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Corneal confocal microscopy detects small fiber neuropathy in CMT1A patients

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Abstract

Although unmyelinated nerve fibers are affected in CMT1A, they have not been studied in detail due to the invasive nature of the techniques needed to study them. We established alterations in C-fiber bundles of the cornea in patients with CMT1A using non-invasive corneal confocal microscopy (CCM).

Twelve patients with CMT1A and twelve healthy control subjects underwent assessment of neuropathic symptoms and deficits, electrophysiology, quantitative sensory testing, corneal sensitivity and corneal confocal microscopy.

Corneal sensitivity, corneal nerve fiber density, corneal nerve branch density, corneal nerve fiber length and corneal nerve fiber tortuosity were significantly reduced in CMT1A patients compared to controls. There was a significant correlation between corneal sensation and CCM parameters with the severity of painful neuropathic symptoms, cold and warm thresholds and median nerve CMAP amplitude.

CCM demonstrates significant damage to C-fiber bundles, which relates to some measures of neuropathy in CMT1A patients.

Keywords

CMT1A; Neuropathy; Electrophysiology; Corneal confocal microscopy; Corneal nerves; C-fibers

Introduction

Charcot-Marie-Tooth (CMT) disease or hereditary motor and sensory neuropathy (HMSN) is the commonest inherited neuromuscular disorder [1]. The diagnosis of CMT is based on family history, clinical evaluation supported by electrophysiology, and genetic testing. Accurate diagnosis of CMT is difficult in many cases due to its extreme genetic heterogeneity. Family history and electrophysiology are useful components in the diagnosis of CMT and allow patients to be classified into different subtypes. CMT1A is the most frequent sub-type (~70–80%). It results from duplication of the peripheral myelin protein

22 (PMP22) gene on chromosome 17p11.2 [2]. Patients with CMT1A typically have uniform slowing of nerve conduction velocities (NCVs) consistent with a demyelination, but they also develop reduced amplitudes of compound motor and sensory nerve action potentials (CMAPs and SNAPs) and progressive muscle weakness suggestive of axonal degeneration [3–5]. Whilst these changes are attributed to primary demyelination with secondary axonal degeneration of the large myelinated fibers [6–8], it is interesting that there is no correlation between motor or sensory NCVs and neurological disability in patients with CMT1A [3, 6].

While small fiber dysfunction has been demonstrated in CMT1 using quantitative sensory tests (QST) [9–11] and sympathetic skin response (SSR) [11], preservation of cerebral potentials following laser and electrical stimulation has suggested preservation of small fibers in a patient with CMT [12]. Sural nerve biopsy studies have demonstrated degeneration and regeneration of the unmyelinated fibers [11, 13, 14], but because this procedure is invasive, it is undertaken less frequently, particularly with the development of genetic analyses. However, pathological studies allow definitive assessment of the primary pathology and also provide insights into underlying pathogenetic mechanisms [15]. As an alternative less invasive approach, a detailed immunohistological and electron microscopic study of dermal nerves in skin biopsies from patients with CMT1A has demonstrated shortening of the internodal length with a loss of Meissner corpuscles and accumulation of intra-axonal mitochondria, suggestive of axonal pathology [16]. Comparable pathology has also been demonstrated recently in dermal nerves of foot skin pad and peripheral nerves in a variety of animal models of CMT [17].

There is now increasing literature on the potential for corneal confocal microscopy (CCM) as a means to quantify C-fiber pathology in peripheral neuropathies. A number of detailed morphometric and immunohistological studies have demonstrated that the subbasal nerve fiber bundles studied by CCM are predominantly nociceptive C-fibers [18–20]. Indeed it has been applied to evaluate diabetic neuropathy [21, 22], idiopathic small fiber neuropathy [23], Fabry disease [24], and a series of conditions that cause small nerve fiber damage, including hereditary sensory and autonomic neuropathy (HSAN) [25], autoimmune neuropathy [26], Crohn's disease [27] and neuropathy associated with chemotherapy [27, 28]. We have also shown that corneal nerve damage assessed using CCM relates to the severity of intraepidermal nerve fiber loss [29], is related to a loss of corneal sensitivity [30], and can detect early small nerve fiber regeneration following pancreas transplantation in diabetic patients [31].

CCM may therefore provide a non-invasive means to determine whether there is small fiber pathology in patients with CMT. We have undertaken a detailed evaluation of neuropathy using conventional neurophysiology and quantitative sensory testing in addition to CCM and non-contact corneal aesthesiometry (NCCA), to quantify nerve damage in patients with CMT1A.

