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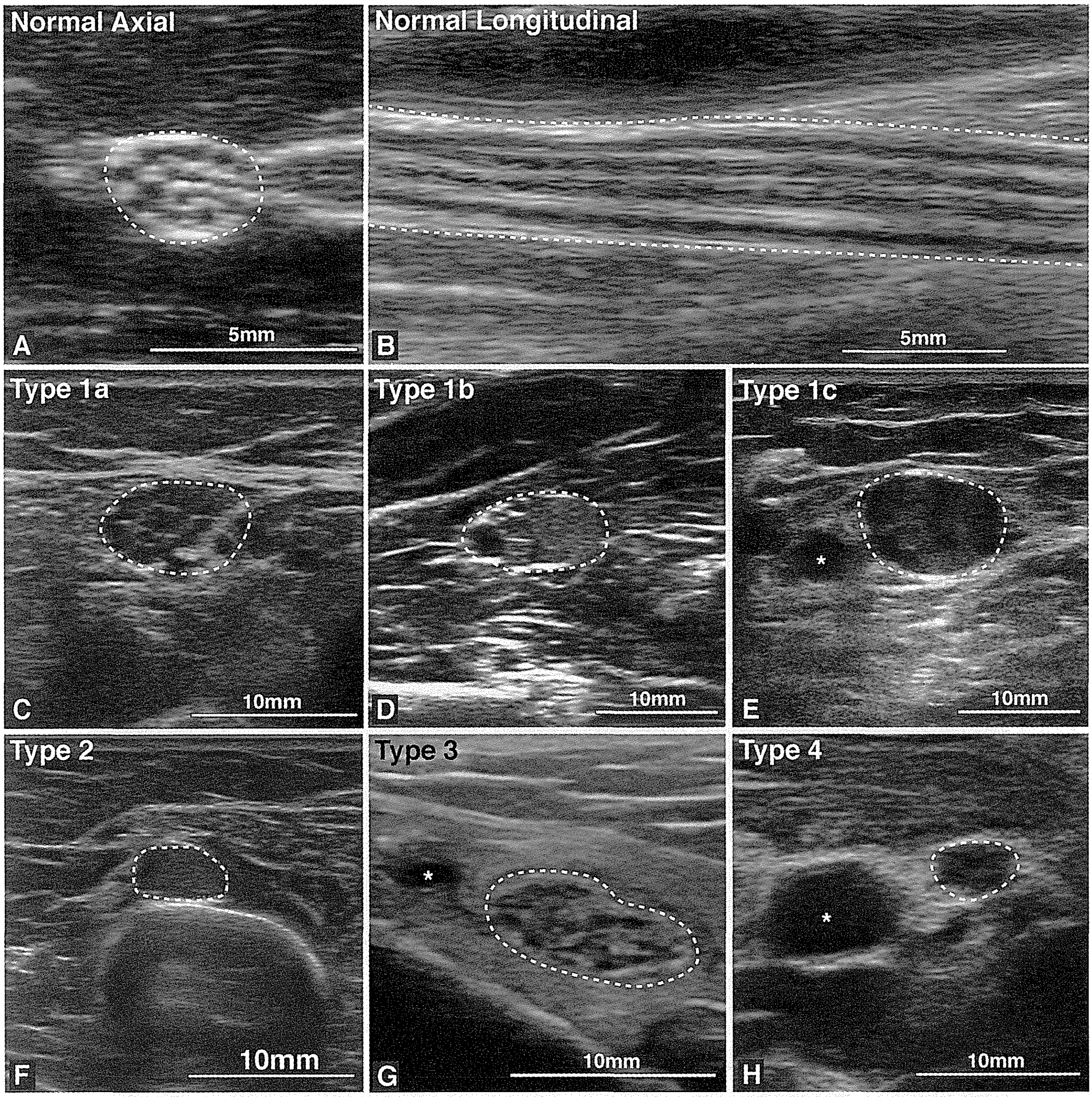

Figure 2 Patterns of nerve ultrasound changes in peripheral neuropathy (PN). Normal nerve ultrasound (US) appearances are shown in A (axial image of the median nerve in the forearm, cross-sectional area (CSA 7 mm^2) and B (longitudinal image of the tibial nerve in the popliteal fossa, CSA 12 mm^2) demonstrating a characteristic fascicular pattern. A number of patterns of nerve US abnormalities may be seen in PN and examples are shown in C–H. (C) An enlarged tibial nerve at the ankle (CSA 49 mm^2) in a patient with CMT1A demonstrating heterogeneously enlarged hypoechoic fascicles (type 1a—uniform or heterogenous enlargement of hypoechoic fascicles). (D) An enlarged median nerve in the forearm (CSA 65 mm^2) with mixed hyperechoic and hypoechoic fascicles in a patient with chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) (type 1b—mixed hyperechoic and hypoechoic fascicles). (E) An enlarged median nerve at the elbow (CSA 91 mm^2) with disruption of the normal fascicular architecture in a patient with multifocal acquired demyelinating sensory and motor neuropathy (MADSAM) (type 1c—obliteration of normal fascicular architecture). (F) An enlarged radial nerve (CSA 27 mm^2) in the spiral groove of a patient with CIDP, a region in which US may demonstrate a monofascicular or oligofascicular appearance of normal nerves (type 2—increase CSA in monofascicular nerve). (G) Enlargement of the tibial nerve at the ankle (CSA 95 mm^2) in a patient with hypertrophic neuropathy, with prominent perineurial connective tissue and relatively normal fascicular calibre (type 3—increased CSA due to increased perineurial connective tissue). (H) The median nerve at the midpoint of the arm of a patient with amyloid neuropathy, demonstrating normal calibre (CSA 8 mm^2) but with loss of normal fascicular architecture (type 4—normal CSA with altered echotexture).

