EXAMENSARBETE INOM BIOTEKNIK,
AVANCERAD NIVÅ, 30 HP
STOCKHOLM, *SVERIGE 2017*

Protein biomarkers analysis within muscular dystrophies

SANDRA MENA

KTH
SKOLAN FÖR BIOTEKNOLOGI

PROTEIN BIOMARKERS ANALYSIS WITHIN MUSCULAR DYSTROPHIES

Master thesis

Author: Sandra Carolina Mena Pérez

Supervisor: Cristina Al-Khalili Szigyarto
Stockholm 2017 Master's program: Medical Biotechnology
Kungliga Tekniska Högskolan

Content

Abstract

Muscular dystrophies represent a group of disorders sharing common symptoms of progressive skeletal muscle degeneration and loss of motor function. This project aimed to investigate if biomarkers from one muscular dystrophy can be translated to other ones. An affinity-based proteomics strategy was carried out to identify significantly elevated levels of biomarkers between samples and controls, as well as to identify distinct protein abundance profiles between samples of different types of muscular dystrophies. Plasma and serum samples from different cohorts were analyzed with a selected panel of biomarkers using a multiplex suspension bead array platform. Proteins as Cadherin-5 (CDH5), Mitogen-activated protein kinase 12 (MAPK12), Fibrinogen (FGB), Malate dehydrogenase (MDH1), Homeobox protein Nkx-6.1 (NKX6-1), Fibrinogen (FGA) and LIM/homeobox protein Lhx5 (LHX5) showed higher signals in DMD, LGMD, GNE and Myotonic dystrophy samples of distinct cohorts, and could be selected for possible further experimentation in a larger set of samples.

Abstrakt

Muskeldystrofi representerar en grupp av sällsynta genetiska sjukdomar som gemensamt karakteriseras av progressiv muskel degenerering och förlust av muskel funktion. Projektet har som mål att undersöka om biomarkörer associerade med muskelfunktion kan överföras från en muskeldystrofi till en annan. En affinitets-baserad proteomik metod har använts för att detektera förekomst av signifikant förhöjda proteinnivåer i blodet hos patienter i jämförelse med friska individer och identifiera distinkta protein profiler i patienter med olika typer av muskeldystrofi. En noggrant utvald set av biomarkörkandidater har analyserats i plasma och serumprover med hjälp av en antikropp array som möjligtgör analys av 96 proteiner i 96 prover simultant. Proteiner som kadherin-5 (CDH5), mitogenaktiverat proteinkinas 12 (MAPK12), fibrinogen B(FGB), Malat dehydrogenas 1 (MDH1), Homeobox-protein Nkx-6.1 (NKX6-1), Fibrinogen A (FGA) och LIM homeobox protein 5 (LHX5) visade högre signaler i Duchenne muskel dystrophy, LGMD-, GNE- och myotoniska dystrofi-prover från olika kohorter. Dessa proteiner utgör lovande biomarkör kandidater som kan vidare studeras i större prov kollektioner.

Introduction

Muscular dystrophies represent a group of pathologies caused by different genetic mutations which lead to similar symptoms of progressive skeletal muscle degeneration; affecting mainly limb, axial, and fascial muscles (Mercuri, 2013). Within each of the diseases different times of onset, progression, severity and phenotypes may be found. The wide occurrence of phenotypes differs also depending on the gene affected.

Within the most common muscular dystrophies we may find: Duchenne muscular dystrophy, Limb-girdle muscular dystrophy, Becker muscular dystrophy, Fascioscapulohumeral muscular dystrophy and GNE myopathy. These muscular dystrophies have been characterized, with respect to the age of onset, genetic mutation, severity and associated symptoms etc. (Leoyklang, 2014). For instance, Limb-girdle muscular dystrophy has been characterized in 25 different subtypes, each one affecting a different primary protein and showing different main symptoms, differing in autosomal or recessive inheritance, onset and severity of disease's progression (See Table 1). Other example is Fascioscapulohumeral muscular dystrophy which is classified into two subgroups, differing on the mutated gene, on the genetic and/or epigenetic origin of the disease as well as the symptoms and course of the disease.

Duchenne muscular dystrophy (DMD) has a prevalence of about 1 in 3,600 to 9,337 boys worldwide (Bushby, 2010). It is caused by a frame shift mutation in the dystrophin gene which leads to the loss of its expression. This disorder is the most common inherited muscular dystrophy and has an onset during early childhood leading to loss of ambulance at the age of 10-14, followed by cardiac and respiratory failure at an early age (Mercuri, 2013).

Becker muscular dystrophy is a mild form of muscular dystrophy with an incidence of 1 in 20,000. This disease is also caused by mutations in the dystrophin gene but with a conserved open-reading frame, resulting therefore in the expression of dystrophin at a reduced level (Mercuri, 2013). As the symptoms are present in a milder form, its onset is delayed until adult age.

Limb-girdle muscular dystrophy includes a wider group of subtypes and it is present both in childhood and adulthood. Mutations in more than 50 loci have been reported for Limb-gridle muscular dystrophy, making its correct diagnosis a challenge (Thompson, 2016). The disease is caused by mutations in different genes and its clinical manifestations lead to muscle degeneration and loss of limb musculature (Pegoraro, 2000). The recessive forms of this dystrophy are more common than the dominant variants; and depending on the type and severity, it might lead to cardiac failure. (Mercuri, 2013)

Fascioscapulohumeral muscular dystrophy has an incidence of 3 per 100,000 births worldwide (Mercuri, 2013). Onset occurs in adult life and it is a disorder of dominant inheritance and slow progression. Symptoms include facial muscle weakness, foot drop and scapular winging. The causes are both of genetic and epigenetic nature. In the case of FSHD1 there is a de-repression of a macrosatellite array of D4Z4 repeats on chromosome 4 which leads to the expression of a toxic transcription factor (DUX4) (Petek, 2016). While in FSHD2; which affects fewer individuals, the disease is caused by mutations in the gene SMCHD1; which is a member of condensin/cohesin family of chromatin compaction complexes, and binds to the D4ZU4 repeat. (Daxinger, 2015)

GNE myopathy has also its onset at adulthood, and it is a progressive myopathy caused by a biallelic mutation in the gene GNE, which expression permits the sialic acid synthesis pathway. This autosomal recessive rare neuromuscular disorder leads to complete loss of ambulance after 10-20 years from onset. (Leoyklang, 2014). Currently, its diagnosis relies on sequencing of the GNE gene and clinical trials for its treatment based on the use of sialic acid are currently under study (Nishino, 2015).

The correct diagnose of these dystrophies require diagnostic tests that usually involve invasive methods such as muscle biopsies, genetic tests and physical tests. Creatinine kinase (CK) levels are used to confirm the disorders; however, CK levels may be affected by other factors and therefore does not offer a reliable diagnostic test. CK serum levels can be easily influenced by trauma or physical exercise, and therefore does not represent a sensitive proof to disease progression either. However, once the diagnosis is performed, the progression of the disease involves unspecific and inconclusive tests; as well as some being unsuitable for young patient groups, such as MRI analysis. Physical stress tests are also commonly used to monitor disease progression, but these are subjective and unable to deliver conclusive results (Baird, 2012). All these dystrophies share characteristics of muscle degeneration symptoms (Appendix- Table 1) and have in common that their diagnosis and prognosis represent a challenge. The lack of specific and sensitive methods for diagnosis may delay proper diagnosis and therefore initiation of treatment regimes.

These dystrophies greatly differ in their clinical manifestations, being characterized by progressive muscle degeneration. Several pathophysiological pathways are involved resulting in a complex panel of symptoms affecting, cardiac function, respiratory functions, ambulation and many other different symptoms (Bakay, 2002) (Table 1-Appendix). Therefore, biomarkers associated with different clinical parameters are needed in order to assess an accurate frame of the pathologies progression.

Biomarker discovery has failed to prove clinical utility of single biomarkers; therefore several efforts have emerged in the past few years shifting towards identifying several biomarker candidates that together, can prove to be clinically useful. A panel with different biomarkers is considered to provide a wider perspective of the muscle tissue state during disease progression and response to therapies. The use of several biomarkers provides more robust and conclusive results regarding diagnosis or prognosis (Hathrout 2014). The study of the transcriptome, proteome for identification of biomarkers, is a research focus that promises clinical utility for the development of diagnostic methods that can aid clinical management of muscular dystrophies.

DMD and BMD are the dystrophies that have been most studied. Several blood biomarker candidates have been identified with differential abundance between healthy and diseased individuals, and a strong correlation to clinical parameters e.g. (1) glycolytic enzymes such as Glycogen phosphorylase (PYGM), lactate dehydrogenase (LDHB), Fructose-bisphosphate aldolase A (ALDOA), Beta-enolase (ENOB); muscle specific proteins such as titin (TITIN), myosin light chain 1 (MYL1), Filamin C (FLNC), Myomesin 3 (MIOM3), (2) muscle-derived proteins, Cytochrome C (CYC), (Thrombospodin 4) TSP4, Matrix metalloproteinase 9 (MMP9), myosin light chain 3 (MYL3), calsequestrin-2 (CASQ2), microtubule-associated protein 4 (MAP4), and (3) proteins involved in metabolism and energy production carbonic anhydrase 3 (CA3) and malate dehydrogenase 2 (MDH2), electron transfer flavoprotein A and B and fatty acid binding protein (FABP) (Ayoglu, 2015; Hathout, 2014).

Biomarker studies in FSHD have also shown promising results. Many of the biomarker candidates present in blood are involved in metabolism and energy production and muscle-derived proteins e.g carbonic anhydrase (CA3), troponin I (TNN1), fatty acid binding protein (FAPB), and matrix metallo proteinase 9 (MMP9) (also abundant in DMD and BMD patients). Other group of proteins identified are involved in protein synthesis, folding and degradation such as the ubiquitin-fold modifier 1 (UFM1), eukaryotic translation initiation factor 4 gamma 2 (IF4G2), vacuolar protein sorting-associated protein VTA1 homolog (DRG-1), ribosome maturation protein (SBDS), and heat shock 70 (HSP70) all of which except for HSP70 appear to be specific of FSHD pathology (Petek 2016). Other proteins involved in cell adhesion and fusion include 2-phosphoinositide-dependent protein kinase 1 (PDPK1), tyrosine-protein kinase (FER), and NSFL1 cofactor p47 (NSFL1C). Finally, proteins involved in the inflammatory response as complement 3b (C3b), B-cell tyrosine-protein kinase (BTK), killer cell immunoglobulin-like receptor 2DL4 (KIR2DL4), tumor necrosis factor receptor superfamily member 11A(TNFRSF11A) and catalase (CAT) have been reported as potential biomarkers for FSHD (Petek, 2016).

In the case of GNE myopathy; no robust biomarkers have been identified, the current proposed ones are not very specific and include the analysis of glycolysation/sialylation of muscle alpha-dystroglycan, neural crest cell adhesion molecule, neprilysin and other O-glycans (Leoyklang, 2014). A table referring to the main biomarkers reported in the literature may be found at the Appendix (Table 2).

Different proteomics methods are being used to identify and validate protein biomarkers. Mass-spectrometry based proteomic for example has been used for biomarkers discovery (Hathout, 2015). However, the analysis of large number of samples remains challenging. Within affinity-based proteomics the suspension bead array technology (LUMINEX Corp) offers the possibility of analyzing several samples at once providing a valid rapid tool for screening and validation of candidate biomarkers. This technology offers up to 500 parallel measurements per run (Fredolini, 2016). Besides the previously mentioned advantages of high throughput and sensitivity, this platform offers automated washing steps, improved kinetics and higher flexibility (Fredolini, 2016). Modified aptamer-based technology; also known as SOMAmer, provides as well as a high specificity and sensitivity (Hathout, 2015). The aptamer-based technology provides the possibility of a higher throughput and better sensitivity but at a larger cost.

A high demand of sensitive biomarkers has arisen in order to provide less invasive, more sensitive and accurate diagnosis and possible tools to monitor prognosis. The purpose of this thesis is to evaluate if candidate biomarkers for one muscular dystrophy are translatable to other muscular dystrophies. The goal of this project is to broaden the use of biomarker from one dystrophy to the others.

Methods and materials

Antibody coupling to the beads

The selection of targets was based in choosing top candidates reported in the literature by transcriptomic data that could be translated between the diseases. After the selection of the targets; corresponding antibodies were selected from the Human Protein Atlas Project repository, based on their quality and previous validation.

The volume of each antibody used, was calculated according to manufacturers instructions using the following formula:

$$Volume\ of\ Ab[\mu L] = (500{,}000\ beads)x1.75\mu g\ /\ (1\ 000{,}000\ beads))/(antibody\ concentration\ mg/ml)$$

MES buffer was used to dilute the antibodies. Negative controls were also prepared with only buffer (bare beads) and α-albumin. Beads with different color IDs were selected for every antibody and control. Vials were vortexed and 40 µl of each was transferred to different wells in an U-shape bottom Greiner plate. The supernatant was removed while the plate was placed on a magnet in order for the beads to form a pellet on the well's bottom. Two consecutive washes with activation buffer were performed. For each wash the plate was sealed, vortex and centrifuged to minimize spilling. Activation buffer was added to get a final concentration of 0.5 mg/well of EDC and NHS. The beads were incubated at room temperature at 630 rpm for 20 min followed by two consecutive washes using the magnet with MES buffer. Diluted antibodies were added to the beads and the plate was incubated for 2 hours at dark, room temperature at 650 rpm. After the incubation 2 washes were performed as previously described with PBS-T (10mM Na_2HPO_4, 2mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 0.05 % Tween20), and final volume of 50 µl of storage buffer (Blocking reagent for ELISA (Roche) 1:9 in miliQ water, 1:1000 ProClin) was added to quench any remaining activity. Incubation for 2 hours at room temperature at 650 rpm was performed and the stock was storage at 4°C at dark. During coupling of antibodies to the beads in all steps performed, the plates were protected by light, sealed, vortexed and centrifuged.

Coupling Efficiency Testing

After coupling of the beads, a coupling efficiency test was performed to evaluate the success of the coupling by checking MFI values on the LUMINEX platform. This test would allow to calculate the number of beads required for each well, and obtain a final count of around 100 beads per antibody for each measurement. The sample tests were also used to assess the background signals.

Detection antibodies were prepared by mixing anti-rabbit IgG and PBS-T 0.05 %. 3 replicates designed by adding 5 µl of each bead stock into 3 different wells in a U-shape bottom Greinder plate. Detection antibody solution prepared by a solution of anti-rabbit IgG coupled to R-PE (Sigma Aldrich) 1:2000 (1ml + 2ml PBS-T 0.05%) was added to each of the wells. Plate was incubated for 20 min at room temperature at 650 rpm followed by 3 washes with PBS-T 0.05 % on magnet. Final volume of 100 µl PBS-T 0.05% was added to the wells before detection using the LUMINEX platform assay. The median fluorescent intensity and the total bead count in each well for each target analyzed; were read for every well and every analyte, which corresponded to each initial bead stock.

Biotin-labelling of samples

Serum and plasma samples from patients affected by different muscular dystrophie,s and healthy donors were thawed and 3 µl were transferred into different wells on a microtiter plate, containing sterile and filtered PBS. Biotin solution was prepared by solubilizing the Biotin in DMSO at a concentration of 0.01 mg/µl biotin, and then diluted to a concentration of 4 µg/µl biotin with filtered PBS. Biotin solution was added to each well containing the diluted samples, plate was sealed, vortexed and centrifuged before incubation for 3 hours at 4° C with vortexing

every 30 min. The reaction was stopped by adding 0.5 M Tris-HCL, pH 8.0 and further incubation at room temperature at 650 rpm for 20 min. Labelled samples were then stored at -20°C.

Incubation of samples with bead stock
1µl of labelled samples was diluted on 50µl of assay buffer consisting in PBS-T 0.05%, 10% v/v rabbit IgG and 1:1000 ProClin (Sigma Aldrich). Diluted samples were heat treated at 56°C for 30 min, and cooled at 23°C for 10min. Vortex and centrifuge of the heated samples followed to then, be incubated with 5µl of the bead stock. Incubation was performed overnight both at room temperature and at 4°C, at dark for 650rpm.

Analysis of samples
Incubated samples were washed 3 times with 60µl of PBS-T 0.05% per well. Then 50 µl of PBS-T 0.05% + SAPE at 1:750 concentration was added to each well and samples were incubated for 20min, at room temperature, dark, 650rpm. After the incubation, the plate was washed again 3 times with 60µl of PBS-T 0.05% per well. Final volume 60µl of PBS-T 0.05% per well was added and assay run in the LUMINEX platform was analyzed. Initial full clean-up of the equipment was performed. Clogs were liberated from the needle, machine was run with sanitizing protocols and all required solutions were freshly made. Calibration and Verification kits were used to test performance of the instrument. *Luminex equipment usage*
Luminex® Xponent® Software was used to run the assays.

