Original Research

Inducible Heat Shock Protein 70 Levels in Patients and the mdx Mouse Affirm Regulation during Skeletal Muscle Regeneration in Muscular Dystrophy

Gwenny Cosemans1,2, Caroline Merckx1, Jan L. De Blecker1,3, Boel De Paepe1,3,*

1Laboratory for Neuropathology, Department of Neurology, Ghent University, 9000 Ghent, Belgium
2Ghent-Fertility and Stem cell Team (G-FaST), Department of Human Structure and Repair, Ghent University, 9000 Ghent, Belgium
3Neuromuscular Reference Center, Ghent University Hospital, 9000 Ghent, Belgium
*Correspondence: boel.depaep@ugent.be (Boel De Paepe)

Abstract

Background: Stress-inducible heat shock protein 70 (HSP70) is both a protective chaperone involved in protein homeostasis and an immune regulator. In both capacities, HSP70 has been implicated in muscle disorders, yet with fragmented and differing results. In this study we aimed to compare results obtained in the mouse model for the severest form of muscular dystrophy (MD) equivalent to Duchenne MD, termed the mdx mouse, with results obtained in human MD. Methods: Skeletal muscle and serum samples were obtained from 11 healthy controls, 11 fully characterized patients diagnosed with Becker MD and limb girdle MD (LGMD), and six muscle disease controls. In addition, muscle extracts were prepared from tibialis anterior of mdx and control mice at ages 4, 8 and 12 weeks. The HSP70 levels were quantified using RT-PCR, western blotting and protein arrays, and localized in muscle tissue sections using double immunofluorescence. Results: We found selective and significant 2.2-fold upregulation of HSP70 protein in mdx tibialis muscle at the earliest disease phase only. In LGMD and Becker MD patients, HSP70 protein levels were not significantly different from those of healthy muscle and serum. HSP70 was localized to regenerating muscle fibers both in mouse and human MD skeletal muscle tissues. Toll-like receptor (TLR) 2 and TLR4 expression was moderately increased on the sarcolemma in MD muscle, yet protein levels were not significantly different from normal controls. Conclusions: HSP70 upregulation in MD appears disease stage-dependent, marking the phase of most active muscle regeneration in the mdx mouse. We postulate that well-timed supportive therapeutic interventions with HSP70 agonists could potentially improve muscle tissue’s regenerative capacities in MD, attenuating loss of muscle mass while we await gene therapies to become more widely available.

Keywords: Becker muscular dystrophy; Duchenne muscular dystrophy; heat shock protein 70; limb girdle muscular dystrophy; muscle regeneration

1. Introduction

Chaperones escort other macromolecular structures whilst the latter acquire their proper conformational structure, through processes of (un)folding and (dis)assembly. Molecular chaperones termed heat shock proteins (HSPs) can be induced by heat stress and are grouped according to molecular weight into different families [1]. The heat shock proteins of 70kd (HSPs70) are structurally conserved proteins composed of an N-terminal ATPase domain, a peptide-binding domain and a C-terminal “lid” domain that can capture substrates at their binding site [2]. In humans, the HSP70 lie encoded within multiple genes and pseudogenes, producing high numbers of mRNA variants and isoforms [3]. Particular HSP70 may have preferential client proteins and/or co-chaperones allowing them to perform, in addition to their recognized redundant functions, also more unique tasks. The seven main isoforms are termed HSP70-1A, HSP70-1B, HSP70-1T, HSP70-2, HSP70-6, HSC70 and HSP70-14. Part of these display transiently increased expression following cellular insults [4]. The HSPA1A gene produces the most abundant stress-induced isoform, which will be referred to as HSP70 in this study.