recent studies using higher frequency probes (≥12 MHz) have provided further information regarding nerve morphology in patients with various subtypes of CMT.

CMT disease type 1A

CMT1A, the most common form of demyelinating CMT, is caused by a duplication of *PMP22* that encodes peripheral myelin protein 22, a transmembrane protein in the compact myelin of the peripheral nerves. Schwann cells and abundant connective tissue around thinly myelinated axons ('onion bulbs') are the main features of pathology in CMT1A. In patients with CMT1A, nerve US reveals that the CSA of peripheral nerves, brachial plexus and nerve roots are larger than those in healthy subjects. Nerve CSA is uniformly increased throughout the course of the nerve, and the CSA and diameter of the C6 nerve root are also larger than those in controls (figure 1). Martinoli

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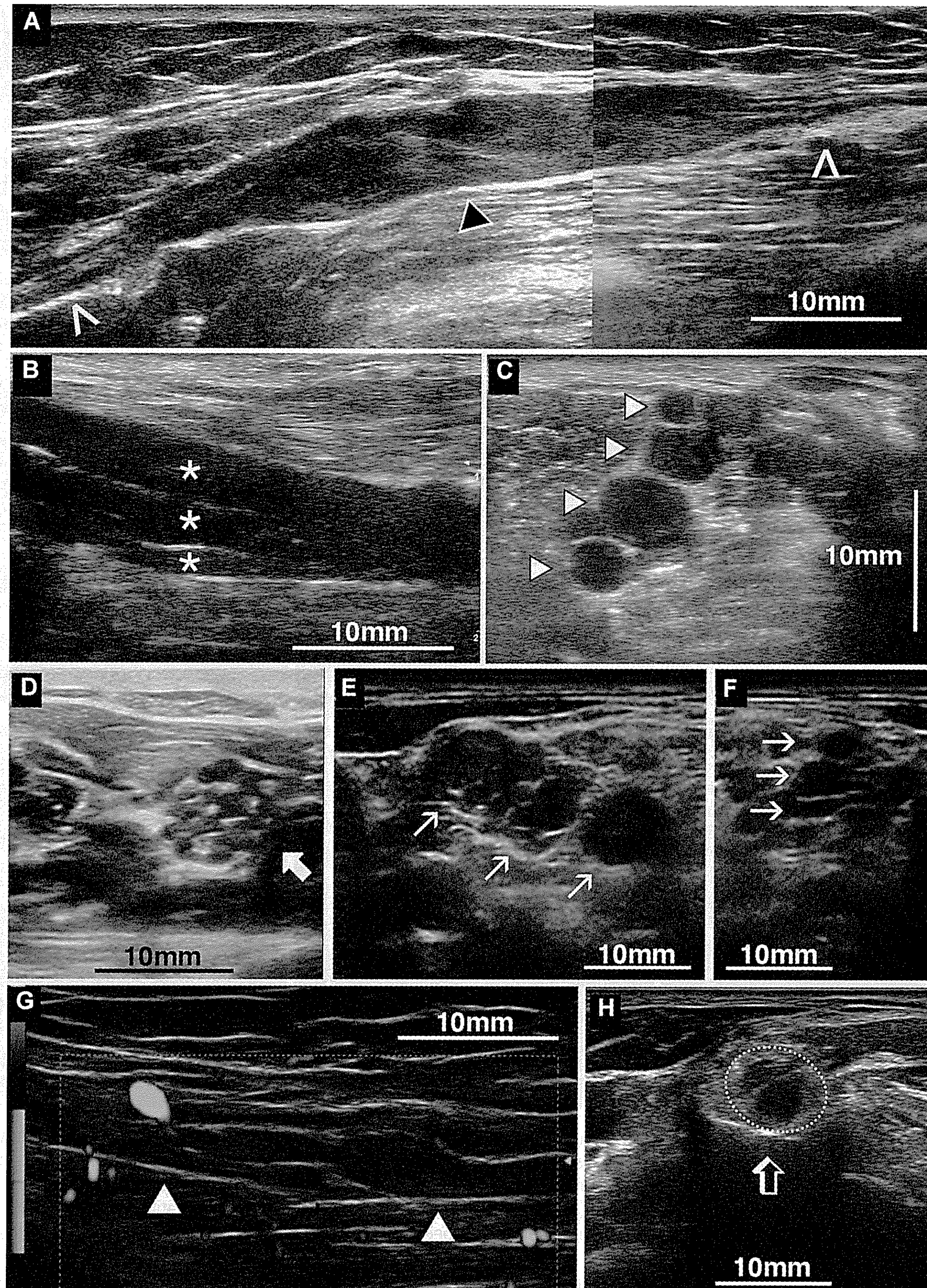

