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MORPHOMETRIC ANALYSIS OF NORMAL MUSCLE BIOPSIES : A STATISTICAL APPROACH

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ABSTRACT

With a Quantimet 720, an automated image analysis of muscle cross-sections has been developed, using the property of muscle tissue to display two main fibre types with the ATPase reaction. 14 parameters, including size, number and spatial distribution, have been investigated on 53 biopsies of normal leg muscles. The data were tested in relation with sex and increasing age. Some trends are apparent which indicate a certain wide variability within the morphometric parameters concerning the normal muscle. A principal component analysis revealed 3 distinct groups of parameters. The most reliable ones are the type I/type II area ratio and the coefficient of variation of the fibre size.

INTRODUCTION

Quantitation of morphological features is essential for a full appraisal of muscle biopsies and this approach can be a useful aid to definite minor changes. The problem is to know whether these small abnormalities are due to physiological variations or to a beginning pathology. For this purpose, it is necessary to obtain the best possible estimate of the limits of normality in healthy individuals.

A lot of papers have dealt with type 1 and type 2 fibre size and number. More recently, attention has been drawn to the spatial arrangement of the two main muscle

fibre types (Morris and Raybould, 1971 ; Jennekens et al., 1971 ; Johnson, 1973 ; Lexell et al., 1983) according to the "enclosed fibre" technique of Jennekens. In a previous paper (Tankosic et al. 1983), we have described a quite different approach to this problem using an electronic image analyser (Quantimet 720). In this study, a series of morphometric parameters, not only fibre size and number, but also spatial distribution criteria, have been determined on 53 biopsies of normal human leg muscles. We will further discuss the correlations between all these parameters and their importance to the diagnosis.

MATERIALS AND METHODS

Biopsies : Muscle samples were obtained from 53 not specially trained male and female patients aged between 2 months and 70 years. 32 open biopsies were taken from peroneus longus and 21 from tibialis anterior. They were considered by the anatomo-pathologists to be normal by classical histological and histochemical criteria. The tissue specimens were frozen in isopentane cooled to -150°C in liquid nitrogen. Ten micrometer-thick cryostat cross sections were stained to demonstrate the myofibrillar ATPase activity at pH 9.4 after an acid preincubation at pH 4.63 according to the technique of Brooke and Kaiser (1969). With this technique, we observed strongly reactive type 1 fibres, non reactive type 2A and weakly reactive type 2B fibres (fig.1). As pointed out by Saltin et al. (1977), the subdivision of type 2 fibres do not give rise to identical sub-groups with regard to the technique used by such and such author. For this reason, and in order to be able to compare our results with others, type 2A and type 2B fibres were grouped and considered as type 2 fibres.

Morphometry : The morphometric system consists essentially of an electronic image analyser Quantimet 720 (Cambridge Instrument) interfaced with a 9825 S Hewlett-Packard calculator. The previously described method (Tankosic et al., 1983) has been used to obtain 14 morphometric parameters :

Size parameters

F1	Mean area of type 1 fibres
F2	Mean area of type 2 fibres
CV1	F1 coefficient of variation

CV2 F2 coefficient of variation
F1/F2 Mean area ratio
DN Ratio of the numerical densities of type 1 to type 2 fibres
DS Ratio of the areal densities of type 1 to type 2 fibres

Spatial distribution parameters

S Number of sequences type 1 - type 2 events
X1 Mean size of type 1 fibre clusters in number of standard frames of mesure (SFM)
X2 Mean size of type 2 fibre clusters in SFM
A1 Size heterogenity of aggregates of type 1 fibres
A2 Size heterogenity of aggregates of type 2 fibres
A1/X1 Spatial distribution index of type 1 fibres
A2/X2 Spatial distribution index of type 2 fibres

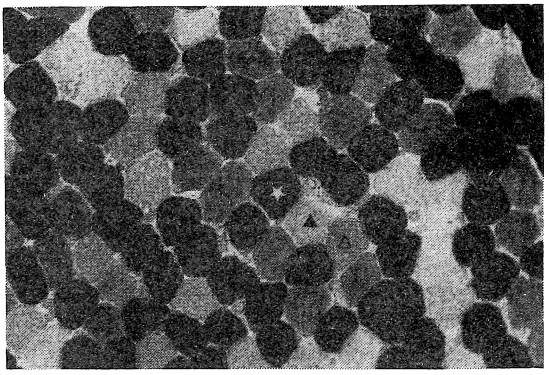

Fig. 1 : Transverse cryostat section of normal peroneus longus. A myosin ATPase reaction at pH 4.63 (x 200).

