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Clinical prospective study on disease variability and score generation in patients with Charcot-Marie-Tooth disease type 1A (HMSN1A)

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from

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Dedicated to my dear parents, in love

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Abbreviation index

AD	average distance
ADM	average distance between means
AFM	Association Française Contre Les Myopathies
AFO	Ankle Foot Orthesis
ANOVA	Analysis of Variance
APN	average proportion of non-overlap
BMBF	Bundesministerium für Bildung und Forschung
BMI	Body-Mass-Index
Buffer-EL	Buffer-Erythrocytes-Lysate
CH	Congenital Hypomyelination
CIDP	Chronic Inflammatory Demyelinating Polyradiculoneuropathy
CMAP	Compound Muscle Action Potential
CMT 1/1X/2	Charcot-Marie-Tooth Type 1, 1X, 2
CMTNS (2)	Charcot-Marie-Tooth Neuropathy Score (2)
cz	Czech Republic
(D)/ (N)	dominant side/ non-dominant side
DSD	Déjerine-Sottas disease
e.g.	for example
EDTA	Ethylenediaminetetraacetic acid
etc.	et cetera
FOM	Figure Of Merit
goe	Göttingen
HMSN1A	Hereditary-Motor-and-Sensory-Neuropathy Type 1A

HNPP	Hereditary Neuropathy with Liability to Pressure Palsies
it	Italy
kDa	Kilo Dalton
LLN	Lower Limit of Normal
NA	not available
NCV	Nerve Conduction Velocity
ONLS	Overall Neuropathy Limitation Scale
PFA	Paraformaldehyde
PMP22	Peripheral Myelin Protein 22
pos/ neg	positive/ negative
QST	Quantitative Sensory Test
RLT	RNeasy Lysis Buffer
RNA	Ribonucleic Acid
SAP	Sensory Action Potential
sd	standard deviation
SF-36	Short-Form 36 Questionnaire
SNAP/ SAP	Sensory Nerve Action Potential
SOP	Standard Operation Procedure
spa	Spain
std	standard
TNS	Total Neuropathy Score
ULN	Upper Limit of Normal
UMG	Universitätsmedizin Göttingen
V	Volt
VAS	Visual Analogue Scale

1 Introduction

1.1 Peripheral Neuropathies

Peripheral neuropathies represent one of the most commonly found symptoms in neurological patients. They are due to various causes including complex medical conditions (e.g. diabetes mellitus), toxic metabolites (e.g. thallium, arsenic), drugs (e.g. fluoroquinolone, vincristine, isoniazid, cisplatin), infections (e.g. borrelia burgdorferi, mycobacterium leprae) and hereditary causes (e.g. Friedreich's ataxia, Charcot-Marie-Tooth disease). Mycobacterium leprae and malnutrition are the most commonly found causes for peripheral neuropathies today in developing countries, while alcohol abuse and diabetes mellitus are the leading causes for these symptoms in industrialized parts of the world (Masuhr and Neumann, 2007; Weiss et al., 2012).

Since many forms of neuropathies are acquired, inherited peripheral neuropathies represent a minority among all forms of neuropathies. The first causative *gene loci* have been discovered within the last two decades. So far, about 50 *loci* and 30 causative genes have been successfully identified and linked to the group of inherited peripheral neuropathies. An updated list on molecular genetic studies and recently identified genes and *loci* can be retrieved on: http://www.molgen.ua.ac.be/CMTMutations/Home/IPN.cfm (information retrieved on 17th October 2012).

Recently, clinicians started to distinguish inherited neuropathies based on their origin, as they classify them in neuropathies, (I) in which the neuropathy is the main symptom of the disease (e.g. Charcot-Marie-Tooth disease (CMT), hereditary neuropathy with liability to pressure palsies (HNPP)) and neuropathies, (II) in which the clinical manifestation is part of a more widespread neurological or multisystem disorder (e.g. Leukodystrophies, Porphyrias, Friedreich's ataxia) (Reilly, 2007).

1.2 Charcot-Marie-Tooth disease

The Charcot-Marie-Tooth (CMT) disease, also known as hereditary motor and sensory neuropathy (HMSN), was first described by J.-M. Charcot, P. Marie and H. H. Tooth in 1886 (Irobi et al., 2004). The Charcot-Marie-Tooth disease affects approximately 1 in 2,500 people (Skre, 1974). More recent investigations indicate an unsuspected higher prevalence of 1 in

1,214 people in Western Europe (Braathen, 2012). There are estimations about 30,000 affected patients suffering from Charcot-Marie-Tooth disease in Germany (Grehl and Rautenstrauß, 1997). Thus, CMT is considered a rare neurological disease (Murphy et al., 2012). The number of un- and misdiagnosed patients remains unknown. Possible differential diagnoses include e.g. poliomyelitis, Friedreich ataxia, hereditary neuropathy with liability to pressure palsies (HNPP), Déjerine-Sottas disease (DSD), congenital hypomyelination (CH), Refsum's disease, chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) and spinal muscular atrophy (Pareyson, 2004; Hanemann and Ludolph, 2002).

CMT is a synonym for a heterogeneous group of inherited neuropathies (Lupski and Garcia, 1992). It can be distinguished in autosomal dominant demyelination (CMT1), axonal (CMT2), X-linked (CMT1X) and autosomal-recessive types of neuropathies (Patzkó and Shy, 2012). Taking into consideration the nerve conduction velocity (NCV), there can be made a clinical assessment and a fairly solid prediction of the type of inheritance. Since NCVs with less than 38 m/s are characteristic of CMT type 1 and NCVs above 38 m/s are typical of axonal CMT2. Intermediate NCVs (25-45 m/s) are often associated with male patients suffering from CMT1X (Patzkó and Shy, 2011; Reilly and Shy, 2009).

Around two third of CMT patients suffer from type 1A, which is therefore the most common subtype (Pareyson, 2004). CMT1A has an autosomal-dominant inheritance pattern and is caused by an intrachromosomal duplication of the gene pmp22 located on chromosome 17p11.2 (Vance et al., 1989; Raeymaekers et al., 1991, Lupski and Garcia, 1992). The encoded protein PMP22 is a small tetraspanned 22-kDa membrane glycoprotein, which plays a crucial role in myelin assembly and synthesis.
Subtle changes in gene-dosages are remarkable, as they are responsible for a range of neuropathy related diseases. While e.g. the duplication is shown to be responsible for CMT1A, a haplo-insufficiency by deletion is associated with hereditary neuropathy with liability to pressure palsies (HNPP), emphasizing the importance of correct dosage of PMP22. The modulation of the PMP22 dosage is now even considered to be a promising therapeutic approach in CMT1A (Jang et al., 2012).
HNPP in contrast to CMT, can be entirely asymptomatic until triggered and then present with episodes of recurrent painless focal motor and sensory peripheral mono-neuropathies. Characteristically, symptoms are limited to areas of so called 'entrapment' like wrists, elbows,

knees and shoulders. Thus, alterations in the same *gene loci* of chromosome 17p11.2 can result in utterly different clinical phenotypes (Rana and Masroor, 2012).

1.2.1 Charcot-Marie-Tooth disease type 1A

CMT1A is a non-lethal, slowly progressive neuromuscular disorder. Age of onset is usually within the first two decades of life (Reilly et al., 2011). Patients suffer from a wide range of symptoms with varying degrees of severity.

Characteristic for CMT1A is distally pronounced muscle wasting and consecutive weakness. Furthermore, typical symptoms include steppage gait, impaired fine motor skills, distal sensory impairment, hyporeflexion (in particular altered tendon reflexes) and skeletal deformities (pes cavus formation **[Figure 1]**) (Pareyson, 2004; d'Ydewalle et al., 2012; Patzkó and Shy, 2011). Apart from peripheral demyelination and consecutive axonal loss, so called onion bulb formations **[Figure 2]** in peripheral nerve biopsies are distinctive findings in CMT1A (Reilly, 2007).

Furthermore, it was recently shown that approximately 16 % of patients with CMT1A suffer from clinical depression due to slowly progressive loss of physical abilities and uncertain predictions regarding their respective future quality of life (e.g. being wheelchair-bound) (Ribiere et al., 2012).

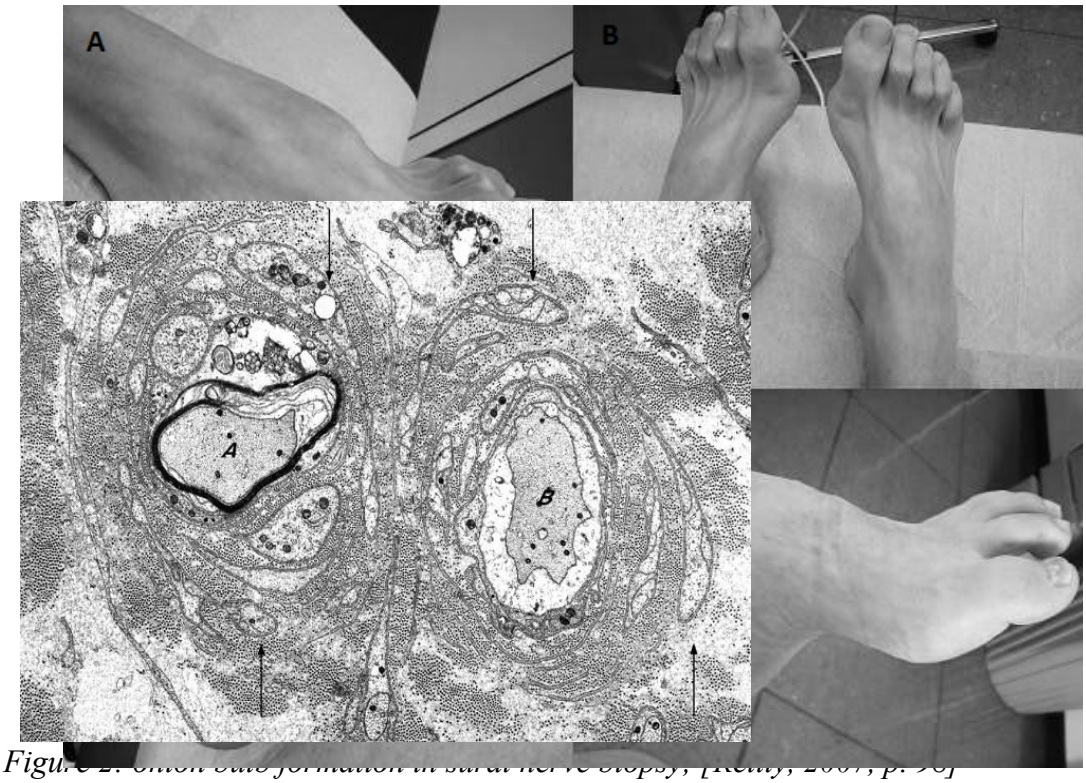

Figure 2: Onion bulb formation in sural nerve biopsy, [Reilly, 2007, p. 98]

(A) thinly myelinated and (B) completely demyelinated axons as characteristic findings in sural nerve biopsies in a patient suffering from CMT1. The onion bulb formation consists of multiple dysfunctional Schwann cell processes (arrows) (Reilly, 2007).

patient to perform a dorsal flexion due to advanced atrophy of peroneus muscles. On this account the patient in A and B suffers from steppage gait. [Photos by M. Mannil]

1.2.2 Charcot-Marie-Tooth Neuropathy Score (CMTNS)

The most common clinical score to classify the severity of patients suffering from CMT1A is so far the *Charcot-Marie-Tooth Neuropathy Score* (CMTNS) **[Table 1]** (Shy et al., 2005). This scoring system consists of nine parameters, which are evaluated on a scale from 0 to 4. The single parameter subscores are added. Thus, the maximum CMTNS-value can amount to 36 points. The disease severity positively correlates with the number of CMTNS points achieved during a clinical examination performed by a physician. The CMTNS (Version 1) is derived from the Total Neuropathy Score (TNS) (Cornblath et al., 1999) [**Table 3**] and adapted to the special symptoms of CMT patients. The ratio between motor and sensory symptoms in the CMTNS is unbalanced in favour of motor symptoms. There are five motor and just four sensory parameters included in this clinical scoring system (Shy et al., 2005).
Recently the CMTNS was published in its second and revised version (CMTNS2) (Murphy et al., 2011) **[Table 2].** In this revised version spoken instructions are provided. The scaling of

the parameters was slightly changed and the often absent Sensory Nerve Action Potential (Ulnar SNAP) parameter was omitted and replaced with Radial Sensory Action Potentials (Radial SAP). Nevertheless, the CMTNS (Version 1) is still the most commonly used scoring system in clinical settings and trials (Pareyson et al., 2011).

