

# Morphometric analyses of normal pediatric brachial biceps and quadriceps muscle tissue

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**Summary.** Pediatric normal brachial biceps (14 specimens) and quadriceps muscles (14 specimens) were studied by immunohistochemistry to quantify fiber-type, diameter and distribution, capillary density, presence of inflammatory cells (CD3, CD20, CD68) and expression of neonatal myosin and MHC class I proteins. Brachial biceps showed more fast-twitch fibers and lower capillary/fiber ratio than quadriceps. The mean diameter of both fiber types was smaller in biceps than quadriceps. Fast-fibers were smaller than slow-fibers, and capillary/fiber ratio was <1.0 in both muscles. Fiber size and capillary / fiber ratio increased with age. Normal limits for infiltrating haematopoietic cells were <4 T lymphocytes, or CD68+ cells, very few B cells, <6 neonatal myosin positive fibers, and no fibers MHC class I positive in one x20 field, for both muscles. The present comparison of quantitative findings between brachial biceps and quadriceps may allow standardization of the assessment of pathological changes in both pediatric muscles.

**Key words:** Skeletal muscle biopsy, Normal morphometry

## Introduction

The assessment of muscle biopsy tissue is an important part of the diagnostic process in the investigation of cases of suspected inflammatory myopathy. In children presenting with suspected juvenile

dermatomyositis (JDM), endothelial cell alterations, a decrease in the number of capillaries, perivascular inflammation, perifascicular atrophy, and muscle fiber degeneration/ regeneration are histopathologic features that may be found in biopsies of skeletal muscle (Brooke and Engel, 1969; Bohan and Peter, 1975; Kissel et al., 1975; Banker, 1975; Crowe et al., 1982; Woo et al., 1988; Emslie-Smith and Engel, 1990; Kissel et al., 1991; Sallum et al., 2002). Although these findings are considered to be important features in the analysis of muscle biopsy material, they may be absent, or not routinely assessed, and muscle tissue taken very early in disease may appear unremarkable especially if analyzed by standard histological techniques only. This may result in up to 20% of cases being reported as normal on routine histopathologic analyses (Li et al., 2004; Pilkington and Wedderburn, 2005). However, when this analysis is refined by including immunohistochemical studies, most of these biopsy samples show increased expression of class I major histocompatibility complex (MHC) on muscle fibers (Topaloglu et al., 1997; Li et al., 2004; Sallum et al., 2009) or deposition of immunoglobulin or complement components in capillaries (Kissel et al., 1986; Gonçalves et al., 2002). Since typical changes in biopsy remain one of the criteria for diagnosis of JDM, we generated a scoring system to assess the severity of pathological changes based on quadriceps (vastus lateralis) biopsy samples (Wedderburn et al., 2007). We have proposed that such a standardized approach to muscle tissue analyses, staining and assessment would yield greater diagnostic information in such cases (Wedderburn et al., 2007, and manuscript in preparation). Such a standardized scoring system could be more widely utilized if it were applicable not only to quadriceps but also to other

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muscles. Normative data for features assessed in this score tool were previously reported for vastus lateralis muscle (Varsani et al., 2008); however, there are no such data for pediatric brachial biceps, which in some clinical centers is the muscle of choice for biopsy.

The objectives of this study were to quantify the fiber-type, diameter and distribution, capillary density, the presence of inflammatory cells (lymphoid and myeloid), and expression of neonatal myosin (NM) and MHC class 1 protein, in morphologically normal pediatric brachial biceps muscle and to compare these features in morphologically normal pediatric quadriceps muscles.