Statistical procedure : The possible correlations on 15 parameters (the 14 measured or calculated ones plus age) have been investigated using a principal component analysis as described by Colgan (1977). With this analytic method, all the biopsies were mathematically classified in a 15-dimensional space and plotted in several 2-dimensional planes which axes form the single components. The results were interpreted looking at the projections on the different axes. Two variables are correlated on a given axis, when their projections are observed far from the centre of gravity, which represents the mean parameter in the 15-dimensional space, on the same side

for a positive correlation, on each side for a negative correlation.

For all the data, the means between the two considered muscles and between male and female patients with regard to age were compared by a two-way analysis of variance. No significant differences have been observed in any age-group. This can be explained in part by the fact that these two leg muscles are antagonist, both having the same activity level in men as well as in women. Consequently, the data listed in table 1 have been considered regardless of the sex of the patient and the muscle sample. For each morphometric parameter, a one-way analysis of variance was performed and the statistical differences were localized with Student's t-test using the common variance of the six age sub-groups.

RESULTS

Principal component analysis : The 15 parameters have been observed on the 3 first single components (SC). The first one (fig. 2a, axis 1), which carries 41.7%

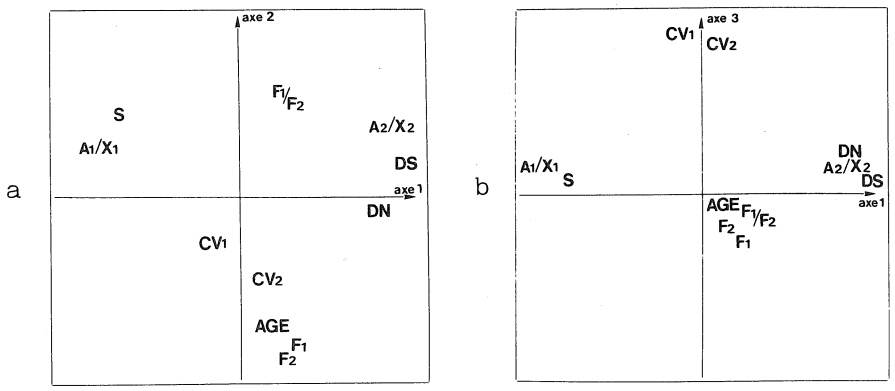

Fig.2 : The first, second (a) and the third (b) single components are considered.

of the total variance of all the results, shows a good correlation with the surface density ratio (DS), the numerical density ratio (DN) and the calculated distribution parameters (S,A,X,A/X). An increase of the number of type 1 fibres (which gives a higher DN ratio) involves a decrease of the A1/X1 ratio, which is interpreted as a presence of type 1 fibre clusters. A decrease of S, indicating a fibre type grouping tendency, is only related to an A1/X1 decrease.

We have to point out that the spatial distribution parameters are little or not correlated with age. Except for the high A1 value in the class 1, the analysis of variance states this independence (table 1).

The second SC (axis 2), of minor importance (22.7% of the total variance), shows an expected positive correlation of the mean fibre area with age and a noteworthy high positive correlation between F1 and F2 ($r = 0.96$). Therefore a normal biopsy which shows large fibres of one type, have also large fibres of the other type and the reverse is true (Fig. 3).