Table 1 *Charcot-Marie-Tooth disease neuropathy score*

	Score				
Parameter	0	1	2	3	4
Sensory symptoms	None	Limited to toes	Extend up to and may include ankle	Extend up to and may include knee	Extends above knees
Motor symptoms					
Legs	None	Trips, catches toes, slaps feet	AFO on at least 1 leg or ankle support	Cane, walker, ankle surgery	Wheelchair most of the time
Arms	None	Difficulty with buttons/zippers	Unable to do buttons or zippers but can write	Can not write or use keyboard	Proximal arms
Pin sensibility	Normal	Reduced in fingers/toes	Reduced up to and may include wrist/ankle	Reduced up to and may include elbow/knee	Reduced above elbow/knee
Vibration	Normal	Reduced at fingers/toes	Reduced at wrist/ankle	Reduced at elbow/knee	Reduced above elbow/knee
Strength					
Legs	Normal	4+, 4, or 4− on foot dorsiflexion	≤3 Foot dorsiflexion	≤3 Dorsiflexion and plantar flexion	Proximal weakness
Arms	Normal	4+, 4, or 4− on intrinsics or finger extensors	≤3 Intrinsics or finger extensors	<5 Wrist extensors	Weak above elbow
Ulnar CMAP	>6 mV	4.0–5.9 mV	2.0–3.9 mV	0.1–1.9 mV	Absent
(Median)	(>4 mV)	(2.8–3.9)	(1.2–2.7)	(0.1–1.1)	(Absent)
Ulnar SNAP	>9 μV	6.0–8.9 μV	3.0–5.9 μV	0.1–2.9 μV	Absent
(Median)	(>22 μV)	(14.0–21.9)	(7.0–13.9)	(0.1–6.9)	(Absent)
Total (max. 36)					

AFO = ankle-foot orthosis; CMAP = compound muscle action potential; SNAP = sensory nerve action potential.

Table 1: Charcot-Marie-Tooth Neuropathy Score (Version 1) [Shy et al., 2005, p. 1210]

The Charcot-Marie-Tooth Neuropathy Score (Version 1) is used to assess the clinical disease severity of CMT patients of all common subtypes. The achieved subscores are added to a total score, which positively correlates with the individual disease affection. Thus, the minimum score is 0 points and the maximum score amounts to 36 points.

Score values of 10 and below, indicate mild affection. Score values between 11 and 20 indicate moderate affection and score values above 20 indicate a severe clinical phenotype (Shy et al., 2005).

Parameter	0	1	2	3	4
Sensory symptoms*	None	Symptoms below or at ankle bones	Symptoms up to the distal half of the calf	Symptoms up to the proximal half of the calf, including knee	Symptoms above knee (above the top of the patella)
Motor symptoms (legs)†	None	Trips, catches toes, slaps feet Shoe inserts	Ankle support or stabilization (AFOs) Foot surgery‡	Walking aids (cane, walker)	Wheelchair
Motor symptoms (arms)	None	Mild difficulty with buttons	Severe difficulty or unable to do buttons	Unable to cut most foods	Proximal weakness (affect movements involving the elbow and above)
Pinprick sensibility*,§	Normal	Decreased below or at ankle bones	Decreased up to the distal half of the calf	Decreased up to the proximal half of the calf, including knee	Decreased above knee (above the top of the patella)
Vibration‖	Normal	Reduced at great toe	Reduced at ankle	Reduced at knee (tibial tuberosity)	Absent at knee and ankle
Strength (legs)¶	Normal	4+, 4, or 4− on foot dorsiflexion or plantar flexion	≤3 on foot dorsiflexion or ≤3 on foot plantar flexion	≤3 on foot dorsiflexion and ≤3 on plantar flexion	Proximal weakness
Strength (arms)¶	Normal	4+, 4, or 4− on intrinsic hand muscles**	≤3 on intrinsic hand muscles**	≤5 on wrist extensors	Weak above elbow
Ulnar CMAP (median)	≥6 mV (≥4 mV)	4–5.9 mV (2.8–3.9)	2–3.9 mV (1.2–2.7)	0.1–1.9 mV (0.1–1.1)	Absent (absent)
Radial SAP amplitude, antidromic testing	≥15 μV	10–14.9 μV	5–9.9 μV	1–4.9 μV	<1 μV

AFO, ankle-foot orthoses; CMAP, compound muscle action potential; SAP, sensory action potential.
*Use the picture below to discriminate the level of the symptoms.
†Uses aid *most of the time.* The patient was prescribed to wear/use or should be wearing/using the aid in the examiner's opinion (see written instructions, Table S2).
‡See written instructions for details of eligible foot surgery.
§Abnormal if patient says it is definitely decreased compared to a normal reference point.
‖Use Rydel-Seiffer tuning fork. Definition of normal: ≥5.
¶Limb strength scores refer to MRC grade.
**Intrinsic hand muscles strength assessment: test only abductor pollicis brevis (APB) and first dorsal interosseus (FDI), then choose the stronger to give the score.

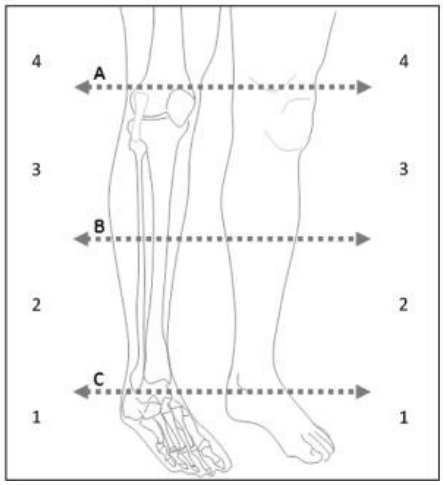

Table 2: CMTNS2 (Version 2) (second and revised version) [Murphy et al., 2011, p. 193]

In the revised version of the CMTNS (Version 2) the parameter Ulnar SNAP was omitted and replaced with Radial SAP. Other parameters e.g. ankle surgery, usage of buttons and AFO were altered. Furthermore, detailed spoken instructions (in English language) are provided.

Parameter	Score				
	0	**1**	**2**	**3**	**4**
QST = quantitative sensory test; ULN = upper limit of normal; LLN = lower limit of normal.					
sensory symptoms	none	symptoms limited to fingers or toes	symptoms extend to ankle or wrist	symptoms extend to knee or elbow	symptoms above knees or elbows, or functionally disabling
motor symptoms	none	slight difficulty	moderate difficulty	require help/assistance	paralysis
autonomic symptoms, n	0	1	2	3	4 or 5
pin sensibility	normal	Reduced in fingers/toes	Reduced up to wrist/ankle	Reduced up to elbow/knee	Reduced to above elbow/knee
vibration sensibility	normal	reduced in fingers/toes	reduced up to wrist/ankle	reduced up to elbow/knee	reduced to above elbow/knee
strength	normal	mild weakness	moderate weakness	severe weakness	paralysis
tendon reflexes	normal	ankle reflex reduced	ankle reflex absent	ankle reflex absent, others reduced	all reflexes absent
vibration sensation (QST vibration)	normal to 125% ULN	126 to 150% ULN	151 to 200% ULN	201 to 300% ULN	>300% ULN
sural amplitude	Normal/ reduced to <5% LLN	76 to 95% of LLN	51 to 75% of LLN	26 to 50% of LLN	0 to 25% of LLN
peroneal amplitude	Normal/ reduced to <5% LLN	76 to 95% of LLN	51 to 75% of LLN	26 to 50% of LLN	0 to 25% of LLN

Table 3: Total Neuropathy Score (TNS) [modified from Cornblath et al., 1999]

The sensory symptoms in the CMTNS (Shy et al., 2005) include the affected area of impairment (limited to toes, ankle, knee or above). Furthermore, it includes the pin sensibility, which is examined by using a sharp edged device and a cotton bud on different areas of a blind-folded patient. Another method being used in the CMTNS to determine sensory symptoms is using a Reidel-Seiffer-Tuning-Fork to determine pallesthesia. Sensory symptoms also include sensory neurological action potentials (SNAP) measured using electroneurography on ulnar or alternatively median nerves. Due to the high incidence of carpal tunnel syndromes in the general population, measurements on the ulnar nerve are preferred. Motor symptoms are assessed by taking the patient's history (usage of walking aids, fine motor skills), by measuring the strength of arms and legs according to the Medical Research Council (MRC) Scale for muscle strength and by measuring the compound muscle action potential (CMAP) by electroneurography on ulnar (preferred) or median nerves.
The overall Charcot-Marie-Tooth Neuropathy Score reliably states the grade of affection. A CMTNS < or = 10 shows mild, a CMTNS from 11 to 20 shows moderate and a score equaling 21 or above indicates severe affection (Shy et al., 2005).

1.3 Secondary clinical outcome measures

Secondary clinical outcome measures represent alternative examination techniques in order to improve the assessment of disease severity in CMT patients. These examinations include the Overall Neuropathy Limitations Scale (ONLS), 9-hole-peg test, 10m-timed walking as well as extensive dynamometry tests. These items were proven to provide sufficient intra- and inter-rater reliability and represent promising additions to future score generations (Solari et al., 2008).