The functionality of HSP70 is highly dependent on its localization. Intracellularly, HSP70 is involved in aiding de novo protein synthesis and protein restorative actions in response to cellular damage. HSP70 interacts with its client polypeptides in a process which is stabilized by complex joint interventions with co-chaperones belonging to the DnaJ family, and specific allosteric regulators such as the C-terminus-HSP70-Interacting Protein (CHIP). In addition, HSP70 activity is further fine-tuned through intracellular re-localization [5] and post-transcriptional modifications [6]. Secreted HSP70 on the other hand has a regulatory role through activation of different signalling pathways. Its immunoregulatory role known as ‘chaperokine’ activity is mediated via its interaction with innate immune receptors. HSP70 released into the extracellular milieu activates Toll-like receptors (TLRs) on antigen-presenting cells, facilitating antigen cross-presentation. Downstream effects include upregulation of adhesion molecules, co-stimulatory
molecule expression, and cytokine and chemokine release [7] achieved through nuclear factor κB (NFκB)-mediated pathways. A protective effect of HSP70 against apoptosis has also been established. HSP70 can directly associate with apoptotic protease activating factor 1 (APAF-1), preventing recruitment of caspases to the apoptosome complex and blocking the latter’s functional assembly [8,9].

Chaperones are abundant in skeletal muscle, which is unsurprising as the tissue is highly metabolically active and susceptible to contraction-induced and other damage, which further increases in both acquired and hereditary muscle disorders [10,11]. Such general upregulation of chaperones in diseased muscle illustrates the tissue’s attempt to restore and regenerate. However, individual studies reported varied regulation and systematic evaluations are few, clouding our understanding of the beneficial potential of these self-protective factors. To elucidate the role of chaperones further, this study zoomed in on the muscular dystrophies (MD). They represent a diverse group of hereditary muscle disorders, with a broad spectrum of severity and common as well as more specific disease features. Duchenne MD is the most frequent and severest form and is, along with milder Becker MD, caused by mutations in the gene encoding the muscle membrane protein dystrophin. Another large subgroup encompasses limb girdle MD (LGMD) characterized by proximal weakness affecting the pelvic and shoulder girdles and caused by more than 30 different genetic defects of both autosomal dominant and recessive inheritance [12].

HSP70 displays a broad spectrum of activity, yet is presumed to display mainly positive effects on muscle fiber recuperation from damage, which makes it a potential target for therapy. Our study aimed to further elucidate its potential role in successful muscle fiber regeneration by investigating the mdx mouse, the most widely used animal model for Duchenne MD. In the time frame between 4 and 12 weeks of age, mdx skeletal muscles experience severe bouts of muscle fiber degeneration and regeneration, yet subsequently evolve to stable disease [13,14]. The mdx model thus displays a milder skeletal muscle phenotype and near normal life expectancy, accomplishing more successful skeletal muscle tissue recuperation than human Duchenne MD. In this study, we confront findings in the earliest phase of disease of mdx mice with results of quantification and localization of HSP70 in patients with genetically characterized Becker MD and LGMD due to ANO5 variants. We observed disease stage- and regeneration-dependent HSP70 expression patterns.

2. Materials and Methods

2.1 Patient Material

This retrospective study included muscle biopsies and sera from 27 individuals: 11 normal controls, 10 fully characterized MD patients, and six muscle disease controls (Table 1). Muscle biopsies from seven individuals without evidence of muscle disease were obtained from the Ghent University Hospital’s files (NC1-7). Sera from healthy controls (NC8-11) were commercially obtained (Zenbio, Durham, NC, USA). Muscle tissues concerned leftovers of diagnostic open muscle biopsies. For western blot analysis, tissues were snap frozen. For histology, muscle was first mounted and frozen in cooled isopentane, transferred to liquid nitrogen and stored at –80 °C.