Results and Discussion

Target and antibodies selection
In order to select candidate muscular dystrophies and candidate protein biomarkers for various muscular dystrophies that could be assessed and analyzed, targets were selected after an extensive literature search on the most common muscular dystrophies: Duchenne muscular dystrophy, Limb-girdle muscular dystrophy, Becker muscular dystrophy, Fascioscapulohumeral muscular dystrophy and GNE myopathy (Burch, 2015; Daxinger, 2015: Hathout, 2016; Leoyklang , 2014; Petek, 2016).
Transcriptome analysis of each disease were used from the literature if available; in order to evaluate expression patterns, that may have been reported as different between normal state and diseased state. 92 biomarkers were found and selected as candidates for this study (See Table 2). The biomarkers selected were considered to be able to provide information for at least, one of the diseases. The antibodies were obtained from the Human Protein Atlas database repertoire. For the 92 selected proteins, validated antibodies were selected against 77 of them by a careful selection criteria. Each antibody was reviewed, analyzed and compared with the other antibodies that were available for the same target. Selection criteria was a balance between value scores on protein array; only antibodies recognizing the antigen on protein arrays were considered; also, only antibodies with results of performed western blots recognizing a protein of correct molecular weight, were accepted in the selection decision. Antibody concentration was taken into account in order to reduce possible impediments in the experiment performance, IH validation score was considered, giving attention to these antibodies stated to be supportive or uncertain and not unreliable; finally, extra information of the antibody state was considered. All antibodies were reviewed and compared one by one to select the top candidates (Appendix.Table 3- Selected biomarkers).

Experimental setup
An approach based on affinity proteomics, using the LUMINEX platform for analysis of multiplex antibody arrays on beads in suspension, was used for the experiments. The main principle of the approach relies in the covalent coupling of selected antibodies to color-coded magnetic beads, each of them having a unique fluorescent ID. These beads create when mixed in a suspension, a multiplex antibody array, that when incubated with biotin-labeled plasma and serum samples, make possible the detection of captured proteins through binding events, using a fluorescent reporter functionalized with streptavidin (streptavidin-phycoerythrin SAPE). The LUMINEX platform provides a capillary system similar to a flow cytometer, that has a dual laser system capable of detecting the bead/antibody ID, as well as the SAPE intensity. Being then a powerful array for rapid screening of candidate biomarkers and offering the advantages of a high sample throughput, flexibility and few quantities of sample needed (Fredolini, 2016).
Samples used in these experiments provided from different cohorts of different clinical sites. The UNIFE cohort provided by the University of Ferrara contained at total of 52 samples of which 30 were obtained from DMD patients, 6 belonged to controls and 16 belonged to different disorders as: Limb-Girdle Muscular Dystrophy, Spastic Ataxia, Facioscapulohumeral muscular dystrophy, Myotonic dystrophy, Episodic ataxia, Spastic parapesis, myofibrillar myopathy, Glycogenosis, Filaminopathy and Collagen VI myopathies. UCL cohort was provided by samples obtained from the University College of London, and included 18 plasma samples (9 controls and 9

samples of Limb-Girdle Muscular dystrophy patients) and 17 serum samples (9 controls and 8 samples of Limb-Girdle Muscular dystrophy patients). LUMC cohort was obtained from the Leiden University Medical Center in the Netherlands and contained 33 serum samples and 34 plasma samples of patients with Becker Muscular Dystrophy however no samples of any control. NEWC cohort was obtained from the Newcastle University and included 33 serum samples of GNE Myopathy patients and 25 serum samples of controls. A selection of samples from the UNIFE cohort was selected in order to optimize the methods as a test sample collection. Replicates of the methodology were performed going from low scale to big scale of number of antibodies coupled. First the coupling was performed with a selection of 9 antibodies and 6 controls in 21 different beads IDs of different concentrations to its assess the effect on the results. In a further experiment, a set of 77 antibodies plus two negative controls and one positive control were then performed on 80 bead IDs divided in two batches of 35 and 44 beads respectively. In every experiment two negative controls were used, one containing bared beads and another containing a-albumin. rIgG was also coupled to a bead ID as a positive control.

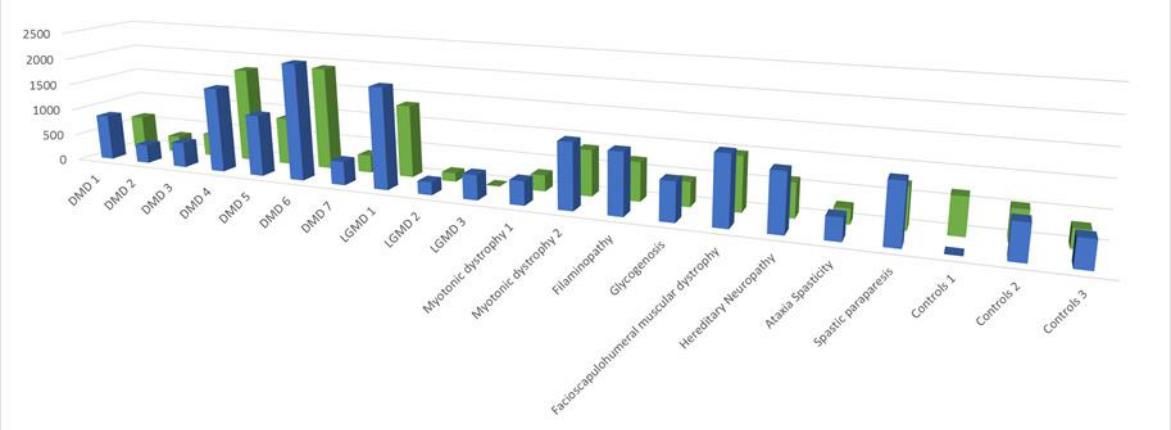
The coupling efficiency was tested and confirmed with R-Phycoerythrin conjugated antirabbit IgG antibody before incubation with the samples. Median Fluorescent intensity values on this coupling efficiency test were obtained as expected to be around 1,000-5,000, considering 50 to 200 as a normal background (bead without antibodies coupled). For all the coupled beads a MFI value was confirmed of being above 1000 for a total 100-150 beads measured, while the negative controls showed significantly lower MFI values between 20-200,

A small set of samples from one cohort was first biotinylated and analyzed by incubation with a bead stock prepared; which contained antibodies against CA3, MDH2, Fibrinogen and a nuclear Factor- erythroid like 2. Changes in the protocol were performed including the usage of a different blocking buffer, and elimination of casein in the assay buffer. This test set was used to optimize assay conditions. Several experiments were performed to test the correct biotinylation of the samples, and the antibody coupling to the beads by changing buffers and incubation conditions. Different concentration of antibodies for successful couplings were tested as well.

The cohort analyzed included samples from UNIFE with samples from patients affected by DMD patients, and other disorders as Filaminopathy, Limb-Girdle Muscular Dystrophy, Spastic Ataxia, Facioscapulohumeral muscular dystrophy, Myotonic dystrophy, Episodic ataxia, Spastic parapesis, myofibrillar myopathy, Glycogenosis and Collagen VI myopathies as well as controls. The samples were incubated with 2 bead stocks containing beads coupled to antibodies at different concentrations.

The first bead stock was performed using 1.75μg of antibody solution for coupling to 500, 000 beads, whereas during the second batch 3 μg of antibody solution was used. The entire UNIFE cohort was then biotinylated and analyzed with a set of 9 antibodies and 2 negative controls to test the blocking and the negative controls (the bare beads and α-albumin). In the bead stock coupled with 1.75μg antibodies a mildly decreased signal was detected (Entire graphics, figure 1 Appendix).

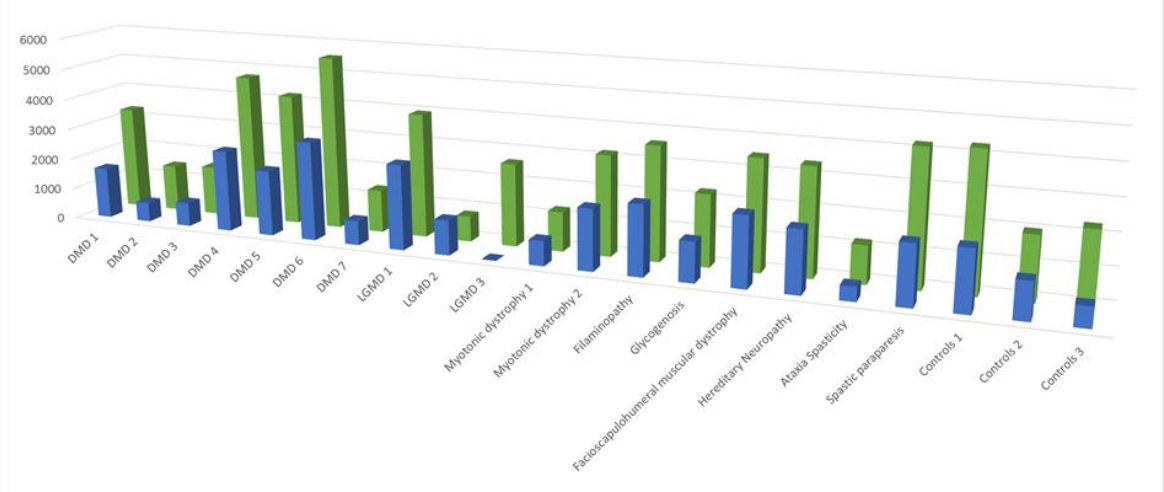
a)



b)

Figure 1. Protein abundance measurements variation with beads coupled at two different concentrations of antibodies. Blue bars represent measurements performed with beads labeled with antibodies in the presence of 1.75 μg whereas green bars represent measurements performed with antibodies in the presence of 3.5 μg. a) MDH2 0507; b) HPA002990-Nuclear factor. MFI values in Y axis

The protocol recommended by the manufacturer was modified also with respect to the assay buffer. Experiments were performed with an assay buffer containing PBS-7 0.05% (9:10), a freshly made rabbit IgG solution at 5mg/ml (1:10), and Proclin (1:1000) with and without casein. When both buffers were used on a small set of beads analyzing the entire cohort, significant changes of high signals were observed, for which it was able to conclude that the presence of casein in the buffer assay, was interfering with the detected signal. The blocking of the beads in the presence of casein resulted in low signals for all targets, including the positive (CA3) and the negative control (Bare bead and a-albumin). This could be due to the possible presence of proteases or interfering proteins, resulting therefore in a lower signal. However, the differences between the signals varied (See Appendix, Figure



1 for detailed graphics). In the case of α-albumin, rabbit IgG, MDH2 0507 targeting malate dehydrogenase, HPA 001275 targeting for a transcription factor and CA3 0121 targeting for Carbonic Anhydrase, no significant difference on the signals was observed even though it was evident a slight higher signal. However, in the case of antibodies CA3 0122 targeting for Carbonic Anhydrase 3, HPA002990 targeting for a Nuclear Factor- erythroid like 2 and HPA007370, a higher contrast was observed in the detected signals. Based on these results performed on a small set of antibodies, it was concluded that a higher concentration of antibody would be more suitable when designing a large-scale experiment, as the optimization for each antibody would be time consuming and almost impossible. Although the protein concentration was provided and confirmed; the discrepancy observed could also be due to the antibody purity level, or an inactivity event due to reagent handling.

The results obtained from the analysis of the UNIFE cohort confirmed what was expected; as antibodies targeting for carbonic anhydrase 3 (CA3) and malate dehydrogenase 2 (MDH2), were found to have a higher abundance in patients than controls; although signals were obtained in the control samples as well; this probably due to unknown underlying condition in the controls, or engagement in physical exercise before the collection of samples (Gianola S, 2013).

Special attention was given to antibodies that showed a higher signal between patients and controls: HPA002990 showed in controls an expression of 14% to 22% over LGMD and myotonic dystrophy samples respectively while almost the same expression when compared to DMD samples. (Figure 2). CA3 showed a fold of 30% to 80% on the 3 groups of patient samples over controls. Cadherin5 showed a fold of 30% to 100% on the 3 groups of patient samples over controls. MDH2 showed a fold of 10% to 70% on the 3 groups of patient samples over controls. HPA 001275 antibody targeting for a transcription factor associated with hypoxia showed a fold of 40% to 80% on the 3 groups of patient samples over controls. HPA064755 and HPA051370 both targeting for fibrinogen showed a higher abundance of 15 to 25% over DMD patients than in controls, however, less abundant in groups of myotonic dystrophy in around 20% to 30% less. (See Appendix, Figure 2)

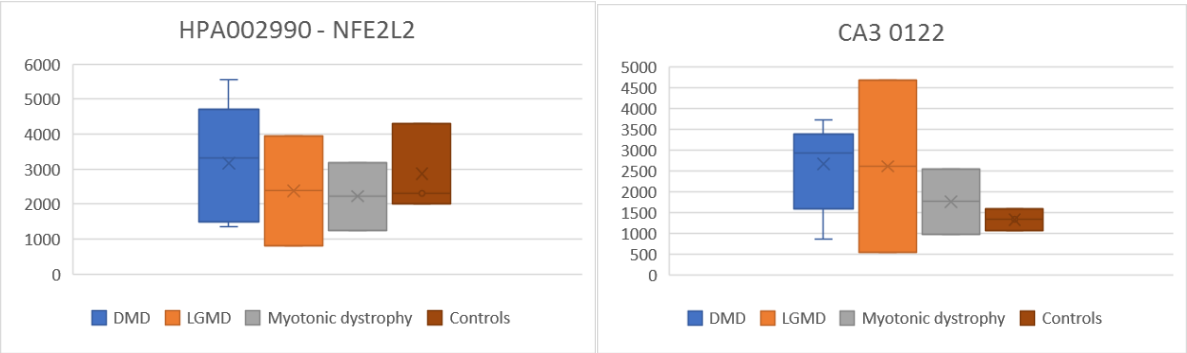


Figure 2. Protein abundance in DMD,LGMD, Myotonic dystrophic patients vs controls in serum samples. MFI values in Y axis. Graph on the left: HPA002990 targeting for a nuclear factor. Graph on the left, CA3 0122 targeting for carbonic anhydrase.

In the case of HPA002990 targeting for the nuclear factor; its high abundance in controls over LGMD and Myotonic dystrophy samples was of interest, as this factor is a protein involved in the regulation of antioxidant proteins expression involved in protection against oxidative damage caused by injury and inflammation (Shelar, 2016). Under normal conditions this protein is kept in the cytoplasm, where it is degraded by other proteins; while under oxidative stress, it travels to the nucleus, where it initiates among with a promoter the transcription of antioxidative proteins (Shelar, 2016). Therefore, its induction may result in cytoprotectively proteins. Its higher expression is found in normal human tissues of kidney, lung, heart, liver and muscle.
Recently it was reported that the genetic ablation of the NrF2 transcription factor, resulted in a compromised expression of antioxidant genes (Shelar, 2016). The disruption of this gene showed in experiments with mice, a resulting impaired mechanism for stem cells activation, and leaded to a delayed skeletal muscle regeneration (Shelar, 2016). The data shown by Shelar and his colleagues in their experiments with mice, leaded to the conclusion that the expression of this factor is required for cell activation, proliferation and differentiation, for an effective healing after injury in muscle fibers. Its high expression in controls could be related to some sort of mechanism involved in regeneration of skeletal muscle and recovery from injury. During a health state, following daily activities that may lead to a muscle deterioration, a regeneration capacity will be regulated by certain factors in order to maintain the muscle function. The nuclear factor targeted by HPA002990 shows the capacity of muscle regeneration and maintenance.
Regarding the high signals obtained in controls for HPA064755 and HPA051370; both targeting for fibrinogen, may be explained by the high presence of fibrinogen in every sample. According to what was reviewed in the literature, fibrinogen is expected to be highly expressed both in controls and patients and increasing with the age (Hathout, 2016). Due to the information regarding age or underlying conditions of the patients is not available, it is not possible to assess if this might be related to the age of both the patient's samples and controls, or the presence of other condition that might cause an inflammation response.
Additionally, it was noticed that one control showed higher signals for all the beads analyzed in comparison to the other control samples, which could affect in the statistical analysis.
After the UNIFE cohort was analyzed with this small set of antibodies and experimental conditions were improved, all the cohorts were biotinylated and prepared, aiming to study the entire set of selected beads on all samples.

Cohorts and samples analysis
Each cohort was analyzed with the 77 antibodies plus 2 negative controls one with albumin and one with bare beads. For each cohort and analysis, the distribution of the signal detected for each antibody within the samples was analyzed. First only DMD patients vs controls were analyzed in the UNIFE cohort to identify the top candidates of antibodies, and then the other disorders signals were included. (Entire graphics, Appendix Figure 3)
The total mean value MFI of patient's vs controls for all antibodies were graphed against each other to assess the highest peak signals, and the most marked differences between patients and controls. The targets with different abundance of target in patients' samples in comparison to controls were selected. The highest signals obtained belonged to the proteins: Fibrinogen alpha chain (FGA), Zinc Family member 3 (ZIC), Protein kinase, cAMP-dependent catalytic alpha-protein kinase, Protein kinase, cAMP-dependent catalytic gamma-protein kinase, Protein kinase, cAMP-dependent catalytic betta-protein kinase (PRKACA, PRKACG, PRKACB), RELT tumor necrosis factor receptor (RELT), (CDK14), serum amyloid A2:A4 (SAA2-SAA4; SAA2; SAA1), Fatty acid binding protein 3 (FABP3), Homeobox protein Nkx-6.1 (NKX6-1) Notch 3 (NOTCH3), Neuronal differentiation 2 (NEUROD2). All of these were found to be higher expressed in patients than in controls. One type of antibody against fibrinogen and for Homeobox protein Nkx-6.1 (NKX6-1). (Figure 3)

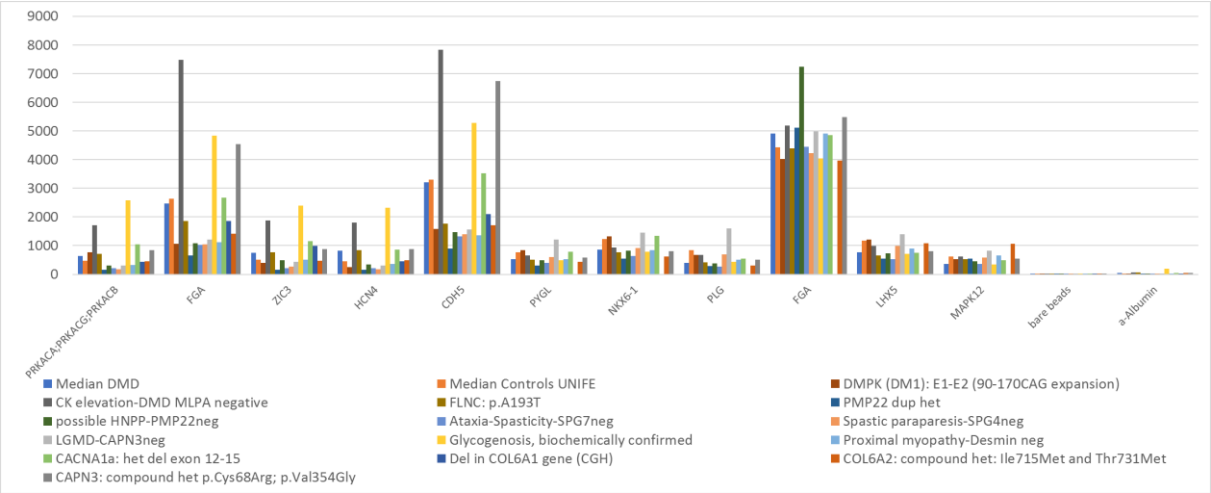


Figure 3. Graphic of entire UNIFE cohort. DMD patients' median vs. controls median vs unique values of other disorders. MFI values in Y axis

In the case of fibrinogen, two different antibodies targeting this one protein were used. Both antibodies recognized different isoforms and epitopes of the protein, which would explain the variability observed in the obtained signals for these two antibodies.