The Overall Neuropathy Limitation Scale (ONLS) includes the patients ability to perform everyday tasks and grades the impairment of lower and upper extremities summarized in a short form (Graham and Hughes, 2006). The 9-hole-peg test is used to evaluate the fine motor skills in patients suffering from various diseases. The patients need to place nine pegs in preformed holes and retrieve them afterwards. The examination is performed with both the dominant and non-dominant hand and is timed (Earhart et al., 2011). The 10m-timed walking is performed barefoot on even ground. For the dynamometry tests, a CITEC©-handheld

dynamometry device was used, performing different subsets of tests, for instance the three-point grip, pinch-grip, fist-grip, foot dorsal and doot plantar measurements (Solari et al., 2008; Spink et al., 2010).

1.4 Aims and perspective

The aim of this prospective, multi-centre clinical trial is to identify new secondary clinical outcome measures and validate them in a subset of CMT1A patients. At a later stage, this very information will be used to clinically validate biomarkers in skin biopsies and whole blood.

New outome measures are needed, since the current scoring systems like the CMTNS are reliable and valid to distinguish between mildly and severely affected patients, but reach their limitations especially with regard to change over time. The CMTNS changes merely 0.2-0.686 points/ year and there are indications that the progression rate increases with age (Shy et al., 2008; Pareyson et al., 2011). In a slowly progressive neuropathy like CMT1A, biomarkers as well as valid and reliable clinical outcome measures are desirable, in order to see a significant effect of therapeutic trials within a reasonable amount of time.

The main goal regarding the clinical outcome measurements is to first establish secondary clinical outcome measures in CMT1A as putative additions to the current scoring systems and to validate the currently frequently used CMTNS. In a second step all patients from Germany will be re-examined after 2.5 to 3 years, in order to investigate the aspect of change over time.

2 Material and methods

2.1 Material for patient examination

sterile surgical gloves	Paul Hartmann AG, Heidenheim
syringe 1 ml	Becton Dickinson GmbH, Heidelberg
disposables needles	Becton Dickinson GmbH, Heidelberg
underlayment sheet, Moli Nea plus L	Paul Hartmann AG, Heidenheim
desinfection spray, Kodan Tinktur forte	Schülke & Mayr GmbH, Norderstedt
disposable tweezers	Department of Neurosurgery, UMG
Biopsy Punch, 3mm diameter	Stiefel GmbH, Wächtersbach
sterile cotton balls	Paul Hartmann AG, Heidenheim
sterile Falcon	Becton Dickinson GmbH, Heidelberg
scalpel, Techno cut	HDM Healthcare, Hereford, UK
Steri Strip	3M Health Care, St. Paul, USA
Durapore patches	3M Health Care, St. Paul, USA
CryoPure container, 1.6 ml (red, yellow)	Sarstedt AG & Co, Nümbrecht
Q-Tips	Karl Beese Verbandstoffe, Barsbüttel

Table 4: Disposable items

Xylocain, 1 %	AstraZeneca GmbH, Wedel
4 % PFA	MPI Experimental Medicine
RNAlater©	AMBION Inc., Austin, USA

Table 5: Chemicals

9 peg hole test	Sammons Preston, Illinois, USA
CITEC© Dynamometry	CIT Technics, Haren, NL
electronic time clock	Aristo, USA

Table 6: Devices

Office Word 2010	Microsoft, Seattle, USA
Office Excel 2010	Microsoft, Seattle, USA
Libre Office 3.4.4	Document Foundation, Open Source
MiKTeX, LaTeX	C/o Christian Schenk, OpenSource
Statistica 9	StatSoft, Hamburg
Statistical Software Package R version 2.15.0	The R Project For Statistical Computing, OpenSource
Adobe Illustrator CS5	Adobe Systems, San Jose, USA

Table 7: Software

2.2 Material for RNA purification from human whole blood

QIAamp® RNA Blood Mini Kit	Qiagen, Hilden
pipets and sterile, RNase-free pipet tips	Qiagen, Hilden
microcentrifuge with rotor for 2 ml tubes	EuroClone, Mini Speedy, Milano, Italy
ethanol (96-100 %)	MPI Experimental Medicine
70 % ethanol in water	MPI Experimental Medicine
14.3 M β-mercaptoethanol (β-ME)	Qiagen, Hilden
sterile, disposable, polypropylene tubes (1.5-15 ml depending on sample size)	Qiagen, Hilden

Table 8: RNA purification material

2.3 Protocol for RNA purification from human whole blood[1]

1. Material: 1.5 ml whole blood in EDTA-K tube (cooled on ice for maximum 4 h, if not purified immediately)
2. Mix 1.5 ml of human whole blood with 7.5 ml of buffer EL
3. Incubate for 10-15 min on ice. Vortex shortly twice during incubation.
4. Centrifuge at 400 g for 10 min at 4°C and completely remove and discard supernatant.
5. Add Buffer EL to the cell pellet (twice the amount of Buffer EL compared to the amount of whole blood). Vortex briefly.
6. Centrifuge at 400 g for 10 min at 4°C and completely remove and discard supernatant.
7. Add Buffer RLT to pelleted leukocytes according to the table below. Vortex or pipet to mix:

Buffer RLT (µl)	**whole blood (ml)**	**No. of leukocytes**
350	Up to 0.5	Up to 2 x 10^6
600	0.5 to 1.5	2 x10^6 to 1 x 10^7

Table 9: Amount of Buffer RLT [Riemann et al., 2007][1]

2.4 Patient collective

The study '*Validation of prognostic and diagnostic markers in Charcot-Marie-Tooth disease type 1A (HMSN1A)*' was initiated in Göttingen in 2009. The patients were examined within Germany in Munich and Göttingen. Apart from that, CMT1A patients from Italy, Spain and Czech Republic were also included for the finding of secondary outcome measures and the establishment of biomarkers. Additionally, baseline data of the ascorbic acid trial in Italy and the United Kingdom were used as well (Pareyson et al., 2011). A total of 479 patients with genetic proof of CMT1A by duplication of pmp22 on chromosome 17p11.2 have been examined. The inclusion and exclusion criteria to participate in the multi-centre, prospective study are mentioned below **[Table 10]**. In near future further patients will also be recruited in France, United Kingdom, Belgium and Münster/ Germany. The eligibility criteria as well as the exact examination protocols remain unchanged.

1 modified from RNA Blood mini kit, Qiagen, Hilden

Inclusion criteria	**Exclusion criteria**
informed consent	other neurological disease (acute or prior)
genetical evidence of CMT1A	age <18 and >70 years
clinical manifestation of CMT1A	(pre-) existence of any prior severe internal or psychiatric diseases
no fulfilment of any exclusion criteria	drug, substance or alcohol dependencies
	receptive or global aphasia
	participation in other clinical trials within the last 8 weeks

Table 10: Inclusion and exclusion criteria for participation

2.4.1 Patient recruitment

Since CMT1A is considered to be a rare neurological disease (Murphy et al., 2012) and due to strict study in- and exclusion criteria, the patient recruitment proved to be cumbersome. In order to still recruit a cohort of 479 patients various sources were used. In Germany for instance, patient initiatives (e.g. *Deutsche Gesellschaft für Muskelkranke e.V.*) and referrals from other medical institutions (University Clinic Münster, Rehabilitation Clinic Hoher Meissner, Klinikum Kassel, etc.) were one source of putative study participants.

The recruitment was extended to announcements in specific internet forums (*http://www.dgm.org/dgm-forum/*; *http://www.hmsn.de; http://www.intakt.info/forum/*) and on the webpage of the Max-Planck-Institute For Experimental Medicine **[Figure 3]** and the Universitätsmedizin Göttingen (UMG) (*http://www.em.mpg.de/index.php?id=279; http://www.neurologie.uni-goettingen.de/index.php/cmt1a-studie.html*) [information retrieved on 10th December 2012].

Apart from patient-focused advertisement, selected medical professionals in the field of rehabilitation medicine, neurology and orthopedic surgery were informed about the prospective clinical trial, in order to raise awareness among physicians and to increase the number of referrals. Due to the autosomal dominant inheritance pattern of CMT1A, relatives

of already recruited patients were an additional source of study participants, while doctor-patient confidentiality was remained at all times. Furthermore, the patient data were pseudonymised, indicating only the country of examination for the purpose of detecting cohort-specific batch effects.

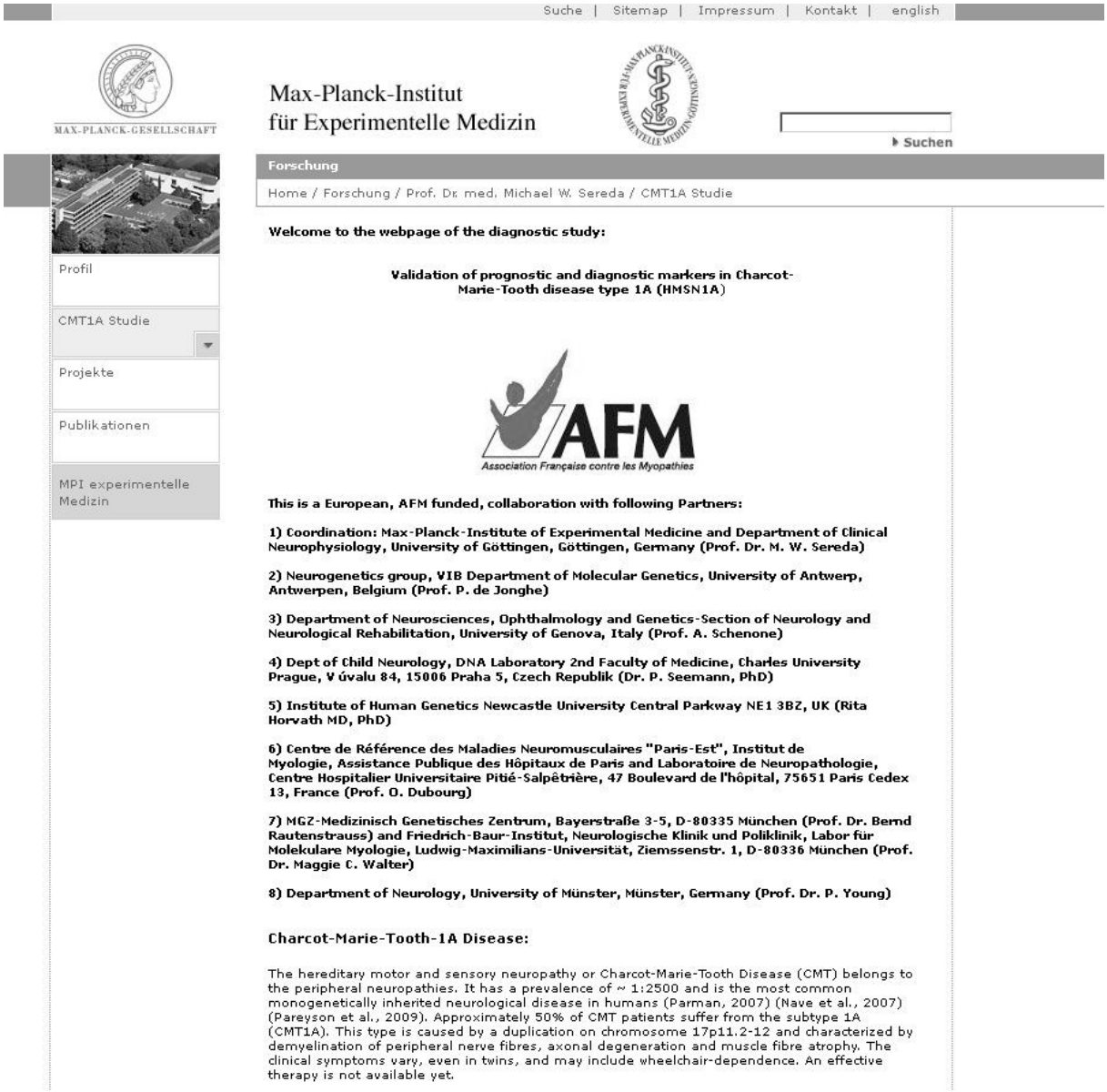

Figure 3. Webpage of CMT1A Trial in Göttingen
information retrieved on 23rd July 2013 under http://www.em.mpg.de/index.php?id=279

Further information on the patient collective is described in section **3.1**.

