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ORIGINAL ARTICLES

Differential diagnosis of vacuolar muscle biopsies: use of p62, LC3 and LAMP2 immunohistochemistry

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Intrafibral vacuoles are the morphological hallmark in a wide variety of human skeletal muscle disorders with different etiology. In most cases, differential diagnosis is feasible with a routine histochemical work up of muscle biopsy. Ultrastructural analysis is an important confirmatory tool, but it is not widely available. Immunohistochemical stainings for p62, LAMP2 and LC3 are commonly available as tissutal marker for autophagy. We compared the immunohistochemical patterns for autophagic markers p62, LC3 and LAMP2 with routine histochemical markers in 39 biopsies from patients with definite diagnoses of glycogen storage disease type 2 (LOPD or Pompe disease, PD), sporadic inclusion body myositis (sIBM), oculo-pharyngeal muscular dystrophy (OPMD) and necrotizing myopathy (NM). Moreover, we also analyzed muscles of 10 normal controls. In PD group, LC3 and LAMP2 showed an higher percentage of positive fibers, whereas p62 was limited to a minority of fibers, thus paralleling the results of histochemical stainings; in NM group, LAMP2 and LC-3 were diffusely and unspecifically expressed in necrotic fibers, with p62 significantly expressed only in two cases. OPMD biopsies did not reveal any significant positivity. The most interesting results were observed in sIBM group, where p62 was expressed in all cases, even in fibers without other markers positivity. There results, although limited to a small number of cases, suggest that the contemporary use of p62, LAMP2 and LC-3 staining may have an adjunctive role in characterizing muscle fiber vacuoles, revealing autophagic pathway activation and providing further clues for the understanding of pathogenetic mechanisms.

Key words: autophagy, Pompe disease, inclusion body myopathy, necrotizing myopathy, immunohistochemistry

Introduction

Autophagy is a highly conserved homeostatic process for lysosome mediated degradation of cytoplasmic components, including damaged organelles and toxic protein aggregates (1). The process of autophagy occurs through a multi-step mechanism, including the formation of a phagophore, which engulfs proteins and organelles destined for degradation, then the production of a membrane-bound vacuole (the autophagosome), which moves along microtubules and fuses with the lysosome to form the autolysosome. Microtubule-associated protein light chain 3 (LC3) is commonly used as a marker of autophagosome formation (2-3). Upon autophagy induction, its modified form LC3-II, associated with autophagic membranes, binds p62/SQSTM1, an adapter protein that targets ubiquitinated protein aggregates (4). The lysosomal-dependent turnover of LC3-II and p62 has emerged as a measure of autophagic proteolysis. Specifically, the accumulation of LC3-II-labeled autophagosomes and/or p62 aggregates is a robust marker of autophagic flux engulfment at any point beyond autophagosome formation (3).

Lysosome-associated membrane protein 2 (LAMP2) is a glycoprotein with a principal role in the adhesion of the lysosome, and therefore in their protection and maintenance studied in human tissues by immunohistochemical markers (5).

Autophagic vacuolar myopathies are a group of muscle disorders characterized by massive autophagic

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buildup. Pompe disease (PD) is due to a defect in lysosomal acid a-glucosidase (GAA), with intralysosomal glycogen accumulation (6); Danon disease is caused by lack of the lysosome associated membrane protein 2 (LAMP2) (7) with increase of dysfunctional lysosomes in skeletal and cardiac muscles; and the X-linked myopathy with excessive autophagy (XMEA) is due to mutations in V-ATPase (8). The exact role of autophagy dysfunction is still debated; however, in a recent work of Nascimbeni at al. (9) other autophagy regulators, like transcription factor EB (TFEB) and vacuolar protein sorting 15 (VPS15), seem to have an active role in the pathogenesis of both Danon disease and PD, showing an autophagy block correlated with the severity of the disease; therefore therapeutic approaches targeted to normalize these factors and restore the autophagic flux should be considered.

