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Evaluation of aggrephagy markers in myofibrillar myopathies

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Abstract

Myofibrillar Myopathies (MFMs) are a growing group of muscular disorders genetically determined, whose diagnosis is based on histological features as myofibrillar degeneration, Z-disk disorganization and protein aggregates' accumulation. Protein aggregates that do not fit the proteasome's narrow pore are targeted for removal via a specialized form of autophagy, called aggrephagy. Our study aims to investigate the potential pathogenic role of aggrephagy in 52 muscle samples from an Italian MFM multicentric cohort. We measured, the percentage of positive areas of key aggrephagy proteins by immunofluorescence staining, of sequestosome 1 (p62/SQSTM1), Neighbor of BRCA1 Gene 1 (NBR1), and ubiquitinated proteins (FK2) in 11 *DES*-, 6 *DNAJB6*-, 5 *FLNC*-, 18 *MYOT*- and 12 *TTN*-mutated patients. We showed that all aggrephagy markers are increased in these patients, regardless of the mutated genes, suggesting a possible common pathomechanism; no positive signal was found in healthy, age-matched controls. We analyzed the association between positivity levels of these markers, measured as percentage of positive areas, and selected clinical features utilizing generalized linear mixed models with gamma distribution as the probability model and center-specific random effects to better capture possible heterogeneity across participating centers. Our findings indicate significant associations between levels of p62, NBR1, and FK2 with age at biopsy (p62 and NBR1 p-values < 0.001, FK2 p-value < 0.05), age of onset (p62 and NBR1 p-values < 0.001, FK2 p-value < 0.01) and disease severity through Walton & Gardner-Medwin (WGM) score at biopsy (all p-values < 0.001) and at the last visit (all p-values < 0.05). Noteworthy, the aggrephagic pathway is mostly activated in *MYOT*-mutated patients compared to the other subgroups. Moreover, the association between aggrephagy and WGM score at biopsy is stronger in this subgroup. Overall, our study emphasizes the role of aggrephagy in MFMs across all patients, and its association with specific clinical parameters.

Keywords Protein aggregation, Myofibrillar alterations, Clinical association, Genetic rare diseases

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Introduction

MFMs are an expanding group of rare and progressive muscular genetic disorders [1], histologically characterized by myofibrillar disorganization beginning at the Z-disk and eventually leading to focal modifications of myofibrils, clearly visible by electron microscopy analysis. Associated features are also the presence of cytoplasmic protein aggregates and autophagic vacuoles. Consequently, MFMs are part of the larger group of protein aggregate myopathies (PAMs) [2].

Most often MFMs present with distal or proximal lower limb weakness, leading to loss of ambulation in a minority of patients over the years. Besides, cardiac involvement is relatively common and can express as structural cardiomyopathy, conduction abnormalities, or a combination of these. The age of onset varies, mostly occurring in middle adulthood [3].

The inheritance pattern is mainly autosomal dominant, although autosomal recessive, X-linked, and digenic inheritance forms have also been identified [2, 4]. However, the causative gene is still unknown in more than 30% of the patients [3].

Currently, 17 genes are associated to MFMs, grouped based on the function of the encoded proteins. Particularly, some of them are important structural proteins that maintain the arrangement of sarcomeres, such as desmin, myotilin, filamin C and Z band alternative spliced PDZ-containing protein (encoded by the *DES*, *MYOT*, *FLNC* and *ZASP* genes). More recently, other structural proteins have been related to MFMs, such as titin, plectin, alpha-actin, lamin, four-and-a-half-LIM-protein-1 (*TTN*, *PLEC*, *ACTA1*, *LMNA* and *FHL1*), all interactors of other MFM's associated proteins, while another group (*CRYAB*, *BAG3*, *DNAJB6*, *HSPB8*) encode for proteins with chaperone functions [4, 5]. Additional proteins related to MFMs are Pyridine Nucleotide-Disulphide Oxidoreductase Domain 1 (*PYROXD1*), involved in the cellular redox regulation, kyphoscoliosis peptidase (*KY*), a muscular protease that interacts with filamin C, titin and lastly, p62 (*SQSTM1*) that causes a form of MFM when combined to mutations in the *TIA1* gene [6].

The aggrephagy is a specialized form of autophagy responsible for removal of protein aggregates that must be degraded and therefore ubiquitinated, called aggresomes. The presence of protein aggregates correlates with neurodegenerative pathologies, like Alzheimer's and Parkinson's diseases [5] and with muscular disorders in which autophagy is altered [7].

The proteins known to be involved in the aggrephagy pathway are p62, NBR1, Autophagy linked FYVE (Alfy/WDFY3), Histone deacetylase 6 (HDAC6), Toll-interacting protein (TOLLIP), Tax1 Binding Protein 1 (TAX1BP1), Optineurin and Chaperonin Containing TCP1 Subunit 2 (CCT2) some of which we investigated in our cohort. Specifically, p62 is the first described selective autophagy receptor with the ability to bind polyubiquitinated proteins and microtubule-associated protein 1 A/1B-light chain 3 (LC3), through its LC3-interacting region (LIR) and a C-terminal ubiquitin-associated (UBA) domain. NBR1 is a soluble selective autophagy receptor with the capability to bind to LC3 because of the presence of the same LIR domain. In muscle tissue, NBR1 is involved in the interaction with the serine/threonine kinase domain (TK), the gene expression regulator domain of titin [7]. TOLLIP enhances the removal of the aggregate of human huntingtin mutant with ubiquitin-binding CUE domain and LIR [8]. Optineurin is a 577 amino acids protein [9] found in the aggregates of various neurodegenerative diseases, known to bind ubiquitin and LC3 to coordinate autophagosome loading. Its function is similar to that one of p62, but the two proteins do not interact [10]. Indeed, optineurin acts as an autophagic receptor interacting with Atg8-related proteins. The optineurin's phosphorylation influences its interaction with LC3, showing a further level of regulation for autophagy receptors [11]. Hitherto, aggrephagy has been still poorly investigated in muscle disorders [7] and no data are available in MFMs.