2.5 SOP – Standard Operation Procedure

1. Provide sufficient information material about the study >72h before the date of the examination. Obtain informed consent at the day of the examination; answer all arousing questions.
2. Take a complete and thorough history of the patient with special focus on age of onset, associated symptoms/ diseases, side medications, inheritance (draw pedigree), prior surgeries and usage of walking aids (AFO etc.).
3. Handheld-dynamometry, CITEC©: start with non-dominant hand; fist grip (patient presses device by closing his/ her fist); three point grip (patient uses Dig. I,II,III); pinch grip (patient uses Dig. I,II); foot plantar flexion; foot dorsal extension[2]; verbal encouragement is mandatory;
4. 9-peg-hole-test: start with non-dominant hand; patient has to fill in all 9 holes with the provided pins and remove them again afterwards; make sure patients do not use more than one pin at a time; measure the time from start of examination till the last pin is being removed; repeat three times per side;
5. Conduct complete neurological physical examination (including reflexes, pallesthesia, etc.)
6. Patient has to fill in the SF-36 questionnaire and mark a visual analogue scale (10cm) corncerning current subjective pain perception)
7. Take blood for general parameters (including VitB12, TSH, fT3, fT4, infectious parameters and HbA1c); Take additional 3ml EDTA Blood and immediately cool on ice; perform RNA purification within 3 hours after drawing blood, utilizing the Quiagen™-QIAmp-RNA-Blood-Mini-Kit; Store in Eppendorf-tube™ at -80°C;
8. Perform electrophysiological examination; preferably with SNAP/ CMAP (base to peak) from Ulnaris nerve, due to high incidence of carpal tunnel syndrome;
9. Perform skin biopsy from medial index finger of the non-dominant hand (diameter 3mm); proceed in a sterile manner (use sterile biopsy punches, scalpels, tweezers, cotton wool, etc.); disinfect finger and surrounding area with disinfectant spray; wipe finger with a sterile cloth once; repeat disinfection three times; give local anaesthesia

2 Usage of a leg fixation device in foot plantar flexion and foot dorsal extension is highly recommended (Solari et al., 2008)

(0.5-2ml lidocaine); wait for several minutes, till the finger becomes numb; repeat disinfection procedure; wear surgical gloves; turn biopsy into the finger without using force; lift the biopsy with sterile tweezers; cut it from below with a disposable scalpel; divide biopsy and put 1st half in RNAlater™ (store for 24 hours in 4°C and afterwards in -20°C); put 2nd half in 4% PFA (max. 1 week old); Close the 3mm wound with 3M-Steri-Strips™, sterile cotton wool and a white plaster;

10. Send a physician report about the findings of the general blood results and the physical examination to the patient's general practitioner;

3 Results

3.1 Descriptive analysis

The BMBF- and AFM- funded clinical prospective study ‚*Validation of prognostic and diagnostic markers in Charcot-Marie-Tooth disease type 1A (HMSN1A)*' was initiated in 2009 in Göttingen and aims at generating biomarkers in skin and whole blood and furthermore at validating secondary clinical outcome measures (e.g. CITEC© handheld dynamometry examinations, 10m-timed walking, 9-peg-hole tests, etc.) as putative additions to current scoring systems.

This thesis emphasizes on the analysis of secondary clinical outcome measures in CMT1A:

479 patients with diagnosed Charcot-Marie-Tooth disease type 1A were included in this clinical prospective trial. These 479 patients were recruited for the biomarker trial in 5 different centers, namely Italy (it), Spain (spa), Czech Republic (cz), Munich (mue) as well as Göttingen (goe) and in context of the baseline assessments of the ascorbic acid trial in Italy and the United Kingdom (Pareyson et al., 2011). Overall there are 58 % female and 42 % male patients, while the median age of examination is 42 years. The median Charcot-Marie-Tooth Neuropathy Score (CMTNS) - as depicted in **[Table 11] -** is 13. The Body Mass Index (BMI) is 25 kg/m² and the vast majority of patients neither suffer from any side diagnoses, nor do they smoke (89 %) or frequently drink alcohol (99 %). The different cohorts represent well the averagely affected patients suffering from CMT1A in Western Europe.

The first 21 patients were already re-examined exactly 3 years after the initial examination and the preliminary results of 10 patients are shown **[Table 12]**. Apart from the analysis of validity and reliability of secondary clinical outcome measures, the item ‚change over time' is of particular interest in score generation as well as in investigation of biomarkers. Even though the final analysis with respect to ‚change over time' will be performed as soon as previous participants from Munich will be re-examined, preliminary findings suggest an increase in the CMTNS and therefore an aggravated clinical phenotype with time.

For better comparison a cohort of 26 healthy controls was recruited additionally for the study **[Table 13]** and reference values of the CITEC© handheld dynamometry were used.

Parameter	Statistics	Median (min.; max.)	NAs
gender			0
| m	202 (42%)		
| w	277 (58%)		
date of Birth	1966-02-12	1965-09-30 (1936-02-20; 1993-12-03)	1
age at examination [years]	42 ± 13	43 (18; 71)	0
genetic proof			0
| no	7 (1%)		
| yes	472 (99%)		
family history			275
| neg	21 (10%)		
| pos	183 (90%)		
imprinting			328
| maternal	67 (44%)		
| paternal	77 (51%)		
| sporadic	7 (5%)		
weight [kg]	70 ± 15	68 (1; 160)	3
height [cm]	168 ± 9.1	168 (147; 193)	13
CMTNS [0/…/36]	14	13 (3; 32)	4
CMAP (medianus) [mV]	2.9 ± 2.5	2.5 (0; 16)	286

Parameter	Statistics	Median (min.; max.)	NAs
SNAP (medianus) [µV]	1.8 ± 4.5	0 (0; 55)	43
10m walk test [sec]	8.4 ± 4.5	7.3 (2.8; 40)	7
9 peg hole test (dominant hand) [sec]	24 ± 11	22 (8.8; 165)	5
9 peg hole test (non-dominant hand) [sec]	26 ± 10	23 (15; 141)	8
visual analogue scale [mm]	32 ± 30	23 (0; 100)	4
fist grip (dominant hand) [kg]	172 ± 85	156 (0; 476)	4
three point grip (dominant hand) [kg]	122 ± 59	116 (6; 320)	6
pinch grip (dominant hand) [kg]	58 ± 37	49 (5.2; 276)	277
foot dorsal (dominant foot) [kg]	119 ± 100	100 (0; 892)	12
foot plantar (dominant foot) [kg]	183 ± 122	160 (0; 910)	9
fist grip (non-dominant hand) [kg]	162 ± 84	141 (12; 486)	276
three point grip (non-dominant hand) [kg]	105 ± 53	100 (8.7; 302)	277
pinch grip (non-dominant hand) [kg]	56 ± 34	48 (0; 236)	277
foot dorsal (non-dominant foot) [kg]	101 ± 86	73 (0; 319)	280

Parameter	Statistics	Median (min.; max.)	NAs
foot plantar (non-dominant foot) [kg]	160 ± 109	133 (0; 437)	278
sensory symptoms [0/1/2/3/4]	1.1	1 (0; 4)	4
motor symptoms legs [0/1/2/3/4]	1.2	1 (0; 4)	4
motor symptoms arms [0/1/2/3/4]	0.65	1 (0; 4)	4
pin sensibility [0/1/2/3/4]	1.5	2 (0; 4)	4
vibration [0/1/2/3/4]	2	2 (0; 4)	4
strength of legs [0/1/2/3/4]	1.5	1 (0; 4)	4
strength of arms [0/1/2/3/4]	0.96	1 (0; 4)	4
Ulnar CMAP (Median) [0/1/2/3/4]	1.8	2 (0; 4)	8
Ulnar SNAP (Median) [0/1/2/3/4]	3.4	4 (0; 4)	8
cohort			0
| cz	27 (6%)		
| goe_1	21 (4%)		
| goe_2	45 (9%)		
| it+uk	271 (57%)		
| mue_1	25 (5%)		

Parameter	Statistics	Median (min.; max.)	NAs
| mue_2	40 (8%)		
| spa	50 (10%)		
age of onset [years]	10 ± 10	6 (0; 40)	417
contraceptive			412
| yes	5 (7%)		
| no	62 (93%)		
problems in pregnancy			408
| yes	4 (6%)		
| no	67 (94%)		
nicotine			409
| no	56 (80%)		
| yes	10 (14%)		
| past	4 (6%)		
packyears	11 ± 15	0 (0; 42)	452
alcohol frequency			408
| no	51 (72%)		
| past	1 (1%)		
| yes_infrequent	19 (27%)		
| yes_often	0 (0%)		
alcohol amount [g/d]	10 ± 19	0 (0; 80)	415
thyroid			429

Parameter	Statistics	Median (min.; max.)	NAs
| no	44 (88%)		
| yes	6 (12%)		
| past	0 (0%)		
family	7.8 ± 3.7	9 (1; 15)	430
orthopedic shoes			429
| cane	1 (2%)		
| crutches	1 (2%)		
|crutches, hip operation	1 (2%)		
| no	46 (92%)		
| orthopedic shoes	1 (2%)		
CMAP (Ulnaris) [mV]	2.2 ± 1.6	2 (0.1; 5.5)	455
SNAP (Ulnaris) [µV]	2.6 ± 3.5	0.5 (0; 10)	463
BMI [kg/(m2)]	25 ± 4.4	25 (11; 49)	16

Table 11: Descriptive statistics

Parameter	Statistics	Median (min.; max.)	NAs
gender			0
| m	6 (60%)		
| w	4 (40%)		
date of birth	1965-01-30	1962-09-04 (1953-07-03; 1985-02-09)	0
age at examination [years]	48 ± 9	50 (27; 58)	0
genetic proof			0
| no	0 (0%)		
| yes	10 (100%)		
family history			0
| neg	1 (10%)		
| pos	9 (90%)		
imprinting			2
| maternal	2 (25%)		
| paternal	6 (75%)		
| sporadic	0 (0%)		
weight [kg]	78 ± 12	83 (56; 96)	0
height [cm]	174 ± 9.9	176 (154; 185)	0
CMTNS [0/…/36]	18	18 (14; 22)	0
CMAP (medianus) [mV]	1.2 ± 0.78	1.3 (0.3; 2.6)	0