Other myopathies are characterized by the presence of intrafibral vacuoles, originated by different pathological processes; these include inclusion body myopathies (IBM), necrotizing myopathies (NM), and oculophayngeal muscular dystrophy (OPMD).

IBMs are classified as sporadic (s-IBM), a relatively common inflammatory myopathy classically presenting in older individuals (10-12), and hereditary (h-IBM), caused by gene mutations producing intrafibral protein storage, with disruption of cell architecture (i.e., UDP-Nacetylglucosamine-2 epimerase/N-acetylmannosamine-kinase or GNE gene).

Necrotizing myopathies (NM) have a multifactorial etiology; they may have an acute or subacute onset, can be severe, may have an autoimmune pathogenesis or be associated to cancer, and may be related to statin therapy. Diagnosis is based on the clinical picture and on muscle biopsy showing minimal or no inflammatory infiltrates and marked muscle necrosis with vacuolated fibers and macrophagic activation, unlike other inflammatory myopathies (10).

Oculopharyngeal muscular dystrophy (OPMD) is a late-onset muscle disease associated with progressive ptosis of the eyelids, dysphagia, and unique histological features, including intracytoplasmic rimmed vacuoles and tubule-filamentous intranuclear inclusions (INIs) in skeletal muscle. Polyalanine [poly(A)] expansion mutations in the polyadenine-binding protein 2 (PABN1) gene have been shown to cause OPMD (11). Since its impairment leads to accumulation of autophagosomes, autophagy can be detected by immunohistochemistry for autophagy proteins LC3 and p62/SQSTM1; immunostaining for either LC3 or p62 was proposed to replace electron microscopy in the diagnosis of autophagic vacuolar myopathies (12-15).

LC3 and p62 have also been evaluated as markers of IBM (16).

In routinary muscle biopsy evaluation, differential diagnosis of vacuolar myopathies can be challenging for the presence of only mild alterations, or unspecific/unrelated tissue changes; moreover, in some cases clinical data may lack or are only partially supportive.

Aim of this retrospective study is to verify the use of a simple immunohistochemical procedure to detect autophagic activation in a series of muscle biopsies with a defined diagnosis of vacuolar myopathy, in order to ameliorate the diagnostic accuracy.

Materials and methods

Ethics statement

All patients had undergone quadriceps muscle biopsy for diagnostic purposes, and signed full informed consent. No individually identifiable patient data are presented in this report.

Objectives

This is a retrospective study on muscle tissue samples stored in liquid nitrogen from patients affected with different types of vacuolar myopathies, namely late onset PD (LOPD), NM, s-IBM, OPMD. Immunohistochemistry for p62, LC3 and/or LAMP2 was compared with routine histological and histochemical staining, in order to evaluate their role as diagnostic tools for the differentiation of autophagic vacuolar myopathies.

Case selection

A search of the database of tissue bank at the Neuromuscular Unit was carried out, spanning the interval between 1988 and 2016. Only patients with complete clinical, genetic and follow up data were included.

Twenty patients with a confirmed diagnosis of LOPD (9 men, 11 women; mean age 44.4 ± 28.8); seven sIBM patients (4 men, 3 women; mean age 65 ± 14.9) and four OPMD patients (1 man, 3 women; mean age 55.2 ± 10.1) were included in the study. Moreover, eight patients with NM were also considered (5 men, 3 women; mean age 51.1 ± 21.4).

Normal controls (5 men, 5 women; mean age 60.5 ± 7.1) were selected from a larger pool of muscle biopsies characterized by lack of pathologic findings

Patients characteristics are reported in Table I; levels of plasma creatine kinase prior to biopsy were available in the clinical record in 47 out of 49 subjects. A review of the original muscle biopsy slides was also made.

Table I. Clinical characteristics in 49 cases.