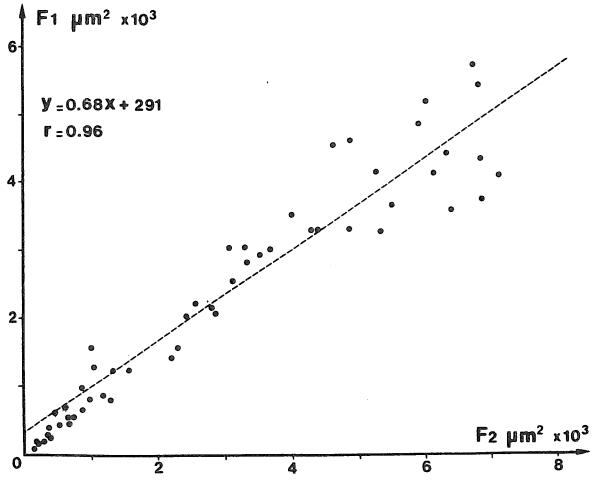

Fig. 3 : Graph of type 1 versus type 2 fibre size within the studied biopsies.

The two last parameters CV1 and CV2 stand apart on the third SC (10.8% of the total variance) (Fig. 2b). Consequently, they seem to be independent of the other parameters, in particular age.

Fibre size (tables 1 and 2) : Whatever the histochemical fibre type or the age of the patient may be, the histograms of the fibre sizes show a more or less symmetric distribution of cross-sectional areas around a modal peak. We restricted the age range of the subjects to 6 groups, taking into consideration the variations of fiber sizes with age observed by Brooke and Engel (1969).

Table 1 : MEAN PARAMETERS AND STANDARD ERROR OF THE MEAN IN THE DIFFERENT MUSCLE BIOPSIES WITH RESPECT TO AGE

Parameters	age classes (years) 1 (0-5)	2 (5-10)	3 (10-20)	4 (20-30)	5 (30-50)	6 (50-70)	F-statistic*
	Number of biopsies 18	7	7	7	6	8	
F1 μm^2	581 ± 87	1453 ± 232	2737 ± 370	4189 ± 345	4226 ± 321	3624 ± 375	$F^{47}_{5} = 45.03$ $p \leq 0.001$
F2 μm^2	609 ± 89	1740 ± 324	3273 ± 365	5679 ± 364	5795 ± 514	4726 ± 644	$F^{47}_{5} = 49.36$ $p \leq 0.001$
CV1 %	27 ± 2	24 ± 2	26 ± 4	30 ± 3	25 ± 3	25 ± 1	n.s.
CV2 %	21 ± 1	25 ± 3	22 ± 5	27 ± 5	23 ± 1	26 ± 3	n.s.
F1/F2	0.96 ± 0.06	0.90 ± 0.13	0.83 ± 0.04	0.74 ± 0.05	0.74 ± 0.05	0.80 ± 0.06	n.s.
DN	1.56 ± 0.26	2.37 ± 0.57	1.97 ± 0.25	2.05 ± 0.22	1.75 ± 0.18	1.30 ± 0.19	n.s.
DS	1.28 ± 0.24	2.21 ± 0.55	1.56 ± 0.20	1.36 ± 0.13	1.10 ± 0.05	0.97 ± 0.15	n.s.
S	97 ± 3	83 ± 6	91 ± 4	90 ± 5	88 ± 4	94 ± 4	n.s.
X1 SFM	2.7 ± 0.3	3.9 ± 0.6	3.1 ± 0.3	3.1 ± 0.3	2.9 ± 0.1	2.5 ± 0.2	n.s.
X2 SFM	2.4 ± 0.1	2.0 ± 0.2	2.2 ± 0.2	2.3 ± 0.2	2.6 ± 0.1	2.7 ± 0.3	n.s.
A1	19 ± 2	12 ± 2	11 ± 1	12 ± 1	12 ± 1	17 ± 1	$F^{47}_{5} = 5.00$ $p \leq 0.001$
A2	19 ± 2	22 ± 2	19 ± 2	20 ± 2	16 ± 1	16 ± 2	n.s.
A1/X1	9.2 ± 1.4	4.1 ± 1.5	3.8 ± 0.5	4.1 ± 0.5	4.3 ± 0.5	7.4 ± 0.8	$F^{47}_{5} = 3.63$ $p \leq 0.01$
A2/X2	9.1 ± 1.5	11.9 ± 2.4	9.2 ± 1.5	8.8 ± 1.2	6.1 ± 0.6	6.3 ± 1.2	n.s.

