disease classes than originally anticipated. Genetic counselling of family members for private class 5 and class 4 mutations in rare genes for which no natural history studies exist are associated with ambiguities regarding prognosis. Challenges also adhere to genetic variants of unknown pathogenicity and it is important that they are published in relevant databases for future use. Last but not least, the genetic counselling capacity, and expertise, should be scaled up to maintain a proportionate relationship between the increased capacity for diagnostic testing of index patients and the subsequent follow up of their family members.

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PAPER I

PAPER II

PAPER III