Subject ID	Group	CK level (x n.v.)	Sex	Age	Clinical involvement
1	LOPD	3X	F	44	Mild
2	LOPD	NA	F	34	Absent
3	LOPD	5X	M	52	Absent
4	LOPD	8X	M	66	Severe
5	LOPD	6X	M	34	Mild
6	LOPD	10X	F	30	Mild
7	LOPD	2X	F	57	Mild
8	LOPD	3X	F	74	Severe
9	LOPD	2X	M	42	Mild
10	LOPD	4X	F	49	Mild
11	LOPD	4X	M	1	Absent
12	LOPD	6X	F	52	Severe
13	LOPD	2X	M	61	Mild
14	LOPD	N	M	74	Severe
15	LOPD	5X	F	39	Absent
16	LOPD	5X	M	1	Severe
17	LOPD	4X	F	61	Absent
18	LOPD	2X	F	49	Absent
19	LOPD	2X	F	15	Absent
20	LOPD	N	M	53	Severe
21	NM	N	F	76	Mild
22	NM	6X	F	53	Mild
23	NM	4X	M	29	Severe
24	NM	N	M	34	Mild
25	NM	2X	M	82	Mild
26	NM	38X	M	25	Mild
27	NM	37X	M	64	Mild
28	NM	11X	M	46	Mild
29	IBMyositis	2X	F	33	Severe
30	IBMyositis	NA	F	61	Mild
31	IBMyositis	3X	F	71	Severe
32	IBMyositis	2X	M	68	Severe
33	IBMyositis	2X	M	77	Mild
34	IBMyositis	5X	M	73	Mild
35	IBMyositis	2X	M	72	Severe
36	OPMD	N	F	59	Mild
37	OPMD	N	F	66	Severe
38	OPMD	N	F	42	Mild
39	OPMD	N	M	54	Mild
40	CONTROL	N	M	47	Absent
41	CONTROL	N	M	54	Absent
42	CONTROL	N	M	58	Absent
43	CONTROL	N	M	65	Absent
44	CONTROL	N	M	71	Absent
45	CONTROL	N	F	62	Absent
46	CONTROL	N	F	58	Absent
47	CONTROL	N	F	61	Absent
48	CONTROL	N	F	59	Absent
49	CONTROL	N	F	70	Absent

Muscle biopsy sections

Serial 7-8 μm sections were cut from muscle samples stored in liquid nitrogen, and parallel processed for hematoxylin and eosin (H&E), modified trichrome Gomori, Periodic acid Schiff (PAS) stain and acid phosphatase stain according with routine procedures.

Immunohistochemistry

Immunoperoxidase staining for LC3 (mouse monoclonal antibody, clone 5F10, Nanotools; 1:100 dilution following antigen retrieval) and p62/SQSTM1 (guinea pig polyclonal antibody, Progen Biotechnik; 1:100 dilution following antigen retrieval) was performed on frozen tissue samples.

For LAMP2 we used purified rat anti-mouse CD107b monoclonal antibody, clone ABL-93, BD Biosciences, 1:100).

Quantification

Quantification was performed on muscle sections using a bright-field light microscope, with the investigator blind to group assignment of each subject. Prior to counting, each slide was viewed at low (2x-20x) and high power (40x) to determine whether positive fibers were present scarcely or in abundance. Muscle fibers containing the characteristic central inclusion, rimmed vacuoles, or punctate staining pattern were counted as positive, while fibers devoid of staining were counted as negative. The same criteria were used for morphological and histochemical stainings. A total of 200 fibers/slide were counted in specimens with abundant positivity, while a total of 600 fibers/slide was counted in specimens with scarce or patchy positivity (to reduce the sampling error). Tissue on the slide was divided into quadrants and randomly selected, non-overlapping fields were counted at high power in each quadrant until the total count was reached. The results were recorded as a percentage (the number of positive fibers divided by the total number of fibers counted).