### Material and methods

Twenty-eight muscle biopsy specimens, consisting of fourteen brachial biceps (9 boys, 5 girls; age at biopsy ranged from 3y to 10y, mean age of 5 years 8 months) from the muscle bank of University of Sao Paulo, and fourteen quadriceps (6 boys, 8 girls; age at biopsy ranged from 2y to 12y, mean age of 5 years 11 months) from the tissue bank of Great Ormond Street Hospital, London were analyzed. The study was approved by the Research Ethics Committees of the two institutions. The gender ratio (male/female) was 1.15, and there was no statistical difference of age distribution between the two groups ( $p=0.891$ , student's *t* test). The muscle biopsies were performed by the open surgical technique, as part of the diagnostic procedure in all cases for children presenting with mild hypotonia, without any detectable muscle weakness at clinical examination, no raised serum CK levels, and had all been reported as morphologically normal by two independent pathologists.

Muscle specimens were snap frozen in liquid nitrogen within 1 hour of biopsy, and stored at  $-80^{\circ}\text{C}$ . Immunohistochemistry was performed on  $7\ \mu\text{m}$  cryostat sections as described (Wedderburn et al., 2007; Varsani et al., 2008; Sallum et al., 2009) using antibodies to myosin heavy chain slow (WB-MHCs, 1:80), myosin heavy chain fast (WB-MHCf, 1:40), CD3 (UCHT1, 1:200) recognizing T cells, CD20 (L26, 1:600) recognizing B cells, CD68 (KP1, 1:400) recognizing myeloid cells, MHC class 1, heavy chain (W6/32, 1:50), neonatal myosin (WB-MHCn, 1:25) (all Novocastra, Newcastle-Upon-Tyne UK), and CD31 (JC/70A, 1:20) recognizing endothelial cells (DAKO, Cambridge, UK). A positive control sample of inflammatory myopathy was included in the immunohistochemistry batch for MHC class 1 staining.

Percentages of fast-twitch and slow-twitch fibers were calculated as fibers stained positive for myosin heavy chain fast- and slow-isoforms, respectively versus total number of fibers. The fiber diameter, defined as the maximum diameter across the smaller aspect of the muscle fiber was measured for slow- and fast-twitch fibers in at least 200 muscle fibers from each sample on digital images photographed using a x20 objective

(Brooke and Engel, 1969). Capillary to fiber ratio was calculated as the number of structures stained for CD31 versus the total number of fibers counted in the same area. At least 200 muscle fibers were counted on 2-3 digital images photographed using the x20 objective. Fibers presenting more than 50% of their surface on the top or left edge on the images were enumerated. Two independent observers performed the counting, and the mean score was used for statistical analysis.

Infiltrating haematopoietic cells, and fibers expressing neonatal myosin and MHC class I, were counted in the whole section by two independent observers using a x20 objective. The results are expressed as mean cells/ $\text{mm}^2$  and mean cells/x20 field ( $0.332\ \text{mm}^2$ ). Student's *t* and Mann-Whitney U tests were applied for parametric and non-parametric data analysis, respectively, for comparison of the diameter of fast- and slow-twitch fibers and the proportion of each fiber type between biceps and quadriceps muscles. Pearson's and Spearman's tests were applied for correlation between parametric and non-parametric data, respectively, using SPSS v.15 (Chicago, IL, USA). Differences were considered statistically significant at  $p<0.05$ .

### Results

Fast-(type IIa and IIb) and slow-(type I) twitch muscle fibers were identified in brachial biceps and quadriceps muscle samples, by staining for myosin heavy chain fast- and slow-isoforms, respectively (Fig. 1) and fiber proportions were calculated for each muscle type. Comparison between brachial biceps and quadriceps muscle showed that significantly more fast-myosin positive fibers were observed in biceps than in quadriceps ( $p=0.021$ , Student's *t*-test), whereas the opposite was seen for slow-myosin positive fibers ( $p=0.042$ , Student's *t*-test) as shown in Fig. 2A,B. Measurement of fiber size in both muscle types showed that fast-twitch fibers were slightly smaller than slow-twitch fibers in each muscle type but this was not significant. The mean diameters of both the fast- and slow-twitch fibers were smaller in brachial biceps than corresponding fiber type to quadriceps, (Fig. 2C,D) but again these differences were not significant. Biceps muscle fast-twitch fiber size correlated with age ( $r=0.785$ ,  $p=0.001$ , Pearson's test) (Fig. 3A). Although no significant correlation was observed between biceps slow-twitch fiber size and age ( $r=0.388$ ,  $p=0.171$ , Spearman's test) a trend of slow-fiber size increase with age was seen (Fig. 3C). For quadriceps muscle, both fast- and slow-twitch fibers showed statistically significant increases of fiber size with age,  $r=0.786$ ,  $p=0.021$ , and  $r=0.761$  and  $p=0.028$ , respectively (Spearman's test) (Fig. 3B,D).