2.2 Animal Model

Mice were Black 10 genetic background mdx C57BL/10ScSn-Dmdmdx/J and C57BL/10SnJ controls purchased from Jackson Laboratories (Bar Harbor, ME, USA), kept with free access to food and water ad libitum. Muscle tissues were collected from male mice at ages 4, 8 and 12 weeks. Eight mice were sacrificed per age group and left and right m. tibialis anterior were dissected from the hind limbs. For western blot analysis, the tissue samples were snap frozen in liquid nitrogen and stored immediately at –80 °C. For histology, muscle was first mounted and frozen in cooled isopentane, transferred to liquid nitrogen and stored at –80 °C.

2.3 Quantitative RT-PCR

Total RNA was prepared from tibialis anterior using the RNeasy Mini kit following the manufacturer’s instructions (Qiagen, Hilden, Germany). RNA concentration was measured with a Nanodrop 1000 (ThermoFisher Scientific, Waltham, MA, USA), and cDNA was prepared from 200 ng of RNA with SuperScript II Reverse transcriptase in the presence of 500 µg/mL oligo dTs, 0.1 M DTT, and 10 mM dNTPs (ThermoFisher Scientific). PCR reactions were run in triplicates on a 7500 Real Time PCR system in Taqman Gene Expression Mastermix (Applied Biosystems, Foster City, CA, USA) using 6-carboxy-fluorescein (FAM)-labeled probes and specific primers for HSPA1A (Mm01159846_s1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Mm99999915_g1). Using Quantstudio software, results were presented as 2-CT relative to GAPDH mRNA expression and statistical analysis was done using the mixed model approach.

2.4 Protein Profiler Arrays

Serum from five MD patients (BM1-3, LG1-2), six disease controls (MT1-3, IM1-3) and four healthy controls (NC8-11) was typed on human apoptosis protein profiler arrays according to the manufacturer’s specifications (R&D Systems - Bio-Techne, Minneapolis, MN, USA). Protein spots were visualized with the Chemidoc, spot volumes were analyzed with Image Lab 6.0 software via the linear quantity regression method with local background subtraction (Bio-rad, Hercules, CA, USA). Protein densities relative to reference spots were calculated as mean ± sd per diagnostic group. T-test for normally distributed independent variables determined p-values.
Table 1. Patient information.

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Abbreviations: creatine kinase in U/L (CK), female (F), male (M), muscular dystrophy (MD). Age is given in years; age of onset is defined as the age of appearance of first symptoms, for patient LG6 this was limited to asymptomatic CK elevation. The normal range of CK values is below 180 U/L for females and below 200 U/L for males. ANOS5 mutations cause limb girdle MD type R12.