Imaging

Images were taken with an AXIO digital camera on a BX41 bright-field light microscope using cellSens Entry 1.4 software (all by Olympus Corp) and were edited with Adobe Photoshop Version 12.0.2.

Statistical methods

Data were analyzed with SPSS statistical software (Version 18). For between-group comparison of the demographic data we used one-way ANOVA with post-hoc Bonferroni test (age and sex). To calculate sensitivity and specificity receiver operating characteristic (ROC) analysis was performed on all muscle biopsies. All tests were 2-tailed with $\alpha = 0.05$.

Results

Detailed results of the percentage of positive fibers for each staining are reported in Table II.

On light microscopy, we identified several histologic patterns suggestive of different cathegories of vacuolar myopathies.

In the LOPD patients group, characterized by variable clinical and muscle tissue involvement, LAMP2 and LC3 were positive in 65% of patients, whereas p62 positivity was seen only in 25% of subjects with a finely punctate staining pattern, paralleling morphological, PAS and acid phosphatase reactions (55%) (Figg. 1A, 2). No correlation with the clinical features was observed.

The NM group presented more extensive alterations with all methods, showing variable and heterogeneous expression of LAMP2 (7 out of 8 cases) and LC3 in 4 subject (Fig. 3), mainly in necrotic fibers, with less specificity; interestingly, p62 positivity was strongly observed only in 2 cases (22 and 27), both of them with a necrotizing myopathy of unknown origin and severe rabdomyolisis.

In IBM group, p62 and LC3 were diffusely expressed; in particular, p62 was positive in all eight subjects (Fig. 4B) respect to LC3 positivity in 6 cases (Fig. 4D). Differently from the NM group, LAMP2 showed a less significant expression in 6 cases (Fig. 4C).

In OPMD group, p62, LAMP2 and LC-3 were substantially negative in all cases, with LC3 mild unspecific staining only in a couple of fibers in 2 patients, LAMP2 in 1 case and no positivity for p62 antibody in all cases (data non shown).

In normal control sample, there was no intrafibral staining (Fig. 5B, C and D); a typical normal nuclear positivity was seen on LC3, LAMP2 and p62-stained sections.

There was no statistically significant difference in the mean age among the five groups (LOPD 44.4 ± 20.83 *vs* VM 51.1 ±21.49 *vs* IBM 65 ± 14.95 *vs* OPMD 55.2 ± 10.11 *vs* CONTROLS 60.5 ± 7.16 years; $p = 0.056$) or sex distribution (LOPD 55% *vs* VM 25% *vs* IBM 43% *vs* OPMD 75% *vs* CONTROLS 50% female, respectively; $p = 0.019$).

Figures 6 to 8 show the percentage of LC-3, LAMP2 and p62 positive fibers respectively in the different patients groups compared with the control group.

As expected, the higher percentage of positive fibers for autophagy markers is observed in LOPD patients, with a major occurrence of LAMP2 and LC3 staining;

Table II. Percentage of positive fibers for morphological, histochemical and immunohistochemial staining in 39 patients. All controls were completely negative.