Capillaries were enumerated by counting structures identified by CD31 staining (Fig. 4A). The capillary/fiber ratio was significantly lower in brachial biceps compared to quadriceps, ( $p=0.044$ , Mann-

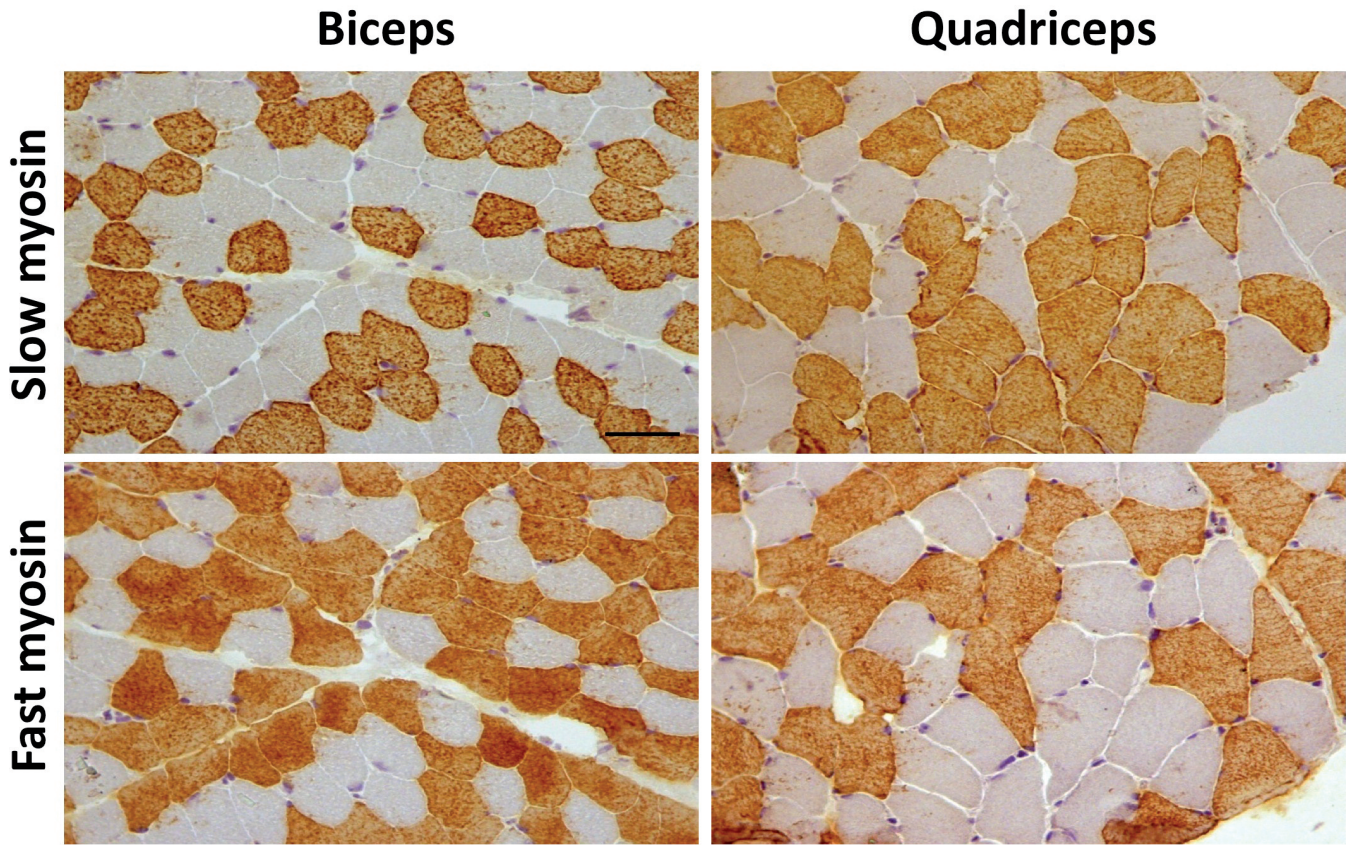