Figure 3 Ultrasound (US) abnormalities in chronic inflammatory demyelinating polyradiculoneuropathy (CIDP). A number of US abnormalities may be detected in patients with CIDP. (A) Focal enlargement and hypoechogenicity of the median nerve in the cubital fossa (black arrowhead) with a relatively normal fascicular pattern proximal and distal to the swelling (^).(B and C) Enlargement of the cervical nerve roots and brachial plexus (thin arrows), which is most commonly symmetric and may be associated with normal nerve calibre in more distal nerves. (D) Increased nerve cross-sectional area with prominent fascicular enlargement (thick arrow). In the patient, nerve enlargement was diffuse and involved all studied upper limb nerves. The cervical nerve roots (between anterior and middle scalene muscles) are depicted in a patient with multifocal acquired demyelinating sensory and motor neuropathy (MADSAM; E and F). US abnormalities were asymmetric with marked enlargement on the right (E) and relatively normal nerve calibre on the left (F). This corresponded with the clinical deficits, which were more severe in the right upper limb. (G) Increased nerve vascularity on Doppler US in the median nerve at the midpoint of the arm in a patient with CIDP. (H) The C6 nerve root in a patient with CIDP showing reduced definition of its epineurial margin.

et al[31] reported that patients with CMT1A can be distinguished from those with other types of CMT (CMT2 and CMTX1) by either a larger CSA or a larger fascicular diameter in median nerves. Likewise, Noto *et al*[32] demonstrated that CSA was also increased in the great auricular nerves and in C6 nerve roots in patients with CMT1A. Thus, nerve US findings demonstrate

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why CMT1A was previously classified as a hypertrophic neuropathy based just on nerve palpation.

Regarding the CSAs of the sural nerves, two conflicting descriptions have been reported. Pazzaglia *et al*[33] found that the CSA in the sural nerve was not increased in the majority of patients with CMT1A. Noto *et al*[32] found that the CSA in the sural nerve was increased in their population with CMT1A. One possible reason for this discrepancy can be attributed to the different methods of CSA measurement between their studies. Pazzaglia *et al* tracked the nerve circumference inside the hyperechoic rim, whereas Noto *et al* traced the nerve circumference including hyperechoic rim.

In terms of the correlation between US, clinical and electrophysiological findings in CMT1A, there is an inverse relationship between the CSA and the nerve conduction parameters, such as motor conduction velocity and compound muscle action potentials amplitude.[23 32] There is also a positive correlation between the CSAs in the median nerve and CMT neuropathy score (CMTNS) that quantifies the disease severity in patients with CMT1A.[32] Taken together, in patients with CMT1A, the extent of nerve enlargement assessed by US paralleled not only the physiological function of peripheral nerves but also the clinical disease severity.

CMT disease type 1B

CMT1B, another demyelinating form of CMT, is caused by mutations of *MPZ* gene that encodes myelin protein zero, a major constituent of peripheral myelin proteins. In a study involving a large family with CMT1B, enlargement of the median and vagus nerves was detected in affected family members.[34] Conversely, sural nerve calibre was reduced, possibly reflecting length-dependent axonal loss.

CMT disease type 2

CMT2, a group of axonal forms of autosomal dominant CMT, includes more than 19 distinct types. Among them, the most common form is CMT2A that is caused by mutations of mitofusin 2 (*MFN2*) gene. In axonal neuropathies, axonal loss is predicted to result in decreased nerve caliber; however, median nerve CSA in patients with CMT2 is slightly larger than that of normal subjects.[23 31] This discrepancy may be related to histopathological findings that include Schwann cell hyperplasia with pseudo onion bulb formations and endoneurial swelling seen in some genetic subtypes of CMT2.[35]

CMT disease type X1

CMTX1, the second most common form of CMT, is caused by point mutations of gap junction-associated protein B1 (*GJB1*) gene, which encodes connexin-32 protein. Although a statistically significant difference was not demonstrated, the median nerve CSAs in patients with CMTX1 were larger than those in healthy subjects in one study, but were smaller in another report.[23 31] Both studies included a small number of patients; therefore, a further study with larger number of patients will be needed.

With the accumulation of future studies of US findings in various types of CMT, nerve US in combination with results of nerve conduction studies may provide tools to facilitate more targeted gene analysis in patients with suspected CMT. Nerve US is also useful for the diagnosis of hypertrophic-type CMT in the rare instance when compound muscle action potentials are not evoked in demyelinating CMT due to severe atrophy in distal muscles or marked increase in stimulation threshold.

