Neuromuscular and Microvascular Changes in Intrinsic Tongue Muscles of Patients with Various Forms of ALS

Students: Ayesha Hayat and Sara Rashid Khadeiar

Tutor: Per Stål

ABSTRACT

The study objective was to investigate whether amyotrophic lateral sclerosis (ALS) affects the intrinsic tongue muscles differently when the symptoms begin in cranial or lower limb muscles. Muscle fiber area, myosin heavy chain (MyHC) composition and vascularization were analyzed in the anterior and posterior region of the tongue with immunohistochemistry and morphometric techniques in 7 patients with classical ALS (limb onset) and 5 patients with progressive bulbar ALS (cranial onset). Samples from 5 previously healthy subjects were used as controls. The morphological results were correlated to the clinical data for each patient.

The results showed that the ALS samples had various degrees of pathological changes in both the anterior and posterior region of the tongue. The fiber area and number of capillaries around fibers was decreased by approximately 50% compared to controls. In both anterior and posterior regions, there was a shift against fibers co-expressing slow and fast MyHC isoforms. Although both classical and bulbar forms of ALS affected the muscles, there were also important differences between the two. In the bulbar form of ALS, the proportion of MyHCI fibers were decreased, a finding in contrast with classical ALS where MyHCII fibers have been reported to be affected preferentially. The slow contracting MyHCI fibers in the bulbar form of ALS differed also by being better supplied by capillaries. Conclusively, ALS had a large impact on the tongue muscle morphology, but both the form of the ALS and inherent factors seem to influence the disease process.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease affecting the motor neurons in the motor cortex, brain stem and spinal cord. The disease survival rate varies normally from three to five years from the onset of disease, though the rate of disease progression differs among individuals (Turner et al., 2002). ALS can be subdivided into three groups according to the progression pathway in the central nervous system. The first form, the classical ALS, affects both lower motor neurons (motor neurons innervating muscles) and upper motor neurons (cortical motor neurons involved in regulation of lower motor neurons). Another form, progressive spinal muscle atrophy (PSMA), affects primary motor nerves in the periphery. The degeneration of the lower motor neurons causes muscular atrophy, decreased muscle force, fasciculation and palsy. A third subtype, progressive bulbar palsy, affects primary motor neurons in the brain stem innervating cranial muscles, which results in degeneration of oro-pharyngeal muscles and difficulty regulating speech, mastication- and swallowing (Depaul et al., 1993; Brown, 2004).

The human tongue motor-system is of considerable importance for oro-facial function. Two groups of muscles, the extrinsic and intrinsic, make up a unique biomechanical system for precise changes in position and shape. The physiological properties of muscles, such as velocity, force properties and fatigue resistance, are reflected by their composition of contractile proteins i.e. myosin heavy chain composition (MyHC). Because MyHC contains both the ATPase and the actin-binding site it is regarded as the best marker of the functional heterogeneity among muscle fibers. The MyHC isoforms can be classified into MyHCI (slow), MyHCII (fast) with subgroups IIa and IIx and into hybrid fibers co-expressing both slow and fast MyHC isoforms - MyHCI+II (Pette et al., 1990; Schiaffino et al., 1994). Normally fibers expressing MyHCI are slow contracting, have numerous capillaries and are fatigue resistant, while fibers expressing MyHCII are fast contracting, have fewer capillaries and lower fatigue resistance (Staron et al, 1997; Andersen et al., 1977; Ingjer, 1979; Hather et al, 1991)

The aim of the study was to determine the differences in form of ALS, classical or bulbar, regarding muscle capillarization and MyHC composition of the intrinsic tongue muscles of ALS patients in the end stage of the disease. The results will be correlated to the region of the tongue where the samples were obtained and to clinical variables for each patient.

MATERIALS AND METHODS

Muscle sampling

Muscle samples from the human tongue were obtained at autopsy from 13 subjects who had suffered of different forms of ALS (for demographic data see table 1). Control samples were obtained from five previously healthy subjects at autopsy (three females and two males, mean age 41y, range 26-57) who suffered a sudden accidental death.