Subject ID	Group	LC-3 (% positive fibers)	p62 (% positive fibers)	LAMP2 (% positive fibers)	HE (% vacuolated fibers)	TRIC (% vacuolated fibers)	PAS (% vacuolated positive fiber)	Acid phosphatase (% positive fibers)
1	LOPD	3,5	4	6.5	3	13.5	5.5	3
2	LOPD	0	0	0	0	0	2.5	0
3	LOPD	0	0	0	0	0	0	0
4	LOPD	37.5	2	25	25	45	35	20
5	LOPD	0	0	0	0	0	0	0
6	LOPD	0	0	0	0	0	0	0
7	LOPD	6	0	1.5	3	23.5	4	3
8	LOPD	7.5	4.5	14	7.5	13	5	2
9	LOPD	0	0	0	1.5	0	0	1.5
10	LOPD	0.5	0	3.5	2	1.5	1.5	3
11	LOPD	0	0	14	5	4.5	8.5	2.5
12	LOPD	12.5	4	26	12,5	10	20	7.5
13	LOPD	28	0	34	26.25	40	25	7.5
14	LOPD	8.5	0	10	6	3.5	3.5	3
15	LOPD	21.5	2	25.5	20	15	18.5	15
16	LOPD	6	0	0	75	75	75	75
17	LOPD	0	0	0	0	0	0	0
18	LOPD	16	0	0	0	0	0	0
19	LOPD	0	0	0.5	0	0	0	0
20	LOPD	0	0	0	0	0	0	0
21	NM	2.5	0	7	15	10	5	2.5
22	NM	0	21	1	0.5	1	1	2.5
23	NM	1	0	1.5	1.5	0	3	0
24	NM	0	0	1.5	10	7.5	10	3
25	NM	2	1	2,5	2,5	1	2	0
26	NM	0	0	9	0,5	0,5	0	12,5
27	NM	19	13	14	4,5	5	2	6,5
28	NM	0	0	0	1	0,5	0	0
29	sIBM	9	6	15	18,5	25	1,5	2
30	sIBM	9	5,5	6,5	6	6	0	1,5
31	sIBM	9,5	7	2,5	5	7,5	1,5	1,5
32	sIBM	5,5	15,5	3,5	2	2	2	0
33	sIBM	8	5,5	4,5	6	3,5	0	1,5
34	sIBM	0	14,5	2,5	1,5	0	0	0,5
35	sIBM	6	5,5	0	2,5	1,5	0	2
36	OPMD	0	0	1	0	1	0	0
37	OPMD	1,5	0	0	0	1	0	0
38	OPMD	1,5	0	0	0	0	0	0
39	OPMD	0	0	0	2	1,5	0	0,5

however, also in NM and in sIBM these three antibodies seem to have a different significance recognizing different patterns. In fact, whereas the percentage of LC3 and LAMP2 positive fibers is not statistically significant between different groups (Figs. 6, 7), the percentage of p62-positive fibers in muscle sections was significantly higher in sIBM group than in LOPD ($p < 0.001$ ANOVA with Bonferroni correction), OPMD ($p < 0.01$ ANOVA with Bonferroni correction) and in the control group ($p < 0.001$ ANOVA with Bonferroni correction) (Fig. 8); p62 positivity was also observed in muscle fibers showing normal histochemical features. ROC analysis of our data indicates a 100% specificity and 75% sensitivity of p62 staining for IBM.

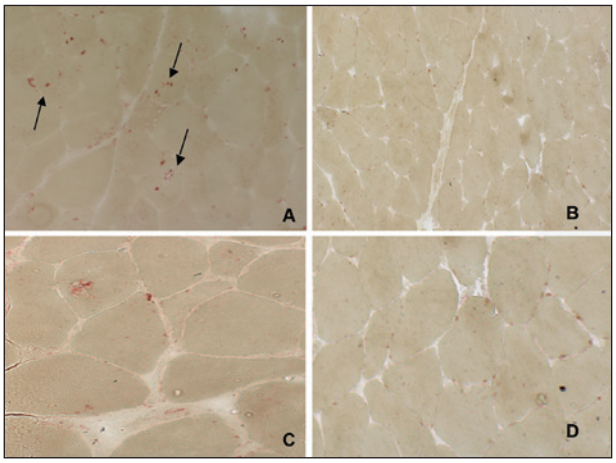

Figure 1. A representative case of acid phosphatase in LOPD (A), in NM (B), in sIBM (C),in OPMD (D) and in a control group (E). In the first three groups acid phosphate is present in focal regions in fibers (arrows).