2.5 Western Blotting

Muscle tissues were grinded in extraction buffer (50 mM tris buffer, 5 mM ethylenediamine tetraacetic acid pH 7.4 with complete protease inhibitor (Merck, Overijse, Belgium), and centrifuged 15 minutes 13,000 rpm at 4°C. The supernatant was harvested and protein concentration was estimated with the Biodrop device (Biochrom, Waterbeach, UK), measuring the UV absorbance at 280 nm. Western blotting was carried out with the NuPAGE system (ThermoFisher Scientific) on 10% bistris gels and nitrocellulose coated membranes. For mouse samples, primary antibodies were either 1 µg/mL polyclonal rabbit anti-HSP70 (ThermoFisher Scientific) or 0.15 µg/mL anti-HSP90 (Sigma Aldrich, St Louis, MI, USA), with 0.2 µg/mL polyclonal rabbit anti-GAPDH (Abcam, Trumpington, UK) simultaneously. Blots were incubated with 0.2 µg/mL peroxidase-labeled goat anti-rabbit (Abcam), stained with clarity substrate, and imaged with a chemidoc. For human samples, blots were incubated with 1 µg/mL IgG1 monoclonal mouse anti-HSP70 (ThermoFisher Scientific) and 0.5 µg/mL mouse monoclonal anti-GAPDH (LS-Bio, Seattle, WA, USA) simultaneously, incubated with peroxidase-labeled donkey anti-mouse (Abcam) stained with clarity substrate, and imaged with a chemidoc. Due to the sample size restrictions, quantification for patient LG7 was done on an earlier blot first incubated with 1 µg/mL IgG1 monoclonal mouse anti-HSP70 (ThermoFisher Scientific) imaged as mentioned above, subsequently re-blocked, incubated with 0.2 µg/mL rabbit polyclonal anti-GAPDH (Abcam) and stained with the anti-rabbit colorimetric western breeze system (ThermoFisher Scientific). The second detection was done with 2 µg/mL polyclonal rabbit anti-TLR2 (ABClonal, Woburn, MA, USA) processed and imaged with a chemidoc, and subsequently re-blocked and incubated with 0.5 µg/mL monoclonal mouse anti-GAPDH and stained with the anti-mouse western breeze colorimetric system (ThermoFisher Scientific). A third detection was done with 3 µg/mL mouse monoclonal IgG2b,k anti-TLR4 (LSBio) and 0.5 µg/mL mouse monoclonal anti-GAPDH (LS-Bio) simultaneously, and stained with the anti-mouse.
colorimetric western breeze system (ThermoFisher Scientific). Blots were imaged with the Chemidoc Imaging System (Bio-Rad Laboratories, Hercules, CA, USA) with automatic exposure times excluding non-quantifiable saturated pixels. Densities of protein bands were determined with Genetools software (Syngene, Bangalore, India). Relative raw volumes were measured for each track and expressed relative to GAPDH as loading control. The data obtained was normally distributed according to the Shapiro-Wilk test. Group comparisons were assessed by a two-sided unpaired t-test with statistical significance defined by p-values below 0.05.

2.6 Immunofluorescent Staining

Eight µm muscle sections were prepared on poly-L-lysine coated slides and acetone fixed for 2 minutes. Mouse tibialis tissues from four animals from each age and diagnostic group were double stained with 4 µg/mL polyclonal rabbit anti-HSP70 (ThermoFisher Scientific), and either 5 µg/mL goat polyclonal anti-CD56 (R&D), 2.5 µg/mL rabbit polyclonal anti-dystrophin (Abcam) or 5 µg/mL polyclonal rat anti-F4/80 (Abcam). Human quadriceps muscle sections were prepared from seven individuals without muscle pathology (NC1-7) and from seven patients with LGMD (LG1-7), and stained with 7.3 µg/mL rabbit polyclonal anti-HSP70 (ThermoFisher Scientific) combined with 1 µg/mL mouse monoclonal IgG1 anti-CD56 (Sanbio, Uden, The Netherlands) or 0.5 µg/mL mouse monoclonal IgG1 anti-dystrophin (DYs2, Leica Biosystems, Machelen, Belgium), and 20 µg/mL rabbit polyclonal anti-TLR2 (ABClonal) combined with 2 µg/mL mouse monoclonal IgG2a,α anti-TLR4 (LS-Bio). Sections were stained with appropriate CY3-labeled (ThermoFisher Scientific) and AlexaFluor488-labeled (Jackson Laboratories) secondary antibodies, fluoromounted (Sanbio) and visualized under a fluorescence microscope.

3. Results

3.1 Muscle HSP70 Protein Levels were Significantly Elevated in the Youngest mdx, with Strong Expression in Regenerating Muscle Fibers