The anterior muscle specimen was obtained 1 cm from the tip of the tongue, and the posterior sample was obtained in the root immediately posterior to the foramen caecum linguae. All specimens were obtained 1-2 days post mortem, a delay acceptable for obtaining reliable fiber typing based on both the staining reaction for myofibrillar ATPase (Eriksson et al., 1980) and expression of MyHC isoforms (Tuttle et al., 2014). The muscle samples were mounted for transverse sectioning of the intrinsic longitudinalis muscle in OCT compound (Tissue Tek®, Miles Inc, Elkhart, IN, USA) and were then rapidly frozen in liquid propane chilled with liquid nitrogen. The tissue samples were stored at –80°C until further processing.

Immunohistochemistry

Serial cross-sections, 5 µm thick, were cut in a cryostat microtome (Reichert-Jung, Leica Heidelberg, Germany) at –20°C and mounted on glass slides with. The sections were processed for immunocytochemistry by using previously characterized monoclonal antibodies (mAbs) against human slow MyHC (mAb A4.840; strong affinity for MyHCI), slow and fast MyHC (mAb N2.261; strong affinity for MyHCIIa, weak for MyHCI, and no affinity for MyHCIIx) and fast MyHC (mAb A4.74; strong affinity for MyHCIIa). The cell border of capillaries and the muscle fibers was marked with two mAbs directed against laminin, the major non-collageneous component of the basement membrane in muscle fibers and capillaries. Monoclonal Ab 4C7 against laminin α5 chain labels capillaries strongly and the basement membrane of fibers weakly, whereas mAb 5H2 against laminin α2 chain labels only the basement membrane of fibers. Double labelling of fibers was achieved by incubating the sections with a solution containing two different primary mAbs. The method has been used previously in morphometric analysis of fiber areas and capillaries in human skeletal muscles (Stål et al., 2000). Immunohistochemical visualization of bound antibody was performed either using indirect peroxidase-antiperoxidase (PAP) staining (Sternberger, 1979) or by indirect immunofluorescence using affinity-purified abs specially prepared for multiple labeling and

conjugated with fluorochrome with different emission spectra, fluorescein (FITC), Rhodamine Red-X (RRX), Alexa 488 and Alexa 647 (Jackson ImmunoResearh Laboratories, Inc West Grove, PA, USA). For details of the staining techniques, see Stål et al., 2000; Liu et al. , 2002 and Lindström et al., 2009.

Muscle fiber classification

Based on the staining pattern for the different MyHC mAbs, the fibers were classified as fibers containing pure MyHCI or pure MyHCII. Fibers co-expressing MyHCI and MyHCII isoforms were classified as hybrid fibers, MyHCI+II. For control, muscle samples were stained and classified in fiber types according to their staining intensity for ATPase (EC 3.6.1.3) at acid and alkaline pH.

Morphometric analysis

Three to four random areas of each muscle cross-section stained for the used antibodies were scanned in a light microscope (Leica DM6000B, Leica Microsystems CMS GmbH, Wetzlar, GER) equipped with a color CCD camera (Leica DFC490) and a digital high-speed fluorescence CCD camera (Leica DFC360 FX). To estimate muscle fiber area (FA) the circumference of each fiber and each capillary was traced along the periphery of the basement membrane on a computer image. All capillaries within a distance of max 5□m from each individual fiber were marked on the image and the number of capillaries on the whole muscle cross-section was counted.

The morphological data was based on the evaluation of a total of 2242 muscle fibers from five normal subjects (average of 448 fibers per subject) and 4064 muscle fibers from the 13 ALS subjects (average of 165 fibers per subject). Fifty fibers have previously been shown to be sufficient to determine the capillarization in fibers in biopsy samples from normal limb muscles (Porter et al., 2002).

Capillary parameters

Capillary density (CD) was calculated as the total number of capillaries per mm^2 muscle cross-sectional area. All capillaries in contact, or nearly in contact, with muscle fibers were present as the number of capillaries around fibers (CAF). The numbers of capillaries related to each

fiber relative to their fiber cross-sectional area (CAFA) were calculated according to the formula; CAF / fiber cross-sectional area x 10^3. This variable relates the number of capillaries around fibers to fiber size and measures the cell area that each capillary supplies.