Methods

Selection of Patients

Twelve patients with CMT1A (6 men, 6 women, average age 43.0 ± 3.5 years) and twelve healthy control subjects (7 men, 5 women, average age: 43.0 ± 3.5 years) were studied. All patients were confirmed to have the *PMP22* duplication by dosage analysis.

The study was approved by the Central Manchester Ethics committee, and written informed consent was obtained according to the declaration of Helsinki. All patients were diagnosed

and referred from the department of Genetic Medicine, Central Manchester University Hospital NHS Foundation Trust.

Assessment of Neuropathy

All patients and controls underwent a detailed evaluation of their neurological symptoms according to the Neuropathy Symptom Profile (NSP) [32]. The McGill pain analogue score was used to assess the severity of pain. Neurological deficits were assessed using the neuropathy disability score (NDS) and included vibration, pin prick and temperature perception as well as the presence or absence of ankle reflexes to establish the severity of neuropathy (NDS 0–2, no neuropathy; NDS 3–5, mild neuropathy; NDS, 6–8, moderate neuropathy; and NDS, 9–10, severe neuropathy [32]). Vibration Perception Threshold (VPT) was measured using a Neurothesiometer (Horwell, Scientific Laboratory Supplies, Wilford, Nottingham, UK). Quantitative sensory testing included assessment of cold sensation (CS) and cold induced pain (CIP) to assess A δ fibers and warm sensation (WS) and heat induced pain (HIP) to assess C fibers on the dorsum of the left foot, using the MEDOC TSA II (Medoc Ltd., Ramat Yishai 30095, Israel). Electrodiagnostic studies were undertaken using a Dantec “Keypoint” system (Dantec Dynamics Ltd, Bristol, UK) equipped with a DISA temperature regulator to keep limb temperature constant between 32 and 35°C. Full electrophysiological assessment in motor (Median, Ulnar, Fibular) and sensory (Radial, Sural) nerves was performed from the right limb. Motor studies were performed using silver-silver chloride surface electrodes at standard sites defined by anatomical landmarks. Compound muscle action potential (CMAP) amplitudes were taken from baseline to negative peak. Motor nerve conduction velocities were calculated after distal and proximal stimulation. Sensory studies were recorded using a bar electrode (cathode-anode distance 3cm) placed at standard sites. Recordings for sural and radial nerves were taken using antidromic stimulation over a distance of 140 and 100mm respectively.

Corneal sensitivity

Corneal sensitivity was quantified using a non-contact corneal aesthesiometer (NCCA) (Glasgow, Caledonian University, UK) which uses a puff of air through a bore 0.5mm in diameter lasting 0.9 seconds and exerting a force expressed in millibars (mbars) [30]. An electronic pressure sensor displays the force exerted in millibars (mbars). The stimulus jet is mounted on a slit lamp. It is positioned 1 cm from the eye, and the air jet is aligned to the centre of the cornea. The subject feels a sensation on the cornea which is most commonly describing as being “cold” or as a “breeze” and acknowledges this. Each subject is presented with a supramaximal stimulus, and the staircase method is employed by reducing the stimulus strength until the patient does not feel the jet. This is then increased to a threshold level and reduced to the point where the stimulus is not felt. The whole process is repeated three times to derive a threshold. The coefficient of variation for NCCA was 5.6%.

Corneal Confocal Microscopy

Patients underwent examination with the Heidelberg Retina Tomograph (HRT III) (Rostock Cornea Module) *in vivo* corneal confocal microscopy. The subject’s eyes were anesthetized using a drop of 0.4% benoxinate hydrochloride, and Viscotears were applied on the front of the eye for lubrication. The patient was instructed to fixate on a target with the eye that was not being examined. A drop of viscoelastic gel was placed on the tip of the objective lens, and a sterile disposable Perspex cap was placed over the lens. The gel optically couples the objective lens to the cornea. Several scans of the entire depth of the cornea were recorded by turning the fine focus of the objective lens backwards and forwards for approximately 2 minutes to acquire satisfactory images of all corneal layers providing *en face* two dimensional images with a lateral resolution of approximately 2 μ m/pixel and final image

size of 400 pixels $\times$ 400 pixels. Images were obtained using the section mode, which enabled manual acquisition and storage of a single image at a time. For the purposes of this study, we obtained high quality images of the sub-basal nerve plexus of the cornea from each patient and control subject. This layer is of particular relevance for defining neuropathic changes, since it is the location of the main nerve plexus that supplies the overlying corneal epithelium. These nerve fiber bundles contain unmyelinated fibers, which run parallel to the Bowman layer before dividing and turning upwards toward the surface to terminate as individual axons underneath the surface epithelium [33,34]. This has been confirmed using electron microscopy, where nerve bundles containing unmyelinated axons were shown to penetrate the Bowman membrane throughout the central and peripheral cornea at approximately 400 sites [35]. Five images per patient from the center of the cornea were selected and examined in a masked and randomized fashion [36].