HSP70 protein levels were determined through quantitative western blotting (for original blots and quantification, consult Supplementary Fig. 1 and Supplementary Table 1) and were found significantly elevated in tibialis anterior of 4-week-old mdx compared to control animals of the same age (p = 0.005), showing a 2.2-fold upregulation (Fig. 1A,B). In extracts from 8-week-old animals, substantial variation of HSP70 levels between individual mdx mice was noted. At 12 weeks of age, HSP70 levels were notably lower in controls and mdx alike. At those later ages, relative expression levels of HSP70 were not different between control animals and mdx mice. In comparison, relative HSP90 expression levels were not significantly increased in mdx compared to control mice of any age (Fig. 1B). Localization studies found HSP70 to be expressed mostly in small fibers, which included regenerating fibers identified as CD56 positive (Fig. 1C). From 4 weeks onward, increasing infiltration of inflammatory cells in tibialis tissue was noted. Part of the macrophages were HSP70 positive, mostly those present in larger collections of inflammatory cells. Many necrotic fibers invaded by macrophages could be observed, which contained high levels of HSP70 (Fig. 1C) and increased in number with advancing age. In comparison, low basal levels of HSP70 were present in tibialis sections from normal control mice (Supplementary Fig. 2). Quantitative PCR for hspal mRNA levels did not detect significant changes in expression levels at 4, 8 and 12 weeks of age compared to control mice (Supplementary Table 2).

3.2 In Human LGMD Tissues, HSP70 Localized to Regenerating and Necrotic Muscle Fibers

Muscle extracts from LGMD patients with ANOS variants displayed HSP70 and TLR2 protein levels similar to levels in healthy controls. TLR4 tended to be elevated, yet not significantly altered compared to levels in normal controls (p = 0.09) (Supplementary Table 3, Fig. 2A). Weak TLR4 staining could be observed in normal muscle sections, while TLR2 staining was absent (data not shown). In LGMD tissues, TLR4 was observed on the sarcolemma of muscle fibers with normal width often appearing in groups, alongside more infrequent and discontinuous TLR2 staining (Fig. 2B). Low homogeneous levels of HSP70 were observed in the muscle fibers of healthy subjects (Supplementary Fig. 2). In LGMD muscle tissues, strong HSP70 expression was present in small fibers, of which part were identified as CD56 positive regenerating muscle fibers (Fig. 2B). Dark field light microscopy confirmed that the positive structures were muscle fibers (data not shown). In these small muscle fibers, HSP70 expression often co-localized with sarcoplasmic expression of major histocompatibility complex-I (MHC-I) (Fig. 2B).

3.3 Serum HSP70 Protein Levels Were Unchanged in MD Patients Compared to Controls

Levels of circulating HSP70 were not different in sera from MD patients compared to healthy controls, nor compared to disease controls consisting of sera from patients with mitochondrial and inflammatory myopathy. To test our experimental setup, we selected two proteins in the array that were most prominently up- and downregulated in patients. We found densities of pro-caspase 3 significantly increased in sera from MD patients (p = 0.04) compared to levels in healthy controls. Catalase levels were significantly reduced in MD (p = 0.007), mitochondrial myopathy (p = 0.007) and inflammatory myopathy (p = 0.007), compared to levels in healthy controls (Supplementary Table 4, Fig. 2C).
Fig. 1. Association of heat shock protein 70 levels with muscle fiber regeneration in mouse tibialis anterior. (A) Cropped western blots showing more prominent protein bands for inducible heat shock protein 70 (HSP70) in eight mdx mouse compared to eight Black 10 control mice at age 4 weeks. Simultaneous detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) allowed correction for protein loading. Full blots and data can be consulted as Supplementary Fig. 1 and Supplementary Table 1. (B) Comparison of HSP70 and HSP90 protein levels in Black 10 (light gray) and mdx (dark gray) of different ages. Mean values of eight mice per group (full data available in Supplementary Table 1) were calculated as percentages of control value ± sd. The HSP70 protein level was significantly increased in 4-week-old mdx compared to age-matched controls (*p = 0.005). (C) Double fluorescent staining in muscle from 4-week-old mdx mouse MDX4-2 shows colocalization of HSP70 (CY3, red) with CD56 (AlexaFluor488, green). AHSP70-positive (CY3, red) necrotic fiber in muscle from mdx mouse MDX4-5 is invaded by macrophages identified with anti-F4-80 antibody (AlexaFluor488, green). Localization to muscle fibers was morphologically checked by imaging the field with dark field light microscopy, as illustrated in the Supplementary Fig. 1. Scale bar = 100 µm.