Literature search

While gathering information we used the PubMed database in order to find related information. The following MeSH-terms were used to find relevant studies:

- *Amyotrophic lateral sclerosis* + *diagnosis*
- *muscle-fiber-type* + *muscle capillary density* + *skeletal muscle degeneration*
- *Amyotrophic Lateral Sclerosis* + *pathology*
- *Tongue* + *muscle* + *human* + *Myosin heavy chain* + *fiber types* + *capillaries*

Ethical reflection

The autopsy specimens were collected in agreement with Swedish laws and regulations on autopsy and transplantation. The National Board of Health and Welfare and the Ethics Committee of the Medical Faculty (Diary no: 94-135), Umeå University, approved the protocol. The tongue muscle samples were collected with a sufficient protection and respect of privacy, confidentiality and anonymity of data by coding the samples so no samples can be traced to any individuals. All samples were collected post-mortem and after verbal and written permission from the family members of the deceased patients. It is also of importance to clarify the potential benefits of all research regarding this area in order to gain further knowledge and understanding of the disease and hopefully improve diagnosis and treatment in the future.

RESULTS

General morphology of ALS samples

The samples from the ALS subjects differed from controls by having areas with severe morphological changes in both the anterior and posterior regions of the tongue. The control samples were generally characterized by a check board pattern of well-packed muscle fibers of about similar size and form. In contrast, the fiber population in the ALS samples was more disorganized and characterized by a large variability in fiber size, fiber shape and packing of

fibers. Fibrosis and clusters of small sized fibers mixed with larger fibers were common in the ALS samples, but not in the controls. Moreover, the vascularization of the intrinsic tongue muscles varied more in the ALS samples than in controls. While the capillary network in the controls was more or less evenly distributed over the muscle cross-section, the ALS samples had commonly a high density of capillaries in some regions whereas other areas were nearly devoid of vessels. The clinical data for each subject and results are summarized in table 1-3. The results are presented in mean values of the compiled results from all subjects in table 1 and 2 as well as individual data from each of the ALS patients in table 3.

Comparison between controls and ALS samples

The mean muscle fiber area values in ALS patients and controls are presented in Table 1 and Table 2. The muscle fiber area in the ALS subjects was approximately 50% smaller than in controls in both the anterior (mean area 503 vs. 916 μm, respectively) and posterior (mean area 831 vs. 1744 μm, respectively) regions. The smallest fiber area was observed for MyHCI fibers in the posterior region of the tongue were the fiber size was 69% smaller than in controls.

The proportion of different muscle fiber types in ALS patients and controls is presented in Table 2. Muscle fibers MyHCI and MyHCII and the hybrid form MyHCI+II were distinguished in all muscle samples, but not in all cases. There were generally an increased number of fibers expressing MyHCI+II in both the anterior and posterior regions. The shift against a higher proportion of MyHCI+II fibers was in the anterior region in expense of a lower proportion of MyHCII fibers. In contrast, in the posterior region, the shift against MyHCI+II fibers was in expense of a lower proportion of MyHCI fibers.

In the ALS tongue samples, the number of capillaries around each individual fiber (CAF) was 52% lower than in controls in the anterior region (1.1 vs. 2.1, respectively). The corresponding value in the posterior region was 44% lower (1.1 vs. 2.5, respectively). When relating CAF to fiber area, the CAFA was 51% lower value in the anterior region than in controls (2.2 vs. 4.3, respectively) and 53% lower in the posterior region (1.6 vs. 3.0, respectively). The capillary density (CD) values were approximately equal between ALS and control subjects in both the anterior (825 vs. 823 cap/mm^2, respectively) and posterior (663 vs. 770 cap/mm^2, respectively) regions. However, the ALS samples showed a large inter-individual variability in capillary density, as observed by the high standard deviations (SD) values. In the anterior region, the SD

values in the ALS samples 2.9 times larger than in controls and in the posterior region the SD was 2.3 times larger (see table 1).

Comparison between the sporadic bulbar (SPBP) and classical forms of ALS (FALS and SALS)

The subjects with bulbar ALS showed more pronounced tissue alterations in the posterior region of the tongue than in the classical form of ALS (FALS and SALS). The proportion of MyHCI fibers was in the posterior region approximately 50% lower in the SPBP than in the classical ALS subjects (40.1% vs. 21.3%, respectively) while the proportion of MyHCII fibers was approximately 40% higher (27.0% vs. 43.3%, respectively). Moreover, in the posterior region, the CAF values for SPBP subjects were higher (0.9 vs, 1.4, respectively), while there were no differences in the anterior region (1.1 vs. 1.1, respectively). However, when relating the capillarization of fibers to its area, the SPBP subjects had in both the anterior and posterior regions higher CAFA values than the classical ALS subjects (see Table 3).