Four corneal nerve parameters were quantified: (i) Corneal nerve fiber density (CNFD) - the total number of major nerves/mm² of corneal tissue; Corneal nerve branch density (CNBD) - the number of branches emanating from all major nerve trunks/mm² of corneal tissue; (iii) Corneal nerve fiber length (CNFL) - the total length of all nerve fibers and branches (mm/mm²) within the area of corneal tissue; and (iv) Corneal nerve fiber tortuosity (CNFT). CNFD and CNFL are considered to reflect overall nerve fiber degeneration, while CNBD reflects nerve fiber regeneration (which is also captured partially by the CNFL).

Statistics

SPSS 16.05.0 for Windows was used to compute the results. Analysis included descriptive and frequency statistics. All data are expressed as mean $\pm$ SEM. One-way analysis of variance (ANOVA) with Scheffe *post-hoc* tests was used to study differences between means. The Pearson test was used to analyze correlations between potentially related variables.

Results

The clinical characteristics and detailed assessment of neuropathy in CMT1A patients and their matched controls are summarized in Table 1.

Symptoms and Neurological Deficits

Neuropathic symptoms assessed with the NSP were significantly increased in CMT1A patients ($P < 0.0001$), as was the severity of pain assessed using the McGill pain analogue ($P = 0.001$). The Neuropathy deficit score (NDS) was significantly increased, consistent with a severe neuropathy in all 12 patients (9.1 ± 0.4 , $P < 0.0001$).

Quantitative Sensory Tests

Vibration perception threshold (VPT) ($P < 0.0001$) and CS ($P = 0.01$) were significantly increased in patients compared to control subjects. However, CIP, WS, and WIP did not differ significantly from control subjects.

Electrophysiology

Sural SNAPs and fibular CMAPs were not elicited in all cases. Low voltage, slowed radial and median SNAPs could be obtained in just four cases. As expected, upper limb motor nerve conduction in all patients with CMT1A showed diffuse and uniform slowing of conduction velocity (less than 30m/s) along with prolongation of distal and F-wave latencies. Upper limb CMAP amplitudes were significantly reduced (Table 2).

Corneal sensation

Corneal sensitivity was significantly reduced in CMT1A patients compared to control subjects ($P=0.01$) (Table 3).

Corneal Confocal Microscopy

Corneal nerve fiber density ($P=0.01$) (Fig. 2.a), nerve branch density ($P=0.02$) (Fig. 2.b), nerve fiber length ($P<0.0001$) (Fig. 2.c) and nerve fiber tortuosity ($P=0.004$) (Fig. 2.d) were significantly reduced in CMT1A patients compared to control subjects (Figures 1, 2 and Table 3). There was no difference in corneal nerve parameters between males and females (NFD- $P=0.8$; NBD- $P=0.6$; NFL - $P=0.9$; NFT - $P=0.5$; NCCA - $P=0.4$).

Correlations

Both NDS and NSP showed no significant correlations with NCCA or corneal nerve morphology. However, the severity of pain as judged by the McGill Pain Analogue score correlated significantly with NCCA, NFD and NFL (Table 4). There was no significant correlation with VPT, but NCCA correlated significantly with CS and WS. CCM demonstrated a correlation with CS and WS and reached statistical significance between NFD and CS. Both Median and Ulnar nerve conduction velocity showed no association with NCCA or CCM. However, NCCA correlated significantly with Ulnar nerve amplitude and showed borderline significance with Median nerve amplitude. While CCM did not correlate with Ulnar amplitude, both NBD and NFL showed a significant correlation with Median nerve CMAP amplitude.

Discussion

The diagnosis of CMT has evolved from a purely clinical approach supported by electrophysiology to the currently employed combined clinical/genetic approach. Despite advances in the identification of many of the causative genes for CMT, accurate diagnosis of CMT still requires a detailed knowledge of the clinical and genetic subtypes and their frequencies in different populations. CMT1A is caused by a 1.4-Mb duplication of the PMP22 gene on 17p11.2 [2, 37]. Electrophysiology is an essential component of the diagnosis of CMT and allows patients to be classified into two types: CMT1 (demyelinating) and CMT2 (axonal) subtypes using upper limb NCVs (median or ulnar nerves), where an $NCV<38$ m/s (commonly around 20 m/s) is strongly suggestive of CMT1A. In this study we have undertaken a systematic evaluation of neuropathy assessing symptoms, neurological deficits, conventional neurophysiology, quantitative sensory testing, NCCA and CCM in patients with CMT1A. The results confirm a significant neuropathy with a markedly elevated NDS and a significant deficit of myelinated nerve fiber function as evidenced by absent lower limb CMAPs and SNAPs and markedly reduced upper limb nerve conduction velocity and amplitudes. Additionally we demonstrate a significant increase in the cold threshold suggestive of A δ , thinly myelinated fiber deficits.