When the signals of the rest of the patients with other type of disorder were included, it was assessed a high variability within the signals available from each sample. The results from the analysis of the samples from the UNIFE cohort cannot statistically support solid conclusions, but can provide information about the feasibility in exploring translation of biomarkers from one muscular dystrophy to another and support selection of targets to be further analyzed. The variability between individuals is high, and possibly a main factor to mask any result. Additionally, this cohort showed high variance of results and difficulties to analyze them due to formation of aggregates.

The analysis of this cohort was limited as it included unique samples, which gives unsatisfactory statistical power of the results. The results should be considered as screening experiments that should be performed on larger sample collections.

Even though some of the signals obtained followed what was expected from the literature, the values between controls and patients were close to each other and the presence of a visible pattern was seen. As the abundance difference is low between patients and controls, the use of these targets as biomarkers might not enable distinction between healthy and diseased state.

Fibrinogen was analyzed in a scatter plot for all the available samples. The high variability between controls and patients showed an abundance of this protein in all samples without a clear tendency (Appendix Figure 4).

When analyzing the NEWC cohort, the samples included only GNE patient's vs controls. Only serum samples were available and analyzed. It was noticed that from all the antibodies used, the signals between patients and controls didn't significantly differ, the obtained signals were low as well; however, the main antibodies with the highest signals were plotted both in the values of median of patients vs. controls, as well as then in its distribution per antibody (Appendix Figure 5). The highest signals were obtained in the antibodies coding for Fibrinogen (FGA), Mitogen-activated protein kinase 12 (MAPK12), Aminoacylase-1 (ABHD14A-ACY1), Neurogenic locus notch homolog protein 3 (NOTCH3), cAMP-dependent protein kinase catalytic subunit α (PRKACA, PRKACG, PRKACB) and Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4). In all cases, there was a higher signal in the GNE Myopathy patient's samples compared with the controls. (Figure 4) However, the slight signal differences observed were compared with the information available in the literature. In the case of fibrinogen, two antibodies targeting different parts of the protein were used, which could explain the different results obtained between both of them. This protein has been reported to be in higher abundance in patients with DMD and its abundance is age dependent (Hathout, 2015). Mitogen-activated protein kinase 12 (MAPK12), was found more abundant in patients in comparison to healthy controls. This is a muscle-enriched, creatine kinase-like protein, and its higher abundance in patient could reflect damage of myofiber membranes and necrosis and its leakage to the cytoplasm. (Fukada, 2013). Aminoacylase-1 was also found to be higher in patients, this protein is involved in the hydrolysis of N-acylated amino acids. In the case of the Neurogenic locus notch homolog protein 3 (NOTCH3), cAMP-dependent protein kinase catalytic subunit α (PRKACA, PRKACG, PRKACB) and Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4), all levels were found as well to be higher in patients than in controls. However, in the case of NOTCH3, it has been reported to be lowered in DMD animal models this protein on studies with mice (Fukada, 2013).

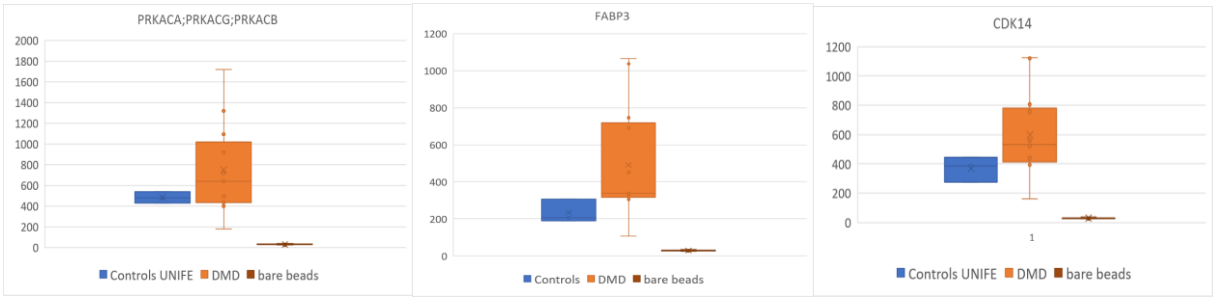


Figure 4. Targets with the highest abundance variation between DMD patients and controls. Graphics of median values and distribution box plots per antibody. Controls in blue, DMD samples in orange and bare beads in red. MFI values in Y axis

The UCL cohort contained both samples from plasma and serum from patients with different types of Limb-Girdle Muscular Dystrophy and controls, for which it was expected a higher difference of abundance of proteins within the patients and samples. It was observed that from the top biomarkers candidates selected for the highest signals, and the higher difference of expression between patients with Limb-Girdle and controls, the samples obtained from Plasma showed a higher signal above those from serum; however, almost the same trend of increased or decreased abundance was present in both types of samples (Figure 5). The selected candidates with the higher difference of detected signals were chosen using the same analysis as in the previous cohorts, being the most significant proteins: Fibrinogen (FGA), LIM/homeobox protein Lhx5 (LHX5), Cadherin-5 (CDH5) and Mitogen-activated protein kinase 12 (MAPK12).

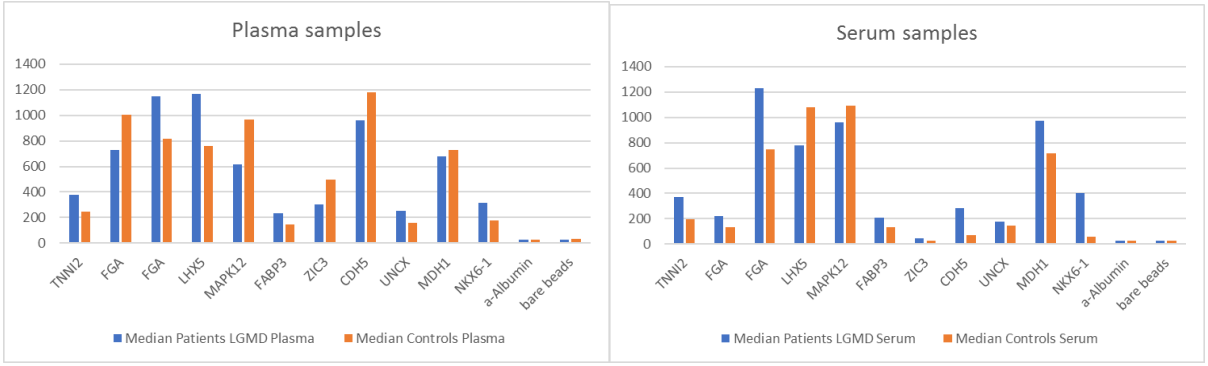


Figure 5. Graphics of UCL Cohort. Top candidate proteins chosen from samples of Limb-Girdle patients vs controls both in serum and plasma samples. MFI values in Y axis

From these analysis, it was assessed that Fibrinogen (FGA), LIM/homeobox protein Lhx5 (LHX5) were considerably higher in plasma samples of Limb-Girdle patients in around 33% to 35% both, while Cadherin-5 (CDH5) and Mitogen-activated protein kinase 12 (MAPK12) were in lower abundance in patients over controls in around 25% and 40% respectively.

In the case of the serum samples, also Fibrinogen (FGA) showed a higher signal in patients over controls of around 40% more, however LIM/homeobox protein Lhx5 (LHX5), showed a distinct pattern as in the plasma samples with a higher signal in controls over patients of around 28%. Additionally, Malate dehydrogenase (MDH1) was detected with a higher signal of around 30% in patients over controls. Malate dehydrogenase (MDH1) and Fibrinogen (FGA) have been reported to be highly abundant in samples of DMD patients (Petek 2016, Hathout 2016).

The high signals detected of Fibrinogen (FGA) and LIM/homeobox protein Lhx5 (LHX5) in patients with Limb-girdle diagnosis, matched the reported high abundance detected in other types of dystrophy as Becker and DMD in the literature. Also, the lower signals in patients' samples of Cadherin-5 (CDH5), matched with previous discoveries on DMD patients (Hathout, 2015), showing the association with the low response in connective tissue remodeling. (Figure 5)

In the case of Fibrinogen, high signals were obtained in all group of patients and to some extent in controls. However, this may be arisen in controls due to this protein is released into the blood as a response to stress, which may be involve exercise or physical activity. However, a higher signal was seen in the group for Limb-Girdle dystrophy patients, confirming previous studies in the literature regarding its abundance in DMD patients (Vidal, 2008). Fibrinogen has a key role in muscle regeneration and maintenance due to its roll in controlling blood loss

after vascular injury. It also has a key role following inflammation and increased vascular permeability by being converted to fibrinogen, which explains the high development of fibrosis in dystrophic muscle found in biopsies (Vidal, 2008).
Regarding the protein Cadherin 5, a lower value in patients than in controls was seen but only in the plasma samples, for which its discrepancies with the serum samples was inconsistent and this could be an artifact for which further experimentation would be required in a bigger set of samples and optimized conditions.
Protein Mitogen-activated protein kinase 12 (MAPK12) showed a similar distribution between LGMD patients and controls. This was contrary to what was expected, as this protein's change of concentration in the blood reflects myofiber membrane instability/damage and necrosis, by leaking of the cytoplasm into the extracellular space.
Finally, within the proteins that rose an interest profile Homeobox protein Nkx-6.1 (NKX6-1) was detected to be higher bin LGMD over controls. This is a transcription factor that has been related to Fascioscapulohumeral muscular dystrophy, although not widely researched.
A new analysis within all the cohorts confirmed the previous experiments with this protein, as it showed results of higher abundance in serum samples of Myotonic dystrophies of UNIFE and plasma samples of LGMD from UCL cohort (Figure 6), (Entire graphics, Appendix figure 6)

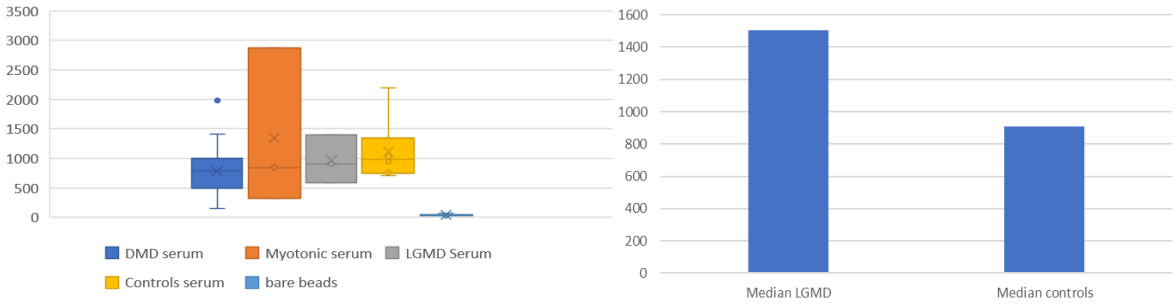


Figure 6. Graphics of analysis of HPA036774 targeting NKX6-1 in UNIFE serum samples and UCL plasma samples. MFI values in Y axis

It was noticed that within all the cohorts only low signals were obtained for the serum samples, the median values showed only signals that could be interpreted as background or absence of the protein; however, in the plasma samples a few proteins showed signals. The results were as expected, since serum has a lower protein concentration than plasma (Lundblad R, 2013). Regarding the plasma samples, a few proteins were identified and compared to what was stated in the literature due to lack of controls within the cohort. From these analyses only an approximation of what was expected to be seen; if controls and a larger group of samples was available, was able to be assessed.
The high variability between individuals could be affecting the results in a bigger way than the pattern given by the disorder, for which results with such small set of samples can only give a hint on further experimentation and research.
After the analysis of all cohorts, it was seen that compared with the signals of the rest of the cohorts, UNIFE samples were significantly much higher. This high variation between the results among cohorts could be explained by several factors among of which are included: the conditions of the experiment, analyzes, incubation of samples, antibody concentration used, and other conditions which were optimized among the experimentation process. For which in future perspectives, the experiment should be repeated to check the reproducibility of the results.
In all cohorts and sample types a discrepancy was seen again with FGA as in the other cohorts, confirming the theory that the different antibodies used for this protein, could be giving different results due to binding to different parts of the protein, and the possibility that the proteins in the samples could be fragmented, degraded or the nature of the different antibodies used would not be the ideal biomarker for these kind of disorders. While the antibody HPA051370 matched a transcript recognizing a sequence of 98 amino acids in positions 371-468, the antibody HPA0064755 binds an epitope of 92 aa in positions 562-653. Further experimentation with a larger group of samples and different antibodies targeting for fibrinogen, could be of interest for a deeper understanding of this behavior and /or provide an explanation to this variability, defining a cause in the nature of the biomarkers or in the experimentation conduction.
Some proteins stated in the literature as Fibrinogen (FGA), LIM/homeobox protein Lhx5 (LHX5), Cadherin-5 (CDH5), Plasminogen (PLG) Homeobox protein Nkx-6.1 (NKX6-1) Mitogen-activated protein kinase 12 (MAPK12), Aminoacylase-1 (ABHD14A-ACY1), Neurogenic locus notch homolog protein 3 (NOTCH3), Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4) , Zinc Family member 3 (ZIC), Protein kinase cAMP-dependent catalytic protein kinase (PRKACA, PRKACG, PRKACB), RELT tumor necrosis factor receptor (RELT), (CDK14), serum amyloid A2:A4 (SAA2-SAA4; SAA2; SAA1), Fatty acid binding protein 3 (FABP3), Homeobox protein Nkx-6.1 (NKX6-1) and Neuronal differentiation 2 (NEUROD2)

were confirmed to be present as it has been previously discussed; however, the analyzed data did not provide a conclusion to categorize the abundance of certain protein depending on the nature of the disorder. Further experimentation should be performed with the top candidate biomarkers in order to study a pattern of expression and assess the importance of such results. Additionally, a wider set of samples would be required in order to relate the presence of higher signals, to the presence of a disorder, and not to variability between individuals of a small sample set.

Conclusion

The completion of this project led to the identification of proteins abundant in plasma and serum samples of patients with different types of dystrophy that could serve as biomarkers when compared to healthy controls.
From the data obtained and its analysis it was also possible to conclude the main factors that represented a challenge during this research. The reduced nature in number of samples available for rare disorders such as muscular dystrophy was a factor that could influence in the results' interpretation. The data seen could have been product of an individual variability instead of an indicator of the pathology.
However, the data analyzed could confirm biomarkers that matched with the previously researched literature, while some others showed a different pattern from what expected. (Hathout, 2016) This discrepancy could be a consequence of factors from both the experimentation and the samples. The possibility of an underlying unknown condition in the controls, could affect the results significantly due to the scarcity of the samples and the high variation on results from few samples available.
Regarding the available samples, conditions that could interfere with the results is the different conditions under which they were obtained; leading to a possible variation between them, reiterating the need of a standardized sample preparation within sites in order to minimize an effect on results (Tawil, 2015).
Regarding the conduction of the experiment, after a series of hypothesis and parallel experiments, it was possible to assess a way of optimizing the experimentation for further results. It was concluded that in order to obtain significant signals, samples should be incubated with the bead stock immediately after their biotinylation and at a temperature of 4°C to prevent degradation. A high concentration of antibody would be preferred when coupling to the beads to assure signal detection of low abundant proteins.
Regarding the results obtained, some biomarkers could be confirmed to be expressed as how it was researched in the literature. Cadherin-5 (CDH5), Mitogen-activated protein kinase 12 (MAPK12), Fibrinogen (FGB), Malate dehydrogenase (MDH1), Homeobox protein Nkx-6.1 (NKX6-1), Fibrinogen (FGA) and LIM/homeobox protein Lhx5 (LHX5) showed the higher signals in DMD, LGMD, GNE and Myotonic dystrophy samples of distinct cohorts, and could be selected for further experimentation within more samples, in order to analyze a specific pattern of expression between the dystrophies in a larger group of samples, if available.
Finally, it was concluded the importance of affinity proteomics in the study of candidate biomarkers in rare diseases. The need for future research and effort to not only detect, but to validate biomarkers to be used in the clinic in the future; the high effort and work required within the field in order to provide conclusive results, and the advantages and challenges from this approach when compared to other technologies. Affinity proteomics provides a window of opportunity to the identification of biomarkers that can lead to non-invasive and robust use on the clinic, and represents a positive impact on treatment, diagnosis and prognosis opportunities, leading to a benefit on social welfare and healthcare, for which still a high effort on this field is required.

