BRAIN
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Skin innervation across amyotrophic lateral sclerosis clinical stages: new prognostic biomarkers

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Over recent decades, peripheral sensory abnormalities, including the evidence of cutaneous denervation, have been reported among the non-motor manifestations in amyotrophic lateral sclerosis (ALS). However, a correlation between cutaneous innervation and clinical features has not been found.

The aims of this study were to assess sensory involvement by applying a morpho-functional approach to a large population of ALS patients stratified according to King's stages and correlate these findings with the severity and prognosis of the disease.

We recruited 149 ALS patients and 41 healthy controls. Patients undertook clinical questionnaires for small fibre neuropathy symptoms (Small Fiber Neuropathy Symptoms Inventory Questionnaire) and underwent nerve conductions studies (NCS) and 3-mm punch skin biopsies from leg, thigh and fingertip. We assessed intraepidermal nerve fibre (IENF) and Meissner corpuscle (MC) density by applying an indirect immunofluorescence technique. Moreover, a subset of 65 ALS patients underwent a longitudinal study with repeat biopsies from the thigh at 6- and 12-month follow-ups. Serum NfL levels were measured in 40 patients.

Sensory symptoms and sensory NCS abnormalities were present in 32.2% and 24% of patients, respectively, and increased across clinical stages. Analogously, we observed a progressive reduction in amplitude of the sensory and motor ulnar nerve potential from stage 1 to stage 4.

Skin biopsy showed a significant loss of IENFs and MCs in ALS compared with healthy controls (all $P < 0.001$). Across the clinical stages, we found a progressive reduction in MCs ($P = 0.004$) and an increase in IENFs (all $P < 0.027$). The increase in IENFs was confirmed by the longitudinal study. Interestingly, the MC density inversely correlated with NfL level ($r = -0.424$, $P = 0.012$), and survival analysis revealed that low MC density, higher NfL levels and increasing IENF density over time were associated with a poorer prognosis (all $P < 0.024$).

To summarize, in patients with ALS, peripheral sensory involvement worsens in parallel with motor disability. Furthermore, the correlation between skin innervation and disease activity may suggest the use of skin innervation as a putative prognostic biomarker.

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Introduction

The selective impairment of motoneurons has been the most recognized clinical characteristic of amyotrophic lateral sclerosis (ALS) for many years. However, the presence of non-motor involvement, such as cognitive, behavioural[1,2] and autonomic dysfunctions,[3] paralleling motor worsening has been increasingly reported in recent years. These observations have challenged the 'motor-centric' theory and initiated a redefinition of ALS as a multisystemic disease. Among non-motor defects, sensory impairment has been described in clinical, neurophysiological and neuropathological studies.[4]

In fact, a growing body of evidence shows the involvement of the somatosensory pathway in human and animal models of ALS at multiple levels, from the very distal endings of sensory nerves in the skin to the cerebral cortex.[5] Regarding the involvement of the peripheral part of the sensory pathway, several neurophysiological studies have revealed abnormalities in patients with ALS. Specifically, in a retrospective study of 103 patients with motor neuron disease and no coexisting conditions that might cause sensory involvement, 32% reported the presence of sensory symptoms (i.e. hypoesthesia, paraesthesia or dysesthesia) and 27% showed sural sensory nerve action potential (SNAP) abnormalities.[6] Moreover, a multicentre study confirmed the presence of sensory nerve conduction abnormalities. Specifically, 22.7% of patients exhibited slowed conduction velocity in at least one nerve, and 12.5% of them met the electrophysiological criteria for sensory polyneuropathy of unknown origin,[7] regardless of age, disease duration and onset. Recently, a Portuguese group[8] found sensory polyneuropathy in 8.6% of ALS patients, which was more frequent in older men and patients with respiratory symptom onset. Consequently, the presence of mild sensory peripheral neuropathy no longer rules out the diagnosis of ALS, as established also by the Awaji guidelines.[9] Pathological studies on sural and superficial peroneal nerves have confirmed the presence of axonal degeneration of large sensory axons in ALS, and up to 91% of the sural nerves of patients show a significant loss of axons.[6,10-12] Besides the impairment of large sensory fibres, an involvement of small sensory fibres has also been demonstrated in ALS patients through skin biopsy[13-16] and corneal confocal microscopy.[17] Across different studies, the analysis of cutaneous innervation has consistently shown a loss of epidermal nerve fibre density. Interestingly, in our previous work, we found, coexisting with nerve degeneration, evident aspects of nerve regeneration.[13] The coexistence of processes of degeneration and regeneration could be the reason for the failed attempts to correlate nerve loss with disease severity in ALS. Therefore, this study aimed to analyse cutaneous innervation in a larger cohort of ALS patients to assess the correlation between morphological findings, disease severity and progression across clinical stages.

Materials and methods

Participants

We recruited 149 patients [males = 90; age = 63 years, interquartile range (IQR) = 16 years] between January 2016 and January 2022 from the Department of Neurology of Telese Terme of the Salvatore Maugeri Foundation and the ALS Center of the University Federico II of Naples, Italy. Patients met a diagnosis of 'probable', 'probable laboratory-supported' or 'definite' ALS, as per the revised El Escorial criteria.[18] Disease severity and clinical staging were assessed with the ALS Functional Rating Scale–Revised (ALSFRS-R)[19] and the ALS King's scale, respectively.[20] The latter is based on the spreading of motor symptoms to three different body regions (bulbar, upper limbs and lower limbs) and on the use of non-invasive ventilation and enteral nutrition. The five stages of the King's staging system are as follows: 1 = one region involved; 2 = two regions involved; 3 = three regions involved; 4A = patient needs gastrostomy; 4B = patient needs non-invasive ventilation.[20] The stage can be derived from direct observation of the patient and the ALSFRS-R scale.[21]

Patients underwent blood analysis to rule out glucose dysmetabolism, dysendocrinopathies, vitamin E, B_{12} and folic acid deficiencies, hepatic and renal failure, or HIV or connective tissue disorders. Patients with electrophysiological evidence of entrapment neuropathy (i.e. carpal tunnel syndrome, ulnar nerve entrapment at the elbow) were also excluded from the study.

Genetic analysis was performed in all patients, exploring *C9orf72* repeat expansion and mutations of *SOD1*, *TARDBP* and *FUS* genes. Written informed consent was obtained from all subjects according to the Declaration of Helsinki before enrolment in the study. The study protocol was approved by the local Ethical Committees of the participating centres.