With regard to small fiber deficits, patients with CMT1A have clinical evidence of moderate painful neuropathic symptoms, which is probably related to a reduction of the A δ afferents [38]. In this study we also show an elevated NSP and McGill pain score, which correlated with damage to corneal nerve fibers. While quantitative sensory testing of small fibers does not demonstrate a significant increase in the thresholds for warmth and heat pain, our studies do demonstrate a significant abnormality in corneal sensation and corneal nerve fiber morphology.

The cornea contains myelinated A δ fibers, which respond primarily to mechanical stimuli, and unmyelinated C-fibers, which respond to thermal and chemical stimuli [19]. In this

study, we scanned the sub-basal layer of the central cornea to image corneal C-fibers. Stromal nerves representing A δ fibers could not be consistently imaged in all patients. Quantitative analysis of stromal nerves imaged using CCM is recognized to be difficult [39], especially to objectively distinguish normal from abnormal nerves [39–41]. In a previous sural nerve biopsy study the number of unmyelinated axons/Schwann cell was reduced in patients with CMT1A [14]. We have now demonstrated significant pathology of the corneal C-fiber bundles located in the sub-basal layer using CCM together with reduced corneal sensation in patients with CMT1A. Importantly, these findings closely correlate, particularly with the severity of painful neuropathic symptoms and small fiber deficits as well as median and ulnar nerve CMAP amplitude, the latter reflecting axonal integrity. This is the first time that a correlation between C-fiber pathology and clinical severity of neuropathy has been established in CMT1A. Thus unlike QST, sural nerve biopsy and dermal skin biopsy, NCCA and CCM can be used as rapid non-invasive objective tools for clinical assessment and quantification of small fiber abnormalities in CMT1A. The explanation of these findings may lie in roles of *PMP22* in processes other than myelination [42]. *PMP22* over expression in myelinating Schwann cells produces abnormal growth and differentiation resulting in defective myelin stability and turnover [43]. We hypothesize that similar *PMP22* over expression in non-myelinating Schwann cells could also be the basis of the observed defects in C-fiber bundles in CMT1A. This would be consistent with previous observations in sural nerve biopsies [14], where a reduction in the number of axons/Schwann cell profile has been demonstrated, thus providing insights into the biological role(s) of *PMP22* and pathological mechanisms involving its protein.

Furthermore, these observations add to our published studies which demonstrate the clinical utility of CCM in the assessment of patients with a range of peripheral neuropathies, including diabetic neuropathy [22, 44], Fabry Disease [24] and idiopathic small fiber neuropathy [45]. These findings provide the basis for further studies to define whether there are differences in corneal nerve morphology of patients with CMT that primarily affect axons. Ideally, detailed ultra structural morphological studies from the cornea of patients with CMT1 should also be undertaken to be absolutely certain that all the fibers assessed using CCM are indeed unmyelinated. We acknowledge that this is a preliminary study, and further studies are required in a larger group of patients to compare corneal nerve morphology in patients with CMT1 and CMT2 with differing severity of neuropathy. Therefore CCM may aid in earlier diagnosis and to undertake longitudinal or interventional studies in patients with CMT.

Acknowledgments

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List of Abbreviations

ANOVA	Analysis of variance
CCM	Corneal Confocal Microscopy
CNFD	Corneal Nerve Fibre Density
CNBD	Corneal Nerve Branch Density
CNFL	Corneal Nerve Fibre Length
CNFT	Corneal Nerve Fibre Tortuosity
CMAP	Compound motor action potential

CMT	Charcot-Marie-Tooth
CS	Cold Sensation
CIP	Cold Induced Pain
HIP	Heat Induced Pain
HSAN	Hereditary sensory and autonomic neuropathy
NCCA	Non Contact Corneal Aesthesiometer
NCV	Nerve Conduction Velocity
NDS	Neuropathy Disability Score
NSP	Neuropathy Symptom Profile
PMP22	Peripheral myelin protein 22
QST	Quantitative Sensory Testing
SEM	Standard error of the mean
SNAP	Sensory Nerve Action Potential
SSR	Sympathetic Skin Response
VPT	Vibration Perception Threshold
WS	Warm Sensation

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Figure 1. CCM images of the sub-basal layer of cornea: (a) control subject, (b) patient with CMT1A. The red arrows indicate main nerve fibers and yellow arrows indicate branches.