Future perspectives

The completion of this project made able to assess future improvements and possibilities of research.
The conditions of the methodology and experimentation could be tested, to establish an optimization of it to obtain higher and more accurate signals. Such changes could be implemented for further experiments targeting a smaller set of antibodies and a higher number of samples.
Additionally, the higher signals obtained in certain proteins led to the establishment of a set of biomarkers that could be studied in larger groups of samples within the disorders, to study a possible pattern of differentiation of abundance within the types of disorders. Finally, further research with affinity proteomics need to be carried out; not only for muscular dystrophies, but for many other rare disorders.

Acknowledgments

I would like to thank my supervisor Dr. Cristina Al-Khalili Szigyarto who mentored me and advised me during the entire planning and conduction of this project. I am thankful for all her support and teachings, her feedback, her corrections, understanding and even moral support. She let this to be my own work while guiding me in the right direction when needed, and inspiring me since the beginning to continue the hard work.

I would like to thank as well to the Biotechnology school of KTH, for giving me an opportunity of carrying this project in their premises and supporting me with everything I needed to complete it.

Finally, I would like to thank all the people involved in the collection of the samples I was granted to work with. To the patients, their families and the staff involved in their care, as their commitment to research made me possible to complete this project.

References

Arvanitidis A, Henriksen K, Karsdal MA, Nedergaard A. (2016). "Neo-epitope Peptides as Biomarkers of Disease Progression for Muscular Dystrophies and Other Myopathies". *Journal of Neuromuscular Disorders.* Vol 3, No. 3. Pp 333-346.

Baird M, Graham S, Baker J, Bickerstaff G. (2012). "Creatine-kinase- and Exercise-Related Muscle Damage Implication for Muscle Performance Recovery". *Journal of Nutrition and Metabolism*

Bakay M, Zhao P, Chen J, Hoffman EP. (2002). "A web-accessible complete transcriptome of normal human and DMD muscle". *Journal of Neuromuscular Disorders.* Vol 12. Pp 125-142.

Burch P, Pogoryelova O, Goldstein R, Bennett D, Guglieri M, Straub V, Bushby K, Lochmüller H, Morris C. (2015). "Muscle-derived proteins as serum biomarkers for monitoring disease progression in three forms of muscular dystrophy". *Journal of Neuromuscular Disorders.* Vol 2, No. 3. Pp 241-255.

Ayoglu B, Chaouch A, Lochmüller H, Politano L, Bertini E, Spitali P, Hiller M, Niks EH, Gualandi F, Pontén F, Bushby f, Aartsma-Rus A, Schwartz E, Le Priol Y, Straub V, Uhlén M, Cirak S, 't Hoen PA, Muntoni F, Ferlini A, Schwenk JM, Nilsson P, Al-Khalili Szigyarto C.. (2015). "Affinity proteomics within rare diseases: a BIO-NMD study for blood biomarkers of muscular dystrophies". *EMBO MolecularMedicine.* Vol 6, No. 7. Pp 918-936.

Bushby K, Finkel R, Birnkrant DJ, Case LE, Clemens PR, Cripe L, Kaul A, Kinnett K, McDonald C, Pandya S, Poysky J, Shapiro F, Tomezsko J, Constantin C; DMD Care Considerations Working Group. . (2010). "DMD Care Considerations Working Group Diagnosis and management of Duchenne muscular dystrophy, part 1. Diagnosis, and pharmacological and psychosocial management". *Lancet Neurol*. Vol. 9. No 1. Pp 77–93

Daxinger L, Tapscott SJ, van der Maarel SM.(2015). "Genetic and epigenetic contributors to FSHD". *Current Opinion in Genetics and Development.* Vol 33. Pp 56-61.

Fukada S, Ma Y, Ohtani T, Watanabe Y, Murakami S, Yamaguchi M. (2013). "Isolation, Characterization and Molecular Regulation of Muscular Stem cells". *Frontier in Physiology*. Volume 4. No 317.

Fuller H, Graham L, Hurtado M, Wishart T. (2016). "Understanding the molecular consequences of inherited muscular dystrophies: advancements through proteomics experimentation". Journal of Expert Review of Proteomics. Vol 13. No 7. Pp 659-671.

Fredolini C, Byström S, Pin E, Edfors F, Tamburro D, Iglesias M, Häggmark A, Hong MG, Uhlen M, Nilsson P, Schwenk J. (2016). "Immunocapture strategies in translational proteomics" *Expert review of proteomics.* Vol 13. pp 83-98.

Gianola S, Pecoraro V, Lambiase S,Gatti R, Banfi G, Moja L.(2013). "Efficacy of Muscle Exercise in Patients with Muscular Dystrophy: A Systematic Review Showing a Missed Opportunity to Improve Outcomes". *Plos One*. Vol 8 No 6.

Hathout Y, Marathi RL, Rayavarapu S, Zhang A, Brown KJ, Seol H, Gordish-Dressman H, Cirak S, Bello L, Nagaraju K, Partridge T, Hoffman EP, Takeda S, Mah JK, Henricson E, McDonald C. (2014). "Discovery of serum protein biomarkers in the mdx mouse model and cross-species comparison to Duchenne muscular dystrophy patients". *Human Molecular Genetics*. Vol. 23. No. 24. Pp 6458-6469.

Hathout Y. et.al. (2015). "Large-scale serum protein biomarker discovery in Duchenne muscular dystrophy". *Proceedings of the National Academy of Sciences of the United States of America*. Vol. 112. No. 23. Pp 7153-7158. Available at < https://www.ncbi.nlm.nih.gov/pubmed/26039989>

Hathout Y, Brody E, Clemens PR, Cripe L, DeLisle RK, Furlong P, Gordish-Dressman H, Hache L, Henricson E, Hoffman EP, Kobayashi YM, Lorts A, Mah JK, McDonald C, Mehler B, Nelson S, Nikrad M, Singer B, Steele F, Sterling D, Sweeney HL, Williams S, Gold L.(2016). "Clinical Utility of serum biomarkers in Duchenne muscular dystrophy". *Clinical Proteomics*. Vol. 13. No. 9.

Leoyklang P, Malicdan MC,Yardeni T, Celeste F, Ciccone C, Li X, Jiang R, Gahl W, Carrillo-Carrasco N, He M, Huizing M. (2014). "Sialylation of Thomsen-Friedenreich antigen is a noninvasive blood-based biomarker for GNE myopathy". *Biomark Med*. Vol 8. No 5. Pp 641-652.

Lopez-Alemany, Suelves M, Diaz-Ramos A, Vidal B, Munoz-Canoves P. (2005). "Alpha-enolase plasminogen receptor in myogenesis". Frontiers in biosciences. Vol 10. No. 30-6.

Lundblad. R. (2013). "Considerations for the Use of Blood Plasma and Serum for Proteomic Analysis". *The Internet Journal of Genomics and Proteomics*. Volume 1 Number 2.

McAdam L. Vajsar J, Biggar WD. (2012). "S.P.52 Troponin T adventures in Duchenne muscular dystrophy". *Neuromuscular disorders*. Vol 22. No 9-10. Pp 884-885.

Mercuri, E., Muntoni F. (2013)."Muscular dystrophies". *The Lancet.* Vol 381. No 9869. Pp 9-15. Available at <http://www.sciencedirect.com/science/article/pii/S0140673612618972>

Mokuno K, Riku S, Matsuoka Y, Sobue I, Kato K.(1985). “Serum Carbonic Anhydrase III in Progressive Muscular Dystrophy”.*Journal of Neuromuscular Disorders.* Vol 67 pp 223-228.

Nishino I, Carrillo-Carrasco N, Argov Z. (2015). “GNE Myopathy current update and future therapy”. *Journal of Neurosurgical Psychiatry*. Vol. 86. No 4. Pp 385-392.

Ohta M, Itagaki Y, Itoh N, Hayashi K, Nishitani H, Ohta K. (1991). “Carbonic Anhydrase III in Serum in Muscular Dystrophy and Other Neurological Disorders: Relationship with Creatine Kinase”. *Clinical Chemistry*. Vol 37. No 1. Pp 36-39.

Pegoraro E, Hoffman E. (2000). “Limb-Girdle Muscular Dystrophy Overview”. Gene Reviews.

Petek L, Rickard A, Budech C, Poliachik S, Shaw D, Ferguson M, Tawil R, Friedman S, Miller D. (2016). ”A cross sectional study of two independent cohorts identifies serum biomarkers for fascioscapulohumeral muscular dystrophy (FSHD)”. *Journal of Neuromuscular Disorders.* Vol 26, No. 7. Pp 405-413.

Shelar SB, Narasimhan M, Shanmugam G, Litovsky SH, Gounder SS, Karan G, Arulvasu C, Kensler TW, Hoidal JR, Darley-Usmar VM, Rajasekaran NS.(2016). “Disruption of nuclear factor (erythroid-derived-2)-like 2 antioxidant signaling: a mechanism for impaired activation of stem cells and delayed regeneration of skeletal muscle. *Federation of American Societies for Experimental Biology Journal*. Vol 5. p1865-1879.

Stapleton D, Lau X, Flores M, Trieu J, Gehrig SM, Chee A, Naim T, Lynch GS, Koopman R. (2014). “Dysfunctional muscle and liver glycogen metabolism in mdx dystrophic mice.” *Plos One*. Vol 9. No 3.

Tawil R, Padbergb G, Shawc D, Van der Maareld S, Tapscotte S. (2015). “Clinical trial preparedness in facioscapulohumeral muscular dystrophy: Clinical, tissue, and imaging outcome measures 29–30 May 2015, Rochester, New York”. *Neuromuscular Disorders.* Vol 26. Pp 181–186.

Thompson T, Chan Y, Hack A, Brosius M, Rajala M, Lidov H, McNally E, Watkins S, Kunkela L.(2000). “Filamin 2 : A Muscle-Specific Sarcoglycan Interacting Protein”. *Cell Biology*. Vol 148. No 1.

Thompson R., Straub V. (2016). “Limb-girdle muscular dystrophies-international collaborations for translational research”. *Nature Reviews Neurology*. Vol. 12. Pp 294-309.

Vidal B, Serrano A, Tjwa M, Suelves M, Ardite E, De Mori R, Baeza-Raja B, Martínez de Lagrán M, Lafuste P, Ruiz-Bonilla V, Jardí M, Gherardi R, Christov C, Dierssen M, Carmeliet P, Degen J, Dewerchin M, Muñoz-Cánoves P. (2008). “Fibrinogen drives dystrophic muscle fibrosis via a TGFβ/alternative macrophage activation pathway”. *Genes and Development*. Vol 22. No 13. 1747-1752.

Wen HL, Lin YT, Ting CH, Lin-Chao S, Li H, Hsieh-Li HM. (2010). “Stathmin, a microtubule-destabilizing protein, is dysregulated in spinal muscular atrophy”. *Human Molecular Genetics*. Vol 2. No 9. Pp 1766-1778.

Appendix

Table 1- Muscular dystrophies and main characteristics

					Clinical features					
Disorder	**Inheritance**	**Gene mutated**	**Subcellular localisation**	**Onset**	**Motor symptoms**	**Weakness distribution**	**Cardio-myopathy**	**Respiratory impairment**	**Disease course**	**Progression**
Duchenne muscular dystrophy	X-linked recessive	Dystrophin (DMD)	Sarcolemma-associated protein	Early onset/ Childhood onset	Loss of ambulation by the age of 13 years	Proximal>distal	Severe	Severe	Progression of motor, cardiac, and respiratory signs	Dilated cardiomyopathy in almost all patients by 18 years of age. Ventricular dysrhythmias occur in older patients
Becker Muscular dystrophy	X-linked recessive	Dystrophin (DMD)	Sarcolemma-associated protein	Childhood onset/ Adulthood onset	Independent ambulation achieved, variable progression	Proximal>distal	Severe	Not frequent	Progressive with high variability	Present in 40% of patients older than 18 years and more than 80% of those older than 40 years. Most patients develop dilated cardiomyopathy followed by ventricular arrhythmias
Fasciocapulohumeral muscular dystrophy										
Type 1	Autosomal Dominant	DUX4 and chromatin rearrangement	Nuclear	Childhood onset/ Adulthood onset	Independent ambulation achieved, variable progression	Distal and oculopharyngeal	Absent	Uncommon and mild	Neurosensory hearing loss and retinal degeneration	Not well characterized
Type 2	Autosomal Dominant	SMCHD1	Structural maintenance of chromosomes flexible hinge domain	Childhood onset/ Adulthood onset	Independent ambulation achieved, variable progression	Distal and oculopharyngeal	Absent	Uncommon and mild	Neurosensory hearing loss and retinal degeneration	Not well characterized
GNE myopathy	Autosomal Recessive	ManAc kinase (GNE)	biosynthesis of N-acetylneuraminic acid (NeuAc), a precursor of sialic acids	Early adulthood	Weakness of distal muscle of the leg (foot drop). Weakness of the anterior compartment of the lower limbs and sparing of the quadriceps muscles.	Distal	Uncommon	Uncommon or mild (Until diseaase sever progression)	asymmetric foot drop or manifestations initially appearing in upper extremities and onset in the proximal leg musculature. scapular weakness (mimicking scapuloperoneal syndrome), or distal weakness of the hands with various degrees of involvement	Slolwly progressive. Average 10 years till the need to use wheelchair
Limb girdle muscular dystrophy										
Type 1A	Autosomal Dominant	Myotilin (MYOT)	Sarcolemma-associated protein (Z disc)	Adult onset	Independent ambulation achieved	Proximal>distal	Absent	Absent	Generally slowly progressive in adulthood	Severe dilated cardiomyopathy and lethal ventricular arrhythmias might occur in patients with Ducherne muscular dystrophy-like dystrophy
Type 1B	Autosomal Dominant	Lamin A/C (LMNA)	Nuclear lamina-associated protein	Early onset/Childhood onset	Independent ambulation achieved, variable progression	Proximal>distal	Severe	In adulthood	Progression of cardiac signs>motor signs	N/A
Type 1C	Autosomal Dominant	Caveolin-3 (CAV3)	Sarcolemma-associated protein	Adult onset	Independent ambulation achieved; rippling might be seen before weakness	Proximal>distal	Mild	Absent	Slowly progressive, variable	Severe dilated cardiomyopathy and lethal ventricular arrhythmias might occur in patients with Ducherne muscular dystrophy-like dystrophy
Type 1D	Autosomal Dominant	Co-chaperone DNA JB6 (DNAJB6)	Sarcolemma-associated protein (Z disc)	N/A	N/A	N/A	N/A	N/A	N/A	Severe dilated cardiomyopathy and lethal ventricular arrhythmias might occur in patients with Ducherne muscular dystrophy-like dystrophy
Type 1E	Autosomal Dominant	Desmin (DES)	Intermediate filament protein	N/A	N/A	N/A	N/A	N/A	N/A	Major cardiac signs, such as atrioventricular block, can be the presenting symptom or occur within a decade of onset of muscle weakness
Type 1F	Autosomal Dominant	Unknown	Unknown	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Type 1G	Autosomal Dominant	Unknown	Unknown	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Type 1H	Autosomal Dominant	Unknown	Unknown	N/A	N/A	N/A	N/A	N/A	N/A	N/A