Our study aims to investigate the involvement of aggrephagic pathway in muscle samples from a multicentric cohort of 52 MFM Italian patients and to correlate the immunofluorescence analysis with clinical and genetic features.

Materials and methods

The study involves seven neuromuscular referral centers in Italy: Fondazione IRCCS Istituto Neurologico Carlo Besta, Fondazione Policlinico Universitario Agostino Gemelli IRCCS, IRCCS Ospedale San Raffaele, ASST Spedali Civili di Brescia, Università degli Studi di Padova, Università degli Studi di Roma La Sapienza and Università degli Studi di Verona.

We investigated the association between positivity level of selected aggrephagy markers in the muscle tissue and molecular and clinical features of included patients. The average percentage of positive areas of p62, NBR1 and FK2 protein staining, was calculated from images obtained via fluorescence microscopy.

Each center provided muscle tissues of patients with histological and genetic diagnosis of myofibrillar myopathy, according to MFM diagnostic criteria [12]. Disease severity at the time of muscle biopsy and at last follow-up was assessed through the WGM score [13], used to evaluate functional activity on a point system that ranges from 0 to 10, with 0 representing normal activity and 10 representing bedridden activity. The Medical Research Council (MRC) scale for muscle strength [14] is a commonly used scale to assess muscle strength from grade 5

(normal) to grade 0 (no visible contraction). For the analysis, the following biopsied muscle were tested with MRC scale: gastrocnemius (2), peroneus (1), tibialis anterior (4), quadriceps (33), deltoid (5) and biceps brachii (5). Some data were incomplete: the MRC scale was missing for 12 patients, the WGM score at biopsy was missing for 6 patients and WGM score at last follow up was missing for 5 patients. The cohort consists of 52 patients, among which 11 (21.2%) mutated in *DES*, 6 (11.5%) in *DNAJB6*, 5 (9.6%) in *FLNC*, 18 (34.6%) in *MYOT*, and 12 (23.1%) in *TTN*. The subgroup of patients mutated in *MYOT* is more uniform since 16/18 (88.9%) patients carry the same mutation (S60F). All data are listed in the Supplementary Table 1.

Muscle biopsy

Skeletal muscle biopsies were obtained at each participating center, and frozen in liquid nitrogen-cooled isopentane. Histological staining for aggrephagy biomarkers was performed at the Fondazione IRCCS Istituto Neurologico Carlo Besta on 8 µm-thick cryo-sections.

Immunofluorescence

For immunostaining, muscle sections were blocked in 1% normal goat serum (Jackson ImmunoResearch), without fixation. Sections were sequentially incubated with primary, secondary fluorescent antibodies (Alexa, Thermo Fisher Scientific; 1:1500) and DAPI. Immunofluorescence was performed using primary antibodies to: p62 (RRID: AB_2687531, GP62-C, Progene; 1:100), NBR1 (RRID: AB_2149402, sc-130380, Santa Cruz Biotechnology; 1:100) poly-ubiquitinylated proteins (RRID: AB_10541840, BML-PW8810, clone FK2, Enzo Life Sciences; 1:100), TOLLIP (RRID: AB_2790586, PA5-83431, Thermo Fisher Scientific; 1:100), Optineurin, (RRID: AB_2156554, sc-166576, Santa Cruz Biotechnology; 1:100). Images were captured using Axioplan fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany) equipped with the Axiovision se64 rel 4.9.1 software.

Quantitation of aggregates in muscle tissue

The area percentage of the aggregates positive to p62, NBR1 and FK2 antibodies, indicating the positivity levels, was determined on muscle tissue sections 20x using the NIH ImageJ software v. 1.53t (http://rsb.info.nih.gov/nih-image/) as described previously [15].

Briefly, three randomly selected fields were photographed and digitized from each patient's section. Images, using the software, were inverted to produce black-and-white representations, where black indicated aggregates and white indicated negative signals. Manual corrections were applied to eliminate non-specific fluorescence. Positivity was quantified as the percentage of the total image area, and the mean percentage across the three fields was calculated for each patient.

Statistical analysis

Given the multicentric and hierarchical nature of our data (with patients clustered within centers), we utilized mixed models to appropriately account for the nested structure of our dataset. This approach allows for the consideration of variability both within and between centers, thereby providing a more robust analysis of the association between aggrephagy markers and clinical features in MFMs.