Hereditary neuropathy with liability to pressure palsies

Hereditary Neuropathy with liability to pressure palsies (HNPP) is caused by a deletion of PMP22 and nerve biopsies in such patients reveal focal thickening of myelin, that is, tomacula. Beekman and Visser[36] first reported focal and multiple nerve enlargements in a patient with HNPP not only at typical nerve entrapment sites but also outside the entrapment sites. Non-uniform nerve enlargement patterns have been reported in some studies of patients with HNPP.[23 37 38] Gianneschi *et al*[37] revealed that no morphometric changes were seen in the distal nerve segments where entrapment is unlikely while the distal motor latencies were increased. Morphological abnormalities identified on US were not always correlated to the neurophysiological parameters in patients with HNPP, unlike those in patients with CMT1A.

Immune-mediated neuropathies

Chronic inflammatory demyelinating polyradiculoneuropathy (CIDP)

Typical CIDP

The clinical features of typical chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) are well recognised, including progressive symmetric weakness involving proximal more than the distal muscles, sensory impairment and reduced or absent deep tendon reflexes. Histopathologically, nerves in patients with CIDP demonstrate segmental demyelination and remyelination resulting in onion bulb formation and varying degrees of interstitial oedema and endoneurial inflammation.[39] There are also a number of atypical or variant presentations, such as multifocal acquired demyelinating sensory and motor neuropathy (MADSAM), sensory-predominant CIDP, and distal forms such as distal acquired demyelinating sensory neuropathy (DADS).[40–42]

In the majority of patients with CIDP, abnormalities are detected on nerve US. However, just as there is clinical variability, there is a wide range of nerve US findings reported in CIDP (figure 3). Increased CSA of peripheral nerves and/or cervical nerve roots is most frequently reported.[43–48] Hypertrophy of the vagus nerve has also been reported in CIDP.[49 50]

While nerve enlargement is frequently identified, there may be marked variability in the nerve CSA both between-patients and within a patient. In some cases there may be massive enlargement (figure 3), but in other patients nerve calibre may be normal or mildly enlarged. Variability in nerve enlargement may be seen when different nerves of the same patient are compared and along the course of the same nerve, and identifying intranerve and internerve variability of CSA may be of diagnostic benefit.[51]

Three separate classes of US morphological findings have been described in CIDP depending on the CSA and echogenicity.[52] Class 1 nerves were enlarged with hypoechoic fascicles. Class 2 nerves were enlarged with mixed hypoechoic and hyperechoic fascicles. Class 3 nerves were of normal calibre but demonstrated abnormal hyperechoic fascicles, which were less easily distinguished from the perineurial connective tissue. The nerve US patterns correlated with disease duration (class 3 was associated with longer disease duration). As such, variations in US findings in CIDP may reflect different pathophysiological stages of the disease, although further histopathological correlation is needed. As CIDP is a chronic, segmental disorder often with a relapsing course, it is expected that different classes of nerve changes may coexist in some patients.

Nerve vascularity may also be increased in patients with CIDP as assessed with Doppler US studies (figure 3).[53] Nerve blood flow strongly correlates with cerebrospinal fluid protein and the number of enlarged nerves, suggesting that nerve vascularity may reflect disease activity.

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The correlation between the US findings and neurophysiology features or functional disability remains controversial,[43 52] although correlation between the extent of nerve enlargement and the duration of the disease has been reported.[15 52] More detailed comparisons between US, clinical and neurophysiological findings are needed to clarify this point. Nerve US abnormalities may improve with treatment response.[45]

CIDP variants

The extent and nature of US abnormalities in CIDP variants are less well defined, although changes overlap with typical CIDP.

Multifocal acquired demyelinating sensory and motor neuropathy

Patients with MADSAM present with asymmetric motor and sensory deficits, often with patchy neurophysiological abnormalities in nerve conduction studies. US demonstrates multifocal nerve enlargements (figure 3) that may be identified at sites of current or previous electrophysiological conduction blocks.[54]

Distal acquired demyelinating symmetric neuropathy

The US features of DADS have not been systematically studied. Neuropathy associated with antimyelin-associated glycoprotein (MAG) antibodies is most commonly categorised with DADS and one study has evaluated US findings in this patient population.[55] Patchy enlargement of nerves was identified most commonly at entrapment sites. Of interest, distal nerve enlargement was not prominent and this contrasts with the characteristic neurophysiological findings of prominent slowing of distal nerve conduction.[56]

POEMS syndrome

The demyelinating neuropathy associated with POEMS (polyneuropathy, organomegaly, endocrinopathy, M-protein, skin changes) syndrome may be confused with CIDP early in the course of the disease. Distinguishing clinical features include poor treatment response and the associated systemic features that contribute to the acronym. Increased serum vascular endothelial growth factor is a marker of the disease. US may also help distinguish POEMS syndrome neuropathy from CIDP. In POEMS syndrome, nerve enlargement may be seen at sites of nerve entrapment but is uncommon in other parts of the nerve,[57] which is distinct from the findings in CIDP.