Fig. 1. Representative immunohistochemical images stained for slow-myosin (upper panels) and fast-myosin (lower panels), on brachial biceps (left panels) and quadriceps (right panels). 200x magnification. Scale bar represents 10  $\mu\text{m}$ .

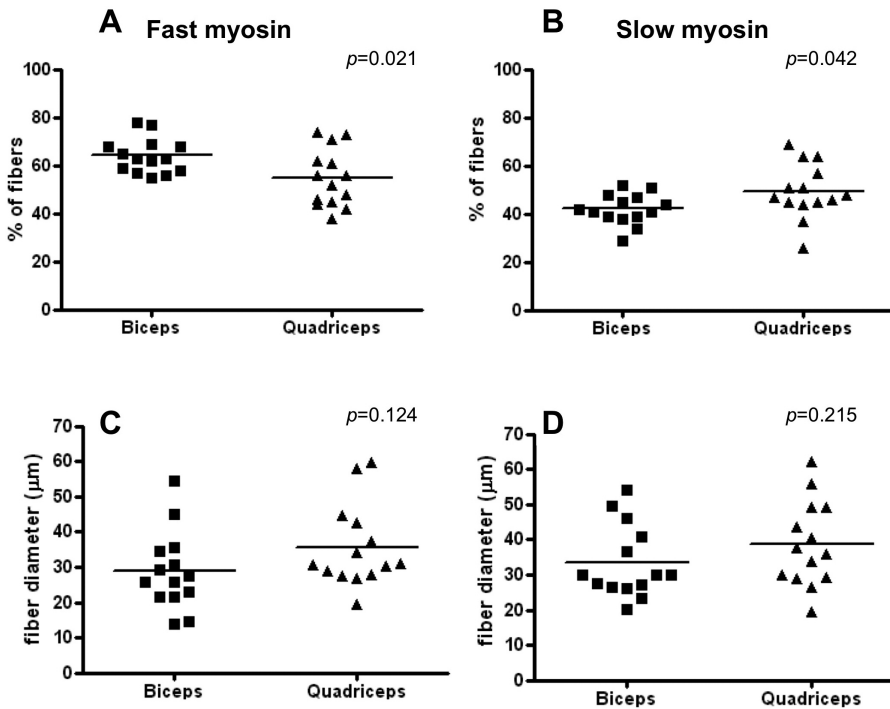
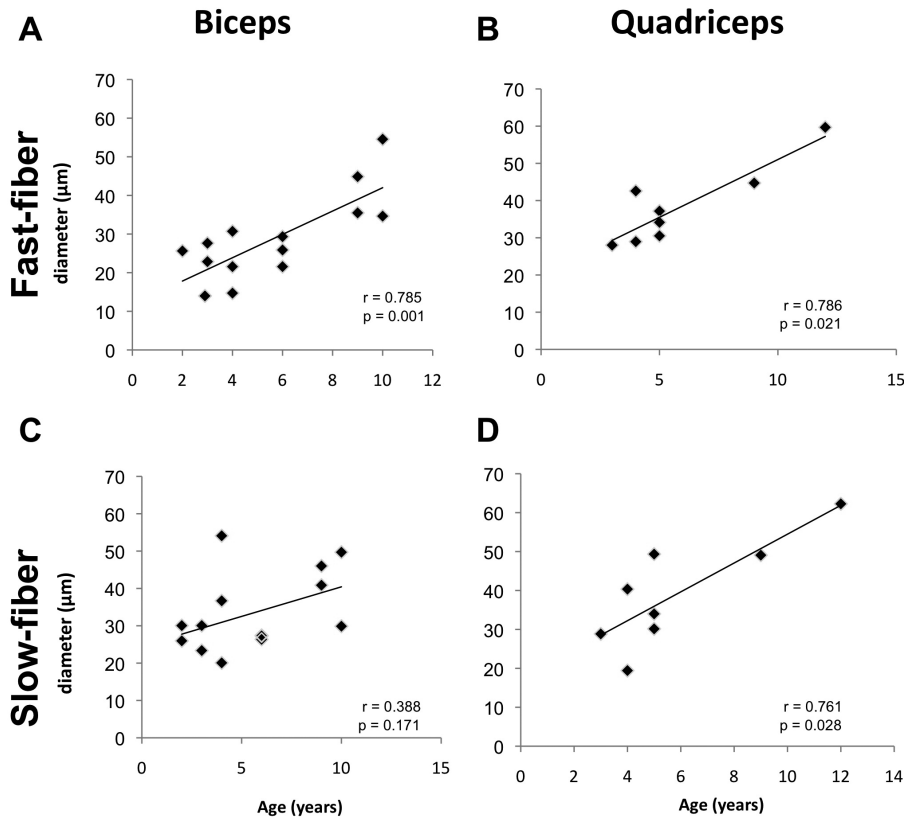