Clinical and instrumental assessment of sensory fibres

All patients underwent clinical assessment of sensory and autonomic symptoms using the Small Fiber Neuropathy Symptoms Inventory Questionnaire (SFN-SIQ).[22] The SFN-SIQ consists of 13 items related to the occurrence of autonomic and sensory disturbances. The overall score ranges from 0 (no symptoms) to 39 (all symptoms, always present). Since the main aim of this study was to assess morphological and functional alterations of sensory fibres, we selected items related to sensory symptoms and considered a sensory item to be involved if it scored ≥2.[23]

Patients also underwent standardized nerve conduction studies as part of the routine assessment protocol. Orthodromic motor conduction studies (amplitude, distal latency and velocities) included bilateral peroneal (recording from extensor digitorum brevis muscles) and ulnar nerves (recording from the abductor digiti minimi),

including F-waves (latency and persistence). Antidromic sensory nerve action potentials (SNAPs) were recorded from ulnar nerve (fifth finger), superficial peroneal nerves (dorsum pedis) bilaterally (conduction velocity and amplitude), and if one or both peroneal nerves were abnormal, additional SNAPs from sural nerves were recorded bilaterally (behind the lateral malleolus).

Stimulation and recording were performed through surface electrodes, and skin temperature was kept above 32°C. SNAP amplitude below the fifth percentile cut-off (compared with age and gender-matched subjects from a historical control group) was considered abnormal.[24] A length-dependent peripheral sensory neuropathy was defined by the presence of at least two abnormal SNAPs recorded from peroneal nerves or sural nerves,[8] while a non-length dependent neuropathy was defined by the presence of at least two abnormal SNAP amplitudes at the upper limbs and normal findings at the lower limbs.

Morphologic evaluation of cutaneous sensory fibres

Skin biopsies were taken from hairy (distal leg and distal thigh, at the union of middle third with distal third) and glabrous skin (at the vortex of fingertip of digit V) with a 3-mm punch in all patients and healthy controls (right side for hairy skin and left hand for glabrous skin). Skin samples were taken from the most affected side of patients with an asymmetric motor phenotype and from the right side of patients without overt clinical asymmetry. Skin samples from all three sites could not be obtained from all patients. Specifically, a total of 354 skin samples were collected: 124 from leg, 130 from thigh and 100 from fingertip.

Specimens were processed with an indirect immunofluorescence technique[25] using a panel of primary antibodies and an endothelium-binding agglutinin (*Ulex europaeus*, Vector) to mark nerves and vessels. Confocal (ApoTome.2, Zeiss) images were analysed by dedicated software. In each sample, intraepidermal nerve fibre (IENF) density per linear millimetre was calculated on four non-consecutive sections double-stained with protein gene product (PGP) 9.5 and collagen-IV, according to published rules.[26]

In glabrous skin, the density of Meissner corpuscles (MCs) per mm^2 was calculated following previously described procedures.[27] A total of 3540 sections were observed, 10 for each sample. Quantitative assessment was performed on a total of 2216 sections (IENFs on 1416 and MCs on 800 sections). For each skin sample, a mean value of IENF and MC (only in glabrous skin) density was obtained. A single operator (I.B.) blinded to diagnosis performed all of the quantitative assessments.

A subgroup of 65 ALS patients also underwent a longitudinal skin biopsy assessment with a follow-up at 6 months (57 patients) and/or 12 months (34 patients). For the longitudinal study, a skin biopsy was taken from the thigh since this site was shown to be the most affected in our baseline data (see 'Results' section). For this part of the study, a further 396 sections were quantified.

Finally, we assessed degenerative and regenerative morphological aspects of 20 patients who underwent the longitudinal study. Skin sections from this group of patients were stained with an extended protocol that also included the marker growth associated protein (GAP) 43 (Millipore, AB5220).

In sections from this group (three non-consecutive sections for each patient at the two time points: baseline and 12 months) we assessed: (i) nerve fibre swellings, by calculating the swelling ratio, defined as the ratio between the number of IENF varicosities >1.5 µm and the number of IENFs; (ii) the presence/absence of regenerative aspects such as clusters of fibres or fibre sprouts; and (iii) the density of IENFs expressing the marker GAP43.

Healthy controls

The fifth percentile cut-off for IENF and MC densities was calculated on our age- and gender-stratified normative database. Normative values of IENF density from leg were derived from a cohort of 528 healthy subjects (age = 47.0 ± 14.3; males/females = 217/311).[28] Values of IENF density from thigh were derived from a group of 207 healthy subjects (age = 49.1 ± 14.2; males/females = 88/119).[29] Values of IENF and MC density from fingertip glabrous skin were derived from a group of 100 healthy subjects (age = 50.0 ± 15.3; males/females = 46/54) (unpublished data). Among them, a group of 41 subjects similar in age and sex were selected for statistical analysis.

Neurofilament light chain analysis

We analysed NfL levels in a subgroup of 40 ALS patients to compare this well-recognized biomarker of axonal degeneration[30] and disease activity with cutaneous innervation, particularly with densities of MCs and IENFs from the three sites.

Sera were obtained the same day on which skin biopsy was performed. Serum samples were centrifuged at room temperature, aliquoted in polypropylene tubes within 1 h of collection and then stored at −80°C. The concentration of NfL protein was determined in duplicate by investigators blinded to the clinical data using a Simoa™ HD-1 immunoassay analyser, (Quanterix), which runs ultra-sensitive paramagnetic bead-based ELISAs.[31]

Statistical analysis

Since the data were not normally distributed (Kolmogorov–Smirnov test: $P < 0.200$), differences in variables between the two groups (patients versus healthy controls) or among patients in the different clinical stages were computed with χ^2 or Mann–Whitney U-test/Kruskal–Wallis as appropriate. *Post hoc* comparisons were run with the Bonferroni test. Demographic and clinical characteristics of the participants were reported as percentages for categorical variables and median (IQR) for non-parametric continuous variables.

A mixed-effect linear regression model was applied for longitudinal analysis of skin innervation (IENF) at thigh, with the setting time (baseline versus post-6 months versus post-12 months) as a fixed factor.