To analyze the association between the positivity levels of aggrephagy markers and clinical features, we employed generalized linear mixed models (GLMMs). Given that our outcome variable, the percentages of positive areas for aggrephagy markers, say y, can only take strictly positive values, we adopted a gamma distribution for our probability model. In detail, we assume that the probability density function of the gamma distribution is given by

$$f\left(y|\mu,\sigma\right)=\frac{y^{1/\sigma^2-1}e^{-y/\left(\sigma^2\mu\right)}}{\left(\sigma^2\mu\right)^{1/\sigma^2}\Gamma\left(1/\sigma^2\right)}$$

for $y>0$, where $\mu>0$ and $\sigma>0$. This parameterization is very useful in a regression context as $E(Y)=\mu$ and thus we can specific the GLMM as

$$\mu_{ic}=\beta_0+\beta_1 x_{i1}+\ldots+x_{iP}+b_c,$$
$$i=1,\ldots,52; c=1,\ldots,7$$

where μ_{ic} is the subject- and center-specific conditional mean and is a function of a set of P subject-specific independent variables, whose effects is given by the β = (β0, β1,... βP), and a Gaussian-distributed center-specific random effect $b_c\sim N(0,\sigma_b)$, capturing unobserved heterogeneity among centers. Parameter estimates were obtained via a maximum likelihood framework using standard statistical software capable of handling GLMMs. All statistical analyses were conducted using R software using the gamlss package [16]. In the estimation step, the random effects are treated as nuisance parameters and integrated out. Nevertheless, the integral does not have a closed form, and we turn to numerical quadrature techniques, namely Gaussian quadrature with seven quadrature points to approximate it.

The gamma distribution is suitable for modelling positive continuous data and is flexible in handling the skewness observed in our measurements. Alternative distributions are available, as discussed by Rigby and colleagues [17], but the gamma distribution was deemed most appropriate for our data.

To ensure the reliability of the estimated results, we performed residual analysis to check for all model assumptions.

The coefficients in the gamma mixed effects regression model quantify the relationship between the predictors and the mean of the response variable. A positive coefficient indicates that as the corresponding predictor increases, the expected value of the response variable also increases (multiplicatively as we are using a log link), while a negative coefficient suggests that an increase in the predictor leads to a decrease in the expected value of the response variable. Statistical significance is indicated as *p-value < 0.05, ** p-value < 0.01, ***p-value < 0.0001.

Results

We performed immunostaining analysis in 52 MFM genetically determined patients (female = 25, male = 27) with available muscle tissue and clinical data. The group's average age of onset was 45.7 ± 22.4 years (range 1–76) and age at biopsy was 53.4 ± 17.1 years (range 7–76). Notably, 2 patients had symptoms' presentation in the first decade of life, one mutated in *TTN* and one in *DNAJB6*. The age average at last visit was 59 ± 20.1 years (range 13–80). The average of WGM at muscle biopsy was 3.1 ± 1.5 years (range 1–8) and the average of WGM at last visit was 3.8 ± 1.6 years (range 1–8). In the entire cohort, the analyzed markers (p62, NBR1, FK2) showed an increased activation hinting that the mechanism of removal of protein aggregates through autophagy is a possible common phenomenon. Nevertheless, 12 (23%) samples showed no positivity for any of the markers investigated (p62, NBR1, FK2). The distribution of mutations among these 12 patients was one (8.3%) in *DES*, 2 (16.7%) in *FLNC*, 4 (33.3%) in *MYOT*, and 5 (41.7%) in *TTN* (Supplementary Table 1). The same markers tested on healthy, age-matched controls did not show any positive signal (data not shown).

We quantified the percentage of positive areas for key aggrephagy proteins p62, NBR1, and FK2 using immunofluorescence staining in 11 *DES*-, 6 *DNAJB6*-, 5 *FLNC*-, 18 *MYOT*- and 12 *TTN*-mutated patients (Fig. 1).

When comparing 18 *MYOT*-mutated patients with all other MFM patients, we observed that the former subgroup showed significantly greater staining positivity for all aggrephagy markers: p62 (p-value < 0.001), NBR1 (p-value < 0.001) and FK2 (p-value < 0.001) (Fig. 2A).

We then analyzed the association between positivity level of these aggrephagy proteins and clinical features. In the entire cohort, regardless of the specific gene or mutation, we found significant associations between the WGM score at muscle biopsy and at last visit with p62 (p-value < 0.001; p-value < 0.05), NBR1 (p-value < 0.001; p-value < 0.05), and FK2 (p-value < 0.001; p-value < 0.05). However, no significant associations were observed between the MRC scale of the biopsied muscle and the positive areas for p62, NBR1 or FK2 (all p-values > 0.05).

The duration of disease at muscle biopsy was significantly negatively associated with FK2 positivity (p-value < 0.05), but no association was found with NBR1 or p62 (both p-values > 0.05). Additionally, significant positive associations were observed between age at biopsy and age of onset with p62 (p-value < 0.001 for both), NBR1 (p-value < 0.001 for both), and FK2 (p-value < 0.05 and p-value < 0.01, respectively) (Fig. 2B).

At last, we performed sub-group analyses selecting those groups with a larger number of patients (*DES*, *MYOT*, *TTN*) (Table 1).

For *MYOT*-mutated patients, we found a significant positive association only when considering WGM score at biopsy and the tested markers: p62 (p-value < 0.01), NBR1 (p-value < 0.05) and FK2 (p-value < 0.01).