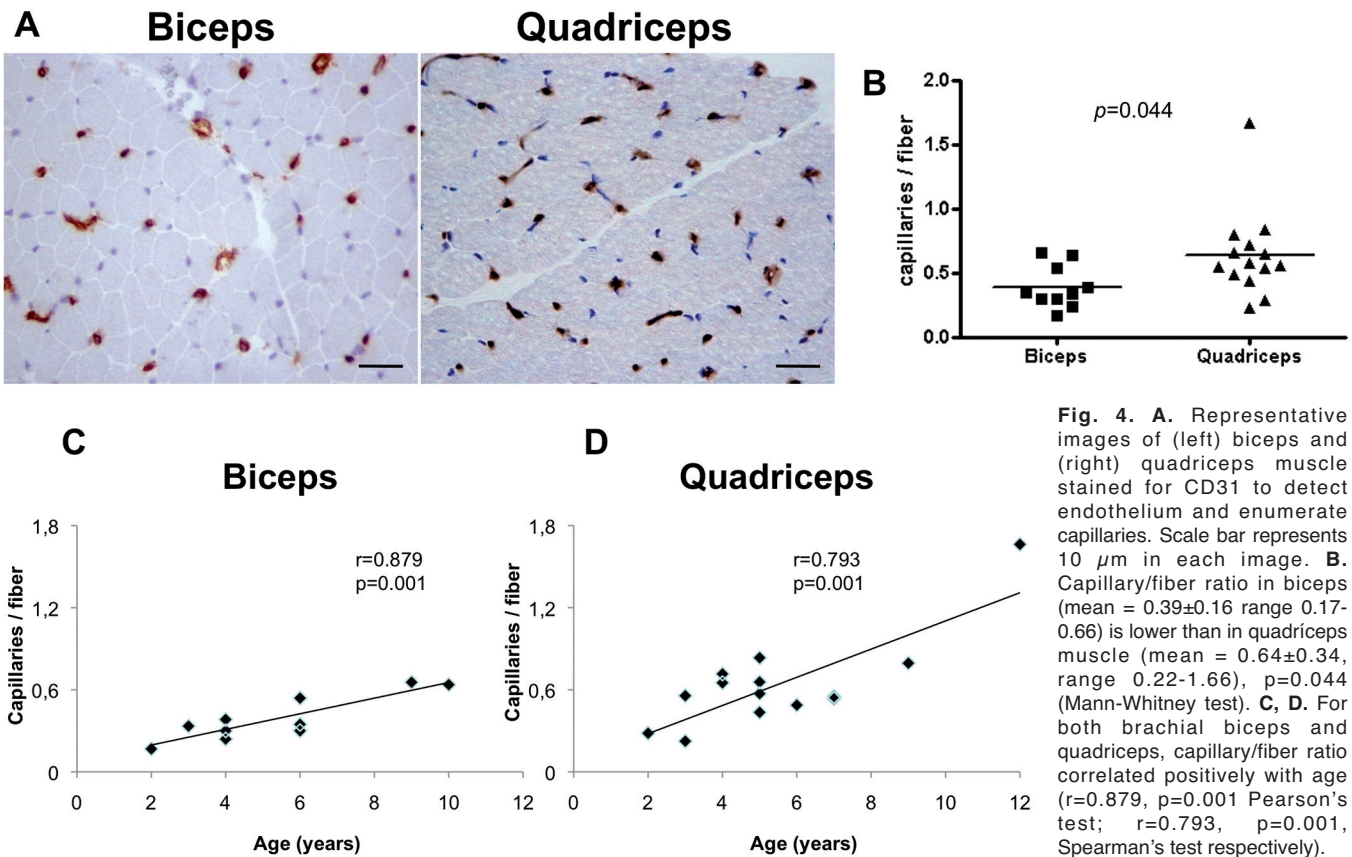
Fig. 2. **A.** Fast-myosin positive fibers represent a larger percentage of fibers in brachial biceps (mean=63%) than in quadriceps muscle (mean=55%),  $p=0.021$  (Student's t test). **B.** Slow-myosin positive fibers represent a smaller percentage of fibers in brachial biceps (mean=41%) than in quadriceps muscle (mean=49%),  $p=0.042$  (Student's t test). **C.** Diameter of fast-twitch fibers in biceps (mean value =  $28.82 \pm 11.03 \mu\text{m}$ , range 13.99-54.55  $\mu\text{m}$ ) is not significantly different from the diameter of fast-twitch fibers in quadriceps (mean value =  $35.65 \pm 11.71 \mu\text{m}$ , range 19.60-59.68  $\mu\text{m}$ ),  $p=0.124$  (Student's t test). **D.** Diameter of slow-twitch fibers in biceps (mean value =  $33.42 \pm 10.41 \mu\text{m}$ , range 20.07-54.10  $\mu\text{m}$ ) is not significantly different from the diameter of slow-twitch fibers from quadriceps (mean value =  $38.74 \pm 12.12 \mu\text{m}$ , range 19.43-60.27  $\mu\text{m}$ ),  $p=0.215$  (Mann-Whitney test).