* The localizations of the significant differences by the Student t-test are reported in table 2.

Table 2 : LOCALIZATION OF THE SIGNIFICANT DIFFERENCES BY THE STUDENT'S t-TEST

F1	t(1-2)=2.69 $p \leqslant 0.02$	t(1-3)=6.66 $p \leqslant 0.001$	t(1-4)=11.14 $p \leqslant 0.001$	t(1-5)=10.63 $p \leqslant 0.001$	t(1-6)=9.39 $p \leqslant 0.001$
		t(2-3)=3.30 $p \leqslant 0.01$	t(2-4)=7.04 $p \leqslant 0.001$	t(2-5)=6.86 $p \leqslant 0.001$	t(2-6)=5.59 $p \leqslant 0.001$
			t(3-4)=3.74 $p \leqslant 0.001$	t(3-5)=3.68 $p \leqslant 0.001$	t(3-6)=2.28 $p \leqslant 0.05$
F2	t(1-2)=2.62 $p \leqslant 0.02$	t(1-3)=6.16 $p \leqslant 0.001$	t(1-4)=11.73 $p \leqslant 0.001$	t(1-5)=11.34 $p \leqslant 0.001$	t(1-6)=9.52 $p \leqslant 0.001$
		t(2-3)=2.96 $p \leqslant 0.02$	t(2-4)=7.60 $p \leqslant 0.001$	t(2-5)=7.51 $p \leqslant 0.001$	t(2-6)=5.76 $p \leqslant 0.001$
			t(3-4)=4.64 $p \leqslant 0.001$	t(3-5)=4.67 $p \leqslant 0.001$	t(3-6)=2.80 $p \leqslant 0.02$
A1	t(1-2)=3.19 $p \leqslant 0.01$	t(1-3)=3.58 $p \leqslant 0.01$	t(1-4)=3.25 $p \leqslant 0.01$	t(1-5)=3.06 $p \leqslant 0.01$	
A1/X1	t(1-2)=2.83 $p \leqslant 0.01$	t(1-3)=3.01 $p \leqslant 0.01$	t(1-4)=2.84 $p \leqslant 0.01$	t(1-5)=2.58 $p \leqslant 0.02$	

t (a-b): calculated t-statistic between class a and class b.

For the 53 subjects, fibre areas display two periods with regard to age (Fig. 4) : a rapid growth until the 20-30 year-old group (F1 : $r = 0.89$ $p \leqslant 0.001$, F2 : $r = 0.90$ $p \leqslant 0.001$) and thereafter a non significant linear decrease (F1 : $r = -0.36$, F2 : $r = -0.33$). In childhood a 4.5 fold increase of the average fibre area is observed between classes 1 and 3. The mean size of type 2 fibres was found to be greater than the mean type 1 area in 46 out of the 53 samples (87%). The fibre area type 1/type 2 ratio shows no significant variation within the studied age groups. The variability of fibre size, as assessed

by the coefficient of variation, is similar in both fibre types and is consistently about 25% (table 3).

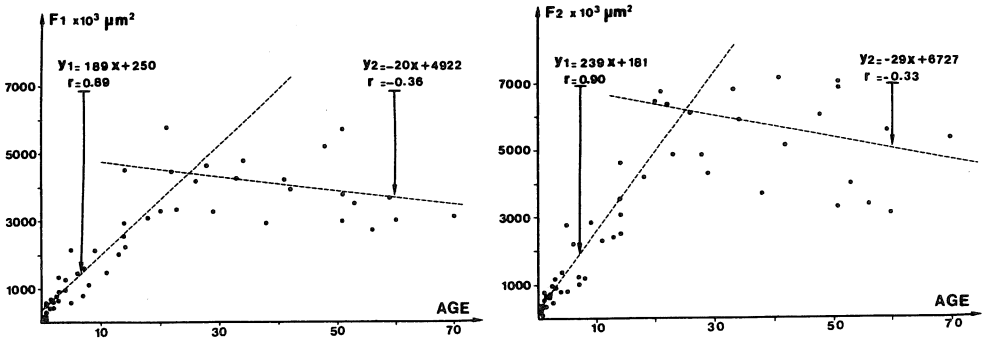

Fig. 4 : Relationship between type 1 (F1) and type 2 (F2) fibre size and age.