Parameter	Statistics	Median (min.; max.)	NAs
SNAP (medianus) [µV]	6.7 ± 6	8.9 (0; 14)	0
10m walk test [sec]	12 ± 4.6	11 (6.3; 21)	0
9 peg hole test (dominant hand) [sec]	28 ± 7.5	28 (21; 40)	0
9 peg hole test (non-dominant hand) [sec]	30 ± 11	28 (19; 51)	0
visual analogue scale [mm]	38 ± 35	30 (0; 99)	0
fist grip (dominant hand) [kg]	145 ± 65	131 (74; 291)	0
three point grip (dominant hand) [kg]	88 ± 53	82 (25; 215)	0
pinch grip (dominant hand) [kg]	55 ± 21	54 (31; 103)	0
foot dorsal (dominant foot) [kg]	37 ± 25	35 (0; 76)	1
foot plantar (dominant foot) [kg]	89 ± 28	99 (41; 122)	1
fist grip (non-dominant hand) [kg]	137 ± 58	116 (73; 265)	0
three point grip (non-dominant hand) [kg]	98 ± 52	93 (17; 217)	0
pinch grip (non-dominant hand) [kg]	51 ± 21	47 (22; 91)	0
foot dorsal (non-dominant foot) [kg]	42 ± 24	40 (0; 80)	1

Parameter	Statistics	Median (min.; max.)	NAs
foot plantar (non-dominant foot) [kg]	86 ± 32	82 (45; 144)	1
sensory symptoms [0/1/2/3/4]	2.5	2 (2; 4)	0
motor symptoms legs [0/1/2/3/4]	1.6	1.5 (1; 3)	0
motor symptoms arms [0/1/2/3/4]	1.4	1 (1; 3)	0
pin sensibility [0/1/2/3/4]	2.1	2 (0; 3)	0
vibration [0/1/2/3/4]	2.8	3 (2; 4)	0
strength of legs [0/1/2/3/4]	1.5	1 (1; 3)	0
strength of arms [0/1/2/3/4]	0.8	1 (0; 2)	0
Ulnar CMAP (Median) [0/1/2/3/4]	2.4	2 (2; 3)	0
Ulnar SNAP (Median) [0/1/2/3/4]	2.7	2 (1; 4)	0
BMI [kg/(m2)]	26 ± 3.2	26 (19; 30)	0

Table 12: *Follow up subgroup;*

Descriptive values of the measured parameters on the follow-up evaluation of the treatment group. The second column contains mean ± sd where appropriate. For scores, the standard deviation was omitted. For categorical variables the fraction of the different levels in absolute values as well as percentages are shown instead.

Parameter	Statistics	Median (min.; max.)	NAs
gender			0
| m	14 (54%)		
| w	12 (46%)		
date of birth	1983-08-07	1984-01-06 (1963-07-06; 2011-06-02)	0
age at examination [years]	28 ± 7.4	27 (20; 47)	0
weight [kg]	72 ± 13	73 (49; 95)	0
height [cm]	177 ± 10	178 (156; 204)	0
10m walk test [sec]	4.7 ± 0.53	4.7 (3.4; 5.6)	1
9 peg hole test (dominant hand) [sec]	16 ± 1.8	16 (14; 20)	1
9 peg hole test (non-dominant hand) [sec]	17 ± 2.1	17 (14; 22)	1
date of inclusion	2011-05-12	2011-05-05 (2011-02-18; 2011-10-11)	0
BMI [kg/(m2)]	23 ± 2.8	23 (17; 29)	0

Table 13: Healthy controls;

Descriptive values of the measured parameters on the control group; The second column contains mean ± sd where appropriate. For scores, the standard deviation was omitted. For categorical variables the fraction of the different levels in absolute values as well as percentages are shown instead.

The remarkable amount of participants (n= 479) enabled the investigation of other aspects of the Charcot-Marie-Tooth disease type 1A as well. While CMT1A is well known for its autosomal dominant inheritance pattern, statements according to imprinting for instance vary. In our analysis, the CMTNS in maternally-inherited CMT1A patients is in tendency lower than in paternally-inherited ones **[Table 14]**, but the effect is not significant (according to $p \leq 0{,}05$). In this model, only age shows a significant impact on the CMTNS as an outcome measure for increasing disease severity.

For the analysis of anticipation, the data set is not sufficient yet, since individual study participants outnumber related ones of different generations. However, literature review indicates the existence of anticipation, which will be a matter of interest in future cohorts (Kovach et al., 2002; Steiner et al., 2008).

Other findings included complications during pregnancy, which 4 out of 71 females (6 %) stated and hypothyroidism, which 12 % of the mainly German cohort suffers from. Nevertheless, severe side effects cannot be seen, since they represent exclusion criteria in the clinical study setting.

	Estimate	Std. Error	t Value	Pr(> | t |)
(intercept)	-22.20	17.83	-1.24	0.22
imprinting	12.53	11.09	1.13	0.26
gender	17.19	10.86	1.58	0.12
age	1.24	0.43	2.87	0.00
imprinting:gender	-5.85	6.62	-0.88	0.38
imprinting:age	-0.41	0.27	-1.52	0.13
gender:age	-0.54	0.27	-2.01	0.05
imprinting:gender:age	0.22	0.16	1.34	0.18

Table 14: Imprinting in CMT1A

3.2 Evaluation of secondary clinical outcome measures

3.2.1 Spearman's ρ correlation

All secondary clinical outcome measurements were correlated and graphically depicted in a correlation matrix according to *Spearman's ρ* **[Figure 4]**. As the dominant and the non-dominant side of the same examination correlate highly, the measurements for the non-dominant limb were discarded from further analysis by agreement. This circumstance fits adequately the natural phenotype of CMT1A, since it is known to cause a symmetrical phenotype. The correlation of Ulnar SNAP (median) does not show strong correlations to any other parameter, while its motor counterpart (CMAP) does.

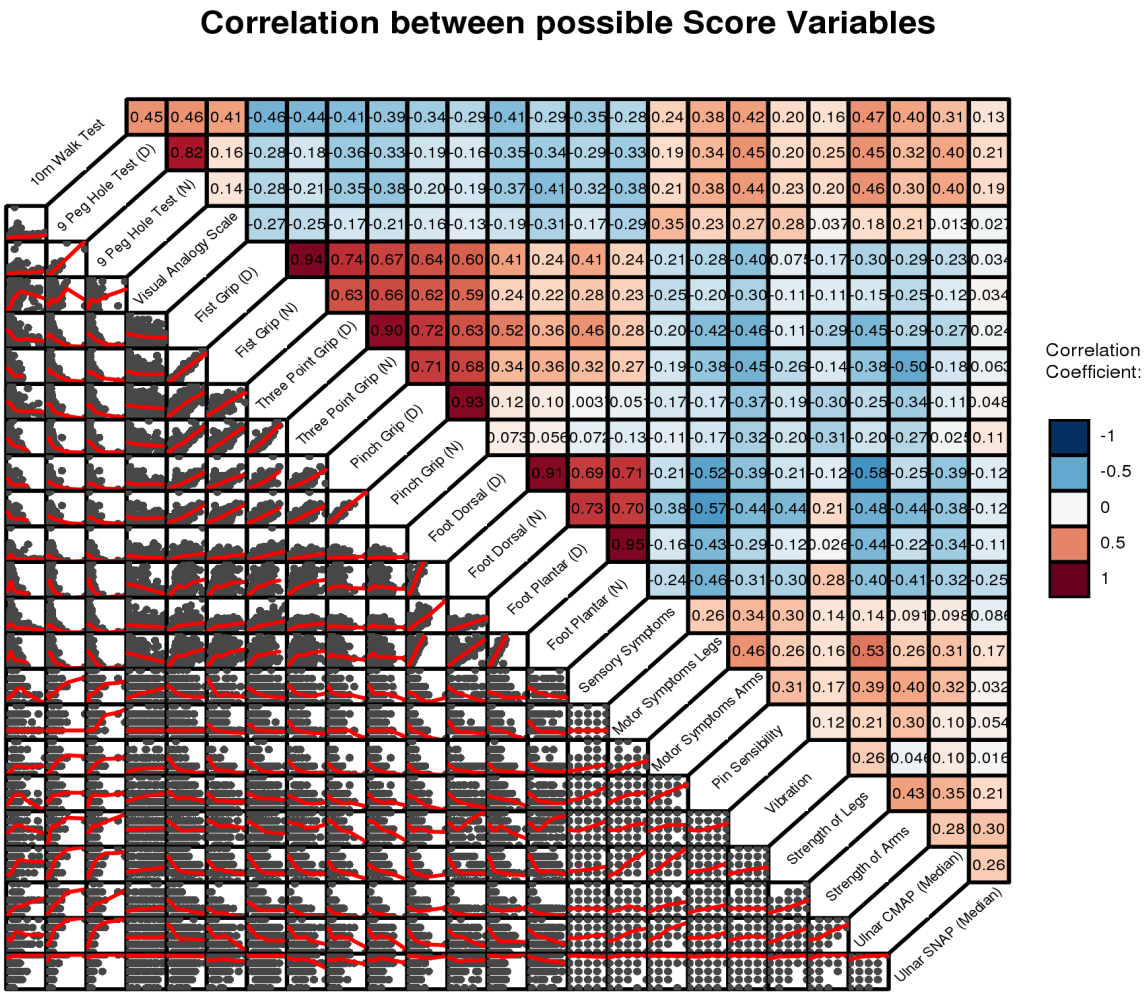

Figure 4: Correlation matrix, Spearman's ρ;

positive correlations are shaded in blue, while negative correlations are shaded in red.

The items sensory symptoms, motor symptoms arms/ legs, pin sensibility, vibration, strength arms/ legs, Ulnar CMAP and Ulnar SNAP are depicted in a binned (0-4) form. High correlations between all CITEC©-handheld dynamometry values of the upper limbs indicate that not all of these measurements are needed to outline the fine motor skills/ strength of the upper extremities.

3.2.2 Initial filtering

The visual analogue scale (VAS) for the perception of pain in everyday life does not show strong correlation to other candidate sub-scores (maximum correlation coefficient 0.41). Therefore, the VAS measurement was discarded from the analysis.

The measurement of the pinch grip was not executed in more than half of the patients, since the Italian cohort was initially recruited in the setting of a therapeutic trial with ascorbic acid with a slightly altered study protocol (Pareyson et al., 2011).

The SF-36 was excluded as a secondary clinical outcome measure, due to its unspecificity towards CMT1A and its too inconvenient interpretation to be considered a feasible clinical score parameter.

3.2.3 Binning of data

In order to achieve comparability with the CMTNS (Version 1), putative secondary clinical outcome measurements were categorized into five subgroups according to the degree of disease affection (0,1,2,3,4) by binning with respect to the 5-quantiles. The quantiles for the grip strength variables were computed separately according to the patients' sex **[Table *15*]** to account for gender-specific differences.