					Clinical features					
Disorder	**Inheritance**	**Gene mutated**	**Subcellular localisation**	**Onset**	**Motor symptoms**	**Weakness distribution**	**Cardio-myopathy**	**Respiratory impairment**	**Disease course**	**Progression**
Limb girdle muscular dystrophy										
Type 2A	Autosomal Recessive	Calpain-3 (CAPN3)	Myofibril-associated proteins	Early onset/Childhood onset	Ambulation achieved	Proximal>distal	Absent	Not frequent	Slow progression	Severe dilated cardiomyopathy and lethal ventricular arrhythmias might occur in patients with Duchenne muscular dystrophy-like dystrophy
Type 2B	Autosomal Recessive	Dysferin (DYSF)	Sarcolemma-associated protein	Childhood onset/ Adulthood onset	Independent ambulation always achieved	Limbs, proximal and distal	Absent	Absent	Progressive in adulthood	Severe dilated cardiomyopathy and lethal ventricular arrhythmias might occur in patients with Duchenne muscular dystrophy-like dystrophy
Type 2C	Autosomal Recessive	g-sarcoglycan (SGCG)	Sarcolemma-associated protein	Childhood onset/ Adulthood onset	Independent ambulation achieved, generally lost in the second decade	Proximal>distal	Severe	Severe	Progression of motor, cardiac and respiratory signs	Severe dilated cardiomyopathy and lethal ventricular arrhythmias might occur in patients with Duchenne muscular dystrophy-like dystrophy
Type 2D	Autosomal Recessive	a-sarcoglycan (SGCA)	Sarcolemma-associated protein	Childhood onset/ Adulthood onset	Independent ambulation achieved, generally lost in the second decade	Proximal>distal	Severe	Severe	Progression of motor, cardiac and respiratory signs	Severe dilated cardiomyopathy and lethal ventricular arrhythmias might occur in patients with Duchenne muscular dystrophy-like dystrophy
Type 2E	Autosomal Recessive	b-sarcoglycan (SGCB)	Sarcolemma-associated protein	Childhood onset/ Adulthood onset	Independent ambulation achieved, generally lost in the second decade	Proximal>distal	Severe	Severe	Progression of motor, cardiac and respiratory signs	Severe dilated cardiomyopathy and lethal ventricular arrhythmias might occur in patients with Duchenne muscular dystrophy-like dystrophy
Type 2F	Autosomal Recessive	d-sarcoglycan (SGCD)	Sarcolemma-associated protein	Childhood onset/ Adulthood onset	Independent ambulation achieved, generally lost in the second decade	Proximal>distal	Severe	Severe	Progression of motor, cardiac and respiratory signs	Severe dilated cardiomyopathy and lethal ventricular arrhythmias might occur in patients with Duchenne muscular dystrophy-like dystrophy
Type 2G	Autosomal Recessive	Titin cap (telethonin) (TCAP)	Sarcolemma-associated protein (Z disc)	Childhood onset/ Adulthood onset	Independent ambulation achieved, generally lost in the fourth decade	Proximal>distal	Mild	Mild	Progressive in adulthood	Severe dilated cardiomyopathy and lethal ventricular arrhythmias might occur in patients with Duchenne muscular dystrophy-like dystrophy
Type 2H	Autosomal Recessive	Tripartite motif-containing 32 (ubiquitin ligase) (TRIM32)	Sarcolemma-associated protein (Z disc)	N/A	N/A	N/A	N/A	N/A	N/A	Severe dilated cardiomyopathy and lethal ventricular arrhythmias might occur in patients with Duchenne muscular dystrophy-like dystrophy
Type 2I	Autosomal Recessive	Fukutin-related protein (FKRP)	Putative glycosyltransferase enzymes	Childhood onset/ Adulthood onset	Independent ambulation achieved, variable progression	Proximal>distal	Severe	Variable	Progressive	Symptomatic cardiac failure over time, at a mean age of 38 years (range 18–58 years)
Type 2J	Autosomal Recessive	TitiN (TTN)	Sarcomeric protein	Childhood onset/ Adulthood onset	Independent ambulation achieved	Proximal>distal	Absent	Absent	Roughly half lose ambulation in adulthood	Severe dilated cardiomyopathy and lethal ventricular arrhythmias might occur in patients with Duchenne muscular dystrophy-like dystrophy
Type 2K	Autosomal Recessive	Protein-1-O-mannosyl-transferase 1 (POMT1)	Glycosyltransferase enzymes	Childhood onset/ Adulthood onset	Independent ambulation achieved, variable progression	Proximal>distal	Severe	Variable	Progressive	Severe dilated cardiomyopathy and lethal ventricular arrhythmias might occur in patients with Duchenne muscular dystrophy-like dystrophy
Type 2L	Autosomal Recessive	Anoctamin 5 (ANO5)	Transmembrane protein, possible sarcoplasmic reticulum	Childhood onset/ Adulthood onset	Independent ambulation achieved, variable progression	Proximal>distal	Severe	Variable	Progressive	Severe dilated cardiomyopathy and lethal ventricular arrhythmias might occur in patients with Duchenne muscular dystrophy-like dystrophy
Type 2M	Autosomal Recessive	Fukutin (FKTN)	Putative glycosyltransferase enzymes	Childhood onset/ Adulthood onset	Independent ambulation achieved, variable progression	Proximal>distal	Severe	Variable	Progressive	Symptomatic cardiac failure over time, at a mean age of 38 years (range 18–58 years)
Type 2N	Autosomal Recessive	Protein-O-mannosyl-transferase 2 (POMT2)	Glycosyltransferase enzymes	Childhood onset/ Adulthood onset	Independent ambulation achieved, variable progression	Proximal>distal	Severe	Variable	Progressive	Severe dilated cardiomyopathy and lethal ventricular arrhythmias might occur in patients with Duchenne muscular dystrophy-like dystrophy
Type 2O	Autosomal Recessive	Protein-O-linked mannose β 1,2-N-aminyltransferase 1 (POMGNT1)	Glycosyltransferase enzymes	Childhood onset/ Adulthood onset	Independent ambulation achieved, variable progression	Proximal>distal	Severe	Variable	Progressive	Severe dilated cardiomyopathy and lethal ventricular arrhythmias might occur in patients with Duchenne muscular dystrophy-like dystrophy
Type 2P	Autosomal Recessive	Dystrophin-associated glycoprotein 1 (DAG1)	Sarcomeric-associated protein	N/A	N/A	N/A	N/A	N/A	N/A	Severe dilated cardiomyopathy and lethal ventricular arrhythmias might occur in patients with Duchenne muscular dystrophy-like dystrophy
Type 2Q	Autosomal Recessive	Plectin 1 (PLEC1)	Sarcolemma-associated protein (Z disc)	N/A	N/A	N/A	N/A	N/A	N/A	Severe dilated cardiomyopathy and lethal ventricular arrhythmias might occur in patients with Duchenne muscular dystrophy-like dystrophy

Table 2- Main proteins studies as potential biomarkers for muscular dystrophies based on transcriptome results

Protein name	Gene name (UniProtKB)	Information	Disorder link	Main disorder with transcriptional evidence
Actin, alpha skeletal muscle	ACTA1	Myofibrillar protein		DMD
Aminoacylase-1	ACY1	Involved in the hydrolysis of N-acylated or N-acetylated amino acids (except L-aspartate).		DMD
Disintegrin & metalloproteinase domain-containing protein 9	ADAM9	protein associated with connective tissue remodeling. Group involved in regulation of connective tissue remodeling in skeletal muscle.	lower expressed in patients of all ages against controls	DMD
Adiponectin	ADIPOQ	Protein involved in extracellular matrix remodeling and cell proliferation. Hormone esclusevely secreted by adipose tissue.	Its increase in sera of older patients may reflect the progressive replacement of muscle by fat.	DMD
Fructose-biSphosphate aldolase A	ALDOA	Myofibril protein. Glycolytic enzyme. Glycolytic enzymes.	Highly abundant in skeletal muscle and their release into blood seems to correlate with CK	DMD
Ankyrin2	ANK2	In skeletal muscle, required for proper localization of DMD and DCTN4 and for the formation and/or stability of a special subset of microtubules associated with costameres and neuromuscular junctions Also binds to cytoskeletal proteins		DMD
Acidic leucine-rich nuclear phosphoprotein 32 family member B	ANP32B	Implicated in a number of cellular processes, including proliferation, differentiation, caspase-dependent and caspase-independent apoptosis, suppression of transformation (tumor suppressor), inhibition of protein phosphatase 2A, regulation of mRNA trafficking and stability in association with ELAVL1, and inhibition of acetyltransferases as part of the INHAT (inhibitor of histone acetyltransferases) complex. Plays a role in E4F1-mediated transcriptional repression		DMD
Carbonic anhydrase type 3	CA3	muscle-enriched protein, creatin kinase-like protein	its high to low change in concentration reflects high myofiber membrane instability/damage, necrosis and leakage of cytoplasm into the extracellular space	DMD
Calcium/calmodulin-dependent protein kinase II α	CAMK2A	muscle-enriched protein, creatin kinase-like protein.	its high to low change in concentration reflects high myofiber membrane instability/damage, necrosis and leakage of cytoplasm into the extracellular space	DMD
Calpain-6	CAPN6	Microtubule-stabilizing protein that may be involved in the regulation of microtubule dynamics and cytoskeletal organization. May act as a regulator of RAC1 activity through interaction with ARHGEF2 to control lamellipodial formation and cell mobility. Does not seem to have protease activity as it has lost the active site residues		DMD, Becker
calsequestrin 2	CASQ2	Calcium-seuqestering protein in the sarcoplasmic reticulum of heart. This isoforms may be shared with slow-twitch muscle, and the mild increases could likely reflect the general trend towards slow -twitch muscle both during regeneration and dystrophy.		DMD
Complement decay-accelerating factor	CD55		Shows a decrease with age in DMD and increases with age in controls. Functionally associated with inflammation and innate immune pathways. Initially at similar levels at a young age between patients and controls, then decrease significantly with age in DMD while increasing with age in controls.	DMD
Cadherin-5	CDH5	protein associated with connective tissue remodeling.	lower expressed in patients of all ages against controls. Group involved in regulation of connective tissue remodeling in skeletal muscle.	DMD
Cyclin-dependent kinase 14	CDK14	Acts as a negative regulator of the proliferation of normal cells by interacting strongly with CDK4 and CDK6. This inhibits their ability to interact with cyclins D and to phosphorylate the retinoblastoma protein		DMD,Becker
Cell adhesion molecule L1-like	CHL1	protein associated with connective tissue remodeling.	lower expressed in patients of all ages against controls. Group involved in regulation of connective tissue remodeling in skeletal muscle.	DMD
C-C motif chemokine 23	CKB	Monokine with inflammatory and chemokinetic properties. Binds to CCR1, CCR4 and CCR5. One of the major HIV-suppressive factors produced by CD8+ T-cells. Recombinant MIP-1-alpha induces a dose-dependent inhibition of different strains of HIV-1, HIV-2, and simian immunodeficiency virus		DMD, Becker
Creatine kinase muscle type	CKM	muscle-enriched protein, creatin kinase-like protein.	its high to low change in concentration reflects high myofiber membrane instability/damage, necrosis and leakage of cytoplasm into the extracellular space.	DMD
Contactin-5	CNTN5	protein associated with connective tissue remodeling.	lower expressed in patients of all ages against controls. Group involved in regulation of connective tissue remodeling in skeletal muscle.	DMD
Cellular retinoic acid-binding protein 1	CRABP1	Receptor for retinoic acid.		FSHD
C-X-C motif chemokine 10	CXCL10	Is an extracellular chemokine and its elevation in serum could be associated with increased T-cell infiltration in inflamed skeletal muscle.	Doesnt show a significant change as function of age. Functionally associated with inflammation and innate immune pathways. Could be an interest pharmacodynamic biomarker to monitor efficacy of anti-inflammatory agents.	DMD
Cytochrome C, somatic	CYCS	Myofibril protein		DMD
Desmin	DES	Desmin are class-III intermediate filaments found in muscle cells. In adult striated muscle they form a fibrous network connecting myofibrils to each other and to the plasma membrane from the periphery of the Z-line structures. May act as a sarcomeric microtubule-anchoring protein: specifically associates with detyrosinated tubulin-alpha chains, leading to buckled microtubules and mechanical resistance to contraction		DMD, Becker
ELAV-like protein 4	ELAVL4	RNA-binding protein that binds to the 3'-UTR region of mRNAs and increases their stability		FSHD
Beta-enolase	ENO3	Myofibril protein. Glycolytic enzyme. Glycolytic enzymes.	Highly abundant in skeletal muscle and their release into blood seems to correlate with CK	DMD, Becker
Electron transfer flavoprotein subunit alpha, mitochondrial	ETFA	The electron transfer flavoprotein serves as a specific electron acceptor for several dehydrogenases, including five acyl-CoA dehydrogenases, glutaryl-CoA and sarcosine dehydrogenase. It transfers the electrons to the main mitochondrial respiratory chain via ETF-ubiquinone oxidoreductase (ETF dehydrogenase).		DMD, Becker
Coagulation factor VII	F7	Factor VIII, along with calcium and phospholipid, acts as a cofactor for F9/factor IXa when it converts F10/factor X to the activated form, factor Xa.		DMD, Becker
Fatty acid binding protein 3	FABP3	muscle-enriched protein, creatin kinase-like protein.	its high to low change in concentration reflects high myofiber membrane instability/damage, necrosis and leakage of cytoplasm into the extracellular space	MD, Becker, LGM
Prolyl endopeptidase FAP	FAP	protein associated with connective tissue remodeling.	lower expressed in patients of all ages against controls. Group involved in regulation of connective tissue remodeling in skeletal muscle.	DMD

Protein name	Gene name (UniProtKB)	Information	Disorder link	Main disorder with transcriptional evidence
Fez family zinc finger protein 2	FEZF2	Transcription repressor. Involved in the axonal projection and proper termination of olfactory sensory neurons (OSN). Plays a role in rostro-caudal patterning of the diencephalon and in prethalamic formation. Expression is required in OSN to cell-autonomously regulate OSN axon projections. Regulates non-cell-autonomously the layer formation of the olfactory bulb development and the interneurons. May be required for correct rostral migration of the interneuron progenitors		FSHD
Fibrinogen	FGA FGB FGG	Functionally associated with inflammation and innate immune pathways	Increases with age in both DMD and controls.	DMD
Filamin C	FLNC	Myofibril protein. Is an actin cross linker protein and is located in the sarcolemma as well as in the sarcomers.	The release of these fragments into blood circulation is likely a result of proteolytic activity during muscle inflammation and necrosis.	DMD
Hepatocyte nuclear factor 3-beta	FOXA2	Constitutively active protein kinase that acts as a negative regulator in the hormonal control of glucose homeostasis		FSHD
Glyceraldehyde 3-phosphate dehydrogenase	GADPH	Myofibril protein		DMD
Growth/differentiation factor 11	GDF11	Exogebous GDF-11 can reverse age-related cardiomyopathy and skeletal muscle deterioration in mice. Candidate for ameliorating the cardiomiopathy as well as skeletal muscle deterioration.	Initially at similar levels at a young age between patients and controls, then decrease significantly with age in DMD while increasing with age in controls	DMD
Glucose-6-phosphate isomerase	GPI	muscle-enriched protein, creatin kinase-like protein.	its high to low change in concentration reflects high myofiber membrane instability/damage, necrosis and leakage of cytoplasm into the extracellular space	DMD
Alanine aminotransferase 1	GPT	Catalitic activity: L-serine + pyruvate = 3-hydroxypyruvate + L-alanine		DMD
Gelsolin	GSN		Initially at similar levels at a young age between patients and controls, then decrease significantly with age in DMD while increasing with age in controls.	DMD
Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4	HCN4	Hyperpolarization-activated ion channel with very slow activation and inactivation exhibiting weak selectivity for potassium over sodium ions. Contributes to the native pacemaker currents in heart (If) that regulate the rhythm of heart beat. May contribute to the native pacemaker currents in neurons (Ih). May mediate responses to sour stimuli		FSHD
Hepatoma-derived growth factor-related protein 2	HDGFRP2	Functionally associated with inflammation and innate immune pathways	Doesnt show a significant change as function of age.	DMD
Heat-shock 70 kDa protein 1A/1B	HSPA1A	muscle-enriched protein, creatin kinase-like protein.	its high to low change in concentration reflects high myofiber membrane instability/damage, necrosis and leakage of cytoplasm into the extracellular space	DMD
Bone sialoprotein 2	IBSP	Group involved in regulation of connective tissue remodeling in skeletal muscle.	protein associated with connective tissue remodeling.it´s lower expressed in patients of all ages against controls.	DMD
Insulin-like growth factor 2 mRNA-binding protein 1	IGF2BP1	RNA-binding factor that recruits target transcripts to cytoplasmic protein-RNA complexes (mRNPs)		FSHD
Interleukin-34	IL34	Functionally associated with inflammation and innate immune pathways	Doesnt show a significant change as function of age.	DMD
Alpha-internexin	INA	Class-IV neuronal intermediate filament that is able to self-assemble. It is involved in the morphogenesis of neurons. It may form an independent structural network without the involvement of other neurofilaments or it may cooperate with NF-L to form the filamentous backbone to which NF-M and NF-H attach to form the cross-bridges		FSHD
Jagged-1	JAG1	Group involved in regulation of connective tissue remodeling in skeletal muscle.	protein associated with connective tissue remodeling.it´s lower expressed in patients of all ages against controls.	DMD
l-lactate dehydrogenase B chain	LDHB	muscle-enriched protein, creatin kinase-like protein.	its high to low change in concentration reflects high myofiber membrane instability/damage, necrosis and leakage of cytoplasm into the extracellular space.	DMD
Lumican	LUM	Protein involved in extracellular matrix remodeling and cell proliferation		DMD
Mitogen-activated protein kinase 12	MAPK12	muscle-enriched protein, creatin kinase-like protein.	its high to low change in concentration reflects high myofiber membrane instability/damage, necrosis and leakage of cytoplasm into the extracellular space	DMD
Myoglobin	MB	muscle-enriched protein, creatin kinase-like protein.	its high to low change in concentration reflects high myofiber membrane instability/damage, necrosis and leakage of cytoplasm into the extracellular space	DMD
Malate dehydrogenase, cytoplasmic	MDH1	muscle-enriched protein, creatin kinase-like protein. Involved in aerobic energy production during muscle contraction by converting malate to oxoaloacetate and transporting the resulting NADH equivalent across the mithocondrial membrane, could be a good marker for dystrophinopathies.	its high to low change in concentration reflects high myofiber membrane instability/damage, necrosis and leakage of cytoplasm into the extracellular space	MD, Becker, LGM
Myosin 4	MYH4	Myofibril protein		DMD, Becker, LGM
Myosin light chain 3	Myl3	Myofibril protein. Myofibrillar protein .	The release of these fragments into blood circulation is likely a result of proteolytic activity during muscle inflammation and necrosis.	MD, Becker, LGM
Myomesin 3	MYOM3	Myofibril protein. MYOM3 is a component of the sarcomeres and interact with myosisns and MYL1 in striated muscle.	Its detection in blood circulation seems likely to indicate several skeletal muscle damage. The release of these fragments into blood circulation is likely a result of proteolytic activity during muscle inflammation and necrosis.	DMD
Osteomodulin	OMD	protein associated with connective tissue remodeling.Group involved in regulation of connective tissue remodeling in skeletal muscle.	it´s lower expressed in patients of all ages against controls.	DMD
Phosphoglycerate mutase 2	PGAM2	Myofibril protein. Glycolytic enzyme. Glycolytic enzymes.	Highly abundant in skeletal muscle and their release into blood seems to correlate with CK. PGAM2 was found elevated as early as in fetal stage, which makes it a candidate biomarker for neonatal screening	DMD
Pyruvate kinase isozymes M1/M2	PKM,PKM2	Myofibril protein		DMD
Phospholipase A2, membrane associated	PLA2G2A	Functionally associated with inflammation and innate immune pathways. Associated with mucsle inflammation	Doesnt show a significant change as function of age. Could be an interest pharmacodynamic biomarker to monitor efficacy of anti-inflammatory agents. Has been reported ro be dramatically increased in skeletal muscle of patients.	DMD
Plasminogen	PLG	Protein involved in extracellular matrix remodeling and cell proliferation		DMD
Persephin	PSPN	Member of the GDNF family of neurotrophic factors.Persephin signals through the RET receptor tyrosine kinase-mitogen-activated protein kinase pathway, and is known to be expressed in skeletal muscle. May be involved in the reinnervation process, as it has been observed to stimulate neurite outgrowth in oculomotor neurosn.	the increased detection of persephin and decreased detection of RET in patients could be a marker of the ongoing denervation/reinnervation that is ocurring.	DMD
Glycogen phosphorylase	PYGL	Glycolytic enzymes. PYGL breaks down glycogen to monomeric glucose molecules and controls glycogen metabolism.	Highly abundant in skeletal muscle and their release into blood seems to correlate with CK	DMD
Tumor necrosis factor receptor superfamily member 19L	RELT	Functionally associated with inflammation and innate immune pathways.	Initially at similar levels at a young age between patients and controls, then decrease significantly with age in DMD while increasing with age in controls.	DMD
Thrombospondin-4	THBS4	Muscle derived protein. Extracellular glycoprotein involved in extracellular matrix remodelling.	Its increase may be associated with muscle degeneration and regeneration.. Could be a pre-necrotic marker	DMD
Troponin 1, fast skeletal muscle	TNNI2	muscle-enriched protein, creatin kinase-like protein.	its high to low change in concentration reflects high myofiber membrane instability/damage, necrosis and leakage of cytoplasm into the extracellular space	MD, Becker, LGM
Troponin 1, cardiac muscle	TNNI3	muscle-enriched protein, creatin kinase-like protein.	its high to low change in concentration reflects high myofiber membrane instability/damage, necrosis and leakage of cytoplasm into the extracellular space	MD, Becker, LGM
Titin	TTN	Myofibril protein. Myofibrillar protein. TITIN is a component of the sarcomeres and interact with myosisns and MYL1 in striated muscle.	Its detection in blood circulation seems likely to indicate several skeletal muscle damage. The release of these fragments into blood circulation is likely a result of proteolytic activity during muscle inflammation and necrosis.	DMD
WAP, Kazal, Ig, Kunitz and NTR domain-containing protein 1	WFIKKN1		Initially at similar levels at a young age between patients and controls, then decrease significantly with age in DMD while increasing with age in controls.	DMD