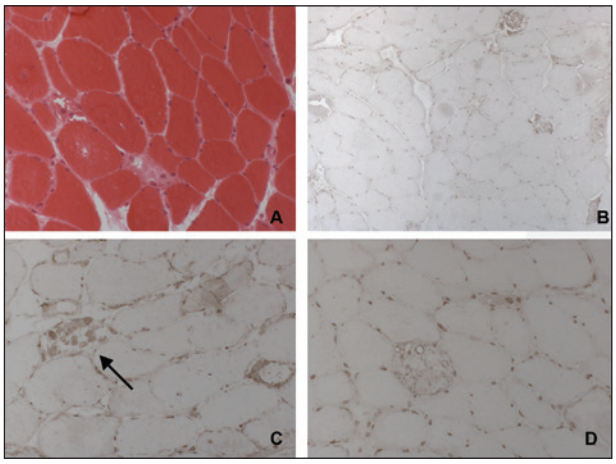

Figure 3. Necrotizing myopathy staining patterns. A representative case of necrotizing myopathy – (A-D) –; subject #25) shows vacuolated fibers on H&E 20X (A), mildly positive with p62 (B), LAMP2 (C) and LC3 (D).

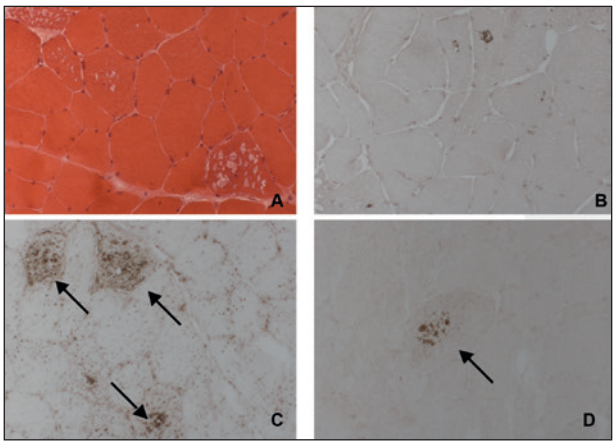

Figure 2. LOPD staining patterns. A representative case of LOPD (A-D; subject #15) shows several autophagic membrane-bound vacuoles seen on H&E 20X (A), LAMP2 (C) and LC3 (D), whereas only a few are p62 (B) positive.

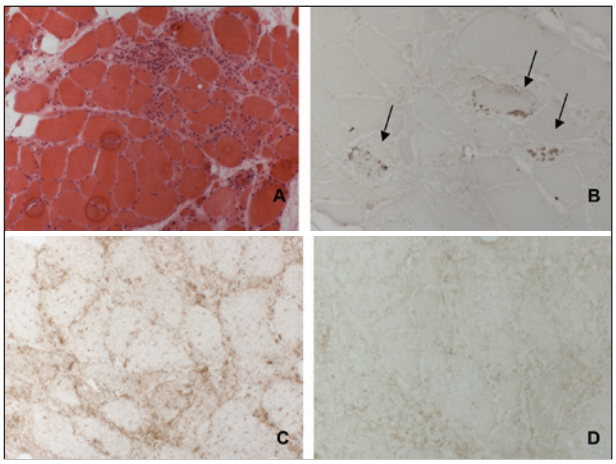

Figure 4. sIBM staining patterns. A representative case of sIBM – (A-D) –; subject #30) shows inflammatory cells and vacuolated muscle fibers seen on H&E 20X (A). There is a significant staining with p62 (B, arrows). Staining for LAMP2 and LC3 are negative.

Discussion

Differential diagnosis of vacuolar myopathies is usually achieved with the routine set of histological and histochemical staining on frozen muscle tissue; congruent clinical data are also necessary to distinguish among the great variety of myopathological entities. In some outlier cases, with only minor changes and with partial or incomplete clinical data, the ultrastructural exam may be necessary to reach a definitive diagnosis. However, this procedure is not diffusely available, is expensive and time-consuming.

In this study, we evaluated only by immunohistochemistry the potential adjunctive utility of p62, LC3 and LAMP2 in four groups of muscle disorders characterized by intrafibral vacuoles, and in a group of normal controls.