There was no significant difference in the CITEC©-dynamometry foot strength variables (foot dorsal and plantar dynamometry) to justify any gender stratification here.

Clinical Parameter	0	1	2	3	4
10m Walk Test [sec]	x ≤ 6	6 < x ≤ 7	7 < x ≤ 8	8 < x ≤ 10	x > 10
9 Peg Hole Test [sec]	x ≤ 19	19 < x ≤ 21	21 < x ≤ 23	23 < x ≤ 27	x > 27
Fist Grip [N] | m | w	 x ≥ 275 x ≥ 208	 275 > x ≥ 208 208 > x ≥ 154	 208 > x ≥ 162 154 > x ≥ 126	 162 > x ≥ 118 126 > x ≥ 92	 x < 118 x < 92
Three Point Grip [N] | m | w	 x ≥ 188 x ≥ 158	 188 > x ≥ 138 158 > x ≥ 120	 138 > x ≥ 112 120 > x ≥ 92	 112 > x ≥ 77 92 > x ≥ 68	 x < 77 x < 68
Foot Dorsal [N]	x ≥ 192	192 > x ≥ 122	122 > x ≥ 72	72 > x ≥ 30	x < 30
Foot Plantar [N]	x ≥ 274	274 > x ≥ 188	188 > x ≥ 136	136 > x ≥ 80	x < 80

Table 15: Binning of data with respect to gender-specific differences

3.2.4 Unbiased approach

In order to evaluate secondary clinical outcome measures in context of the Charcot-Marie-Tooth Neuropathy Score, the binned data of 479 patients is being divided into significant amounts of clusters using a heat diagram and various validation methods *(average proportion of non-overlap (APN), average distance between means (ADM), average distance (AD), figure of merit (FOM), Connectivity, Dunn, Silhouette)*. Ideal parameters of putative scoring systems are intended to distinguish better between these aforementioned clusters.

Furthermore, the information gain of the parameters of the classical CMTNS and the putative secondary clinical outcome measures is shown by an Analysis of Variance (ANOVA). The parameters are being added one at a time for further analysis, in order to explore the significance ($p \leq 0.05$) of the information gain in distinguishing between the various clusters.

In a last step, different modified versions of the Charcot-Marie-Tooth Neuropathy Score are being compared with regard to scope of distribution.

Ideally, a new modified score can add valuable information, shown by better distinguishment of natural clusters and also distributes more evenly and widely than an un-modified CMTNS.

3.2.5 Hierarchical clustering

The heat map **[Figure 5]** depicts the binned data of primary (CMTNS) and secondary clinical outcome measures in 479 patients suffering from CMT1A. The colour-coding represents the degree of severity (0-4). While dark-blue shaded fields represent normal performance (0), lighter shades indicate impairment in performance of the respective clinical parameter (graded 1-4). A vertical section of the heat map shows the performance of a random single study participant. Horizontal sections feature the various score items.

A hierarchical clustering was performed on all study participants and all available parameters, followed by certain validation techniques (*average proportion of non-overlap (APN), average distance between means (ADM), average distance (AD), figure of merit (FOM), Connectivity, Dunn, Silhouette*) (Brock et al., 2011) **[Table 16]**. The results of these validation methods indicate two clusters of patients as confirmed by rank aggregation (Pihur et al., 2009).

The graphical depiction of the two clusters in comparison to the classical CMTNS as box-plots **[Figure 5]**, reveals that the two clusters represent a weakly and a severly affected cohort of CMT1A patients. The density plots emphasize the limitations of the current CMTNS, since it shows a significant overlap of the two clusters and therefore poor discrimination between these clusters. This finding justifies the need for a modified scoring system.

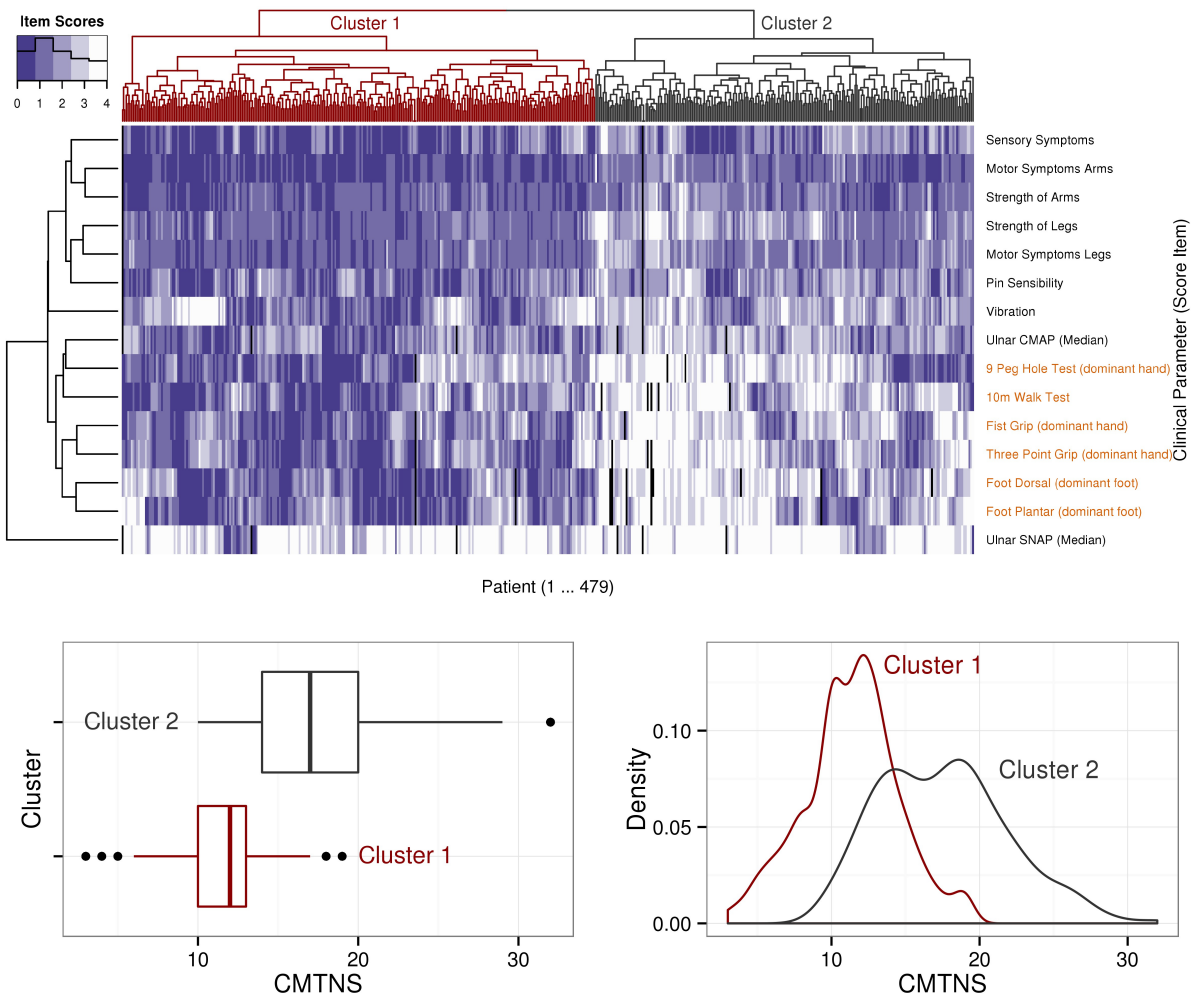

Figure 5: Hierarchical clustering

The hierarchical clustering of the 479 CMT1A patients results in 2 cohorts. They represent a mildly and severely affected set of patients. The CMTNS (Version 1) is unable to distinguish well between these two cohorts. Future outcome measures will be tested for their discriminatory power to distinguish between cluster 1 and 2.

Validation Method	Clusters
APN	3
AD	6
ADM	2
FOM	6
Connectivity	2
Dunn	2
Silhouette	3

Table 16: Validation methods for number of clusters

Due to various validations methods and following rank aggregation, the statistically best fitting model consists of 2 patient clusters.

3.2.6 Analysis of Variance (ANOVA)

An Analysis of Variance (ANOVA) is conducted on the scored primary and secondary clinical parameters of all CMT1A patients with the two main clusters as targets **[Table 17]**. The parameters are added one at a time starting with the primary parameters and followed by the secondary ones (shaded blue). The analysis shows the significance of the information gain in cluster differentiation.

Parameter	p-value
Strength of Legs	**< 0.001**
Motor Symptoms Arms	**< 0.001**
Sensory Symptoms	**< 0.001**
Ulnar CMAP (Median)	**< 0.001**
Pin Sensibility	**0.005**
Strength of Arms	0.041
Motor Symptoms Legs	0.130
Vibration	0.291
Ulnar SNAP (Median)	0.468
Foot Dorsal (dominant foot)	**< 0.001**
Fist Grip (dominant hand)	**< 0.001**
10m Walk Test	**< 0.001**
Foot Plantar (dominant foot)	**0.005**
Three Point Grip (dominant hand)	0.673
9 Peg Hole Test (dominant hand)	0.717

Table 17: Analysis of Variance (ANOVA)

By an Analysis of Variance the discriminatory power between the 2 clusters of all outcome measures was tested. The primary outcome measures of the CMTNS (Version 1) were given priority. Still, four secondary outcome measures provide additional significant information in differentiation between the two clusters.

The Analysis of Variance displays that five out of nine parameters of the original CMTNS, namely *Strength of Legs, Motor Symptom Arms, Sensory Symptoms, Ulnar CMAP (Median)* and *Pin Sensibility* actually contribute significantly - in this ranked order - to distinguish between the two aforementioned clusters.

Four out of six tested secondary clinical outcome measures contribute additional information in distinguishing between the two patient clusters, after already taking into account the information of the nine parameters of the classical CMTNS.

3.3 Score Hypotheses

Several score hypotheses are postulated regarding the results of the Analysis of Variance (ANOVA) **[Table 17]**.

1. Score hypothesis 1 (CMTNS_full) includes all nine primary and the whole set of secondary clinical outcome measurements (max. 60 points).
2. Score hypothesis 2 (CMTNS_signif) contains all primary outcome measurements of the classical CMTNS and adds the significant parameters of the secondary clinical outcome measurements (max. 52 points).
3. Score hypothesis 3 (CMTNS_mod) restricts the score to parameters that significantly add information in distinguishing between the two clusters. Therefore, the four primary and two secondary clinical outcome measurements that do not show any significant information gain are left out (max. 36 points).