Table 3- Selected biomarkers

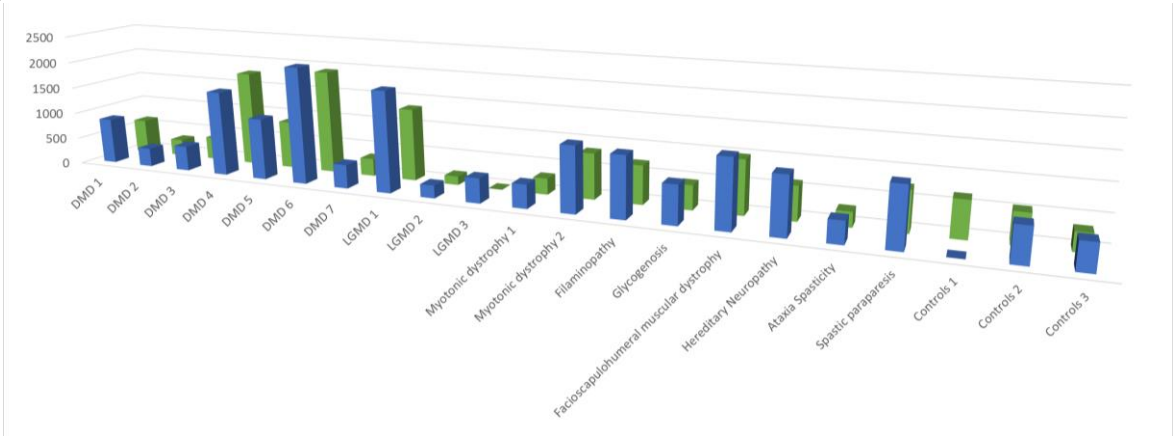
Antibody	Ab conc (µg/ml)	Gene	ENSG	Coded Protein name
HPA071834	101	ABHD14A-ACY1;ACY1	ENSG00000114786;ENSG00000243989	Aminoacylase-1
HPA035970	159	ANK2	ENSG00000145362	Ankyrin2
HPA047760	56	ANP32B	ENSG00000136938	Acidic leucine-rich nuclear phosphoprotein 32 family member B
HPA067561	92	ANP32B;ANP32A;ANP32D;ANP32E	ENSG00000136938;ENSG00000139223;ENSG00000140350;ENSG00000143401	Acidic leucine-rich nuclear phosphoprotein 32 family member B
HPA051783	101	CAMK2G;CAMK2D;CAMK2B;CAMK2A	ENSG00000058404;ENSG00000070808;ENSG00000145349;ENSG00000148660	Calcium/calmodulin-dependent protein kinase II α
HPA040383	198	CAPN6	ENSG00000077274	Calpain-6
HPA027285	245	CASQ2	ENSG00000118729	calsequestrin 2
HPA002190	55	CD55	ENSG00000196352	Complement decay-accelerating factor
HPA075875	68	CDH5	ENSG00000179776	Cadherin-5
HPA021655	196	CDK14	ENSG00000058091	Cell adhesion molecule L1-like
HPA065097	68	CDK14	ENSG00000058091	Cyclin-dependent kinase 14
HPA039492	232	CNTN5	ENSG00000149972	Contactin-5
HPA054954	64	CXCL10	ENSG00000169245	C-X-C motif chemokine 10
HPA000793	54	ENO3	ENSG00000108515	Beta-enolase
HPA068721	305	ENO3;ENO2;ENO1	ENSG00000074800;ENSG00000108515;ENSG00000111674	Beta-enolase
HPA024089	235	ETFA	ENSG00000140374	Electron transfer flavoprotein subunit alpha, mitochondrial
HPA004826	83	F7	ENSG00000057593	Coagulation factor VII
HPA063808	157	F7	ENSG00000057593	Coagulation factor VII
HPA069239	139	FABP3	ENSG00000121769	Fatty acid binding protein 3
HPA061480	170	FEZF2	ENSG00000153266	Fez family zinc finger protein 2
HPA051370	113	FGA	ENSG00000171560	Fibrinogen
HPA064755	302	FGA	ENSG00000171560	Fibrinogen
HPA001901	35	FGB	ENSG00000171564	Fibrinogen
HPA074638	116	FGG	ENSG00000171557	Fibrinogen
HPA071007	82	FLNC	ENSG00000128591	Filamin C
HPA038858	131	FOXA2	ENSG00000125798	Hepatocyte nuclear factor 3-beta
HPA069609	199	GDF11	ENSG00000135414	Growth/differentiation factor 11
HPA052171	94	GPI	ENSG00000105220	Glucose-6-phosphate isomerase
HPA031060	287	GPT	ENSG00000167701	Alanine aminotransferase 1
HPA070538	69	GSN	ENSG00000148180	Gelsolin
HPA073174	105	HCN4	ENSG00000138622	Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4

Antibody	Ab conc (μg/ml)	Gene	ENSG	Coded Protein name
HPA052504	193	HSPA1L;HSPA1A;HSPA1B;HSPA2;HSPA8;HSPA6	ENSG00000109971;ENSG00000126803;ENSG00000173110;ENSG00000204388;ENSG00000204389;ENSG00000204390	Heat-shock 70 kDa protein 1A/1B
HPA059068	132	IBSP	ENSG00000029559	Heat-shock 70 kDa protein 1A/1B
HPA065978	86	IBSP	ENSG00000029559	Bone sialoprotein 2
HPA021367	60	IGF2BP1	ENSG00000159217	Insulin-like growth factor 2 mRNA-binding protein 1
HPA055971	111	IL34	ENSG00000157368	Interleukin-34
HPA008057	88	INA	ENSG00000148798	Alpha-internexin
HPA053908	51	LHX5	ENSG00000089116	LIM/homeobox protein Lhx2
HPA068814	212	LIN28A	ENSG00000131914	LIM/homeobox protein Lhx5
HPA054562	156	MAPK12	ENSG00000188130	Mitogen-activated protein kinase 12
HPA027296	425	MDH1	ENSG00000014641	Malate dehydrogenase, cytoplasmic
HPA046859	171	MYL1;MYL4;MYL6B;MYL6;MYL3	ENSG00000092841;ENSG00000160808;ENSG00000168530;ENSG00000196465;ENSG00000198336	Myosin light chain 3
HPA016564	85	MYL3	ENSG00000160808	Myosin light chain 3
HPA028132	53	MYOM3	ENSG00000142661	Myomesin 3
HPA060457	183	NEUROD2	ENSG00000171532	Neurogenic differentiation factor 2
HPA055750	127	NEUROD4	ENSG00000123307	Neurogenic differentiation factor 4
HPA036774	128	NKX6-1	ENSG00000163623	Homeobox protein Nkx-6.1
HPA067424	248	NOTCH3	ENSG00000074181	Neurogenic locus notch homolog protein 3
HPA005731	125	OMD	ENSG00000127083	Osteomodulin
HPA003457	85	ONECUT1	ENSG00000169856	Hepatocyte nuclear factor 6
HPA042528	52	PGAM4;PGAM1	ENSG00000171314;ENSG00000226784	Phosphoglycerate mutase 2
HPA052820	96	PHOX2B	ENSG00000109132	Paired mesoderm homeobox protein 2B
HPA048823	302	PLG	ENSG00000122194	Plasminogen
HPA043178	77	PLG;PLGLB1;PLGLB2	ENSG00000122194;ENSG00000125551;ENSG00000183281	Plasminogen
HPA065013	92	PRKACA;PRKACG;PRKACB	ENSG00000072062;ENSG00000142875;ENSG00000165059	cAMP-dependent protein kinase catalytic subunit α
HPA069769	105	PRMT1;PRMT8	ENSG00000111218;ENSG00000126457	Protein arginine N-methyltransferase 8
HPA039747	103	PRMT8	ENSG00000111218	
HPA008188	63	PSMA2	ENSG00000106588	Proteosome subunit α type-2
HPA048536	164	PVALB	ENSG00000100362	Parvalbumin alpha
HPA000962	110	PYGL	ENSG00000100504	
HPA004119	200	PYGL	ENSG00000100504	Glycogen phosphorylase
HPA062824	161	RELT	ENSG00000054967	Tumor necrosis factor receptor superfamily member 19L
HPA056586	62	RPS7	ENSG00000171863	40S ribosomal protein S7

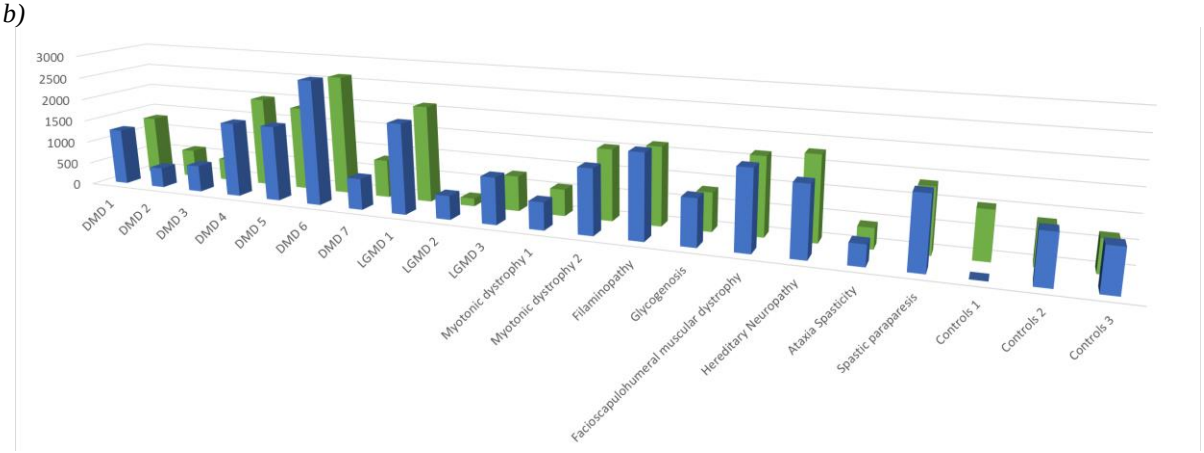
Antibody	Ab conc (µg/ml)	Gene	ENSG	Coded Protein name
HPA059733	116	SAA2-SAA4;SAA2;SAA1	ENSG00000134339;ENSG00000173432;ENSG00000255071	Serum amyloid A-2 protein
HPA055767	143	SERPIND1	ENSG00000099937	Heparin cofactor 2
HPA063683	175	SET;SETSIP	ENSG00000119335;ENSG00000230667	Protein SET
HPA055147	51	SLC18A3	ENSG00000187714	Vesicular acetylcholine transporter
HPA006903	148	SPINT1	ENSG00000166145	Kunitz-type protease inhibitor 1
HPA066783	118	ST8SIA3	ENSG00000177511	Sia-alpha-2,3-Gal-beta-1,4-GlcNAc-R:alpha 2,8-sialyltransferase
HPA026922	113	STMN2	ENSG00000104435	Stahtmin 2
HPA018927	78	THBS4	ENSG00000113296	Thrombospondin-4
HPA055938	480	TNNI2	ENSG00000130598	Troponin 1, fast skeletal muscle
HPA030048	269	TTN	ENSG00000155657	Titin
HPA012086	278	UNC5C	ENSG00000182168	Netrin receptor UNC5C
HPA063294	242	UNCX	ENSG00000164853	Homeobox protein unc-4 homolog
HPA039104	50	WIF1	ENSG00000156076	Wnt inhibitory factor 1
HPA069523	161	ZIC3	ENSG00000156925	Zinc finger protein ZIC 3
HPA041264	97	ACTA2;ACTG2;POTEF;POTEI;POTEJ;POTEE;ACTG1;ACTB;ACTC1;ACTA1;ACTBL2	ENSG00000075624;ENSG00000107796;ENSG00000143632;ENSG00000159251;ENSG00000163017;ENSG00000169067;ENSG00000184009;ENSG00000188219;ENSG00000196604;ENSG00000196834;ENSG00000222038	Actin, alpha skeletal muscle
HPA026700	55	CA3	ENSG00000164879	Carbonic anhydrase type 3
HPA000633	100	OTX2	ENSG00000165588	Homeobox protein OTX2

Figure 1. Protein abundance measurements variation with beads coupled at two different concentrations of antibodies. Blue bars represent measurements performed with beads labeled with antibodies in the presence of 1.75 μg whereas green bars represent measurements performed with antibodies in the presence of 3.5 μg. a) MDH2 0507; b) CA3 0121; c) CA3 0122; d) HPA002990-Nuclear Factor; e) HPA007370-Transcription factor; f) HPA001275-Transcription factor, g) α-albumin, h) rIgG. MFI values in Y axis

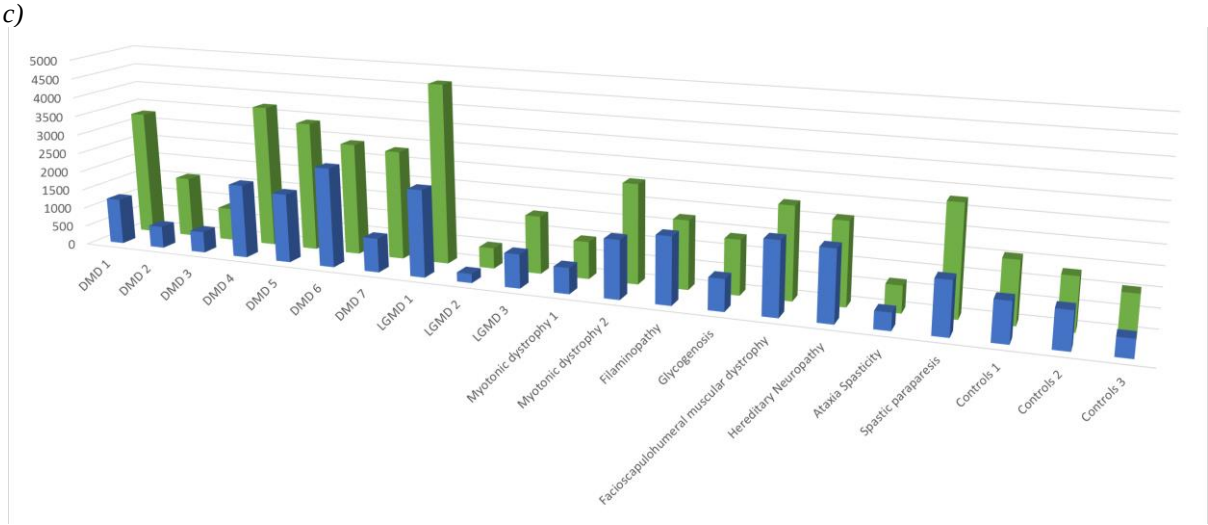
a)