In *DES*-mutated patients, none of the considered parameters was significantly associated with p62 staining (all p-values > 0.05). However, for NBR1 staining, a significant negative association was found with disease duration before biopsy (p-value < 0.05) and with age at biopsy (p-value < 0.05) while for FK2 a significant negative association was detected with WGM at last visit (p-value < 0.01).

In *TTN*-mutated patients, FK2 was significantly positively associated with the increase of MRC scale at biopsy (p-value < 0.05), and NBR1 staining was positively associated with age at biopsy (p-value < 0.001). None of the considered parameters showed a significant association with p62.

Furthermore, we performed immunofluorescence staining for TOLLIP and optineurin to check their involvement in our MFM cohort. We did not include these markers in our statistical analysis, since only 18 (28% *DES*, 33% *MYOT*, 11% *FLNC*, 28% *TTN*) and 14 (21% *DES*, 43% *MYOT*, 7% *FLNC*, 29% *TTN*) patients showed positivity for TOLLIP and optineurin staining respectively. Furthermore, this positivity was often only localized in one or few fibers. Notably, the group in which more samples were positive for these antibodies is the one with *MYOT*-mutated patients. Representative results in Fig. 3.

Discussion

Our study investigates for the first time, the involvement of aggrephagy in MFMs, establishing clinical associations in a large cohort of patients. Aggrephagy is a mechanism whose involvement in muscle diseases has already been reported, yet not fully explored. As described by Askanas' group [18], inclusion body myositis (IBM) muscles have increased levels of p62, LC3, and NBR1 proteins. Bonaldo's group reported co-localization between FHL1 and p62 in Rigid spine syndrome [19]. Furthermore, Lin

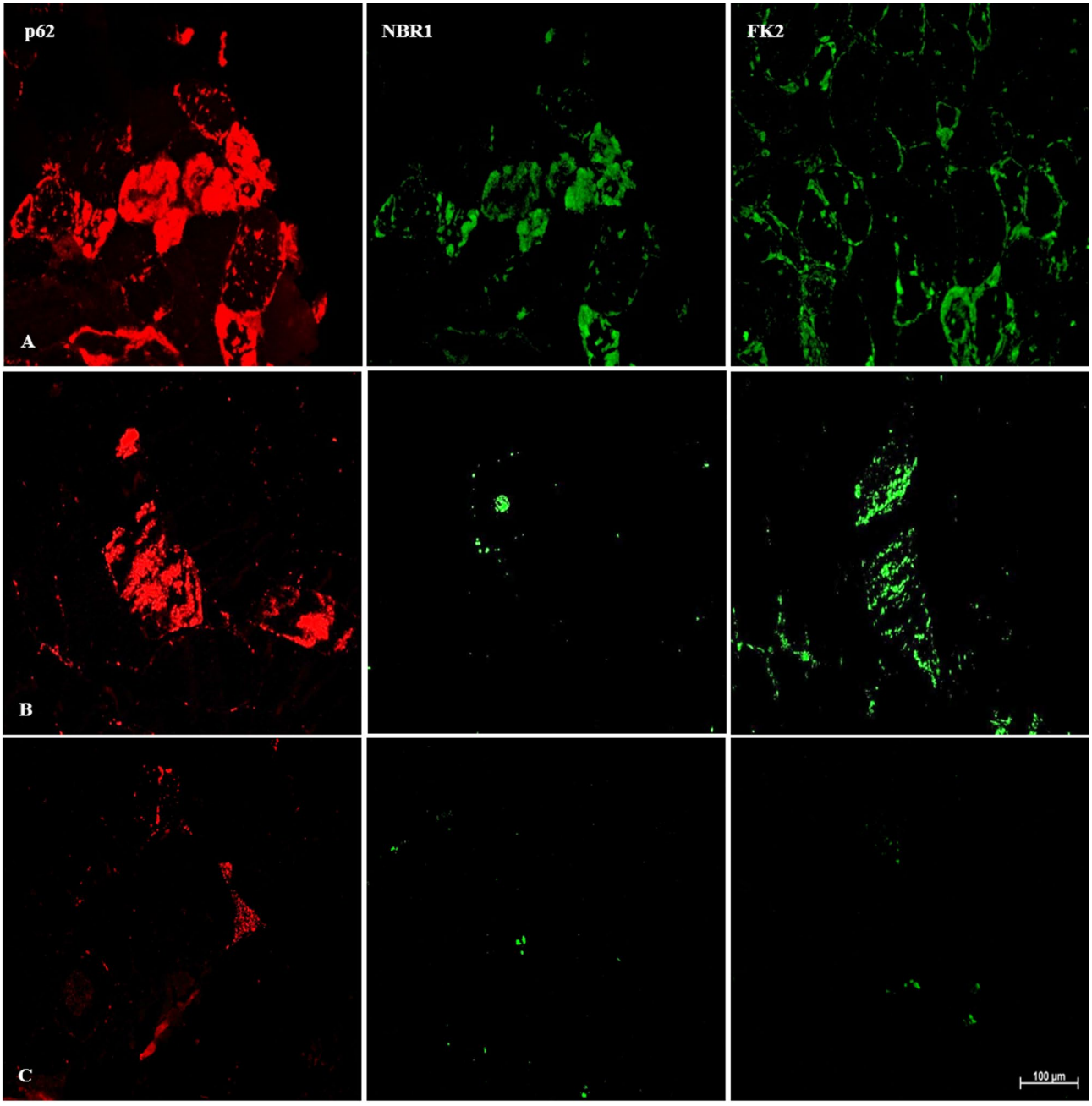

Fig. 1 Immunohistochemistry of aggrephagy markers showing affected fibers. **(A)***MYOT*-mutated patient (S60F) with larger area of signal, **(B)***FLNC*-mutated patient (W2710X) with intermediate area of signal, **(C)***TTN*-mutated patient (V26358Ffs*4/Q35879X) with lower signal's positivity