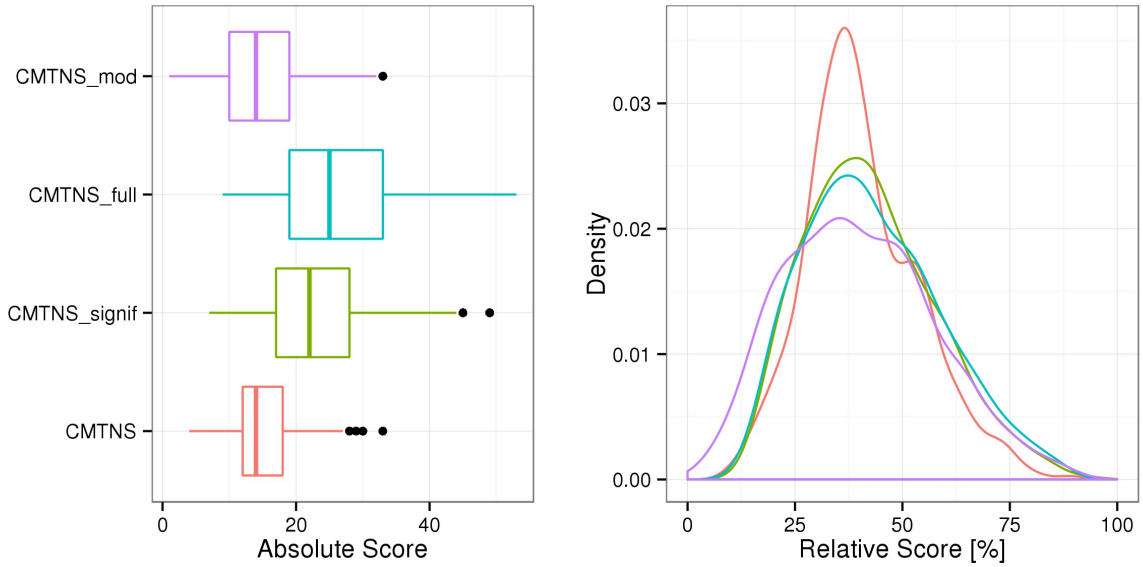

Figure 6: Score Hypotheses

The box plots show the different score hypotheses in absolute values. For better comparison a density plot with relative score values is shown. The CMTNS_mod provides the widest distribution and reaches both extreme points (0 and 36 points in absolute score values).

The graphical depiction of the absolute scores as box plots can only be utilized to compare the width/ distribution of the classical CMTNS and the CMTNS_mod, since both are based on nine score items (max. 36 points) **[Figure 6]**. In order to evaluate the distribution of the other two score hypotheses in this context, density plots are being generated using relative scores. The illustration displays that the classical CMTNS is focused on medium relative scores with a narrow distribution. The CMTNS_signif and CMTNS_full show a wider distribution, while the CMTNS_mod shows the widest distribution with least focus on medium relative scores. Furthermore, the CMTNS_mod is the only score that stretches to both its extreme points (0 and 100%), which represents a valuable quality criteria in score generation. Based on these results the modified score represents the best alternative to the classical CMTNS and is depicted below **[Table 18]**.

Parameter	0	1	2	3	4
strength of legs	*normal*	*4+, 4, 4- on foot dorsiflexion*	*</= 3 on foot dorsiflexion*	*</= 3 on foot dorsiflexion and </= 3 on foot plantar flexion*	*proximal weakness*
motor symptom arms	*none*	*difficulty with buttons/ zippers*	*unable to do buttons or zippers but can write*	*can not write or use keyboard*	*proximal arms*
sensory symptoms	*none*	*limited to toes*	*extend up to and may include ankle*	*extend up to and may include knee*	*extend above knee*
pin sensibility	*normal*	*reduced in fingers/ toes*	*reduced up to and may include wrist/ ankle*	*reduced up to and may include elbow/ knee*	*reduced above elbow/ knee*
fist grip (D) |m |f [Newton]	*x ≥ 275* *x ≥ 208*	*275 > x ≥ 208* *208 > x ≥ 154*	*208 > x ≥ 162* *154 > x ≥ 126*	*162 > x ≥ 118* *126 > x ≥ 92*	*x < 118* *x < 92*
10m walking test [seconds]	*x ≤ 6*	*6 < x ≤ 7*	*7 < x ≤ 8*	*8 < x ≤ 10*	*x > 10*
foot plantar (D) [Newton]	*x ≥ 274*	*274 > x ≥ 188*	*188 > x ≥ 136*	*136 > x ≥ 80*	*x < 80*
foot dorsal (D) [Newton]	*x ≥ 192*	*192 > x ≥ 122*	*122 > x ≥ 72*	*72 > x ≥ 30*	*x < 30*
Ulnar CMAP (Median)	*> 6 mV* *(> 4 mV)*	*4.0 - 5.9 mV* *(2.8 - 3.9 mV)*	*2.0 - 3.9 mV* *(1.2 - 2.7 mV)*	*0.1 - 1.9 mV* *(0.1 - 1.1 mV)*	*absent* *(Absent)*

Table 18: Modified CMTNS (CMTNS_mod)

The modified CMTNS consists only of significant parameters of the CMTNS (Version 1) and selected secondary clinical outcome measures. Non-significant parameters were omitted. Direct comparibility to the CMTNS (Version 1) is possible, due to the same amount of parameters and score levels (0-36).

4 Discussion

4.1 Study participants

The present analysis focuses on patients suffering from Charcot-Marie-Tooth disease type 1A in Western Europe. A total of 479 CMT1A patients were clinically examined within five European clinical centres (Italy, Spain, Czech Republic and Germany) and in context of baseline assessments of the ascorbic acid trial in Italy and the United Kingdom (Pareyson et al., 2011). The participants can be considered a representative cohort of CMT1A patients compared to the general population, since age, BMI and gender ratio reflect average values **[Table 11]**. The fact that the average CMTNS displays medium affection, needs to be interpreted carefully, since the CMTNS in general tends to vary little **[Figure 5]**. Taking into account the slow progression of the disease, its non-lethal nature, strikingly varying degrees of disease affection and the limited number of specialised study centres per country, CMT1A patients are possibly only willing to participate in non-therapeutic trials like these, if the psychological strain exceeds the effort of travelling to a remote location to be examined. Therefore, the allegedly averagely-affected cohort, is possibly more severely affected than expected. Apart from that, the cohort cannot be examined in terms of associated diseases/ complications. Even though minor side diagnoses and medications were noted, severe co-morbidities constituted exclusion criteria to the prospective clinical trial to avoid confounding factors. Nevertheless, the number of participants appears sufficient for score generation and represents one of the largest CMT1A cohorts ever examined.

4.2 Secondary clinical outcome measurements

Selected secondary clinical outcome measures are proven to be valid and to possess excellent inter- and intrarater reliability (Solari et al., 2008). These tools are easy to perform and do not need any invasive or potentially harmful diagnostic examinations. The use of secondary clinical outcome measures still needs to be restricted, in order to avoid redundancy, to test only characteristic features of the disease of interest and to retain the feasibility in an everyday clinical setting. Moreover, the quality of secondary outcome measures varies and some outcome measures, e.g. the dynamometry of the lower extremities, are controversial, since the examiner needs to fixate the patient's leg. Even though, the dynamometry usage in

CMT patients shows low measurement errors and an excellent retest-reliability (Burns et al., 2005), I highly recommend the usage of a leg fixation device (Solari et al., 2008) to avoid inaccuracies. Furthermore, secondary clinical outcome measures test only the consecutive loss of function and do not give insights into the affection of the nerve itself, as e.g. electro-physiology and magnetic resonance imaging studies do.

4.3 Score generation

4.3.1 Approach

The statistical approach included initial filtering, Spearman's ρ, binning of data with respect to gender specific differences, hierarchical clustering and an Analysis of Variance (ANOVA) followed by comparison of different score hypotheses.

Statistically, the comparison of every outcome measurement with its conformity towards the CMTNS is inaccurate, since according to this approach one would assume the CMTNS to represent the 'gold standard' and any further improvement of a modified score can - in its best case scenario - merely reflect the classical CMTNS. Therefore, in an unbiased approach towards score generation, the standard of reference is for instance the evidence-based existence of two patient clusters after various examinations. These clusters represent in this case a mildly and a severely affected group of patients. Testing the discrimination power of the classical CMTNS in context of these two clusters, is supposed to analyse its own accuracy. The disability of the classical CMTNS to distinguish between the two clusters without a significant overlap [**Figure 5]**, reveals its imprecision and justifies a modification, while still acknowledging its power to differentiate between extremes of mild and severe affection. An Analysis of Variance (ANOVA) **[Table 17]** starting with the primary outcome measures of the CMTNS and followed in the ranked order of secondary clinical outcome measurements, reflects the information gain in discriminating between these two referred clusters. Furthermore, it reveals unnecessary secondary clinical outcome measures as well as the redundant primary score parameters, with their information already being incorporated by other parameters of the CMTNS (Version 1).

4.3.2 Score comparison

In a clinical setting, the framework of a scoring system contains the advantage of being end-user friendly and self-explanatory. The results are given in absolute integral numbers and allow direct comparison irrespective of e.g. age-specific effects. In order to implement the mentioned results of significant clinical outcome measures in a new/ modified scoring system, various hypotheses were formed. In anticipation of a wider distribution outreaching to its both extreme endpoints, the various score hypotheses (full, sign., mod.) and the classical CMTNS are graphically depicted in form of box and density plots **[Figure 6]**. The best overall performance is achieved in the mod_CMTNS by not only adding significant secondary clinical outcome measures, but also by removing redundant primary score parameters. A length-dependent analysis will evaluate the important aspect of 'change over time', which is being examined at the moment. The preliminary results of 10 patients show that all patients' symptoms deteriorated. While the CMTNS (Version 1) increased about 1.2 points, the modified CMTNS value increased to 2.9 points. While, these data give promising insights to the 'change over time' of the modified CMTNS, only a more powerful length-dependent analysis will justify the replacement of any other scoring system. Furthermore, the secondary clinical outcome measures are proven to be inter- and intrarater reliable (Solari et al., 2008), but the modified CMTNS as a whole also needs to be tested for these quality criteria.

4.4 Clinical relevance

The modified Charcot-Marie-Tooth Neuropathy score (mod_CMTNS) is of distinctive clinical relevance, since it represents an appropriate clinical outcome measurement to asses the disease severity of Charcot-Marie-Tooth patients with focus on subtype 1A. Furthermore, the mod_CMTNS is relatively easy to perform and does not require any further invasive diagnostic tools. This evidence-based scoring system is supposed to provide the necessary setting for future therapeutic trials. Since the classical CMTNS depicts the disease progression merely in a range between 0.2. and 0.686 points/ year (Shy et al., 2008; Pareyson et al., 2011), clinical trials can not be realized within a reasonable amount of time at the moment.

Moreover, the classical CMTNS declares the majority of patients as averagely affected and is not able to distinguish well between the two aforementioned patient clusters. Thus, an alteration of the existing score in addition to the establishment of biomarkers is needed, in

order to avoid systematic trial errors. A first approach was the testing of biomarkers of the CMT rodent model in a subset of 46 CMT1A patients. A correlation between the CMTNS and two genes of interest in human skin biopsies could be detected (Fledrich et al., 2012). This approach will be expanded to a bigger cohort and compared with the modified CMTNS.