Fibre distribution (table 3) : A slightly larger numerical percentage of type 1 fibres is observed. The mean distribution is 64% of type 1 fibres and 36% of the other type. The data for the surface density area are more or less similar. The coefficient of variation of S (number of type 1 - type 2 fibre alternances) is low, indicating a quite similar arrangement in all the studied biopsies.

Table 3 : THE MEAN AND STANDARD DEVIATION OF SIZE AND DISTRIBUTION PARAMETERS FOR THE 53 STUDIED BIOPSIES.

SIZE PARAMETERS			DISTRIBUTION PARAMETERS	
F1/F2	0.83 ± 0.20		S	90 ± 11
CV1	26.5 ± 7.5		X1	2.9 ± 1.3
CV2	23.6 ± 7.4		X2	2.4 ± 0.5
DS	1.39 ± 0.88	57 % F1 33 % F2	A1 A2	18 ± 4 22 ± 4
DN	1.77 ± 0.96	64 % F1 36 % F2	A1/X1 A2/X2	6.19 ± 4.9 8.67 ± 5.0

The A2/X2 value is observed to be generally higher than the A1/X1 value. The former parameter is constant in all age-groups. The latter one shows a significant higher value in the 0-5 year-old group ($F^{5}_{47} = 3.63$ $p \leqslant 0.01$ table 2). The individual A1/X1 values range from 1.1 to 22.9 and A2/X2 values from 2.1 to 23.6.

Therefore, wide variations are observed because, though the majority of the values (90%) are grouped (Fig. 5), some of them stand out by a high proportion of one of the two histochemical fibre types and a consequent presence of clusters. Obviously the fibres of the other type become more and more isolated. This fact has been especially observed in the young patients.

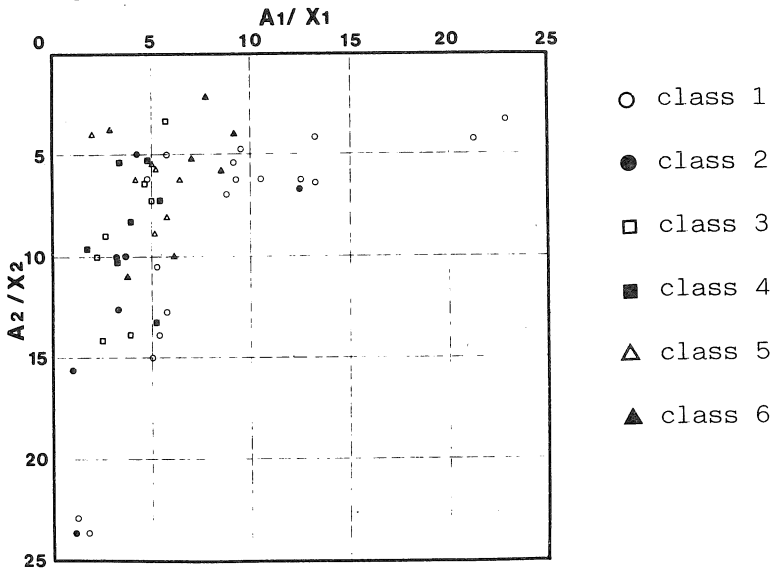

Fig. 5 : Graph of the spatial distribution index with reference to fibre type and age groups.

DISCUSSION

The vast majority of the individual data of fibre size fits neatly within the normal range for peroneus longus and tibialis anterior with regard to age of the patients as defined by previous studies (Edström and Nyström, 1969 ; Reniers et al., 1970).

Slightly lower values have been found by Polgar et al. (1973), but they used an autopsy material where a shrinkage is observed (Larsson, 1978). In agreement with these authors the mean area of type 2 fibres was found

to be greater than the mean type 1 area in 87% of the biopsies. However, the tendency for type 2 fibres to be larger in male subjects as compared with female subjects in biceps brachii (Brooke and Engel, 1969), in deltoideus (Schmitt, 1978), in quadriceps (Larsson, 1978) is not found in our muscle samples.