Fig. 2. Association of heat shock protein 70 expression with immune factors in human muscular dystrophies. (A) Inducible heat shock protein 70 (HSP70) and Toll-like receptor (TLR) 2 and 4 protein levels were unchanged in limb girdle muscular dystrophy (LG) patients (n = 5) compared to normal controls (NC) (n = 4), as quantified relative to glyceraldehyde-3-phosphate dehydrogenase protein levels. For full blots and data, consult Supplementary Fig. 3 and Supplementary Table 3. (B) Double immunofluorescent staining in quadriceps of patient LG6. A small fiber stains for HSP70 (CY3, red) and major histocompatibility complex I (AlexaFluor488, green). Infrequent discontinuous TLR2 staining (CY3, red) (arrowhead), coinciding with more pronounced TLR4 staining (AlexaFluor488, green) (arrowhead). Localization to muscle fibers was morphologically checked by imaging the field with dark field light microscopy, as illustrated in the Supplementary Fig. 1. Scale bar = 100 µm. (C) Levels of soluble HSP70 (sHSP70) measured in sera from normal controls (NC) (n = 4), muscular dystrophy (MD) (n = 5), mitochondrial myopathy (MT) (n = 3) and inflammatory myopathy (IM) (n = 3) were not significantly different (p > 0.05). Array images and data can be consulted as Supplementary Fig. 4 and Supplementary Table 4.
4. Discussion

HSP70 dysfunction has been firmly associated with reduced capacity of muscle to recover from damage [15]. However, there is little consensus on the importance of HSP70 in disease progression of MD, due to reported age- and individual patient-related differences. Interestingly, dominant LGMD type 1 (LGMD1D) is caused by mutations in the HSP70 co-chaperone gene DNAJB6 and is characterized by protein aggregation and vacuole formation, illustrating the potency of HSP70 to prevent proteinopathy [16]. Earlier, we reported elevated levels of both HSP70 and HSP90 in two 9-year-old patients diagnosed with Duchenne MD, the severest form of MD [10]. In the current study, we analysed muscle from patients with milder Becker MD, and LGMD due to ANO5 defects, and found HSP70 levels to be unchanged compared to normal controls. We confirmed earlier reports of low TLR expression in muscle biopsies of healthy individuals, with TLR4 the most abundant [17], and found their levels did not significantly increase in LGMD. In the mdx mouse model for Duchenne MD, we could show a transient upregulation of HSP70 specifically during the earliest phase of MD at 4 weeks of age. Increased protein levels were not accompanied by increased hspala expression, suggesting regulation downstream from transcription. Similarly, hspala mRNA levels have been reported unchanged during proliferation and differentiation of C2C12 cells in culture, while protein levels increased 2-fold at day 2 of differentiation [18]. Such increased protein translation unparallelled by increased gene transcription is quite common in stimuli-induced expression [19]. From murine models, the picture is emerging of subtle and transient HSP70 induction in young mice, which vary between studies and disease stages [20,21]. In addition, HSP70 muscle levels have been proposed a valuable mdx disease severity marker, decreasing with therapeutic response [22]. The elevated HSP70 levels in mdx of the youngest age we reported here, later returning to levels of healthy controls, possibly mark the most active phase of muscle fiber regeneration and degeneration. Such small window of HSP70 upregulation in mdx fits with in vitro studies that determined HSP70 levels increased when cells differentiate from myoblast to myotube [18], a process impaired when cells were treated with HSP70 siRNA [23] further illustrating the positive role HSP70 plays in myogenic differentiation and regeneration.