DISCUSSION

The findings of the study showed that the samples from ALS patients generally expressed muscle fiber degeneration, fiber regeneration, increased proportion of connective tissue, as well as an overall loss of capillary supply when compared to normal reference muscles. However, there was a large inter-individual variability and some differences in changes were observed between the SPBP and classical form of ALS.

The subjects with the SPBP form of ALS had in the posterior region of the tongue a smaller proportion of MyHCI fibers than in both the classical form of ALS and in normal controls. Moreover, MyHCI fibers in the posterior region of the tongue were generally supplied by more capillaries in the SPBP than in the classical form of ALS, although the capillary supply, with one exception, was still lower than in controls. The finding that the SPBP ALS form affected the tongue muscles more than the classical form is in a first glance not particularly surprising. However, the larger loss of muscles fibers belonging to slow motor-units (MyHCI) together with the significantly higher capillary supply of the surviving MyHC fibers in the SPBP than classical form of ALS is highly interesting.

The loss of MyHCI fibers in the SPBP form of ALS reflects a process of denervation and degeneration that is the opposite of what has been previously reported occurring in limb

muscles in classical ALS, where fibers of fast motor-units (MyHCII) are preferentially affected (Gordon et al., 2007). A higher loss of fast motor-units in the classical form of ALS is in line with our results from the tongue muscles. The new finding of a opposite situation indicate that the SPBP form, in contrast to the classical form, targets motor neurons innervating slow contracting and endurance resistant fibers. Although the surviving MyHCI fibers in the SPBP samples had lost capillaries compared to controls, the loss was smaller than in the classical ALS form. One explanation for the differences may be that the bulbar form of ALS denervates the cranial muscles at an earlier stage compared to the classical form. Since the bulbar form initially targets the motor-nerves that innervate cranial muscles, including the tongue, the deleterious neuromuscular process might be in a more advanced stage in bulbar ALS. However, since the mean duration of survival was twice as long in the classical (mean 8.7y) than in the SPBP form of ALS (mean 2.8y), the type of ALS seems to be more important for the changes than the duration of the disease.

Although the disease caused denervation and degeneration of muscle fibers in both the bulbar and classical forms of ALS, the large inter-individual variability in fiber size and capillary supply shows that the disease also affects the patients individually.

In consideration of this, an interesting finding was that two of the deviant cases, one with FALS (no 5) and one with SPBP (no 9), differed from the other by having a very high capillarization, even higher than in controls. Since these cases also had pronounced muscle fiber degeneration, this pattern was unexpected. Interestingly, both these patients differed from the other by having a mutation in the C9ORF72 gene, which have been reported to be a cause for ALS. The C9ORF72 mutation has been reported to be a genetic link between ALS and familial front temporal dementia (FTD) (Renton, 2011), a progressive brain disorders that affects personality, behavior, and language. If there is a connection between the divergent results with high capillarization and the C9ORF72 mutation in these subjects is unclear, but the results are interesting and should be followed up in an extended study of both cranial and limb muscles.

An explanation to the high content of both extremely small as well as very large fibers in some areas of the ALS samples is probably related to the fact that denervation of motor units leads to degeneration of muscle fibers, which in turn cause an increased use of the remaining motor units to uphold oro-pharyngeal function. The increased use and higher load of the survival motor-units up-regulate the protein synthesis and stimulate to muscle fiber growth. The small

fibers might be a mix of denervated/degenerating fibers and newly formed fibers deriving from activated satellite cells (myogenic stemcells). Myogenic satellite cells are known to be up-regulated after muscle overload and muscle injury (Bishop, 1994). Some of the extremely large fibers may also be a result of newly formed fibers that have fused together, as evidence by the presence of large fibers with a "split" appearance in the ALS samples.