Spearman's test was used to explore the relationship between NfL levels and the z-score of cutaneous innervation at each site. Survival curves were constructed with the Kaplan–Meier method, and patients were stratified by the median value of the IENF density at leg, thigh, fingertip, number of MCs/mm^2 or the presence/absence of IENF increase (regenerating/non-regenerating patients) at thigh over time for patients who underwent the longitudinal study. Specifically, we defined a difference higher than 1 fibre/mm[32] between the follow-up and baseline assessments as an increase in IENFs; for patients receiving two assessments during the follow-up (i.e. post-6 and post-12), we considered the average value.

Survival time was defined as the time from symptom onset to the time of death.

A log-rank test was applied to compute differences between the curves.

For all analyses, a two-tailed *P*-value <0.05 was considered statistically significant. Alpha inflation due to multiple comparisons

Table 1 Demographic and clinical variables according to king's clinical staging

	King's clinical stage				P-value	All patients
	1	2	3	4		
Age at testing, years	61 (15)	62.5 (23)	65 (15)	65.5 (15)	0.414	63 (16)
Sex, male/female	17/6	22/12	33/23	18/18	0.296	90/59
Disease duration from onset, months	18 (12)	16 (12)	12 (12)	14 (15)	0.479	14.3 (12)
ALSFRS-R score	42 (4)	38.5 (6)	34 (6)	31.5 (18)	**<0.001**	37 (7)
Disease progression rate, ALSFRS-R points lost/month	0.25 (0.12)	0.46 (0.43)	0.92 (0.93)	1.1 (0.96)	**<0.001**	0.72 (0.99)
Onset, spinal/bulbar	19/4	29/5	46/10	30/6	0.984	124/25
Patients with SNAP abnormalities in at least one nerve, %	9% (2/23)	25% (7/28)	24% (12/50)	50% (6/12)	0.059	24% (27/113)
SFN-SIQ: total score	4 (8)	5 (3)	9 (8)	10 (3)	**0.003**	6 (7)
SFN-SIQ: sensory subscore symptoms, %	22% (5/23)	15% (5/34)	36% (20/56)	61% (22/36)	**<0.001**	35% (52/149)
SFN-SIQ: large sensory fibre subscore symptoms, %	13% (3/23)	15% (5/34)	27% (15/56)	31% (11/36)	0.236	23% (34/149)
SFN-SIQ: small sensory fibre subscore symptoms, %	9% (2/23)	12% (4/34)	18% (10/56)	50% (18/36)	**<0.001**	23% (34/149)

Values are expressed as median (interquartile range) or as percentage (frequencies). Comparisons between the four groups were performed by means of non-parametric Kruskal–Wallis test; frequencies were compared by means of χ^2 tests. Values in bold type indicate significance $P < 0.05$. ALS = amyotrophic lateral sclerosis; ALSFRS-R = ALS Functional Rating Scale-Revised; SNAP = sensory nerve action potential; SFN-SIQ = Small Fibre Neuropathy Symptoms Inventory Questionnaire.

was controlled according to Bonferroni's approach when appropriate. All statistical analyses were carried out using IBM SPSS statistics software (Version 25 for Windows, New York, NY, USA).

Results

Demographic and clinical data

The demographic and clinical characteristics of patients at the various stages according to the King's classification system are reported in Table 1. Median age at onset, sex distribution, disease duration and the percentage of bulbar onset patients were not significantly different across the clinical stages, while patients belonging to the more advanced clinical stages displayed higher disease progression rates. All but seven patients tested negative for the most common genetic mutations. Of the seven patients with genetic mutations, four had *C9orf72* gene expansion, two had *SOD1* gene mutations and one had a *FUS* gene mutation.

Peripheral sensory symptoms and nerve function

Sensory symptoms of any kind were reported by 35% (Table 1) of our ALS patient cohort, with large-fibre sensory symptoms such as paraesthesia reported by 23% of them (34/149) and small-fibre sensory symptoms (i.e. pain-burning, allodynia, diminished temperature and pain sensation) reported by a similar percentage (34/149). The most reported small-fibre sensory symptom was burning-pain in 10.1% of patients (15/149), followed by diminished sensation of temperature (8.7%, 13/149) and pain (6%, 9/149) and allodynia (3.4%, 5/149). The median SFN-SIQ total score was 6 (IQR 7), whereas the median SFN-SIQ sensory subscore was 1 (IQR 2.75).

Stratifying patients according to King's staging, the frequency of patients complaining of small-fibre sensory symptoms increased significantly ($\chi^2 = 17.74$, $P < 0.001$) from stage 1 to stage 4 (Table 1). Analogously, the frequency of large-fibre sensory symptoms increased across clinical stages (Table 1), although not significantly ($\chi^2 = 4.24$, $P = 0.236$).

Nerve conduction study disclosed SNAP abnormalities in 23.9% (27/113) of patients in at least one nerve. The percentage of patients fulfilling the neurophysiological criteria for peripheral sensory neuropathy was 15% (17/113), with a length-dependent pattern in 10.6% (12/113) and a non-length dependent distribution in 4.4% (5/113) of patients (Supplementary Tables 1 and 2). Interestingly, the frequency of patients with at least one abnormal sensory nerve tended to increase across clinical stages ($\chi^2 = 7.43$, $P = 0.059$). In addition, considering the nerve supplying the territory used for morphological evaluation (i.e. fifth digit fingertip), we found a significant reduction in the absolute value of SNAP amplitude from stage 1 to stage 4 for the ulnar nerve ($P = 0.034$; Fig. 1A). The same result was observed for the amplitude of the compound muscle action potential recorded from the abductor digiti minimi after stimulation of the ulnar nerve at wrist ($P = 0.007$; Fig. 1B).

Skin biopsy findings

Epidermal nerve fibres

Quantitative nerve analysis (Table 2) revealed in the leg, thigh and fingertip of ALS patients a marked loss of IENFs compared with the healthy controls (all $P < 0.001$).

The percentage of ALS patients showing an IENF density below the fifth percentile cut-off was 58% (72/124) at leg, 78% (101/130) at thigh and 30% (30/100) at fingertip. IENF density at each site was not different in patients with bulbar onset compared with spinal onset patients (all $P > 0.44$).

We observed a significant increase in IENF density from King's stage 1 to stage 4 at all sites (leg: $P = 0.021$; thigh: $P = 0.02$; and fingertip: $P = 0.027$) (Figs 1D–F and 2A–C). Overall, in the sections with increased IENF density, the signs of chaotic nerve regeneration were also more evident in the subepidermal plexus (sprouts) and epidermis, with evidence of IENF branching and clustering (Fig. 2A–C and K).