In addition to intracellular protein salvage activities, extracellular HSP70 may have immune modulatory effects which could be relevant to the inflammatory components of MD [24]. A large biomarker study identified HSP70 among the proteins significantly elevated in Duchenne MD sera [25]. Our study found unchanged HSP70 levels in Becker MD and LGMD sera. It is proposed that circulating HSP70 may activate immune cell responses via TLRs on antigen-presenting cells [15]. Circulating HSP70 levels correlated with markers of inflammation in neurological disease [26], and TLR activation has been implicated in MD-associated muscle damage [27]. Muscle is a possible source of circulating HSP70, as challenged muscle cells may induce chaperone expression with regeneration and in response to pro-inflammatory cytokines, releasing HSP70 when they become injured. In addition, muscle cells represent facultative antigen presenting cells through induction of MHC-I (and II). While HSP70 levels were unchanged, we did observe prominent regulation of two proteins involved in apoptotic processes. Fitting with an earlier study in Duchenne MD and FSHD [28], pro-caspase 3 was elevated while the levels of the protective factor catalase were significantly decreased, pointing to nonspecific apoptosis activation in muscle disease.

While we await proliferation of genetic therapies aimed to restore the underlying molecular defect, current treatment available to MD patients mostly aims to alleviate symptoms. Since genetic approaches will not be able to cure all patients in the foreseeable future, further development of alternative supportive approaches remains relevant. Of note, chaperone expression studies harbour yet again strong arguments to advocate for physical exercise to promote health. HSP70 levels decline with disuse [29], athletes exhibit 2.8-fold higher basal serum levels than sedentary age-matched controls [30], and levels significantly increase after high-intensity exercise [31]. In addition, reduced circulating HSP70 has been observed in old age [32,33] possibly explaining the decline in the body’s resilience to counteract damage [34]. Higher circulating HSP70 levels reported in young Duchenne MD patients compared to healthy age-matched controls diminishing with advancing age [25] could therefore point to an accumulating inability to resolve progressive muscle damage. It has been established that mdx muscle benefits from enhancing HSP70 [35,36]. Stimulating chaperone activity thus could represent a therapeutic strategy to be explored further for MD, and scientific evidence has emerged appointing protective effects to HSP70 agonists. The pharmacological HSP70 inducer BGP-15 improved dystrophic pathology in mdx treated between ages 4 to 9 weeks, yet interestingly not at a later stage of the disease progression [34,35], again pointing to a therapeutic ‘window of opportunity’ early in dystrophinopathy. The question remains whether this might translate in an advantageously larger therapeutic timeframe for human Duchenne, which exhibits more constant deterioration of muscle function, or whether or not other MD patient subgroups could benefit.

5. Conclusions

This descriptive study of chaperone expression in muscular dystrophies found disease stage-associated HSP70 variations without overt activation of HSP70/TLR immune-regulatory pathways. Therapeutic interventions stimulating HSP70 function could therefore hold potential to improve patients’ quality of life in an attempted to attenuate loss of muscle mass and improve repair and regenera-
tion, while we await gene therapies to become available to more patients.

**Author Contributions**

BDP designed the research study. GC, CM, JLDB and BDP performed the research. GC, CM, JLDB and BDP analyzed the data. BDP wrote the manuscript. All authors revised the manuscript and read and approved the final version of the manuscript.

**Ethics Approval and Consent to Participate**

Human experimentation adhered to ethical and privacy regulations, all patients consented to participate to the study of which procedures had been approved by the Ghent University Hospital Ethics Committee (B670201836756, B670201938779). Animal experiments were conducted at the certified specific pathogen free animal facility of Ghent University (ECD17/130), approved by the Animal Ethics Committee of Ghent University, faculty of Medicine and Health Sciences (ECD19/77, ECD19/110) in accordance with European guidelines (Directive 2010/63/EU).

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**Conflict of Interest**

The authors declare no conflict of interest.

**Supplementary Material**

Supplementary material associated with this article can be found, in the online version, at [https://doi.org/10.31083/j.fbs1403019](https://doi.org/10.31083/j.fbs1403019).

**References**