Multifocal motor neuropathy

Multifocal motor neuropathy (MMN) is a rare neuropathy characterised by slowly progressive limb weakness, most commonly starting in the distal upper limb, with most patients responding to treatment with intravenous immunoglobulin. From a practical perspective, in some cases MMN can be difficult to distinguish from patients with progressive muscular atrophy. US studies identify focal nerve enlargement in the majority of patients with MMN, including in limbs without neurophysiological dysfunction.[45 58 59] This is in contrast to the mild reduction of nerve CSA seen in MND.[60] As such, US studies have been suggested as one method to select appropriate patients for treatment.[28]

Guillain-Barré syndrome

Presently, there are few studies reporting the US findings in patients with Guillain-Barré syndrome (GBS) and no studies to date comparing demyelinating with axonal GBS variants. Nerve enlargement has been reported in 47–83% of patients with early GBS, and may be present in peripheral nerves and/or cervical nerve roots.[15 61] The distribution of nerve changes may be patchy within an individual[61] and may be seen early before neurophysiological changes have developed.[15] Alterations of the fascicular architecture have been reported, with heterogeneous focal enlargement of single fascicles noted in one case report.[62]

In a detailed study, Gallardo *et al*[61] described clinical, neurophysiological and US findings in six consecutive early GBS patients, with pathological correlation with autopsy material in two patients. US of the cervical nerve roots, and major upper and lower limb peripheral nerves was reported. US abnormalities were only detected in 8.8% of the scanned nerves, however, cervical nerve root abnormalities were identified in the majority of patients, consisting of increased CSA and reduced definition of epineurial margins. Indistinct margins of cervical nerve roots was a novel US finding and was correlated with nerve oedema demonstrated on corresponding pathological studies, which suggested that US findings may reflect the pathogenesis of the disease.

While longitudinal studies are generally lacking, a single case report demonstrated that US changes normalised during recovery, in keeping with clinical and neurophysiological improvement.[62] However, increased nerve CSA was identified in patients with residual deficits years after the onset of GBS, but these US changes did not correlate with functional disability.[63]

Infectious polyneuropathies

Leprosy is the most common infectious cause of neuropathy worldwide. Nerve enlargement and loss of fascicular pattern are seen on US.[27 64] These abnormalities are most frequent at common sites of nerve entrapment, in particular the cubital tunnel. However, generally the nerve enlargement tends to be more extensive and less circumscribed. Thickened and hypoechoic epineurium is a characteristic finding. Immunologically mediated reversal reactions are a common cause of skin and nerve injury in leprosy, and Doppler US at this stage may demonstrate increased nerve vascularity, which suggests rapid progression of nerve damage and a poor prognosis.[64]

Axonal neuropathy

The role of US may be less well defined in axonal PN. Intuitively, one may expect reduced CSA in axonal PN due to loss of myelinated fibres. However, this is seldom apparent with the exception of modest reduction of nerve calibre in amyotrophic lateral sclerosis (ALS).[60 65 66] In fact, US of axonal PN may detect nerve enlargement in approximately 20% of patients.[15]

Studies of US in diabetic PN, most of them focused on the evaluation of tibial and median nerves, have demonstrated evidence of nerve and fascicle enlargement, and loss of fascicular pattern.[67–70] Correlation with electrophysiological parameters has been noted in some but not in all studies. Specifically, inverse relationships between nerve CSA and compound muscle action potential amplitude and motor nerve conduction velocity have been identified.[69] Increased water content due to conversion of glucose into sorbitol in the nerve was suggested as a cause of the increased nerve CSA.[67] Nerve enlargement may be an interesting marker of diabetic PN severity in future studies, although it is noted that this finding has not been reported in all studies of diabetic PN.[15 71]

Oxaliplatin-induced neuropathy, which has axonal features on neurophysiological studies,[72] is not associated with reduced CSA on US but rather nerve enlargement at sites of nerve entrapment—a finding suggesting increased susceptibility to mechanical nerve injury.[73]

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Nerve tumours

When only imaging data is considered, hypertrophic neuropathy may be mistaken for a peripheral nerve tumour and vice versa, particularly when nerve enlargement is segmental or when tumours are multifocal, for example, neurofibromatosis, multiple schwannomatosis and intraneural perineurioma.[74 75] A comprehensive review of US in peripheral nerve tumours is beyond the scope of this review (and readers are directed to specific reviews on the topic[76 77]). However, characteristic lesion features are noted in some peripheral nerve tumours, which may help distinguish them from hypertrophic neuropathy. A comprehensive US study of proximal and distal peripheral nerves combined with clinical and neurophysiological information should satisfactorily distinguish each disease process; however, fascicular nerve biopsy may sometimes be needed.

PRACTICAL APPROACH TO US DIAGNOSIS

Mapping nerve abnormalities

An important aspect of the US examination of suspected PN is analysis of the topographic distribution of nerve abnormalities. This includes: the number of nerves involved (diffuse, multifocal or localised); the presence of a proximal or distal predominance; and the uniformity of the involvement along the course of the nerve. Comprehensive US assessment of the peripheral nervous system is the recommended approach, which may include examination of the brachial plexus, upper extremity nerves (median, radial and ulnar) and lower extremity nerves (femoral, sciatic, peroneal, tibial and sural), with the composition of the study guided by the clinical phenotype but also including clinically unaffected regions.