There are several processes that could explain the changes in fiber type proportions in the ALS samples. Firstly, denervation of a specific motor-unit type may reduce the number of a specific MyHC fiber type in the muscle. Secondly, muscle fibers have a high plasticity and the myosin molecule and metabolic compartment can adapt to changed use. Denervation and loss of muscle fibers may increase the use of the surviving motor-units to uphold the function, which in turn caused an adaptive transformation of the fiberphenotype. Alternatively, re-innervation of denervated fibers by a specific motor-unit type may also cause transformation of the contractile proteins in the fibers (Pette et al., 1997).

Normally there is an adaptation of the capillary network to match the size of the muscular fibers (Cebasek, 2005) and the requirement imposed on them for work under aerobic condition. Thus, large sized slow contracting muscle fibers (MyHCI) are surrounded by a larger number of capillaries than small sized fast contracting muscle fibers (MyHCII) (Hudlicka 1991). The lower number of capillaries per fiber (CAF) as well as the lower value of capillaries per fiber area (CAFA) in most cases showed that the denervation/degeneration process of muscle fibers in ALS are followed by a parallel degradation of the capillary network. This finding is in accordance with prior studies of denervated muscles after spinal cord injury or stroke (Pontén, 2003, Martin et al., 1992). However, an interesting finding was that the degradation of capillaries, with exception of the ALS cases with a C9ORF72 mutation, was proportionally larger than the degree of fiber degeneration. This finding might be explained by the fact that a large part of the existing fibers lack innervation and therefore ability for contraction. These fibers probably have a nearly non-existent need for aerobic metabolism, which might result in partial to total loss of capillaries in some areas of the muscle. As a consequence, the mean values for capillary supply of fibers will be very low. Based on this theoretical argument, the lower capillary supply of fibers in the classical than SPBP form of ALS, suggests that the number of muscle fibers that lost their innervation at the end stage of the disease are larger in classical than bulbar ALS.

The small difference in capillary density between ALS samples and controls, despite loss of capillaries, can be explained by the fact that smaller fiber size will increase the total number of fibers per muscle area unit. As a consequence, the capillaries surrounding each "new" fiber per area unit will contribute to the increase of the total number of capillaries per mm^2 muscle cross-section. This knowledge emphasizes the importance of using different capillary parameters in the evaluation of changes in capillarization after muscle denervation.

The high inter-individual variations in pathological changes and the large span in survival time after diagnosis (ranging from 0.8 to 26, 7 years) indicate that inherent differences influence the disease progression and pathogenesis of the disease may be dependent on several factors. A limitation of the study is that the results in this investigation are on only based 13 subjects. A higher number of ALS subjects have to be included in the study before significant conclusions can be drawn.

In conclusion, our study shows that the intrinsic muscles of the tongue are severely affected at the end stage of the disease, independently of form of ALS. However, this study also shows that there are pathognomonic differences in how the tongue muscles are affected by classical and bulbar ALS. The indication of preferential loss of MyHCI fibers in the bulbar form of ALS, which is in contrast to the consequences of the classical form of ALS, is a novel finding that has to be further investigated. The differences in how bulbar and classical ALS affects the tongue muscles, the high inter-individual variability in tissue changes, and the large variability in survival time further demonstrate the complex nature of the ALS disease.

Acknowledgements

We would like to express our gratitude to our supervisor and tutor Professor Per Stål for the useful comments, remarks and engagement throughout the learning process of this master thesis. Furthermore we would like to thank biomedical scientist Anna-Karin Olofsson for technical assistance. We would also like to thank our beloved ones, who have supported us throughout this entire process, both by keeping us harmonious and helping us putting the pieces together.

REFERENCES

Andersen P, Henriksson J (1977). Capillary supply of the quadriceps femoris muscle of man: adaptive response to exercise. *J Physiol* 270(3): 677-90.

Bottinelli R, Schiaffino S, Reggiani C (1991). Force-velocity relations and myosin heavy chain isoform compositions of skinned fibres from rat skeletal muscle. *J Physiol* 437: 655-72.

Brown R H, Jr (2004). Amyotrophic Lateral Sclerosis and Other Motor Neuron Diseases - *principles of internal medicine 14*: Chapter 365.

Cebasek V (2005). Capillary network in slow and fast and in oxidative and glycolytic muscle fibres. *Image Anal Stereol* 24: 51-58.

DePaul R and Brooks B R (1993). Multiple orofacial indices in amyotrophic lateral sclerosis, *J Speech Hear Res* 36(6): 1158-67.