et al. investigated the accumulation of PAPBN1 and its co-localization with p62, NBR1, FK2, and LC3 in Oculopharyngeal Muscular Dystrophy [20]. Likewise, in *PLIN4*-related myopathy, co-localization of perilipin 4, FK2, p62, and NBR1 and an elevated WDFY3 signal indicate that the aggrephagy pathway has been activated [21]. However, clinical meaning of these findings in the muscle tissue in different myopathies has never been investigated.

Notably, our data showed a significant increased activation of the analyzed aggrephagy markers (p62, NBR1, FK2) in our MFM cohort, regardless of the mutated gene, suggesting that activation of aggrephagy is a shared phenomenon in these diseases and may represent a possible common pathomechanism. No comparable signal was evident when staining control tissues. However, 23% of the samples, among which 4 cases mutated in *MYOT*, showed no expression of aggrephagy biomarkers, hence aggrephagy activation is not a fixed process in MFM.

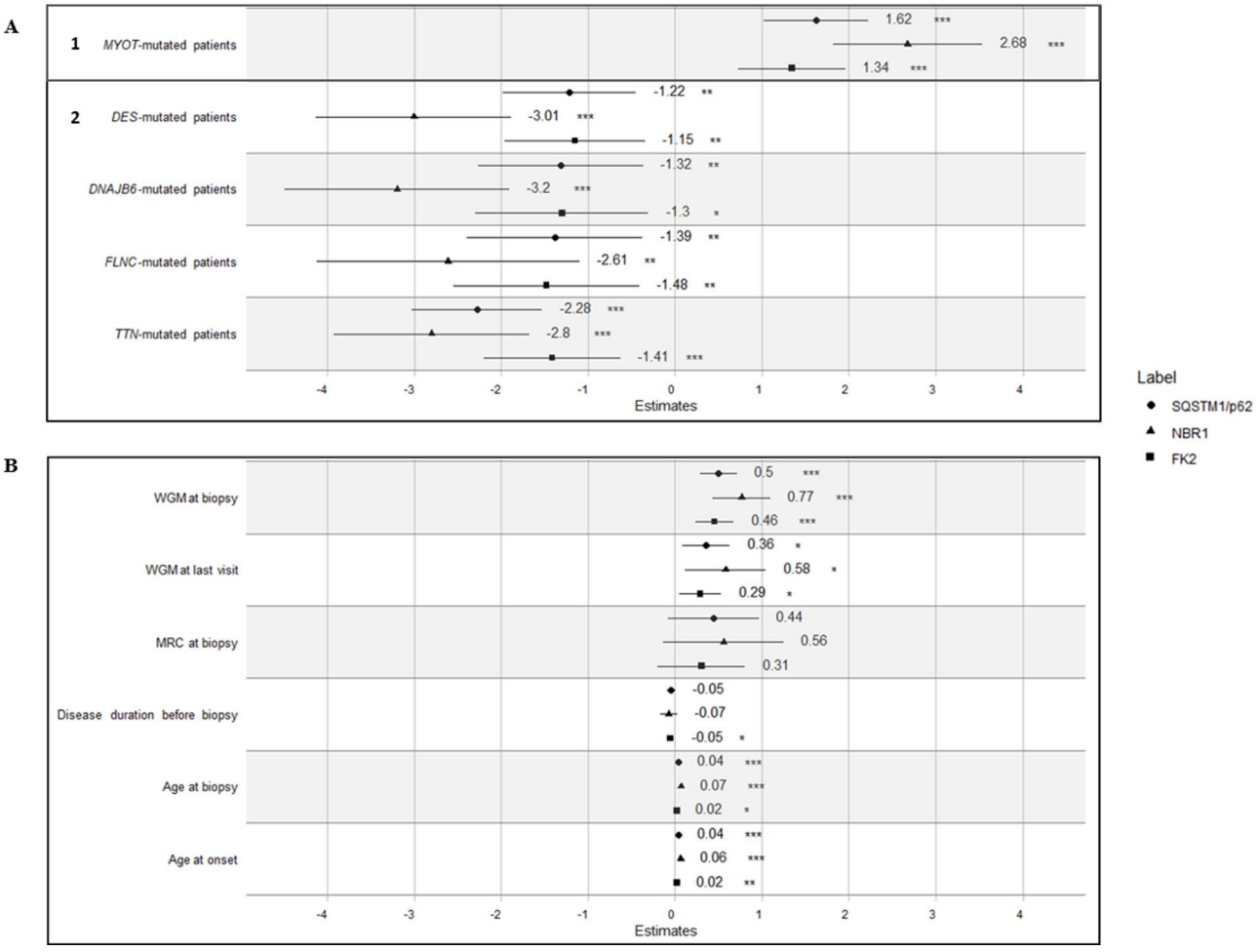

Fig. 2 Multiple plots representing association analysis between aggrephagy markers and clinical data, determined by generalized linear mixed models. (**A.1)** Comparison of the areas of positive staining for p62, NBR1 and FK2 antibodies in *MYOT*-mutated samples versus all the other patients. (**A.2)** Comparison of the positivity areas in *DES*-, *DNAJB6*-, *FLNC*-, *TTN*- mutated patients, each of them compared versus *MYOT*-mutated samples. (**B)** Estimate of the association between WGM at biopsy and at last visit, MRC at biopsy, disease duration before biopsy, age at biopsy and age of onset with the areas of positivity for the markers investigated, calculated in the entire cohort of patients

As expected, p62 shows a greater area of positivity among the entire cohort, underlying its critical role as a chaperone protein. Indeed, p62 binds the ubiquitinated-misfolded proteins, allowing recruitment of the autophagic machinery [22]. FK2 positivity is comparable to that of p62, while NBR1 positive area is less extended, most likely due to its subsequent involvement in this pathway. Despite NBR1 and p62 interacting and forming oligomers together, they can operate independently in the degradation of ubiquitinated proteins [10].

When considering the entire cohort, age at biopsy as well as age of onset are significantly associated with all the proteins investigated, which could directly relate to the progression of these disorders, suggesting a cumulative aggrephagic process. However, disease duration at muscle biopsy is negatively associated with FK2 marker, showing that the longer is the disease duration the smaller is the averaged areas of FK2 positivity. The rationale behind this association is not clear. Nevertheless, an explanation could be found in the fact that FK2 antibody identifies the ubiquitin bound to proteins targeted for degradation, hence may be involved in early stages of the aggrephagy pathway.