The increasing size with growth and the following little size decrease after the third decade is more pronounced in the type 2 fibres. The invariability of F1/F2 ratio involves that this size variation occurs with the same proportion in the two fibre types. This ratio seems to be a better size parameter to define a normal muscle as suggested by Maunder-Sewry and Dubowitz (1981) and Cornelisse et al. (1979).

It has been stated that coefficients of variation of the mean cross-sectional area of normal muscle fibres lie below 30% for Pongratz and Bodechtel (1976) and Cornelisse et al. (1979), 25% for Dubowitz and Pearse (1960) and Brooke and Engel (1969). Suchenwirth et al. (1969) consider a CV of 35% as the upper limit for normal tissue, which is in agreement with our results. There is no significant difference between the CV of the two fibre types: CV1 = 26.5% $\pm$ 7.5, CV2 = 23.6% $\pm$ 7.4.

In a previous paper (Tankosic et al., 1983) we have pointed out that fibre type grouping was characterized by a decrease of the S value, the A/X parameter indicating the size (X) and the heterogenity (A) of the aggregates.

In this study, for the evaluation of the spatial parameters, the linear contiguity of the fibres is considered, whereas the majority of authors (Telerman-Toppet and Coers, 1973, Lexell et al., 1983) adopts the measure of counting the number of enclosed fibres defined by Jennekens et al. (1971) as fibres completely surrounded by fibres of their own histochemical type. Though the approach of the problem is different, it is reasonable to think that one enclosed fibre is never observed in an "alignment" smaller than 3 or 4 frames of measure. With our technique, a mean A1 value of 18 corresponds to a mosaic pattern where 68% of the aggregates are equal or smaller than 3 frames of measure, that is about 3 type 1 fibres, and a mean A2 value of 22 to 88% of aggregates equal or smaller than 3 type 2 fibres. The method takes into consideration the fact that a fibre type can differ in size from the other type.

From the analysis of variance, no grouping tendency is observed with increasing age in our studied population. These results are apparently not in agreement with those of Tomonaga and Maki (1980) who studied a large sample of old patients, and with Bass et al. (1975) who studied rat muscle biopsies.

The main objective of this study was to obtain all the possible morphometric parameters and their limit of variability with our image analysis system. Resulting from the principal component analysis, a normal muscle must be necessarily characterized by three groups of parameters :

- size parameters
- coefficient of variation of fibre areas
- spatial distribution parameters.

In the first group, it is well known that fibre sizes vary considerably with many factors : age, training, disuse.... (Larsson, 1978). Our study shows that the most constant and therefore the most reliable size parameters is the fibre area ratio.

The clear-cut advantage of the CV consists in its low variability and its independence towards the other parameters.

For the third group, we must keep in mind that the spatial distribution parameters show the greatest variability (41.7% on the first SC). Therefore, in order to interpret the spatial distribution in a muscle biopsy, S, A1/X1, A2/X2 need to be considered conjointly. This study indicates that the vast majority of normal biopsies shows a mosaic pattern with small aggregates and preferably of type 1 fibres.

Actually, we focus our attention on various muscular diseases. Our aim is to know what parameters are susceptible of variation and if they remain correlated on the same way with the others. We think that such a multiparametric study is able to detect minor changes caused by a beginning pathology.

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REFERENCES

Bass A, Gutmann E, Hanzlikowa V. Biochemical and histochemical changes in energy-supply-enzyme pattern of muscles of the rat during old age. Gerontologia 1975 ; 21 : 31-45.

Brooke MH, Engel WK. The histographic analysis of human muscle biopsies with regard to fiber types, Part 1 : adult male and female. Neurology (Minneap) 1969 ; 19 : 221-233.

Brooke MH, Engel WK. The histographic analysis of human muscle biopsies with regard to fiber types, Part 4 : children's biopsies. Neurology (Minneap) 1969 ; 19 : 591-605.

Brooke MH, Kaiser KK. Some comments on the histochemical characterization of muscle adenosine-triphosphatase. J Histochem Cytochem 1969 ; 17 : 431-432.