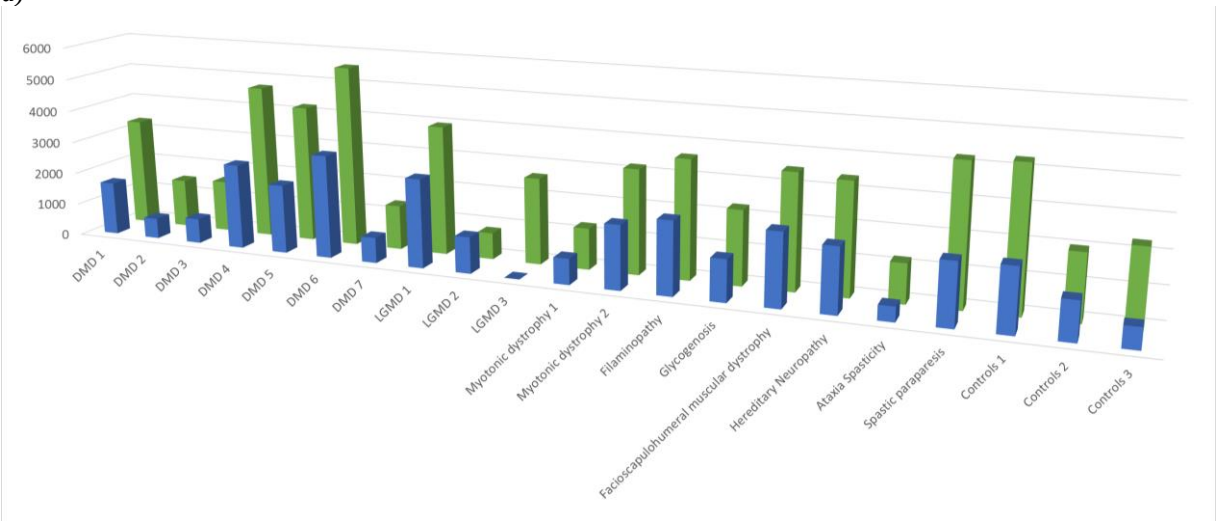
b)



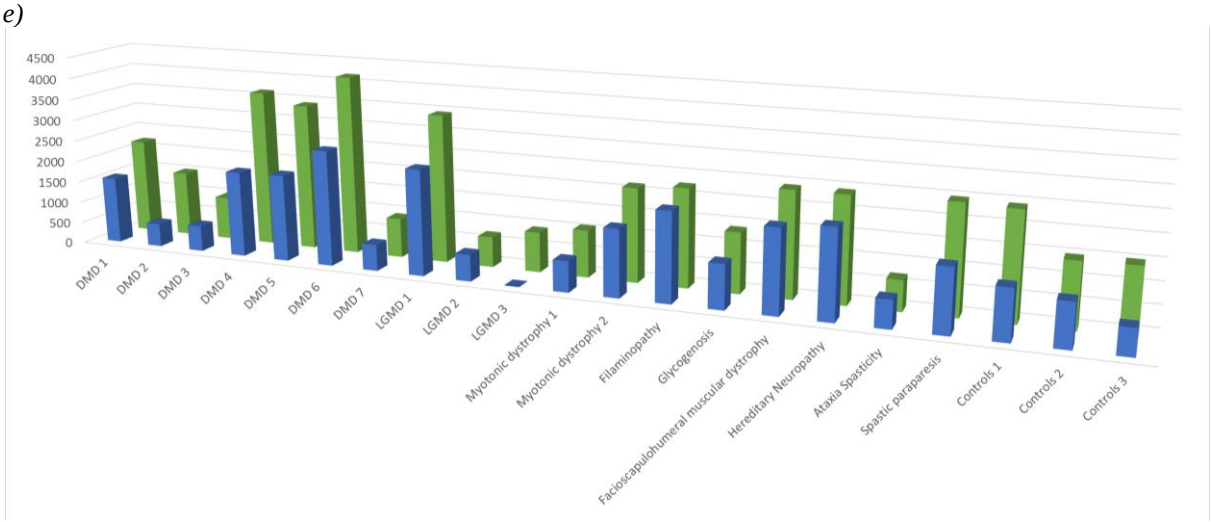
c)



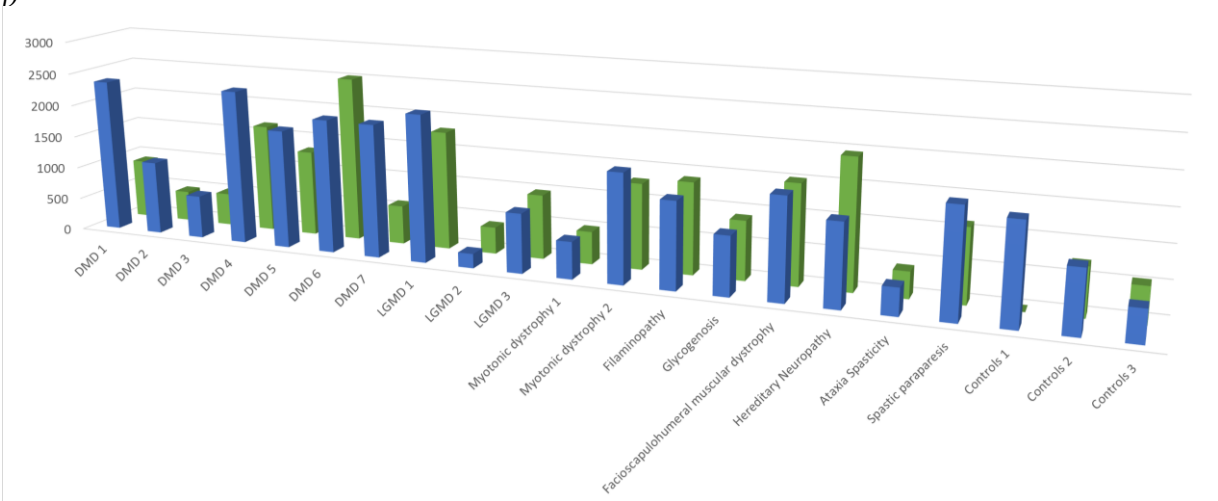
d)


6000
5000
4000
3000
2000
1000
0
DMD 1
DMD 2
DMD 3
DMD 4
DMD 5
DMD 6
DMD 7
LGMD 1
LGMD 2
LGMD 3
Myotonic dystrophy 1
Myotonic dystrophy 2
Filaminopathy
Glycogenosis
Facioscapulohumeral muscular dystrophy
Hereditary Neuropathy
Ataxia Spasticity
Spastic paraparesis
Controls 1
Controls 2
Controls 3

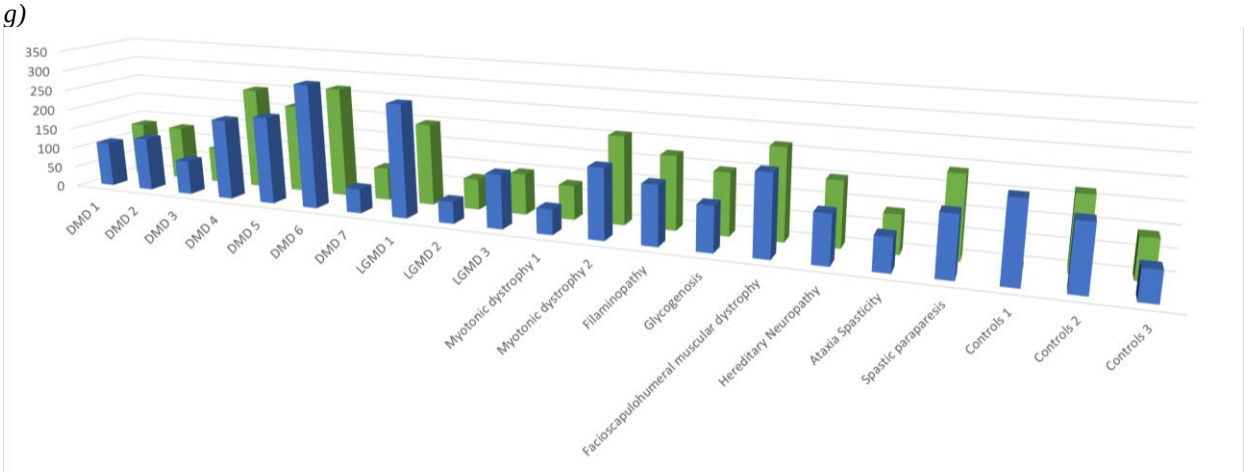
e)


4500
4000
3500
3000
2500
2000
1500
1000
500
0
DMD 1
DMD 2
DMD 3
DMD 4
DMD 5
DMD 6
DMD 7
LGMD 1
LGMD 2
LGMD 3
Myotonic dystrophy 1
Myotonic dystrophy 2
Filaminopathy
Glycogenosis
Facioscapulohumeral muscular dystrophy
Hereditary Neuropathy
Ataxia Spasticity
Spastic paraparesis
Controls 1
Controls 2
Controls 3

f)


3000
2500
2000
1500
1000
500
0
DMD 1
DMD 2
DMD 3
DMD 4
DMD 5
DMD 6
DMD 7
LGMD 1
LGMD 2
LGMD 3
Myotonic dystrophy 1
Myotonic dystrophy 2
Filaminopathy
Glycogenosis
Facioscapulohumeral muscular dystrophy
Hereditary Neuropathy
Ataxia Spasticity
Spastic paraparesis
Controls 1
Controls 2
Controls 3

g)



h)

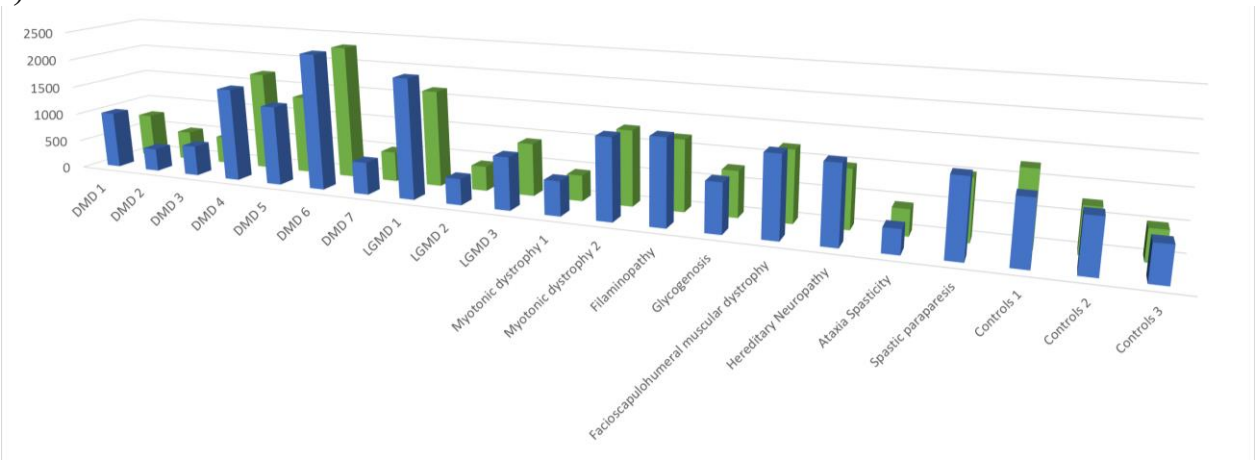
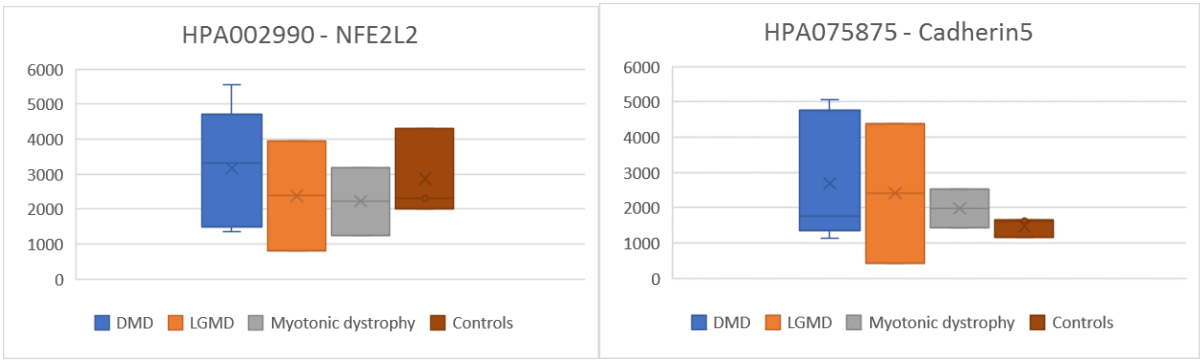


Figure 2. Protein abundance in DMD,LGMD, Myotonic dystrophic patients vs controls in serum samples. MFI values in Y axis



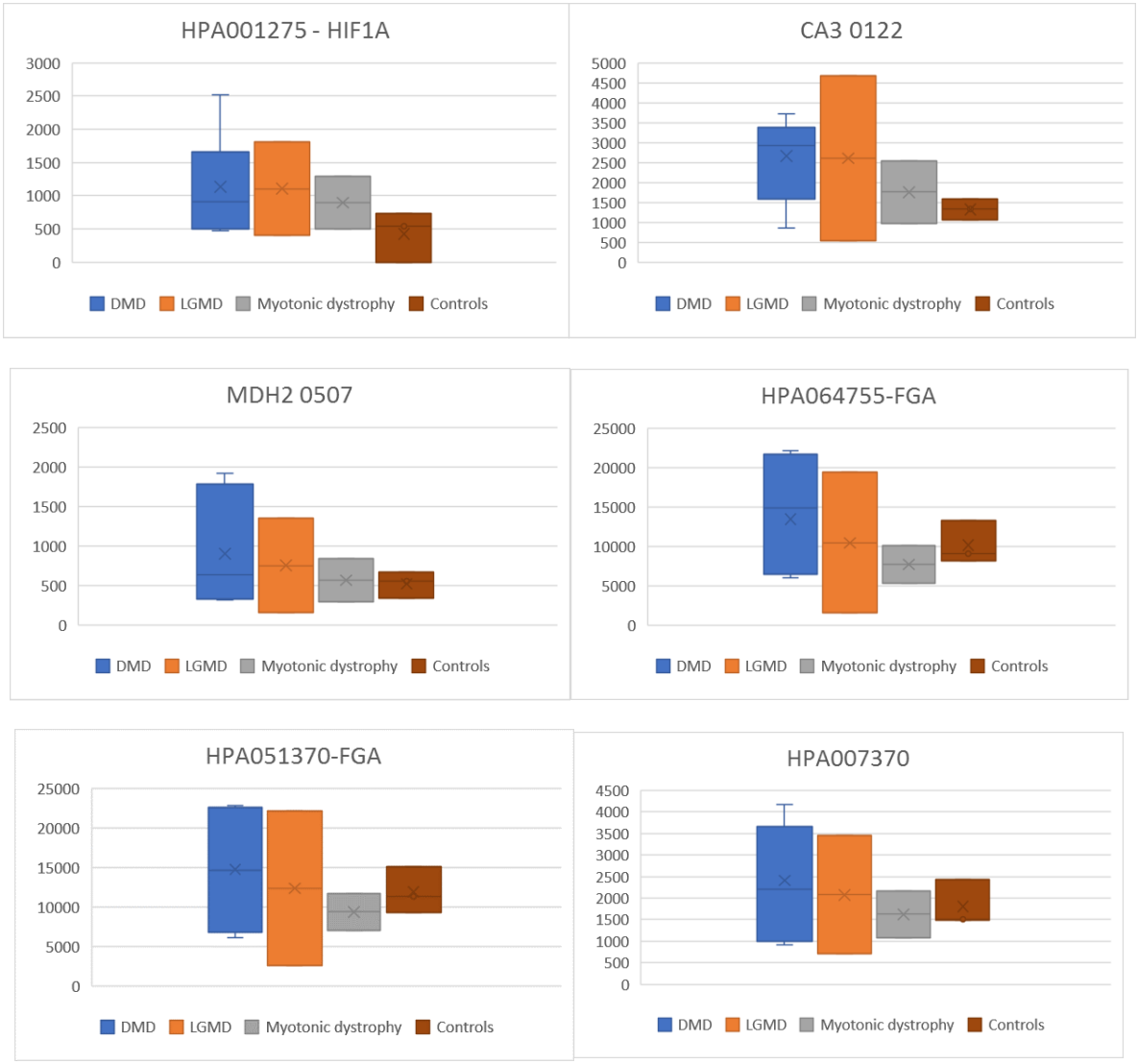
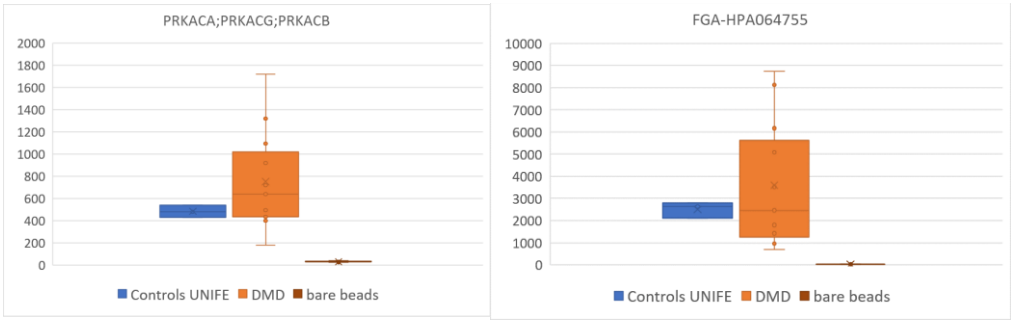
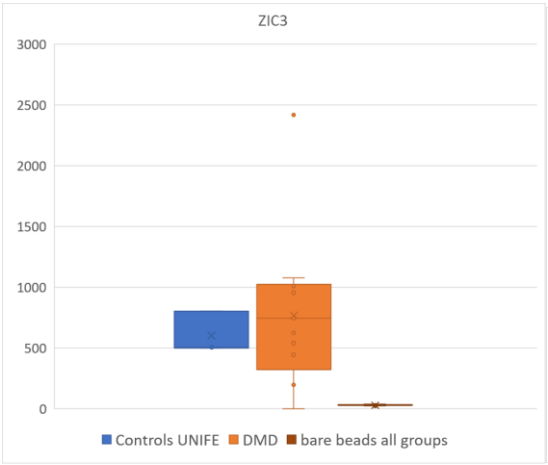
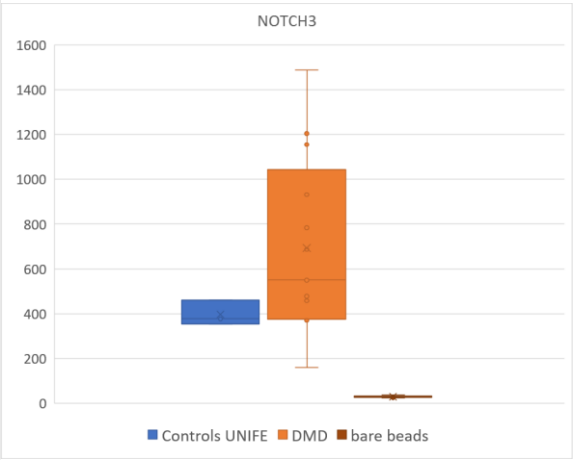
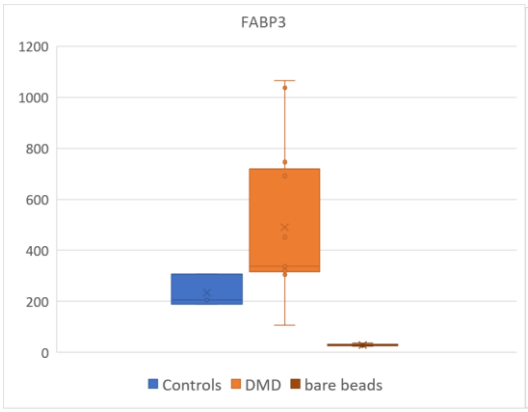