Figure 2. (a) Corneal nerve fiber density (b) corneal nerve branch density (c) corneal nerve fiber length (d) corneal nerve fiber tortuosity (e) corneal sensitivity.

Table 1

Clinical and neurological assessments in control subjects and CMT1A patients. Data are expressed as Mean $\pm$ SEM

Parameter	Control	CMT1A	P-Value
No (F/M)	12 (5/7)	12 (6/6)	-
Age (yrs)	43.0 $\pm$ 3.5	43.0 $\pm$ 3.5	-
NDS (0–10)	0	9.1 $\pm$ 0.4	<0.0001
NSP (0–38)	0	9.0 $\pm$ 2.1	<0.0001
McGill Pain Analogue (0–10)	0	6.3 $\pm$ 1.9	0.001
VPT (V)	3.6 $\pm$ 0.3	33.3 $\pm$ 4.4	<0.0001
CS (°C)	28.2 $\pm$ 0.5	18.6 $\pm$ 6.3	0.01
WS (°C)	37.9 $\pm$ 1.0	41.4 $\pm$ 2.3	0.12
CIP (°C)	11.0 $\pm$ 2.4	5.7 $\pm$ 3.2	0.26
HIP (°C)	45.3 $\pm$ 0.9	47.6 $\pm$ 1.2	0.19

Table 2

Electrophysiology in control subjects and CMT1A patients. Data are expressed as Mean $\pm$ SEM

Parameter	Control	CMT1A	P-Value
Sural Amplitude (μ m)	21.0 $\pm$ 4.7	No response	-
Sural Velocity (m/s)	47.9 $\pm$ 2.6	No response	-
Fibular Amplitude(mV)	5.6 $\pm$ 0.8	No response	-
Fibular Velocity (m/s)	48.5 $\pm$ 1.5	No response	-
Median Amplitude (mV)	9.0 $\pm$ 0.6	3.3 $\pm$ 0.7	<0.0001
Median Velocity (m/s)	53.7 $\pm$ 0.7	21.8 $\pm$ 1.9	<0.0001
Median Latency (ms)	3.0 $\pm$ 0.2	10.3 $\pm$ 0.8	<0.0001
Median F-wave (ms)	27.3 $\pm$ 0.9	63.5 $\pm$ 4.6	<0.0001
Ulnar Amplitude (mV)	8.9 $\pm$ 0.7	3.8 $\pm$ 0.6	<0.0001
Ulnar Velocity (m/s)	59.9 $\pm$ 1.7	20.9 $\pm$ 2.5	<0.0001
Ulnar Latency (ms)	2.4 $\pm$ 0.1	6.2 $\pm$ 0.8	<0.0001
Ulnar F-wave (ms)	28.2 $\pm$ 0.9	63.2 $\pm$ 5.3	<0.0001

Table 3

Corneal sensitivity and corneal nerve morphology in control subjects and CMT1A patients. Data are expressed as Mean $\pm$ SEM

Parameter	Control	CMT1A	P-Value
NCCA (mbar)	0.4 $\pm$ 0.1	1.4 $\pm$ 0.3	0.01
NFD (no/mm ²)	39.5 $\pm$ 1.4	31.6 $\pm$ 2.6	0.01
NBD (no/mm ²)	60.1 $\pm$ 11.8	29.9 $\pm$ 4.7	0.02
NFL(mm/mm ²)	26.7 $\pm$ 1.3	15.8 $\pm$ 1.5	<0.0001
NFT (TC)	14.1 $\pm$ 0.7	11.1 $\pm$ 0.6	0.004

Table 4

Correlations between corneal sensitivity and corneal nerve morphology with neurological parameters in CMT1A patients.

	NDS	McGill Pain Analogue	VPT	CS	WS	Median nerve Amplitude	Median nerve Velocity	Ulnar nerve Amplitude
NCCA	r	.314	.452	-.997	.974	-.800	.320	-.851
	P	.377	.261	.003	.026	.056	.536	.032
NFD	r	-.194	-.300	.954	-.932	.758	-.486	.729
	P	.545	.470	.046	.068	.080	.328	.100
NBD	r	-.510	-.078	.769	-.646	.898	-.768	.647
	P	.090	.854	.231	.354	.015	.075	.165
NFL	r	-.522	-.220	.911	-.793	.842	-.689	.662
	P	.082	.601	.089	.207	.036	.130	.152