Furthermore, since we observed a close correlation between degree of aggrephagy and disease severity at muscle biopsy according to the WGM, we might consider all analyzed aggrephagy mediators as marker of disease severity when collecting the muscle sample; among them, NBR1 showed the strongest association. However, the role of aggrephagy as primary mediator of muscle fibers impairment or secondary phenome needs to be yet clarified.

Moreover, albeit to a lesser degree, also the WGM score at the last follow up visit is significantly associated with the aggrephagy markers in the whole cohort of patients, suggesting a possible clinical prognostic role, especially

Table 1 Table of association analysis for each subgroup (*MYOT*-, *DES*-, *TTN*- mutated patients)

Antibodies	Clinical data	Estimate (± SD) in MYOT-mutated patients	Estimate (± SD) in DES-mutated patients	Estimate (± SD) in TTN-mutated patients
p62	WGM at biopsy	0.790 (± 0.21)**	0.214 (± 0.12)	-0.371 (± 0.44)
	WGM at last visit	0.545 (± 0.25)	-0.131 (± 0.17)	0.046 (± 0.42)
	MRC at biopsy	-0.219 (± 0.76)	-0.061 (± 0.42)	0.129 (± 0.16)
	Disease duration before biopsy	-0.026 (± 0.07)	0.082 (± 0.04)	-0.014 (± 0.03)
	Age at biopsy	0.037 (± 0.05)	0.021 (± 0.02)	0.000 (± 0.03)
	Age at onset	0.029 (± 0.04)	0.006 (± 0.03)	0.050 (± 0.04)
NBR1	WGM at biopsy	0.808 (± 0.34)*	0.197 (± 0.12)	-0.293 (± 0.46)
	WGM at last visit	0.484 (± 0.37)	0.174 (± 0.25)	1.027 (± 0.46)
	MRC at biopsy	-0.095 (± 1.01)	-0.297 (± 0.45)	-0.770 (± 0.38)
	Disease duration before biopsy	0.017 (± 0.11)	-0.101 (± 0.03)*	0.137 (± 0.05)
	Age at biopsy	-0.000 (± 0.07)	-0.054 (± 0.02)*	0.081 (± 0.02)***
	Age at onset	-0.016 (± 0.06)	-0.054 (± 0.04)	0.066 (± 0.03)
FK2	WGM at biopsy	0.894 (± 0.23)**	-0.135 (± 0.12)	-0.393 (± 0.34)
	WGM at last visit	0.497 (± 0.27)	-0.451(± 0.12)**	-0.042 (± 0.37)
	MRC at biopsy	-0.399 (± 0.81)	-0.501 (± 0.31)	0.564 (± 0.10)*
	Disease duration before biopsy	-0.055 (± 0.07)	0.007 (± 0.04)	-0.028 (± 0.02)
	Age at biopsy	0.016 (± 0.05)	-0.012 (± 0.02)	-0.000 (± 0.02)
	Age at onset	0.029 (± 0.04)	-0.013 (± 0.03)	0.031 (± 0.03)

The table shows the evaluation of the subgroups using generalized linear mixed models to estimate association between immunofluorescence positive areas and the clinical parameters. To perform this analysis, we selected only those groups with a sufficient sample size for statistical analysis *(MYOT* 18 patients, *DES* 11 patients, and *TTN* 12 patients). Abbreviations: WGM = Walton & Gardner-Medwin; MRC = Medical Research Council; p62/SQSTM1 = Sequestosome 1; NBR1 = Neighbor of BRCA1 Gene 1

when considering NBR1 versus p62 and FK2. Indeed, with the increase of the WGM score, the NBR1 positivity area increments more than p62's and FK2's, indicating a stronger association between NBR1 positivity and the severity of the phenotype. Hence, NBR1 seems to be the aggrephagy biomarker better correlating with disease severity.