Distinguishing CMT from CIDP

One issue experienced in the neuromuscular clinic is distinguishing some acquired neuropathies from hereditary demyelinating neuropathies and broad diagnostic test batteries and empirical treatment trials are often employed to confirm a diagnosis. Nerve US may contribute to the diagnosis of demyelinating neuropathies in a number of ways.

US may help differentiate CMT from mimicking acquired demyelinating neuropathies, such as in those patients in whom clinical features and nerve conduction studies remain inconclusive. Evaluation of CSAs at intermediate nerve segments helped distinguish demyelinating CMT from CIDP because the CSAs in patients with demyelinating CMT were uniformly enlarged while those in patients with CIDP demonstrated variable enlargement.[78] However, care must be taken because patients with demyelinating CMT other than CMT1A do not always exhibit nerve enlargement.[32]

In addition, US studies facilitate assessment of proximal nerve segments that may be difficult to assess with nerve conduction studies, and hence may improve the detection of PN with demyelinating features in a predominantly proximal distribution.

Identifying the contribution of nerve compression

Despite non-specific findings in patients with axonal PN, US does have an important role in the diagnosis or exclusion of superimposed entrapment neuropathy in these patients, which can be difficult to diagnose using electrophysiological studies alone. US is able to confirm the diagnosis of compressive neuropathy and to rule out anatomical contributions to nerve injury.

As an example, increased CSA of the median nerve without change in wrist-to-forearm ratio might be compatible with diabetic PN, while it would not be indicative of carpal tunnel syndrome.[79] Many causes of PN do not lead to altered nerve morphology on US, but may predispose to secondary entrapment neuropathy. For example, 70% of patients with systemic sclerosis and sensory complaints have US evidence of carpal tunnel syndrome or ulnar neuropathy at the elbow.[80]

FUTURE DIRECTIONS: BEYOND CSA

Recent publications have demonstrated the utility of US in the work-up of patients with PN. However, many studies to date have examined heterogeneous populations. In addition, most studies have focused on the measurement of CSA at different sites, and there is overlap of values with healthy populations. Unlike findings in entrapment mononeuropathy, there is frequently no correlation between the changes in nerve calibre in PN and clinical severity, although detailed clinical information is often not reported. More detailed exploration of the US changes in PN may provide a more powerful assessment of nerve pathology, which may help in the diagnosis of PN.

The high resolution of US allows accurate assessment of different morphological characteristics of the nerve independent of CSA and these features have seldom been mentioned in published studies. The features that may warrant further exploration include: fascicle diameter, fascicle-to-connective tissue ratio, epineurial demarcation and nerve blood flow.[23 52 53]

Proposed classification of nerve US abnormalities

Although the angle of insonation and other technical factors impact on the nature of the nerve image acquired by US, information regarding the histopathological processes occurring within the nerve may be available with closer scrutiny of the US appearance of the nerve. Taking into account the different morphological aspects that can be evaluated at present, the previous reports and our personal observations, we propose the following patterns of nerve involvement (figure 2):

- Type 1: Increased CSA in multifascicular nerves due to fascicular enlargement.
  - Type 1a: With uniform or heterogeneous enlargement of hypoechoic fascicles as seen in hereditary demyelinating neuropathies and CIDP.
  - Type 1b: With mixed hyperechoic and hypoechoic fascicles as seen in longstanding CIDP.
  - Type 1c: With obliteration of normal sonographic fascicular appearance as seen in inflammatory PN, nerve trauma, HNPP, leprosy and some axonal neuropathies; mild examples of this pattern are also common in entrapment sites in nerves of asymptomatic normal individuals.
- Type 2: Increased CSA in monofascicular nerves. This pattern may be seen in the brachial plexus and cervical nerve roots in inflammatory neuropathies, such as GBS and CIDP, and hereditary neuropathies such as CMT.
- Type 3: Increased CSA in multifascicular nerves due to increased perineurial connective tissue. This may be seen in unusual neuropathies, such as hypertrophic mononeuropathy and leprosy, and may contribute to US changes in diabetic neuropathy.
- Type 4: Normal CSA with fascicular enlargement or altered echotexture as seen in CIDP and deposition disorders such as amyloid neuropathy.
- Type 5: Decreased CSA. Reduced CSA has been reported in ALS and rarely in studies of patients with axonal PN.

CONCLUSIONS

US has a complementary role in the diagnosis of PN. US has the advantage of excellent resolution of superficial nerves,

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and the dynamic nature of image acquisition makes it a natural fit for the neuromuscular and electrodiagnostic clinics. Neurophysiological studies have long been considered to be an extension of the clinical examination.[81] It is expected that a similar assertion will be increasingly relevant for US, and the addition of anatomic and structural information may provide important complementary diagnostic information.

Further, US may be useful to distinguish between different types of neuropathy, in particular the demyelinating neuropathies, by identifying patterns of morphological changes. Incorporating US features into diagnostic algorithms may rationalise the process of diagnostic testing with inherent time and cost savings. Exploration of nerve US features in addition to CSA may provide additional pathophysiological and diagnostic insights. Further correlation between US and MRI in PN may allow the roles of each of these techniques to be better delineated.