Eriksson O, Eriksson A, Ringqvist M, Thornell LE (1980). The reliability of histochemical fibre typing of human necropsy muscles. *Histochemistry* 65(3): 193-205.

Gordon T, Charles T. Putman, Hegedus J (2007). Amyotrophic lateral sclerosis - evidence of early denervation of fast-twitch muscles. *Basic Applied Myology 17*: 141-145.

Hather BM, Mason CE, Dudley GA (1991). Histochemical demonstration of skeletal muscle fibre MyHCs and capillaries on the same transverse section. *Clin Physiol* 11(2): 127-34.

Hudlicka (1991). What makes blood vessels grow? *J Physiol* 444: 1-24.

Ingjer F (1979). Effects of endurance training on muscle fibre ATP-ase activity, capillary supply and mitochondrial content in man. *J Physiol* 294: 419-32.

Pedrosa-Domellöf F, Holmgren Y, Lucas CA, Hoh JF, Thornell LE (2000). Human extraocular muscles: unique pattern of myosin heavy chain expression during myotube formation. *Invest Ophthalmol Vis Sci* 41(7): 1608-16.

Pette D, Staron RS (1990). Cellular and molecular diversities of mammalian skeletal muscle fibers. *Rev Physiol Biochem Pharmacol* 116: 1-76.

Pette D, Staron RS (1997). Mammalian skeletal muscle fiber type transitions. *Int Rev Cytol* 170: 143-223.

Pontén E (2003). Tendon transfer mechanics and donor muscle properties: implications in surgical correction of upper limb muscle imbalance (dissertation). Umeå, Sweden: Umeå University.

Porter MM, Stuart S, Boij M, Lexell J (2002). Capillary supply of the tibialis anterior muscle in young, healthy, and moderately active men and women. *J Appl Physiol* 92: 1451-1457.

Renton AE, Majounie E, Waite A, Simón-Sánchez J, Rollinson S, Gibbs R *et al* (2011). A hexanucleotide repeat expansion in *C9ORF72* is the cause of chromosome 9p21-linked ALS-FTD. *Neuron* 72:257–68.

Schiaffino S, Reggiani C (1994). Myosin isoforms in mammalian skeletal muscle. *J Appl Physiol* 77(2): 493-501.

Staron RS (1997). Human skeletal muscle fiber MyHCs: delineation, development, and distribution. *Can J Appl Physiol* 22(4): 307-27.

Sternberger LA (1979). Immunocytochemistry. Wiley Medical 2nd ed.

Stål PS, Lindman R (2000). Characterization of human soft palate muscles with respect to fibre MyHCs, myosins and capillary supply. *J Anat* 197: 275-90.

Turner M, Bakker, Sham P, Shaw CE, Leigh PN, Al-Chalabi A (2002). Prognostic modeling of therapeutic interventions in amyotrophic lateral sclera. *Amyotroph Lateral Scler Other Motor Neuron Disord.* 3(1): 15-21.

Tuttle L, Alperin M, Lieber R (2014). Post-mortem timing of skeletal muscle biochemical and mechanical degradation. *Journal of Biomechanics* 47: 1506–1509

Table 1. Summary of fiber area (FA) and capillary parameters (CAF, CAFA, CD) in the anterior and posterior parts of the tongue muscle from ALS patients and control subjects.

	Anterior		**Posterior**	
	ALS	Control	ALS	Control
FA (μm^2)	503 ± 187	916 ± 680	832 ± 324	1 744 ± 876
CAF	1.1 ± 0.9	2.1 ± 0.5	1.1 ± 0.5	2.5 ± 0.8
CAFA	2.2 ± 1.8	4.3 ± 2.1	1.6 ± 0.8	3.0 ± 1.2
CD (cap/mm^2)	825 ± 680	823 ± 233	663 ± 325	770 ± 139

Table 2. Summary of variables in frequency (%), fiber area (FA) and capillary parameters (CAF, CAFA) relating to fiber types in the anterior and posterior parts of the tongue muscle from ALS patients and control subjects.