Lastly, activation of aggrephagy was detected regardless to the muscle biopsy site (Supplementary Table 1).

Interesting results were obtained from the analysis on the subgroups of *MYOT*-, *DES*-, *DNAJB6*-, *FLNC*- and *TTN*-mutated patients. Notably, activation of aggrephagy was particularly evident in the *MYOT* subgroup, with NBR1 as the most involved biomarker. Similarly, Selcen and colleagues [23], showed a higher fluorescence intensity of the congophilic aggregates in the muscle tissues of patients carrying *MYOT* mutations compared to the *DES*-mutated patients. Additionally, in a 2016 proteomic study, Maerkens et al. reported an enrichment of quality control and degradation proteins in the aggregates of *MYOT* patients [24]. Moreover, the Olive's group showed that Thioflavin T positive aggregates were visible only in myotilinopathy patients and not in *DES* mutated patients [25], highlighting, as we found, a difference between myotilinopathy and desminopathy patients. The stronger positivity found in *MYOT* samples could be related to the function of myotilin as cross-linker of actin filaments, and to its interactors alpha actinin and filamin c, critical structural proteins, that accumulate in aggregates [2].

Likewise, to the entire cohort, although to a lesser extent probably due to lower sample size, there is a significant positive association between WGM at muscle biopsy and all three aggrephagy markers in the *MYOT* group, highlighting that connection between aggrephagy and disease severity is particularly evident in this specific subgroup of MFM. Nevertheless, this correspondence needs further investigation with in vitro and in vivo models.

Aggrephagy activation hence is less pronounced in *DES* samples. According to previous studies, the formation of protein aggregates in *DES* patients is related to mutation-specific effects. It is reported that aggregates are more pronounced when mutations are located in the Rod domain [26], since promoting the aggregates accumulation and basal autophagy induction [27]. However, in our *DES* patients with mutations located in the Rod domain (4 out of 11 patients) we did not observe increase in markers' positivity, suggesting a great variability intra-*DES* subgroup which will require further investigation. Of note, in the *DES* group we observed a significant negative association between NBR1 and disease duration at biopsy and age at biopsy and FK2 and WGM at last follow-up. These results, in contrast with what observed on the entire cohort of patients, could indicate that the *DES* group is enriched in younger patients, or alternatively that NBR1 may be activated earlier in *DES* mutated patients, but this hypothesis remains to be clarified. No other association between disease severity at last visit