Pediatric muscles normative data



**Fig. 3.** Correlation between fast (**A, B**) and slow (**C, D**) fiber mean diameters with age on brachial biceps (**A, C**) and quadriceps (**B, D**) muscles. Spearman's test was applied for correlations on quadriceps muscle, and brachial biceps slow-fiber diameter vs age, and Pearson's test for brachial biceps fast-fiber diameter vs age.



**Fig. 4. A.** Representative images of (left) biceps and (right) quadriceps muscle stained for CD31 to detect endothelium and enumerate capillaries. Scale bar represents  $10 \mu\text{m}$  in each image. **B.** Capillary/fiber ratio in biceps (mean =  $0.39 \pm 0.16$  range 0.17-0.66) is lower than in quadriceps muscle (mean =  $0.64 \pm 0.34$ , range 0.22-1.66),  $p = 0.044$  (Mann-Whitney test). **C, D.** For both brachial biceps and quadriceps, capillary/fiber ratio correlated positively with age ( $r = 0.879$ ,  $p = 0.001$  Pearson's test;  $r = 0.793$ ,  $p = 0.001$ , Spearman's test respectively).

Whitney U test) (Fig. 4B). In both quadriceps and brachial biceps muscles we noted that the capillary to fiber ratio showed a mean value lower than 1.0. Both biceps and quadriceps capillary/ fiber ratios correlated significantly with age ( $r=0.879$ ,  $p=0.001$ , Pearson's test; and  $r=0.793$ ,  $p=0.001$ , Spearman's test, respectively) (Fig. 4C,D).

As in our previous study of 'normal' quadriceps tissue, in the biceps muscle samples a few scattered T cells and myeloid cells were observed, but no focal inflammation or clusters were seen in either muscle type (a focus or cluster was defined as  $>10$  inflammatory cells clustered together) (Wedderburn et al., 2007). MHC class 1 up regulation on muscle fibers was not detected in any sample. Expression of neonatal myosin was seen in occasional fibers, and as expected a non-significant trend towards a higher number of neonatal myosin-positive fibers was noted in younger children (data not shown). Mean values of CD3+ or CD68+ infiltrating cells, and neonatal myosin-positive muscle fibers in normal biceps samples were not significantly different to our previously published data in normal quadriceps samples (Varsani et al., 2008). Thus, mean values (1SD) per 20x field in normal biceps tissue for CD3+, CD68+ and NM+ were: 0.196 (0.26), 1.010 (0.83), and 0.016 (0.05), respectively.

## Discussion

Vascular abnormalities have been reported in several studies of muscle tissue from patients with inflammatory myopathies, particularly JDM where endothelial abnormality, capillary loss and even infarction have been described (Banker, 1975; Crowe et al., 1982; Woo et al., 1988; Kissel et al., 1991; Sallum et al., 2002). To incorporate such findings, a vascular domain with capillary dropout was included in our recent proposal of a score system for muscle biopsy evaluation in patients with JDM (Wedderburn et al., 2007). However, few studies have quantified muscle capillary density, and to our knowledge no such study has been performed in children. In order to assess pathological abnormality it is vital to have reference data on normal tissue for comparison. We have generated normative pediatric data of capillary density, fiber-type diameter and the distribution and number of inflammatory cells in morphologically normal pediatric brachial biceps muscle and compared these to quadriceps muscle. Capillary to fiber ratio was significantly lower in biceps compared to quadriceps. Previous studies have shown that capillary density in mixed muscles varies with the specific type of muscle fiber, being higher in muscles with slow fiber predominance (Mathieu-Costello et al., 1991; Murakami et al., 2010). We observed significantly more fast-fibers in biceps than in quadriceps, which may partially explain the detected difference in the capillary/fiber ratio. Of note, the capillary to fiber ratio was less than 1.0 in this pediatric series consisting of children of 12 years or under. This contrasts with previous reports in adult

muscle suggesting that the ratio of muscle fibers to capillaries in healthy muscle is 1.0 (Emslie-Smith and Engel, 1990). Therefore, patient age is an important parameter to consider when assessing capillary density in pediatric myopathies. Slow-fibers were more numerous in quadriceps, an anti-gravitational muscle, than in brachial biceps as expected. However, fast- and slow-fiber mean diameters did not differ significantly between both muscles. On the other hand, the fast- and slow-fiber diameters correlated with age, as previously described (Miles et al., 2007).

Absence of MHC Class I overexpression, the presence of a very low number of inflammatory cells (CD3, CD68 and CD20), and fibers expressing neonatal myosin were observed in brachial biceps, similar to the data previously reported for quadriceps.

Our study had some limitations, inherent to the difficulty of the study of 'normal' muscle tissue and the fact that samples came from centres in two different countries, with distinct ethnicity, and reasons for patient referral. However as far as possible, the analyzed cases were equivalent in all parameters that could be matched.

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