4.5 Perspective

The perspective of this clinical trial includes the examination of length-dependent alterations in the modified CMTNS (mod_CMTNS). For this reason, CMT1A patients will be re-examined exactly three years after their initial study participation. Aim of this investigation is to test, whether the alterations over time in the mod_CMTNS exceed the ones of the classical CMTNS per year (Shy et al., 2008; Pareyson et al., 2011). Preliminary findings in the first 10 study participants have to be confirmed in a larger cohort. In that respect the mod_CMTNS, which was specifically designed for subtype 1A, will also be tested for other subtypes of CMT in order to evaluate its respective validity and reliability on a larger scope.

Furthermore, the new modified CMTNS (mod_CMTNS) will be used to re-evaluate all study participants according to their degree of disease affection. This data set will be linked to individual gene expression levels in skin biopsies and whole blood samples. Sample candidate genes have been already identified in a transgenic rodent model and translated into a small cohort of CMT1A patients (Sereda et al., 1996; Fledrich et al., 2012). The larger cohort is anticipated to enable the final validation of biomarkers in respect to disease severity. These biomarkers in addition to the new clinical scoring system are needed to start therapeutic trials with CMT1A patients. So far, appropriate outcome measurements for the therapeutic effect of drugs in CMT1A patients do not exist. It is planned to pursue an investigator-driven trial with either a progesterone antagonists or curcumin supplements in the long run (Sereda et al., 2003; Meyer zu Hörste et al., 2007; Carter et al., 2008).

Furthermore, the cumbersome patient recruitment of the past is being optimized at the moment by establishing a Europe-wide online patient registry (CMT-NET).

Apart from pharmacological ways of intervention, physical therapy is currently still the best symptom alleviating treatment for CMT1A patients. It is even recommended that CMT

patients undergo rehabilitation treatment twice per year (Maggi et al., 2011). Recently, a collaboration was initiated with the Neurology Department of the rehabilitation clinic in Bad Karlshafen/ Germany and the Max-Planck Institute for Experimental Medicine in Göttingen/ Germany. Its aim is to offer the study participants from Germany a new evidence-based approach on rehabilitation with special focus on proprioception, stretching, fine motor skills and CMT1A-specific exercises. The outcome of this treatment will again be measured with the help of primary and secondary clinical outcome measurements (including the modified CMTNS).

Finally, the skin biopsies in 4% PFA will be subject to various histological/ immunohistological investigations. Special interest lies in the quantification of Meissner corpuscles as a histological outcome measure in CMT1A (Saporta et al., 2009). Preliminary trials with hematoxylin and eosin staining **[Figure 7]** and immunohistochemistry trials with PGP9.5 antibodies have been performed.

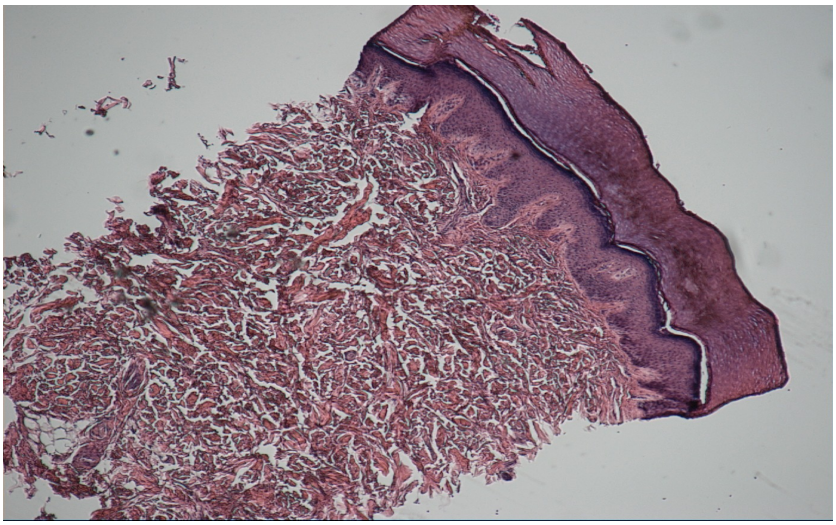

Figure 7: hematoxylin and eosin staining of a human skin biopsy

The sample skin biopsy was performed with a 3 mm biopsy punch device in the medial index finger of the non-dominant hand of a healthy control.

5 Summary

This dissertation is based on a clinical prospective study and represents the data of a multi-centre, prospective European clinical trial on the hereditary peripheral neuropathy Charcot-Marie-Tooth disease subtype 1A (CMT1A)/ (HMSN1A). Additional patients were included through the baseline assessment of the ascorbic acid trial in Italy and the United Kingdom (Pareyson et al., 2011).

The objective of the biomarker trial is to validate new secondary clinical outcome measurements and to generate biomarkers in skin biopsies as well as in whole blood. While the analysis of biomarkers via gene chip analysis is in progress, the validation of secondary clinical outcome measures has led to a critical analysis of clinical scoring systems, with special regard to the most commonly used Charcot-Marie-Tooth Neuropathy Score (CMTNS Version 1) (Shy et al., 2005).

In course of analysing the accuracy of primary and secondary outcome measurements, one of the largest cohorts of patients with proven CMT1A was recruited. 479 patients from Italy, the United Kingdom, Spain, Czech Republic and Germany were examined. Secondary clinical outcome measurements, including the 9-hole-peg test, 10m-timed walking, VAS, SF-36, as well as extensive dynamometry tests (pinch grip, three-point-grip, fist-grip, foot plantar and dorsal flexion) were evaluated.

Based on these results, different score hypotheses were tested to facilitate future therapeutic trials in CMT1A patients and to establish an improved clinical assessment of disease severity. This assessment will further allow a more accurate correlation between the clinical phenotype of patients and their respective gene expression for the establishment of biomarkers. A new evidence-based clinical scoring system (modified CMTNS) with significantly contributing secondary clinical outcome measures was designed. The modified CMTNS consists of 9 parameters, which add significant information to differentiate between two new found clusters of patients. These clusters divide the cohort into mildly and severely affected patient groups. The established Charcot-Marie-Tooth Neuropathy Score (Version 1) is unable to differentiate clearly between these two groups of patients. Furthermore, certain parameters of the existing

score (strength of arms, motor symptoms legs, vibration and Ulnar SNAP) were found to be redundant and therefore omitted in the modified version of the score. The left out parameters were replaced with significant secondary clinical outcome measures (foot dorsal, foot plantar, fist grip and 10m walk test), which are proven to be reliable and valid (Solari et al., 2008). A length-dependent analysis is still necessary to detect the *change over time* in this slowly progressive neuropathy. A preliminary analysis of patients, who were recruited at a second time point approximately three years after the initial assessment, shows that the modified CMTNS depicts the deterioration of the disease better than the CMTNS (Version 1).

The modified CMTNS will help to establish biomarkers, it will facilitate the more accurate assessment of disease severity and therefore lay the foundation of future therapeutic trials in patients suffering from CMT.

6 Appendix

6.1 Case Report Form

Case Report Form

study:

Validation of prognostic and diagnostic markers in CMT1A disease (HMSN1A)

Date of consent

Date of participation

Patient data:

Initials:	**Patient number:**	
birthdate:	sex:	☐ female ☐ male
age:	weight:	Kg
diagnosis:	height:	cm

Name of examiner (Physician):

Name of clinic:

Informed consent available: ☐ yes ☐ no

(see attached)

Head of the study: name, address, telephone, email

Patient history:

Diagnosis

Clinical manifestation since:

Proof of disease since:

Affected family members:

Occupation:

Current medication:

Physical therapy:

Inclusion Criteria		
Informed consent	☐ yes	☐ no
Genitial evidence of subtype 1A	☐ yes	☐ no
Clinical manifestation of CMT1A	☐ yes	☐ no
Patient > 18 years and < 70 years	☐ yes	☐ no

Exclusion Criteria		
Other neurological or psychiatric disorders	☐ yes	☐ no
Drug-, medication- and/or alcohol abuse	☐ yes	☐ no
Other symptoms	☐ yes	☐ no
Lack of cooperation	☐ yes	☐ no

CMTNS: (Shy et al., 2005)

Table 1 *Charcot-Marie-Tooth disease neuropathy score*

	Score				
Parameter	0	1	2	3	4
Sensory symptoms	None	Limited to toes	Extend up to and may include ankle	Extend up to and may include knee	Extends above knees
Motor symptoms					
Legs	None	Trips, catches toes, slaps feet	AFO on at least 1 leg or ankle support	Cane, walker, ankle surgery	Wheelchair most of the time
Arms	None	Difficulty with buttons/zippers	Unable to do buttons or zippers but can write	Can not write or use keyboard	Proximal arms
Pin sensibility	Normal	Reduced in fingers/toes	Reduced up to and may include wrist/ankle	Reduced up to and may include elbow/knee	Reduced above elbow/knee
Vibration	Normal	Reduced at fingers/toes	Reduced at wrist/ankle	Reduced at elbow/knee	Reduced above elbow/knee
Strength					
Legs	Normal	4+, 4, or 4− on foot dorsiflexion	≤3 Foot dorsiflexion	≤3 Dorsiflexion and plantar flexion	Proximal weakness
Arms	Normal	4+, 4, or 4− on intrinsics or finger extensors	≤3 Intrinsics or finger extensors	<5 Wrist extensors	Weak above elbow
Ulnar CMAP	>6 mV	4.0–5.9 mV	2.0–3.9 mV	0.1–1.9 mV	Absent
(Median)	(>4 mV)	(2.8–3.9)	(1.2–2.7)	(0.1–1.1)	(Absent)
Ulnar SNAP	>9 μV	6.0–8.9 μV	3.0–5.9 μV	0.1–2.9 μV	Absent
(Median)	(>22 μV)	(14.0–21.9)	(7.0–13.9)	(0.1–6.9)	(Absent)
Total (max. 36)					

AFO = ankle-foot orthosis; CMAP = compound muscle action potential; SNAP = sensory nerve action potential.

Dynamometer:

HAND-HELD DYNAMOMETRY CITEC				
non-dominant side:		☐ right ☐ left		
	1. trial	2. trial	3. trial	mean
fist Grip				
three point grip				
pinch grip				
foot dorsiflexor				
foot plantarflexors				
dominant side		☐ right ☐ left		
	1. trial	2. trial	3. trial	mean
fist grip				
three point grip				
pinch grip				
foot dorsiflexor				
foot plantarflexors				

10m-walking test:

1	1. trial:	time (in sec.):	_____
2	2. trial:	time (in sec.):	_____
3	3. trial:	time (in sec.):	_____

- mean: time (in sec.): _____

9-peg-hole-test:

1.: non-dominant hand:

- 1. trial: time (in sec.): _____
- 2. trial: time (in sec.): _____
- mean: time (in sec.): _____

2.: dominant hand:

- 1. trial: time (in sec.): _____
- 2. trial: time (in sec.): _____
- Mean: time (in sec.): _____

3mm skin biopsy:

- location: _____
- code of biopsy: CMT1A – I_I_I_I – I_I_I_I

Documentation of unexpected incidents:

Documentation of change in protocol:

Other:

1 SF-36 filled-in: yes ☐ no ☐

2 VAS marked: yes ☐ no ☐

- VAS-value: I_I_I_I mm

date: I_I_I.I_I_I.I_I_I examining physician:

name: _____

signature: _____

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11 Publications

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