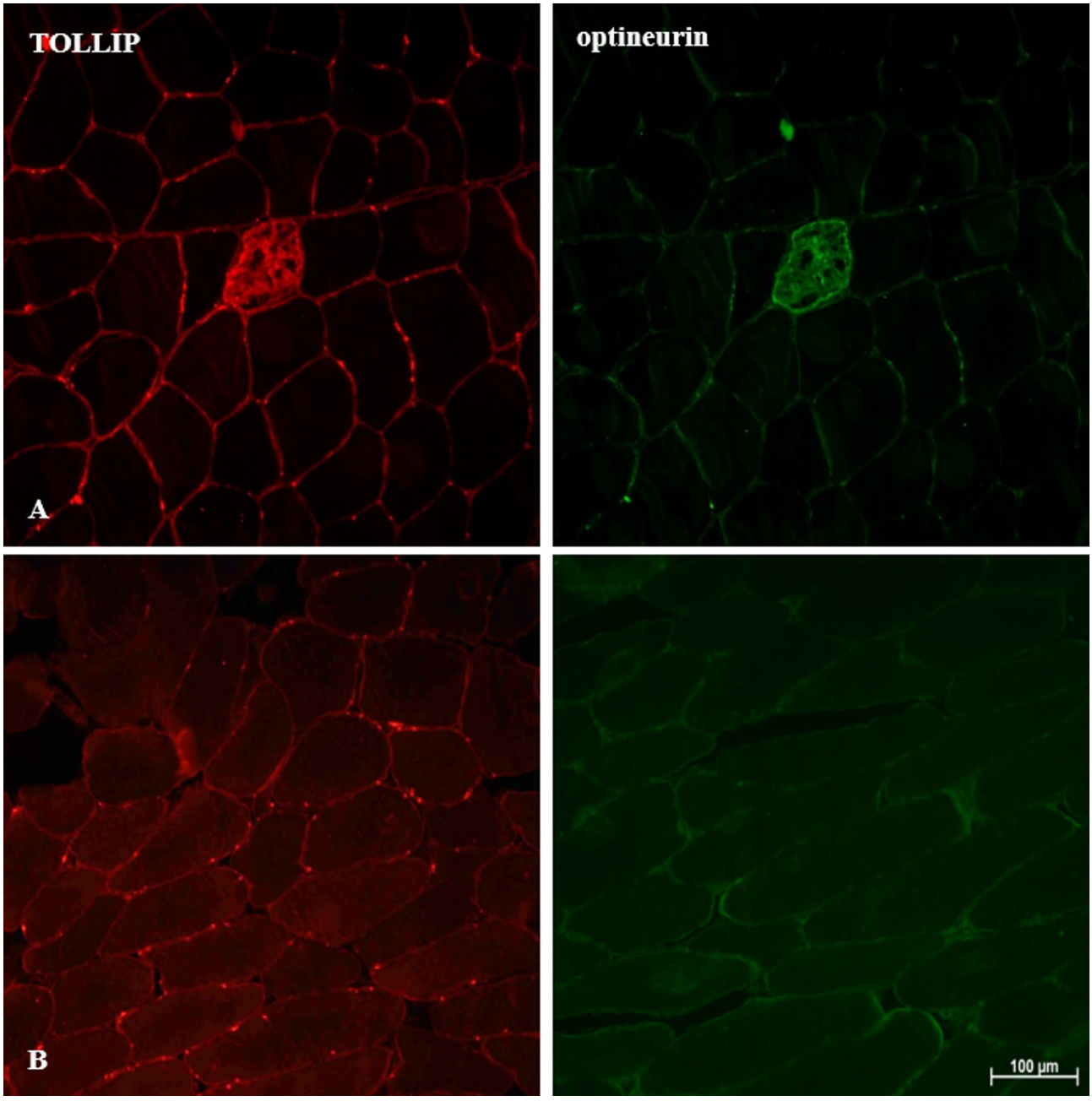

Fig. 3 Immunohistochemistry of TOLLIP and optineurin showing affected fibers. **(A)***MYOT*-mutated patient (S60F) and **(B)***TTN*-mutated patient (C31712R) with no positivity

and aggrephagy biomarkers was observed in specific MFM subgroups, whose small sample sizes may explain this observation.

Moreover, our analysis did not show any association between aggrephagy activation and the MRC score of the biopsied muscle, except for FK2 in the subgroup of *TTN* patients. This could be related to the variability of the biopsy site and the relatively poor validity of the MRC assessment when testing single muscle/movement weakness. In addition, MRC testing is usually focused on group of muscles that may be differently involved by MFMs.

Both TOLLIP and optineurin have been linked to aggrephagy, especially in the central nervous system, but their role in muscle disorder have not been clarified. We observed a less extended positivity with two third of the patients showing no detection of these proteins in the muscle, hinting a possible subsequent enrolment or only a marginal role in the pathomechanisms of MFMs, however, these hypotheses require further analyses.

The applied analysis in our study might be affected by its retrospective nature as well as by the lack of a few clinical data. Moreover, the statistical model based on averaged values might not accurately describe the non-homogeneous pattern within the muscle fibers which is a characteristic phenomenon in muscle disorders. Lastly, WGM as a tool to assess disease severity may be poorly sensitive, being also relatively unchanged between muscle biopsy and last visit and overall, substantially mild, although it was the best scale to retrospectively evaluate patients in our opinion.

Conclusions

In conclusion, our data show that aggrephagy biomarkers are highly detected in muscle tissue from MFM patients and correlate with disease severity at the time of the muscle biopsy, mainly in *MYOT* patients. Further studies are needed to better characterize their involvement in the pathogenesis and their role as therapeutic targets or disease biomarkers in MFMs.

Abbreviations

Alfy/WDFY3	Autophagy Linked FYVE
TOLLIP	Toll-interacting protein
CCT2	Chaperonin Containing TCP1 Subunit 2
HDAC6	Histone Deacetylase 6
KY	Kyphoscoliosis Peptidase
MFMs	Myofibrillar Myopathies
NBR1	Neighbor of BRCA1 Gene 1
NGS	Next Generation Sequencing
p62/SQSTM1	Sequestosome 1
PAMs	Protein Aggregate Myopathies
TAX1BP1	Tax1 Binding Protein 1
WGM	Walton & Gardner-Medwin
LIR	LC3-Interacting Region
UBA	Ubiquitin-Associated
MRC	Medical Research Council
GLMMs	Generalized Linear Mixed Models
LC3	Microtubule-Associated Protein 1 A/1B-Light Chain 3
TK	Threonine Kinase domain
IBM	Inclusion Body Myositis

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40478-025-02041-9.

Supplementary Material 1

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Author contributions

E.I., A.R. and S.G. conducted the experiments, analyzed the data and wrote the manuscript; A.M. conducted the statistical analysis and reviewed the manuscript; F.S. and S.V. prepared muscle samples and supported in collection data; M.C., S.B., P.R., G.M., L.B., M.G., M.F., S.C.P., G.T., G.V., P.T. and E.P. collected material and clinical data; A.C., L.NDG. and G.R. supported in collection and data analysis; A.R., S.G. and L.M. conceived and designed the experiments, L.M. collected clinical data and wrote the manuscript. All authors reviewed the manuscript.

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Data availability

All data is provided within the supplementary information files. Anonymized data not published within this article will be made available for scientific purposes by request from any qualified investigator.

Declarations

Ethics approval and consent to participate

This study was approved by the Institutional Review Board at the Fondazione IRCCS Istituto Neurologico Carlo Besta (Project identification code 84/2022) in compliance with the current version of the Declaration of Helsinki as well as all national legal and regulatory requirements. Due to the observational, retrospective nature of the study and since the study is based on already available anonymous data, a written informed consent procedure is not needed.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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