In LOPD, a lysosomal disease with defective autophagy, LC3, LAMP2 and p62 stainings were comparably positive with a punctate pattern, reflecting the association of LC3-II with the membranes of early autophagosomes, whereas p62 puncta correspond to the accumulation of protein aggregates within early autophagic (LC3-positive) vesicles; hence, the increased punctate staining seen with these markers corresponds to autophagosome

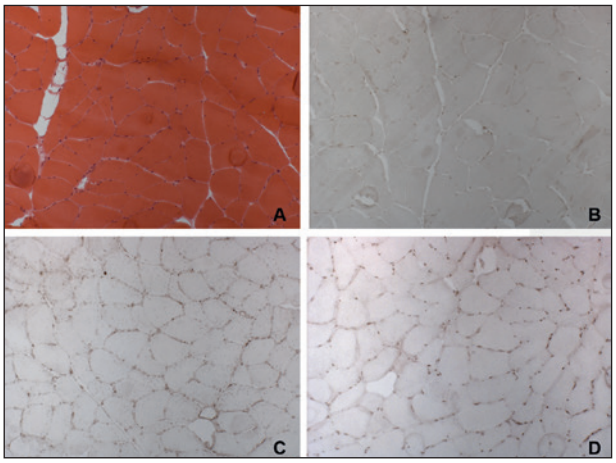

Figure 5. A representative case of normal control group – (A-D) –; subject #47). Lack of sarcoplasmic staining of p62, LAMP2 and LC3. There was background nuclear positivity wit all markers (B-D).

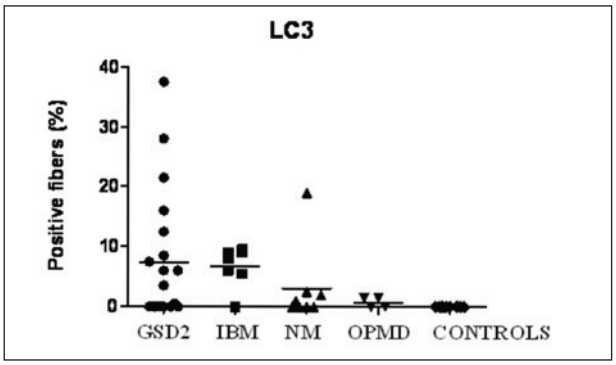

Figure 6. Quantification of LC3-positive fibers in frozen sections in different groups. The percentage of LC3-positive fibers was not statistically significant in LOPD, NM, sIBM, OPMD and in control group. Each study subject is represented with a symbol, lines indicate group means.

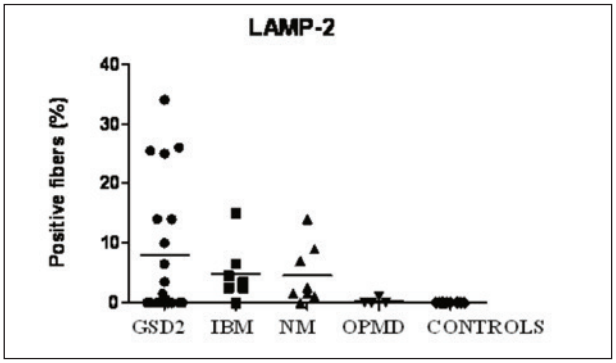

Figure 7. Quantification of LAMP2-positive fibers in frozen sections in different groups. The percentage of LAMP2-positive fibers was not statistically significant in LOPD, NM, sIBM, OPMD and in control group. Each study subject is represented with a symbol, lines indicate group means.