Regarding the possible association between IENF density and survival, the Kaplan–Meier curves did not show significant differences between the groups of patients with an IENF density below versus above the IENF median value at any site (leg: $P = 0.467$; thigh: $P = 0.816$; and fingertip: $P = 0.559$).

Large sensory fibres

In addition to the loss of IENFs, we found a significant reduction of MCs in glabrous skin compared with the HCs ($P < 0.001$; Table 2),

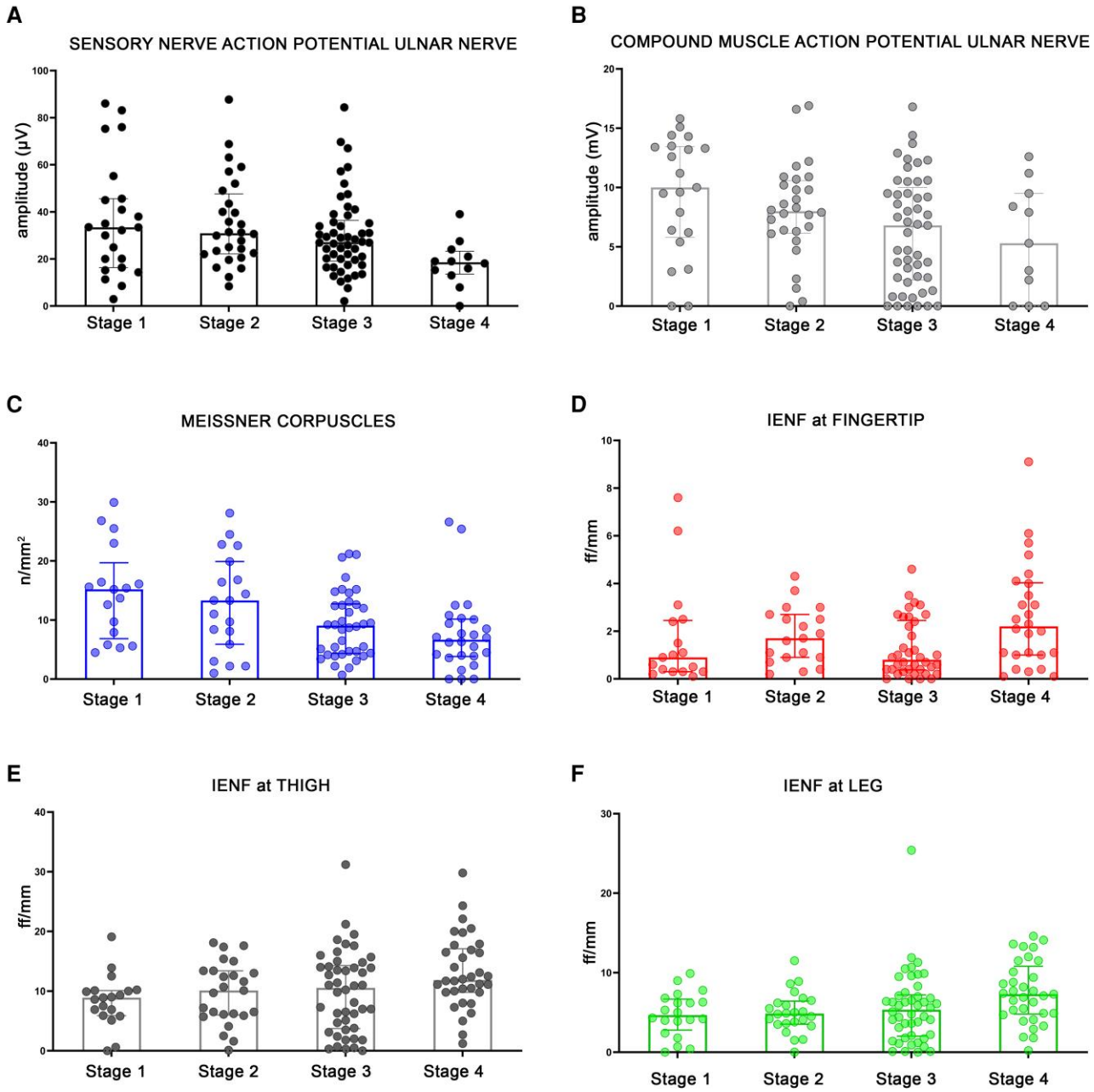

Figure 1 Morpho-functional assessment of peripheral nerve fibres across the clinical stages of amyotrophic lateral sclerosis. Bar dot plots showing (**A**) a significant reduction from King's stage 1 to stage 4 in the sensory nerve action potential (SNAP) recorded from the fifth finger ($P = 0.034$), and (**B**) the compound muscle action potential (CMAP) recorded from the abductor digiti minimi ($P = 0.007$) and stimulating the ulnar nerve at wrist. Bar dot plots showing (**C**) a significant decrease ($P = 0.004$) in Meissner corpuscles (MC) and increase in intraepidermal nerve fibres (IENF) at (**D**) fingertip ($P = 0.027$), (**E**) thigh ($P = 0.02$) and (**F**) leg ($P = 0.021$) across King's clinical stages. Significance, $P < 0.05$.

implying the additional involvement of large fibre endings. The density of MCs was below the fifth percentile cut-off in 53% of ALS patients (53/100). Bulbar onset patients showed lower values of MC density compared with spinal onset patients, but this difference was not significant (5.56, IQR 5.78 versus 9.35, IQR 10.8; $P = 0.07$). According to King's staging, we observed a significant reduction in MCs across stages ($P = 0.004$; Figs 1C and 2D–F). Kaplan–Meier curves of survival probability showed that patients with a MC density below the median value of 9.05/mm^2 displayed a worse prognosis than the patient group with higher MC density ($P = 0.024$; Fig. 3)

Morphological abnormalities

As described in our previous work on 41 ALS patients, we confirmed the frequent occurrence of morphological abnormalities in epidermal and dermal nerves in this cohort of 149 ALS patients (Fig. 2). We observed aspects of nerve degeneration such as varicosity of IENFs and axonal swellings often present in nerve fascicles in the upper and in the lower dermis. Such swellings in some cases were particularly large (Fig. 2G and H) and associated with an abnormal aspect of Schwan cells (Fig. 2I).