Presently, correlations between nerve morphology and electrophysiological function are emerging in demyelinating neuropathies, such as CMT and CIDP, and diabetic neuropathy; however, further exploration is needed to determine the relationship between structure and function in other neuropathy subtypes. Understanding of this emerging diagnostic tool will be better developed by detailed comparisons of US findings with clinical, neurophysiological and histopathological features, and this is recommended as a focus of future research.

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Ultrasound in the diagnosis of peripheral neuropathy: structure meets function in the neuromuscular clinic

Elena Gallardo, Yu-ichi Noto and Neil G Simon

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Identification of *ter94*, *Drosophila VCP*, as a strong modulator of motor neuron degeneration induced by knockdown of *Caz*, *Drosophila FUS*

Yumiko Azuma[1], Takahiko Tokuda[1,2,†,*], Mai Shimamura[3,4], Akane Kyotani[3,4], Hiroshi Sasayama[1], Tomokatsu Yoshida[1], Ikuko Mizuta[1], Toshiki Mizuno[1], Masanori Nakagawa[1], Nobuhiro Fujikake[5], Morio Ueyama[5], Yoshitaka Nagai[5] and Masamitsu Yamaguchi[3,4,†]

[1]Department of Neurology and [2]Department of Molecular Pathobiology of Brain Diseases, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kamigyo-ku, Kyoto 602-8566, Japan, [3]Department of Applied Biology and [4]Insect Biomedical Research Center, Kyoto Institute of Technology, Hashikami-cho, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan and [5]Department of Degenerative Neurological Diseases, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawa-Higashi, Kodaira, Tokyo 187-8502, Japan

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In humans, mutations in the *fused in sarcoma* (*FUS*) gene have been identified in sporadic and familial forms of amyotrophic lateral sclerosis (ALS). *Cabeza* (*Caz*) is the *Drosophila* ortholog of human *FUS*. Previously, we established *Drosophila* models of ALS harboring *Caz*-knockdown. These flies develop locomotive deficits and anatomical defects in motoneurons (MNs) at neuromuscular junctions; these phenotypes indicate that loss of physiological FUS functions in the nucleus can cause MN degeneration similar to that seen in FUS-related ALS. Here, we aimed to explore molecules that affect these ALS-like phenotypes of our *Drosophila* models with eye-specific and neuron-specific *Caz*-knockdown. We examined several previously reported ALS-related genes and found genetic links between *Caz* and *ter94*, the *Drosophila* ortholog of human *Valosin-containing protein* (*VCP*). Genetic crossing the strongest loss-of-function allele of *ter94* with *Caz*-knockdown strongly enhanced the rough-eye phenotype and the MN-degeneration phenotype caused by *Caz*-knockdown. Conversely, the overexpression of wild-type *ter94* in the background of *Caz*-knockdown remarkably suppressed those phenotypes. Our data demonstrated that expression levels of *Drosophila* VCP ortholog dramatically modified the phenotypes caused by *Caz*-knockdown in either direction, exacerbation or remission. Our results indicate that therapeutic agents that up-regulate the function of human VCP could modify the pathogenic processes that lead to the degeneration of MNs in ALS.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease that is characterized by degeneration of motoneurons (MNs); this degeneration leads to progressive muscle weakness and eventually fatal paralysis typically within 1–5 years after disease onset (1). Frontotemporal lobar degeneration (FTLD) is a dementia syndrome with clinically diverse phenotypes that include behavioral changes, semantic dementia and progressive non-fluent aphasia (2). It is well established that ALS and FTLD form a clinical disease continuum (3,4). Up to 15% of ALS patients meet the clinical criteria of FTLD and 30–50% has subtle cognitive deficits (5); likewise, up to 15% of FTLD patients meet the clinical criteria for ALS and up to one-third have at least minor MN dysfunction (5).

* To whom correspondence should be addressed at: Department of Molecular Pathobiology of Brain Diseases, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kamigyo-ku, Kyoto 602-8566, Japan. Tel: +81 752515793; Fax: +81 752118645; Email: ttokuda@koto.kpu-m.ac.jp
†Co-corresponding authors.

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A substantial number of proteins linked to ALS are directly or indirectly involved in RNA processing (6). Mutations in genes encoding two such RNA-binding proteins—transactive response DNA binding protein 43 kDa (TDP-43, gene *TARDBP*) and fused in sarcoma (FUS, gene *FUS*)—have been identified as major genetic causes in both familial and sporadic ALS (2,7–15). TDP-43 and FUS are RNA-binding proteins implicated in multiple aspects of RNA metabolism including transcriptional regulation, mRNA splicing and shuttling of mRNAs between the nucleus and the cytoplasm (16,17).