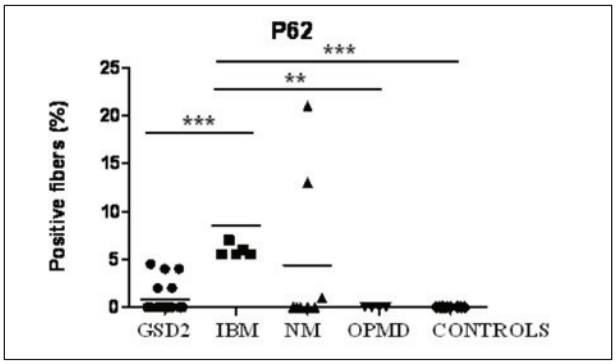

Figure 8. Quantification of P62-positive fibers in frozen sections in different groups. The percentage of p62-positive fibers was significantly higher in sIBM group than in LOPD ($p < 0.001$ ANOVA with Bonferroni correction), OPMD ($p < 0,01$ ANOVA with Bonferroni correction) and in the control group ($p < 0,001$ ANOVA with Bonferroni correction). p62 was marked positive only in two cases in NM group. Each study subject is represented with a symbol, lines indicate group means.

buildup (17). The observed minor incidence of p62 fibers in LOPD group may reflect the different stages of the autophagic process in these patients. In LOPD, LC3 seems to have the higher sensibility, in comparison to conventional stainings and the other markers. However, no LOPD case was detected by immunohistochemistry alone associated to a negative histochemical staining. In fact, in six out of 20 LOPD cases (30%), quadriceps muscle biopsy was totally normal, confirming the need to perform the biochemical test in all cases with a clinical suspect.

In most cases of clinically defined sIBM, intrafibral vacuoles show the typical 'red rim' staining with Gomori's trichrome and are easily recognized. However, non-rimmed vacuoles are also observed, and a possible activation of autophagic pathway is also hypothesized (18). Immunohistochemistry in sIBM biopsies is often characterized by aspecific and variable staining by a variety of antibodies utilized in the routinary muscle biopsy diagnostic study, making this procedure less significant in sIBM diagnosis. In all sIBM cases of our study, we found a significant positivity only for p62, with small positive puncta distributed throughout the sarcoplasm of a higher number of fibers in comparison to the other markers, supporting the hypothesis of a specific autophagic activation in these cases. (Fig. 4B, C and D). In LOPD cases, the puncta were larger and primarily (although not exclusively) located in the center of a reduced number of fibers. Several earlier studies have examined LC3, p62 or LAMP2 staining in the setting of IBM; however, no single work quantitatively compared all three markers on the same set of well-defined specimens (19). In our study p62, but not LC3 and LAMP2, effectively distinguished

the sIBM subject group from other vacuolar myopathy subject; moreover, p62 immunohistochemistry showed the best tradeoff between sensitivity and specificity for sIBM as a diagnostic test applied to an individual case. The p62 staining was qualitatively similar to LC3 staining, consistent with the idea that accumulation of either LC3-labeled autophagosomes or p62- positive aggregates are a marker of autophagic flux inhibition in sIBM.

In OPDM, also characterized by the presence of intrafibral vacuoles, the presence of autophagic activation was excluded in all cases; therefore immunohistochemistry may be useful in the differential diagnosis when clinical data are lacking or unsupportive, in particular in the presence of rimmed vacuoles.

Immunohistochemistry for autophagic markers did not add any additional information in necrotizing myopathies, since necrotic fibers showed a variable and unspecific staining with all antibodies. Interestingly, two patients presented a strong autophagic activation, thus challenging the diagnosis, and a specific follow up is ongoing.

Based on these findings, we can conclude that p62, LAMP2 and LC3 immunohistochemistry have a significant role in the routinary study of muscle when clinical data are not supportive, and could be included in the panel of antibodies when a vacuolar myopathy is observed with histochemical procedure. In particular, LC3 antibodies have a slightly higher specificity in LOPD biopsies, whereas a strong selective p62 positivity seem to be more indicative of sIBM. On the contrary, LAMP2 does not add important clues in differential diagnosis of these pathologies.

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