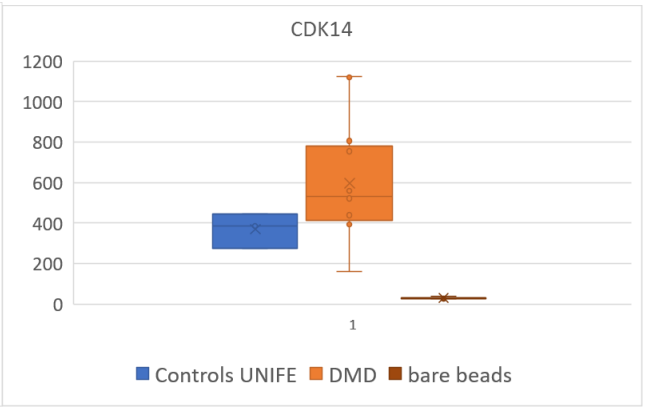
Figure 3. Targets with the highest abundance variation between DMD patients and controls.
Top candidate proteins chosen from the analysis of DMD patient serum samples and controls. Graphics of median values and distribution box plots per antibody. Controls in blue, DMD samples in orange and bare beads in red. MFI values in Y axis

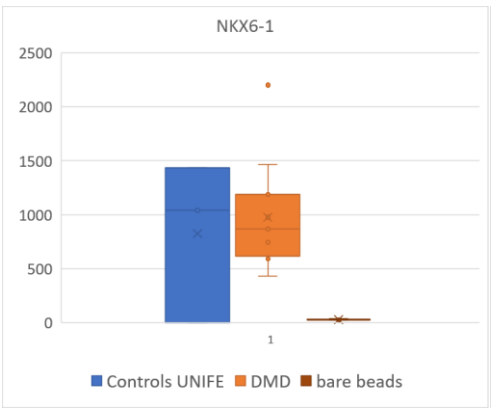



ZIC3
3000
2500
2000
1500
1000
500
0
Controls UNIFE
DMD
bare beads all groups


NOTCH3
1600
1400
1200
1000
800
600
400
200
0
Controls UNIFE
DMD
bare beads


FABP3
1200
1000
800
600
400
200
0
Controls
DMD
bare beads


CDK14
1200
1000
800
600
400
200
0
1
Controls UNIFE
DMD
bare beads


NKX6-1
2500
2000
1500
1000
500
0
1
Controls UNIFE
DMD
bare beads

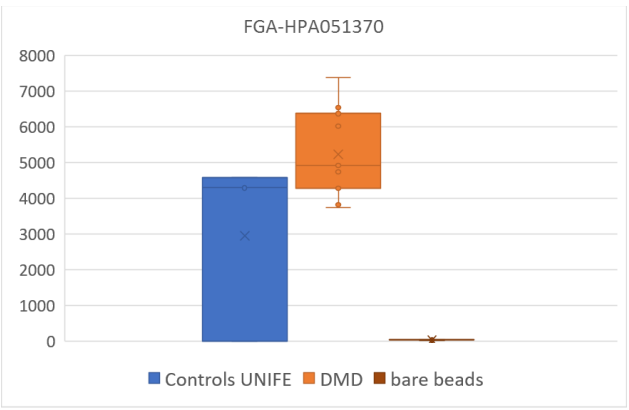

FGA-HPA051370
8000
7000
6000
5000
4000
3000
2000
1000
0
Controls UNIFE
DMD
bare beads

Figure 4. Graphic of the abundance of Fibrinogen in the UNIFE serum samples between DMD patients, controls, and other diseases samples. MFI values in Y axis

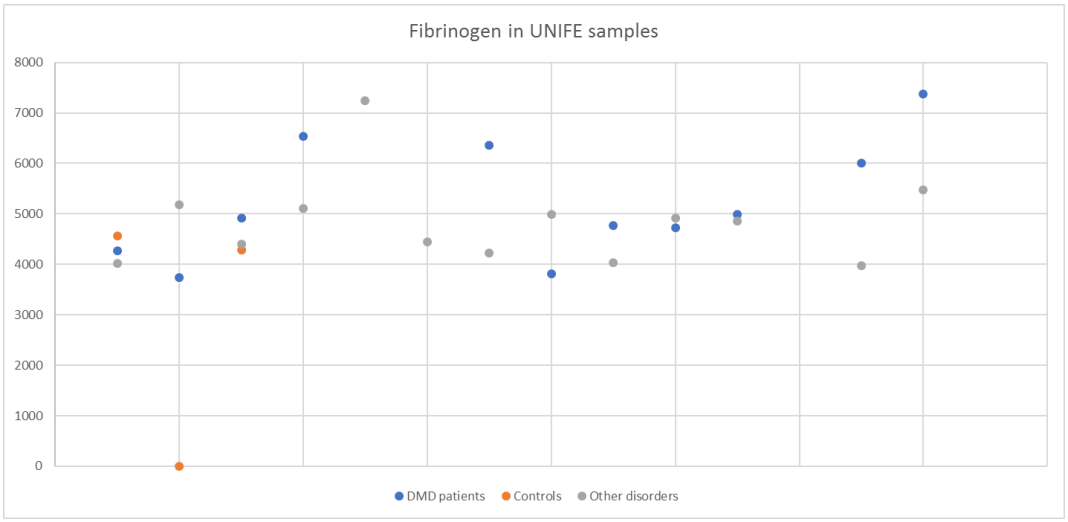
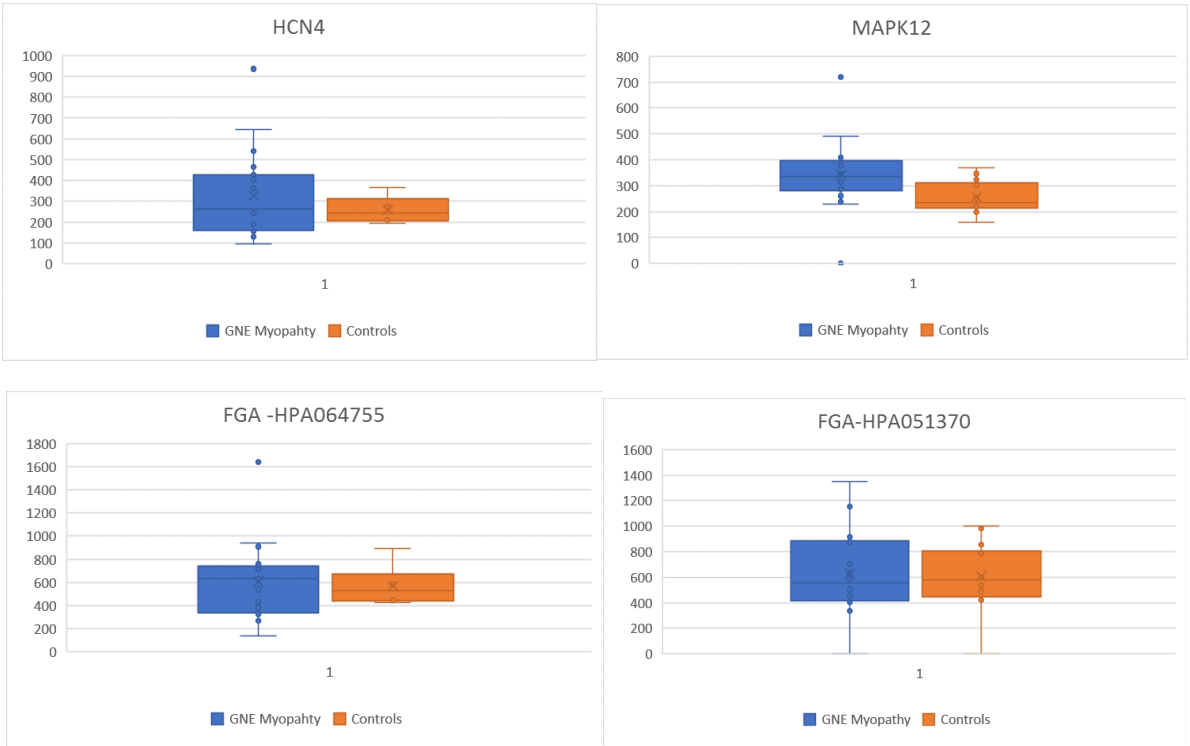


Figure 5. Abundance of fibrinogen in GNE patients in comparison to controls. Median plots and Box Plot of distribution. MFI values in Y axis



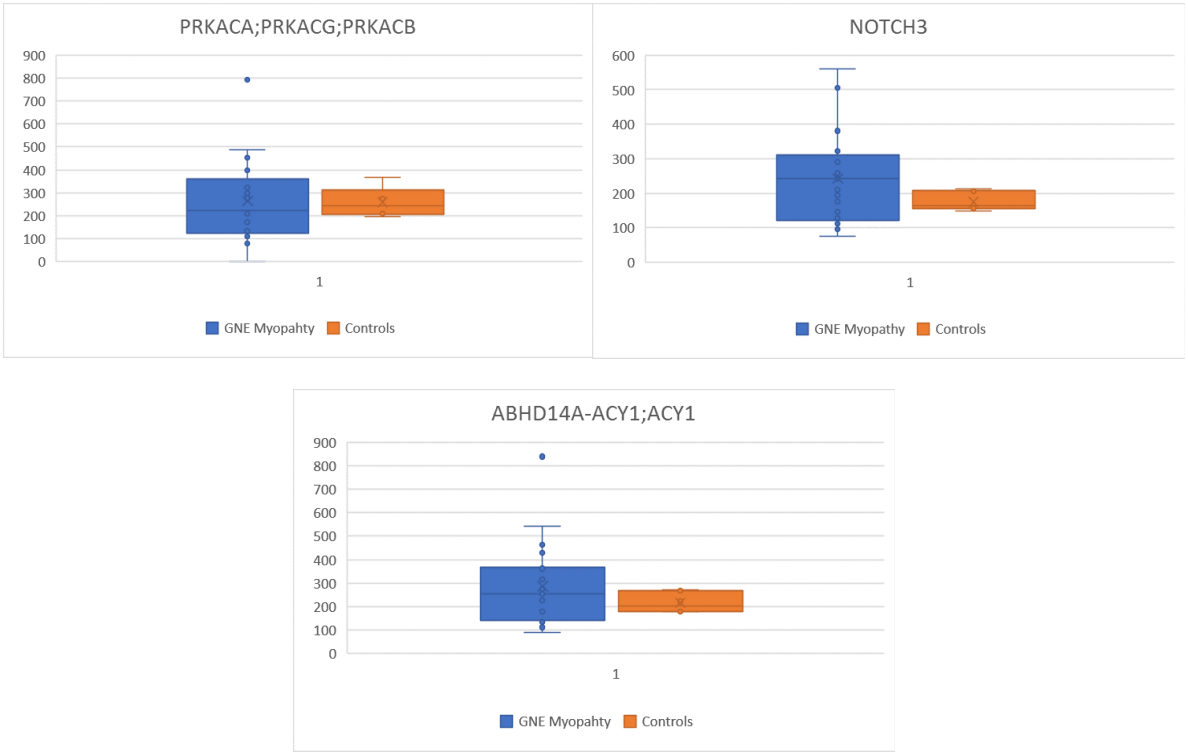
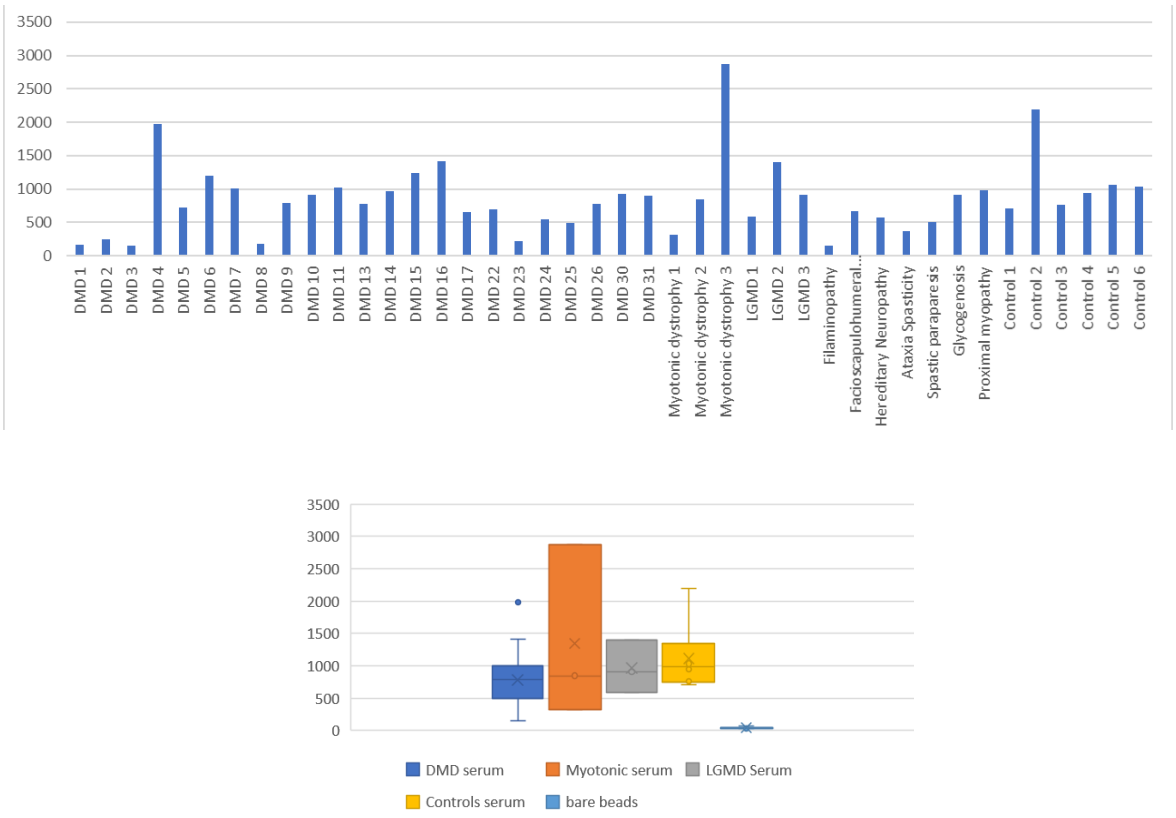


Figure 6. Graphics of analysis of HPA036774 targeting NKX6-1 in UNIFE serum samples and UCL plasma samples. MFI values in Y axis

UNIFE Serum Samples



UCL plasma samples