Colgan PW. Quantitative ethology. PW Colgan, Willey Interscience, Toronto, 1977.

Cornelisse CJ, Bots GTHAM, Wintzen AR, Ploem JS, Van den Brock K. Real-time morphometric analysis of type I and type II fibers in cryostat sections of human muscle biopsies. In Morphometry in Pathology, V.N. Riede and A. Reith, Gustav Fischer Verlag, Stuttgart, New York 1979, 218-238.

Dubovitz V, Pearse AGE. A comparative histochemical study of oxidative enzyme and phosphorylase activity in skeletal muscle. Histochemie 1960 ; 2 : 105-117.

Edström L, Nyström B. Histochemical types and sizes of fibres in normal human muscles. Acta Neurol Scand 1969 ; 45 : 257-269.

Jennekens FG, Tomlinson BE, Walton JN. Data on the distribution of fibre types in five human limb muscles. J Neurol Sci 1971 ; 14 : 245-257.

Johnson MA, Sideri G, Weightman D, Appleton D. A comparison of fibre size, fibre type constitution and spatial fibre type distribution in normal human muscle and muscle from

cases of spinal muscular atrophy and from other neuromuscular disorders. J Neurol Sci 1973 ; 20 : 345-361.

Larsson L. Morphological and functional characteristics of the ageing skeletal muscle in man. A cross-sectional study. Acta Physiol Scand Supl 1978 ; 457 : 3-46.

Lexell J, Dowsnham D, Sjöström M. Distribution of different fibre types in human skeletal muscle. A statistical and computational model for the study of fibre type grouping and early diagnosis. J Neurol Sci 1983 ; 61 : 301-314.

Maunder-Sewry CA, Dubowitz V. Needle muscle biopsy for carrier detection in Duchenne muscular dystrophy. J Neurol Sci 1981 ; 49 : 305-324.

Morris CJ, Raybould JA. Fibre type grouping and end-plate diameter in human skeletal muscle. J Neurol Sci 1971 ; 13 : 181-187.

Polgar J, Johnson MA, Weightman D, Appleton D. Data on fibre size in thirty six human muscles. An autopsy study. J Neurol Sci 1973 ; 19 : 307-318.

Pongratz D, Bodechtel G. Differentialdiagnose der Erkrangungen der Skeletmuskulatur und Hand von Muskelbiopsen. Sammlung Psychiatricher und neurologischer Einzeldarstellungen Stuttgart Georg Thieme Verlag 1976.

Reniers J, Martin L, Jorris C. Histochemical and quantitative analysis of muscle biopsies. J Neurol Sci 1970 ; 10 : 349-367.

Saltin B, Henriksson J, Nygaard E, Andersen P. Fiber types and metabolic potentials of skeletal muscles in sedentary man and endurance runners. Ann New York Acad Sci 1977 ; 301 : 3-29;

Schmitt HP. Quantitative analysis of voluntary muscles from routine autopsy material with special reference to the problem of remote carcinomatous changes (neuromyopathy). Acta Neuropathol (Berlin) 1978 ; 43 : 143-152.

Suchenwirth R, Reichenmuller HE, Bundscher HD. Muscle histochemistry in relation to motor conduction velocity in cases of polyneuropathy. Muscle diseases. Proc. Internat. Congress Milan, ed. Walton Canal and Scarlato 1969, 56-60.

Swash M, Schwartz MS. Neuromuscular diseases. A practical approach to diagnosis and management. Berlin, Heidelberg, New York, Springer-Verlag 1981.

Tankosic P, Marchal C, Floquet J, Burlet C. Morphometric analysis of human muscle biopsies by use of an electronic image analyser (Quantimet 720). Acta Stereol 1983 ; 2/1 : 135-143.

Telermann-Toppet N, Cöers C. Histochimie des fibres musculaires normales. Ann Anat Pathol Paris 1973 ; 1 : 35-48.

Tomogana M, Maki T. Histochemical study on the senile changes of skeletal and ocular muscles. Brighton Abstract of the VIth international histochemistry and cytochemistry Congress 1980.

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