MCs showed frequent morphologic abnormalities such as elongation and simplification, with a marked loss of the neural

Table 2 Demographic and morphological data

	Age, years	Sex, M/F	IENF LEG	IENF THIGH	IENF FT	MC
ALS	63 (16)	90/59	5.4 (4.1)	10.1 (7.7)	1.1 (2.7)	9.0 (9.9)
HC	63 (14)	21/20	13.1 (4.7)	21.9 (2.5)	7.3 (2.8)	25.3 (9.4)
P-value	0.45	0.162	**<0.001**	**<0.001**	**<0.001**	**<0.001**

Comparisons between the two groups were made using the Mann–Whitney U-test or χ^2 test. Values in bold type indicate significance, $P < 0.05$. ALS = amyotrophic lateral sclerosis; F = female; HC = healthy controls; IENF = intraepidermal nerve fibres; FT = fingertip; M = male; MC = Meissner's corpuscles.

component. Moreover, we confirmed the observation of nerve vessel abnormalities, suggesting neoangiogenesis (megacapillary, capillary tortuosity, capillary anastomosis, pseudohypertrophy of the vascular bed) and elongation of dermal papillae (Fig. 2L).

Neurofilament analysis

The serum NfL concentration was very heterogeneous among the 40 patients examined, varying from 11.56 to 348.06 pg/ml (54.75 pg/ml, IQR 88.14). Kaplan–Meier curves of survival probability showed that patients with values of NfL concentration higher than the median value displayed a worse prognosis ($n = 20$ patients) than the patient group with lower NfL values ($n = 20$ patients; $P = 0.008$; Fig. 3).

Correlation analysis with morphological findings displayed an inverse relationship between the MC z-score and serum NfL concentration ($r = -0.424$, $P = 0.012$), while no significant association was found with the IENF z-scores from the three sites ($P > 0.4$).

Longitudinal assessment of epidermal nerve fibres

In the 65 patients who underwent skin biopsy from thigh at 6–12 months follow-up, we observed an overall increase in IENFs over time (baseline: 8.1, IQR 5.78 versus 6-month follow-up: 8.8, IQR 8.1, versus 12-month follow-up: 10.5, IQR 6.03), which however did not reach statistical significance ($P = 0.345$) due to the high variability. Indeed, 25 patients showed increases in IENFs, namely >1 fibre/mm increase in IENFs over time (Fig. 2J and K) and 40 did not (Fig. 3C).

We identified two groups of patients, one with increased IENFs over time and the other without and tested the possibility that the presence or absence of an IENF increase over time may predict the prognosis. The Kaplan–Meier survival probability curves showed that patients with evidence of IENF increase had a shorter survival time than the group of patients without IENF increase(Fig. 3D).

Among the 40 patients without the increase in IENFs, 30 patients showed a continuous reduction (i.e. more than 1 fibre/mm reduction) in IENFs and 10 had stable innervation over time (i.e. within 1 fibre/mm variation over time). The percentage of patients with increased IENFs ($n = 25$ patients), who progressed to higher clinical stages at follow-up, was significantly higher (80%, 20/25) than patients with continuous IENF reduction (43.3%, 13/30) or stable innervation (20%, 2/10, $\chi^2 = 10.65$, $P = 0.002$).

Swelling ratio, clusters/sprouts and GAP43 immunoreactive IENFs in regenerating/non-regenerating patients

The quantification of degenerative/regenerative aspects (Supplementary Fig. 1) was applied in a subgroup of 20 patients who underwent the longitudinal study: 10 patients showing an increase of >1 fibre/mm (regenerating patients) and 10 patients showing no change or a reduction of IENF density (non-regenerating patients) over time.

Our analysis showed that:

(i) Swellings were present in all patients with a median swelling ratio of 0.50 (IQR 0.50) at baseline and 0.47 (IQR 0.37) at follow-up. The median swelling ratio in non-regenerating patients at baseline tended to be higher compared with regenerating patients (0.68, IQR 0.55 versus 0.45, IQR 0.37), although not significantly different. Differences between the two groups were even smaller at the follow-up (0.37, IQR 0.31 versus 0.48, IQR 0.27).

(ii) Clusters/sprouts were present in 30% of patients at baseline and 65% at follow-up. In particular, the percentage of patients with IENF clusters and/or nerve sprouts had increased at 12 months in regenerating patients (2/10 at baseline versus 7/10 at follow-up) but remained quite stable (4/10 at baseline versus 6/10 at follow-up) in non-regenerating patients.

(iii) We found an overall increase in the density of GAP43 immunoreactive IENFs that was higher in regenerating patients (4.64 fibres/mm, IQR 3.36), although not significantly different, compared with non-regenerating (0.95 fibres/mm, IQR 8.77) patients, due to the high variability observed in the latter group.

Detailed quantitative data on degenerative/regenerative morphological parameters are reported in Supplementary Table 3.

Discussion

In this study, we confirmed the involvement of the peripheral sensory pathway in ALS by applying a clinical, neurophysiological and morphological approach. Furthermore, we demonstrated for the first time a correlation between skin innervation and disease severity and prognosis. Notably, in stratifying patients by King's stages, we found a progressive increase in IENFs and a progressive loss of MCs across clinical stages, paralleling disease progression and severity. Moreover, we identified MC density and the changes in IENF density over time as predictors of short survival times.

Regeneration and degeneration of cutaneous sensory endings as new prognostic biomarkers

During recent decades, skin biopsy has had a relevant role in revealing peripheral sensory endings involvement in several neurodegenerative disorders like Parkinson's disease, progressive supranuclear palsy and multiple system atrophy, in which cutaneous denervation is shown to reflect the severity of the disease.[23,33-35]

Our group and others[13-16] have previously described a small fibre pathology also in ALS. In the present work, we confirmed this finding in a larger cohort of ALS patients, demonstrating, compared with controls, a significant loss of IENFs at all of the examined sites, with the thigh being the site most involved (i.e. 78% of patients). This result further underlines the notion that cutaneous denervation is an intrinsic feature of this multisystem degenerative