There is a single *Drosophila* ortholog each for human FUS and TDP-43 named Cabeza (Caz) and TBPH, respectively. Reportedly, *Drosophila* lacking TBPH presented deficient locomotive behaviors, reduced life span and anatomical defects at neuromuscular junctions (NMJs); these phenotypes indicate that a loss of TDP-43 nuclear functions could be a causative factor for the neurodegeneration observed in patients with ALS/FTLD (18). Recently, we reported a fly model of ALS based on neuron-specific Caz-knockdown, which developed locomotive deficits and anatomical defects in MNs at NMJs; these findings demonstrate that loss of physiological functions of FUS in the nucleus could cause the neurodegeneration in FUS-related ALS/FTLD (19). There is substantial evidence that depletion of FUS in zebrafish or in *Drosophila* also causes MN degeneration that can be rescued by the respective wild-type but not mutant *FUS* (20,21). These findings suggest that FUS is important for the survival of MNs and that a "loss-of-function" mechanism could be the fundamental pathogenic mechanism causing FUS-related ALS/FTLD.

Using our established fly model of FUS-ALS induced by *Caz*-knockdown, we aimed to explore molecules that affect phenotypes presented by the model. Specifically, we intended to elucidate the molecular mechanisms leading to neuronal dysfunction in FUS-related ALS/FTLD in order to facilitate the development of disease-modifying therapies, which are eagerly desired for the treatment of those relentless neurodegenerative diseases. Initially, we focused on determining whether or not several ALS-related genes affect the phenotypes presented by our fly models, and we found a genetic link between *Caz* and *ter94*, which is the *Drosophila* ortholog of human *Valosin-containing protein* (*VCP*).

VCP is a member of the AAA (ATPase associated with a variety of cellular activities) family of proteins, which are implicated in a large variety of biological functions including the regulation of ubiquitin-dependent protein degradation, control of membrane fusion and of dynamics of subcellular components, vesicle-mediated transport and nucleocytoplasmic shuttling (22–24). Association of *VCP* mutations with human disease was first identified in patients with IBMPFD (inclusion body myopathy with early-onset Paget disease and frontotemporal dementia) (25) and more recently in those with ALS (26). There is a single ortholog of human *VCP* in *Drosophila,* named *ter94,* which is predicted to share ~83% amino acid sequence identity with human VCP.

Here, we found that genetic crossing the strongest loss-of-function allele of *ter94* with *Caz*-knockdown severely enhanced the *Caz*-knockdown phenotypes in flies; it severely exacerbated locomotive disabilities and the degeneration of MNs induced by neuron-specific *Caz*-knockdown. Conversely, the over-expression of *ter94* rescued those phenotypes.

RESULTS

Knockdown of *Caz* in eye imaginal discs induces morphologically aberrant rough eyes

To investigate the molecular mechanisms of FUS-related neurodegeneration, we have already generated *Caz*-knockdown fly models of ALS by using the highly versatile GAL4/UAS-targeted expression system (19). To eliminate the possibility of off-target effects, we generated 11 independent transgenic fly lines and obtained one fly line from the Vienna *Drosophila* RNAi center (VDRC; Table 1); *Caz* double-stranded RNA (dsRNA; inverted repeats, IRs) targeted to the different region of the *Caz* mRNA is expressed in those fly lines as described in our previous study (19). The RNAi of the fly lines we generated (responder controls) was targeted to the region corresponding to residues 1–167 (four lines, UAS-*Caz*-IR_{1-167}) and 180–346 (seven

Table 1. Associated phenotypes of fly strains carrying UAS-Caz-IR crossed with different GAL4 driver strains

	Transgene strain	Chromosome linkage	Act5C-GAL4> 28°C	25°C	GMR-GAL4>	elav>GAL4
UAS-*Caz*-IR_{1-167}	3	III	Lethal	Lethal		
	4	III	Lethal	NE	Mild rough eye	ND
	11	II	Lethal	NE	Mild rough eye	ND
	21	III	Lethal	NE	Mild rough eye	ND
UAS-*Caz*-$IR_{180-346}$	11	II	NE	NE	Mild rough eye	NE
	12	II	NE	NE	Mild rough eye	NE
	17	II	NE	NE	Mild rough eye	ND
	22	III	NE	ND	Mild rough eye	NE
	24	III	NE	NE	ND	NE
	32	III	NE	ND	Mild rough eye	ND
	33	III	NE	NE	Mild rough eye	ND
UAS-*Caz*-$IR_{363-399}$		II	NE	ND	Mild rough eye	LD

We used two independent Caz-RNAi constructs, UAS-*Caz*-IR_{1-167} and UAS-*Caz*-$IR_{180-346}$, to generate 11 independent transgenic fly lines. UAS-*Caz*-$IR_{363-399}$ was obtained from VDRC. To drive the expression of *Caz* dsRNA in the whole body of the flies, or specifically in the eye imaginal discs or neuronal tissues, we cross UAS-*Caz*-IR flies with *Act5C-GAL4*, *GMR-GAL4* or *elav-GAL4* flies, respectively. Phenotypes associated with the resultant genotypes are summarized. Each transgenic strain shows a consistent phenotype. NE, no effect; ND, not determined; LD, locomotive dysfunction.