	MyHC I		MyHC II		MyHC I+II	
	ALS	Control	ALS	Control	ALS	Control
Anterior						
Frequency (%)	23.7 ± 19.3	21.4 ± 7.6	61.4 ± 18.2	70.7 ± 8.8	14.8 ± 12.4	7.9 ± 4.0
FA (μm^2)	404 ± 226	906 ± 571	512 ± 186	1158 ± 551	554 ± 219	1144 ± 830
CAF	0.9 ± 0.6	2.3 ± 0.6	1.1 ± 0.4	2.0 ± 0.5	0.9 ± 0.6	1.9 ± 0.5
CAFA	2.7 ± 1.5	5.2 ± 2.4	2.4 ± 0.7	3.9 ± 2.1	2.3 ± 1.3	3.9 ± 1.6
Posterior						
Frequency (%)	31.7 ± 32.0	45.5 ± 17.3	33.0 ± 21.0	34.9 ± 14.5	35.3 ± 33.6	19.6 ± 27.0
FA (μm^2)	495 ± 337	1579 ± 814	798 ± 591	1956 ± 1020	948 ± 417	1905 ± 950
CAF	1.0 ± 0.6	2.4 ± 0.6	1.0 ± 0.6	2.4 ± 0.9	1.1 ± 0.7	2.6 ± 0.9
CAFA	1.9 ± 1.8	3.4 ± 0.8	2.3 ± 1.7	2.3 ± 1.0	2.1 ± 1.8	3.2 ± 1.4

Table 3. Summary of patient characteristics, forms of ALS, including familial ALS (FALS), sporadic ALS (SALS) and sporadic progressive bulbar palsy (SPBP) and results relating to frequency of fiber types, fiber area and capillary parameters in the anterior and posterior parts of the tongue muscle in 13 ALS patients.

Pat	Sex	Age	Diagn	Dur. (yr)	Frequency (%) MyHC I		Frequency (%) MyHC I+ II		Frequency (%) MyHC II		FA		CAF		CAFA		CD	
					Ant	Post	Ant	Post	Ant	Post	Ant	Post	Ant	Post	Ant	Post	Ant	Post
1	m	78	FALS	U	34.7	27.0	14.6	6.75	50.6	66.3	354	166	1.2	0.5	3.3	3.0	1146	1134
2	m	75	FALS	26.7	25.4	53.5	4.7	7.0	69.8	39.6	545	930	0.9	1.1	1.7	1.3	663	592
3	f	64	FALS	10.1	47.1	93.4	5.9	0	47.1	6.7	451	1110	0.7	1.3	1.7	1.2	620	605
4	m	59	FALS	0.8	10.3	0.6	13.9	74.0	75.8	25.4	620	1092	0.6	0.7	0.9	0.6	382	305
5*	m	54	FALS	3.7	0.7	3.2	32.2	79.3	67.0	17.6	607	990	2.6	0.9	4.1	1.0	1446	358
6	m	U	SALS	18.9	4.4	9.0	0	63.9	95.7	27.1	323	621	0.3	0.8	0.8	1.3	321	535
7	f	80	SALS	1.0	59.9	93.9	4.8	0	35.4	6.1	512	844	1.1	1.3	2.1	1.8	813	701
					26.1	40.1	10.9	33.0	63.1	27.0	488	822	1.1	0.9	2.1	1.5	770	604
8	m	66	SPBP	1.1	27.4	36.8	11.0	13.2	61.5	50.0	578	889	0.9	1.6	1.5	1.8	697	958
9*	f	74	SPBP	3.0	21.1	3.9	25.2	74.5	54.7	22.6	439	736	3.1	2.0	7.2	3.3	2799	1309
10	m	77	SPBP	1.5	13.6	12.3	17.1	16.0	69.3	71.3	421	522	0.4	0.5	1.0	1.0	307	282
11	m	U	SPBP	3.6	49.4	51.3	8.6	1.1	41.9	47.6	935	506	0.9	0.9	1.0	1.9	568	898
12	f	U	PBP	5.0	1.5	2.4	11.5	72.7	87.1	24.9	153	1430	0.2	1.8	1.4	1.3	320	554
					22.6	21.3	14.7	35.5	62.9	43.3	505	816	1.1	1.4	2.4	1.9	938	800
13	f	U	U	U	13.2	24.4	44.4	52.0	42.4	23.6	545	977	0.9	0.9	1.7	0.9	664	382

*C9ORF72 gene mutation

U= Unknown