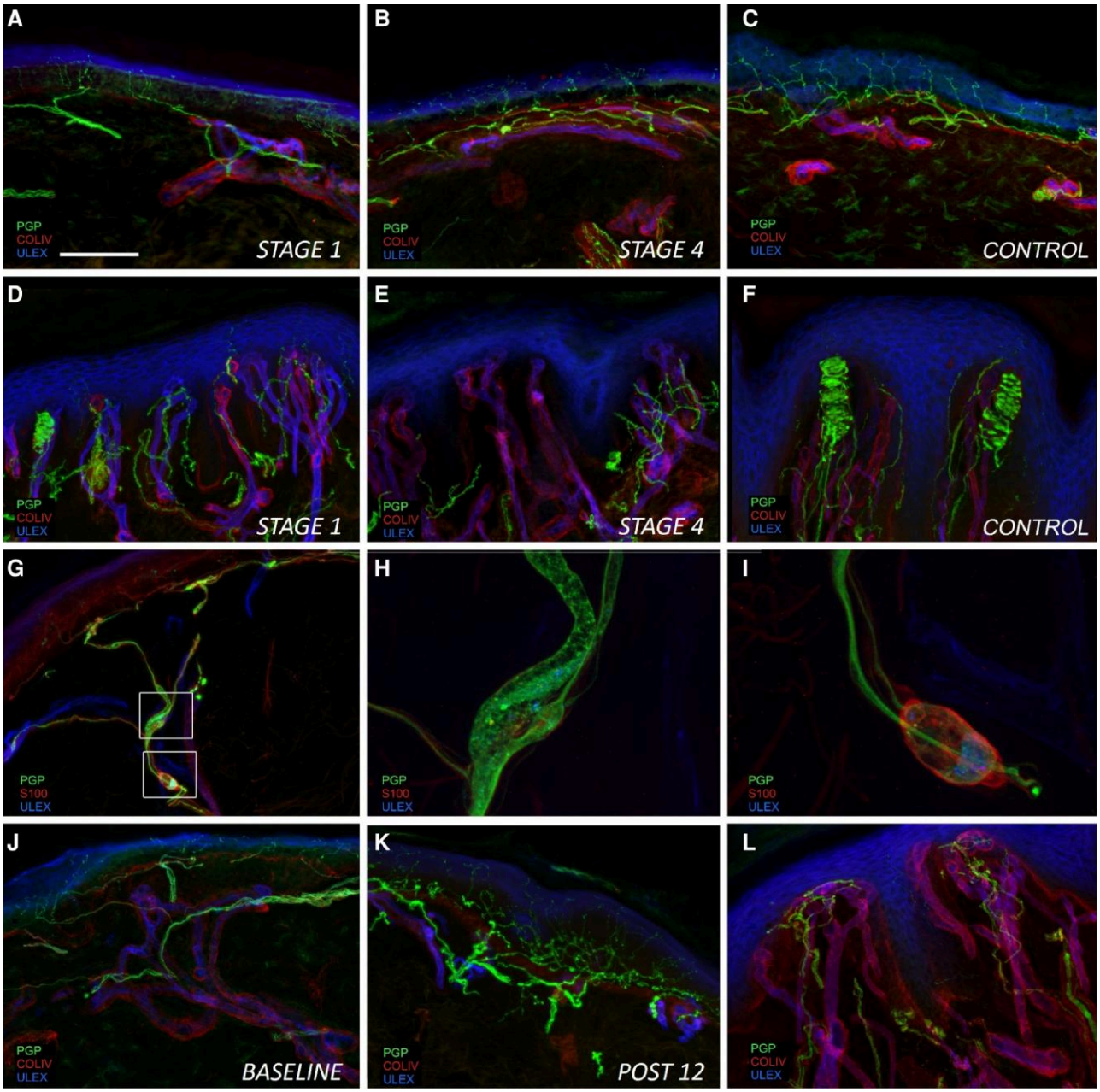

Figure 2 Cutaneous innervation across King's amyotrophic lateral sclerosis clinical staging and in the longitudinal study. Confocal images of punch biopsies from hairy and glabrous skin in amyotrophic lateral sclerosis (ALS) patients (**A**, **B**, **D**, **E**, **G**, **H**, and **J**–**L**) versus controls (**C** and **F**). Green, nerve fibres; blue, epidermis and endothelia; red, basement membranes and vessels in all except **G, H** and **I**, in which Schwann cells are in red. Increased IENF density in King's stage 4 patient compared with a patient in stage 1 (**B** compared with **A** and control in **C**). Conversely, a progressive loss of Meissner's corpuscles is evident in glabrous skin of a stage 1 patient versus a stage 4 patient (**D** compared with **E** and to control in **F**). Morphological abnormalities, such as swellings of dermal fibres are common in ALS and sometimes particularly large (**G** and detail magnified in **H**) and involve Schwann cells (detail of **G** magnified in **I**). An increase in intraepidermal nerve fibres (IENFs) over time along with subepidermal sprouting, epidermal nerve clustering and branching is evident in a patient undergoing repeated biopsies (see **K**, 12-month biopsy compared with **J**, baseline biopsy). In **L**, evident signs of the complexity and elongation of the capillary loops in dermal papillae, together with signs of severe nerve degeneration. Scale bar = 100 µm in **A**–**F** and **J**–**L**; 200 µm in **G**; 25 µm in **H** and **I**. ColIV = collagen-IV; PGP = protein gene product; Post 12 = 12 months after baseline assessment; ULEX = *Ulex europaeus*.

condition. However, as we have previously observed, a regenerative process coexisted with nerve degeneration, with evident signs of nerve remodelling. This process affected particularly the count of IENFs, making it difficult to find any correlation between IENF density and disease severity and progression. In this study, we overcame this difficulty by enlarging the cohort of patients and stratifying them with the King's clinical stages. We observed, consistently in all three examined sites, higher IENF values in patients in the more advanced King's stages compared with patients in the early ones. In other words, we found higher IENF density in patients with greater motor impairment and a higher disease progression rate.

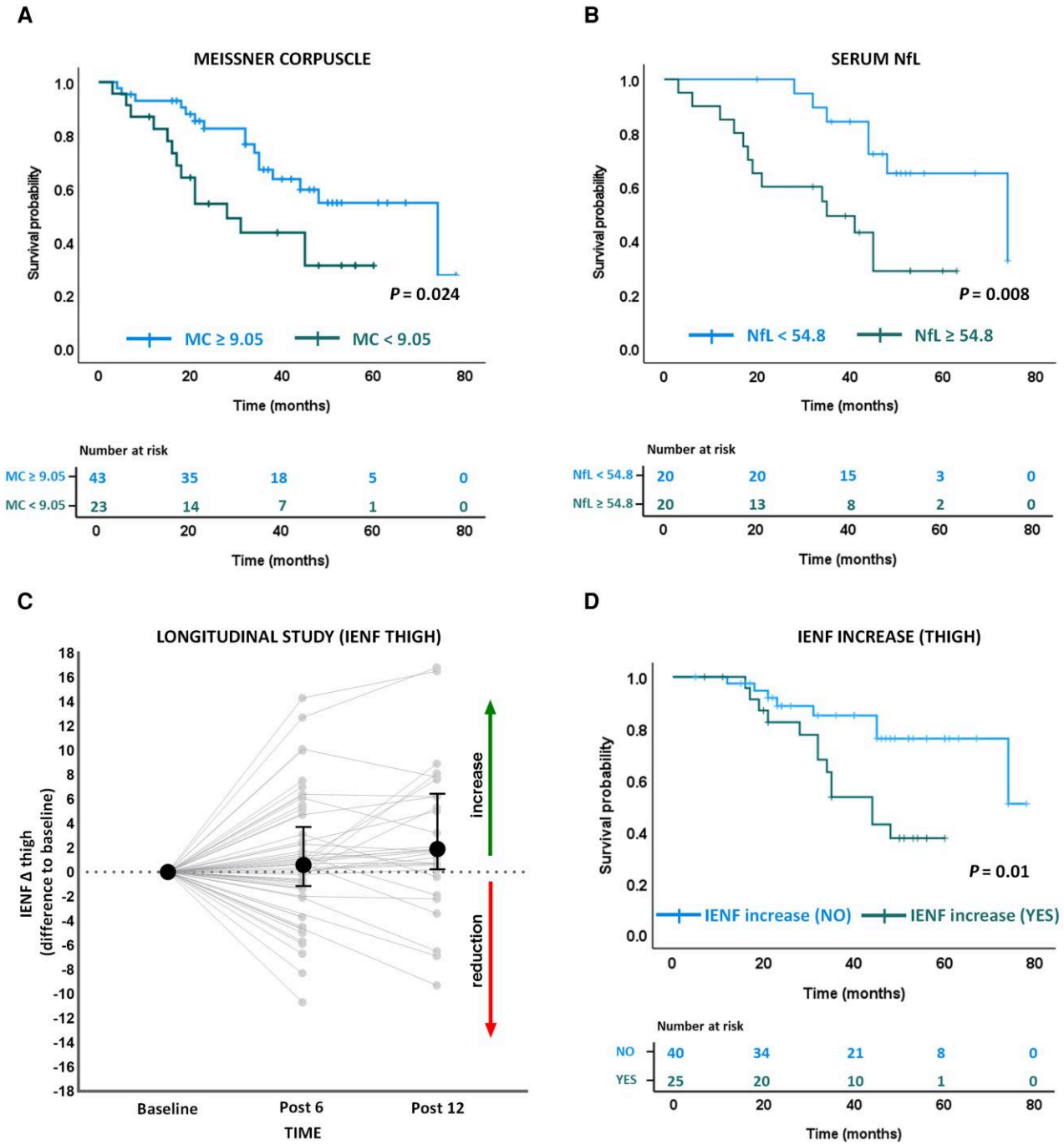

Figure 3 Kaplan–Meier survival curves based on morphological and NfL data, and skin biopsy longitudinal study. Kaplan–Meier curves of survival probability among ALS patients stratified by median value of (**A**) Meissner corpuscle (MC) density (n/mm^2), (**B**) serum neurofilament (NfL) levels (pg/ml) and (**D**) patients with and without evidence of an intraepidermal nerve fibre (IENF) increase at thigh over time. Lower MC values (<9.05 n/mm^2), higher NfL levels (≥54.8 pg/ml) and increasing IENFs over time are associated with shorter survival periods. Time is from symptom onset to death. (**C**) Overall increase in median value of IENFs at 6 (post 6) and 12 (post 12) months after baseline assessment. Note that the IENF value at each time point (post-6 and post-12) is expressed as the difference from the baseline assessment (IENF Δ thigh). Filled grey and black circles indicate individual and median IENF Δ thigh values, respectively. The green and red lines indicate the increase and decrease in IENFs over time from baseline, respectively. Significance, $P < 0.05$.

The higher IENF density across the stages, together with the more chaotic aspects of cutaneous innervation, suggested an upregulation of reparative pathways with increased release of growth factors or neurotrophin receptors affecting predominantly small fibres that may parallel the aggressiveness of the disease.

The morphological analysis of cutaneous innervation in the longitudinal part of the study further supported the link between IENF regeneration and disease aggressiveness. In fact, the increase in IENF density over time allowed us to identify patients with shorter survival times. Objective morphological signs of an

upregulation of the reparative pathway were, in addition to the increase in IENFs over time, the increased percentage of clusters/sprouts and increased density of GAP43 immunoreactive IENFs. GAP43 is a neuronal membrane phosphoprotein highly expressed during development and during nerve regeneration to guide axonal outgrowth.[36,37]

Overall, these findings may reflect a greater regenerative drive over time, likely due to the higher accumulation or upregulation of the nerve growth factor (NGF). This is an important factor regulating survival during development and in regenerative or neuroinflammatory processes. Therefore, NGF accumulation or upregulation may be linked to neuroinflammation, one of the pathogenetic mechanisms involved in ALS.[38,39] Also, the abnormalities of the vascular bed, which we assessed in our previous work and confirmed in the present cohort of patients, although only qualitatively, may be linked to the direct or indirect effects on angiogenesis of NGF.[40] Several studies have reported increased expression of NGF or NGF receptors in ALS patients and experimental models. An increase in proNGF in the CSF has been demonstrated in ALS, together with an increased expression, by spinal motor neurons, of the p75 receptor,[41] a receptor for neurotrophins expressed during the developmental phases of the nervous system to promote survival and differentiation. Increased NGF production and p75NTR-dependent death signalling have been found in cocultured motor neurons and reactive astrocytes.[42] We can therefore hypothesize that higher aggressiveness of the disease, sustained by the greater involvement of reactive astrocytes, might be associated with greater production of neurotrophins and relative receptors as reflected by the higher evidence of regenerative signs in the skin. Interestingly, keratinocytes and skin fibroblasts may contribute locally to higher production of NGF/proNGF, likely stimulated by neurodegeneration[43] but also by the abnormal accumulation of TDP-43 as recently reported in ALS patients.[44] Moreover, p75NTR signalling has been implicated in the pathology observed in mice overexpressing hSOD1G93A, the best characterized ALS mouse model.[45]

In addition to the morphological changes linked to regeneration, we observed axonal swellings along IENFs in all our patients. The assessment of the swelling ratio was not substantially different in our subgroups of patients at follow up, but tended to be higher in non-regenerating patients at baseline, probably predicting nerve loss. In fact, axonal swellings were previously described[46,47] as pre-degenerative signs in neuropathies and found to contain an accumulation of mitochondria, vesicular organelles and neurofilaments. Therefore, their occurrence may be the result of defective axonal transport, very likely associated with the pathogenetic mechanisms underlying ALS. The morphological changes that we observed over time are the result of degenerative and regenerative drives that affect the plasticity of cutaneous innervation and may lead to a different picture. Depending on the predominance of one of the two drives, incomplete or aberrant reinnervation may occur. Our results showed that the predominance of the regenerative drive is associated with greater aggressiveness of the disease. Therefore, studying the mechanisms underlying cutaneous nerve remodelling could be useful to understanding some pathogenetic mechanisms underlying ALS, which could potentially represent new therapeutic targets.

Another relevant finding of our work came from the analysis of glabrous skin innervation that provided the unique possibility to assess, in addition to IENFs, the very distal part of the large sensory fibres with corpuscolated endings, the MCs. These are capsulated, rapidly adapting mechanoreceptors, crucial for the perception of fine touch and sensorimotor control during object manipulation.[48] The loss of MCs, with values below the fifth percentile cut-off in more than 50% of ALS patients, implies an involvement of large sensory fibres which, often, remain subclinical and neurophysiologically silent as observed in other pathological conditions.[33,49] Indeed, mechanoreceptors and their myelinated afferences are not explored by routine neurophysiological assessment of sensory nerves, since electrical stimuli depolarize the nerve trunk more proximally. Therefore, the morphological evidence of MC loss may be an early sign of large sensory fibre involvement in ALS that neurophysiologically can only be detected later with the progressive loss of the largest axons in the nerve trunk.

Interestingly, across the King's stages, while IENFs increased, MCs decreased, with the lowest values in the more advanced stages, reflecting, then, the severity of the disease. Moreover, a density of MCs below the median value allowed us to identify a group of patients with shorter survival times. This finding, apparently in contrast to that for IENFs, most likely reflects the prevalent impairment of large fibres driven by the neurodegenerative mechanisms underlying ALS. Moreover, the large fibre population is not under the control of NGF and therefore should be spared by the hypothesized mechanism linked to NGF upregulation.[50] As an expression of the prevalent large fibre involvement in ALS, the MC count provides a more defined and linear picture of progressive nerve degeneration across the King's stages. Moreover, MCs inversely correlated with serum NfL levels, for which a role as a prognostic biomarker has recently been demonstrated in several studies of patients with ALS[30] and further confirmed in our cohort of patients.

Indeed, the group of patients with a MC density lower than 9.05/mm^2 displayed a higher concentration of serum NfL (135 pg/ml, IQR 104.5 versus 66.2 pg/ml, IQR 56.93). This result is in line with the notion that NfLs are neuronal cytoplasmic proteins highly expressed in myelinated axons,[30] and their levels are negatively associated with the myelinated but not unmyelinated fibre density at sural biopsy in patients with peripheral neuropathy.[51] Thus, the clear correlation of NfL with MCs in our study further supports the role of MCs as a prognostic biomarker.

Functional correlates of peripheral sensory pathology across clinical stages

In our cohort of patients, about 25% exhibited at least one abnormal sensory nerve, in agreement with previous studies,[6,7] and this percentage tended to increase in the more advanced stages. Moreover, of those patients, 15% fulfilled the neurophysiological criteria for peripheral sensory neuropathy, with a length-dependent pattern in most of them, confirming previous literature.[7,8]

In line with the progressive loss of MCs (biopsy from the fingertip of the fifth finger, ulnar territory), we also found a progressive reduction of ulnar nerve SNAP through the clinical stages, implying that the sensory neurodegenerative process, during the course of the disease, did not remain confined to the very distal part of the sensory nerve but induced a progressive axonal loss more proximally in the ulnar nerve trunk. The reduction in the ulnar sensory nerve amplitude, with values that, often, remained within the normal range, paralleled the reduction in ulnar nerve motor potential amplitude across the stages of the disease. This implies that, although often subclinically, sensory involvement is associated with lower motor neuron disease, indexed by distal motor potential amplitude, along the disease course. Our results agree with

pathological studies[12] demonstrating on the sural nerves the involvement of unmyelinated and myelinated fibres with frequent aspects of demyelination-remyelination and finally of nerve fibre degeneration, findings which support a common metabolic disorder in ALS affecting both motor and sensory neurons. On the other hand, patients are often overwhelmed by motor disability and may report sensory symptoms only when specifically questioned. However, when this occurs, with the help of questionnaires, as in our patient cohort, the increase in sensory symptoms along the progression of the disease is evident.

In fact, sensory symptoms were present overall in one third of our large cohort of patients and in most of those in King's stage 4. This finding underlies the clinical burden of sensory impairment in ALS that becomes more relevant in patients in the advanced stages of disease. This was particularly evident for symptoms such as burning pain or reduced sensitivity to thermal stimuli, attributable to small fibre involvement, implying that the newly regenerated fibres were dysfunctional and/or hyperexcitable.

We acknowledge some limitations of our study, including the lack of a careful psychophysical assessment of large and small sensory fibres by *ad hoc* testing (e.g. quantitative sensory testing, tuning curves to vibrotactile stimuli and so on) to find possible perceptual correlates of the morphological abnormalities that we observed in this study. Moreover, longitudinal assessment was not performed for NfL or for cutaneous innervation from other body sites. In particular, it would have been interesting to evaluate the density of MCs over time. Hopefully this aspect can be addressed in future studies by applying less accurate but non-invasive techniques such as *in vivo* confocal microscopy.[52] Finally, the innervation of autonomic structures and the density of dermal vessels were not quantified in the present study, which mainly aimed to evaluate sensory involvement in ALS patients.

Conclusion

In this work, we demonstrated that there is sensory involvement in ALS progression across the King's clinical stages, and we identified novel biomarkers of disease progression and prognosis through skin biopsy analysis. Finally, we suggested the possibility that the study of skin innervation may contribute to understanding the pathogenetic mechanisms underlying ALS, which could be the target of new pharmacological treatments.

Data availability

Anonymized data may be shared on request to the corresponding or senior author from a qualified investigator for non-commercial use, subject to restrictions according to participant consent and data protection legislation.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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