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DEVELOPMENT OF IMMUNOASSAYS FOR MUSCLE DIALYSIS SAMPLES FROM PATIENTS AFFECTED BY FACIOSCAPULOHUMERAL MUSCULAR DYSTROPHY

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Master of Science Thesis Report

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Abstract

Interstitial Fluid is a complex sample, highly abundant in the human body that can give information regarding tissue secretion, intracellular signaling and tissue health status. The composition of the interstitial fluid can give information regarding the processes occurring in muscles and alterations due to pathological changes occurring during disease progression. Currently this sample has not yet been characterized within rare diseases like muscular dystrophies. Facioscapulohumeral Muscular Dytrophy is an inherited progressive myopathy, characterized by the degeneration and progressive muscular fiber necrosis of muscles from the face, upper arms and lower limbs. It can be diagnosed; but in an advanced stage where weakness in the muscles have already occur. Meanwhile there is no current understanding of the mechanisms happening in the muscle. In this project an immunoassay protocol was developed using suspension bead array technology to create an optimal method to analyze the protein content of these samples. The technological platform allows antibody-based capturing and detection of protein targets from biotinylated biological samples. By modifying an existing protocol for analysis of serum and plasma samples abundance of 63 protein targets was measured in muscle interstitial fluid from healthy individuals and patients affected by facioscapulohumeral dystrophy (FSHD), The optimized steps were the sample pre-treatment, the assay buffer dilution ratio and the incubation time for capturing the protein targets. The findings of this project indicate that using 1 μl of muscle interstitial fluid sample with minimized dilution factor and 60-fold molar excess biotin relative to sample protein concentration enables detection of Interstitial fluid protein components. The proteins detected are ret finger protein-like 4B (RFPL4B) and albumin in from affected muscle and histone cluster (HIST1H3A) and albumin in non affected muscle.

Introduction

Facioscapulohumeral Muscular Dystrophy (FSHD) is a common progressive myopathy with an incidence of more than 1 in 10,000 people affected (Deenen et al. 2014). In this specific type of muscular dystrophy, the pattern of distribution for the muscle degeneration involves the muscles of the face, shoulder and upper arms (Mercuri et al. 2013). This disease is characterized by the progressive muscular fiber necrosis and degeneration leading to atrophy (Mul et al. 2016). Besides the common symptoms, patients may develop complications of the central nervous system (CNS), retinal telangiectasia and hearing loss (Sacconi, Salviati, and Desnuelle 2015). According to the symptoms, FSHD can be classified in two groups, the adult-onset and the infantile onset. In both forms (FSHD1 and FSHD2) genetic and epigenetic alterations are involved (Daxinger et al. 2015). In FSHD1, these alterations are transmitted as an autosomal dominant trait. The locus is mapped in the subteloremic region of the chromosome 4q. In this region classical genes are lacking but there is a macro satellite of repeated 3.3 kb, called D4Z4. In the general population this repeat varies among 11 and 110 while in FSHD patients have 1 to 10 repeated units. It is shown that every D4Z4 repeat contains an open reading frame that encodes for a retro gene called DUX4, which when expressed in muscle cells induces the expression of germ line genes and immune mediators, in addition to retro transposons. Due to mutations FSHD patients fail in maintaining the complete suppression of DUX4 in skeletal muscle (Yao et al. 2014). For this reason there is a common understanding that there is a relationship between the DUX4 gene and FSHD. In the case of FSHD2 the clinical phenotype has a more complex pattern of inheritance. It shows a mutation in chromosome 18p in a gene that is essential for inactivation of X chromosome (SMHD1) in addition to the permissive 4q allele (Lemmers et al. 2012). This means that genetic alteration in both forms of FSHD result in the inadequate expression of DUX4 in differentiated tissues. DUX4 expression is supressed in differentiated tissues leading to splicing of the residual DUX4 transcripts so it can remove the carboxyterminal domain; this process is associated to cell toxicity. In patients affected by FSHD the full-length DUX4 transcripts fail to supress the expression in skeletal muscle in addition to differentiated tissues leading to decreased expression of the full-length of DUX4 mRNA and protein (Snider et al. 2010).

The increased expression of DUX4 leads to an interference with myogenic differentiation, which leads to apoptotic cell death making the cells more susceptible to oxidative stress (Daxinger et al. 2015). DUX4 inadequate expression is also related to inflammation associated and muscle degeneration due to the processes that occurs as consequence of this (Frisullo et al. 2011, Hauerslev et al. 2013). Currently FSHD can be diagnosed when the patient shows symptoms of weakness of their muscles, which is confirmed with direct DNA testing. Currently muscle biopsies are not sufficient to positive diagnose FSHD. The pathological changes are not completely understood and there are altered cellular mechanisms that need further investigation (Mul et al. 2016). As other rare genetic disorder, few studies have been performed and our current understanding of the FSHD is limited.

Samples such as serum or plasma have been often used for biomarker discovery and validation for the most common disorders(Lea et al. 2011, Ayoglu et al. 2014, Qundos et al. 2014). Serum and plasma are complex

biological samples gathering biomolecules like proteins leaking from all organs (Gianazza et al 2016) . They can be obtained in a non-invasive manner from patients, and in a high quantity for further testing. These samples are being used widely in diagnosis, DNA analysis, protein quantification, biomarkers discovery and validation (reference is neede here) . However, to collect samples in the vicinity of a specific tissue the interstitial fluid can be a more suitable alternative to investigate pathological changes occurring in muscle degenerative disorders. This sample contains secreted proteins from cells located in the proximity of where the sample is collected. In the context of muscular dystrophies in which muscle groups are affected at different degree, interstitial fluid is therefore, a more appropriate sample to be analysed. Proteins and signalling molecules leaking from affected and non-affected muscle groups from the same patient can be analysed. Being mainly only in direct contact with myocytes, the samples can be very informative since biomolecules from other organs and cells are lacking in comparison to serum and plasma. Another advantage of IF is that you can analyse samples collected at different time points and study pathological changes directly. To understand biological processes involved in muscle function interstitial fluid content would be interesting to characterize since their retrieval is less painful for the patient in comparison to muscle biopsies. Another advantage is that the sample is less complex, and more prone for analysis.

Analysis of protein profiles is a viable option to map the protein content of biological samples and understand the mechanisms involved in disease progression. One of the most popular approaches used is the affinity-based proteomics, a technique that is being employed for the analysis of disorders using different body fluids as samples An example of this technique is the multiplex antibody suspension bead array, which can be used on different platforms (Schwenk et al. 2008, Uhlén 2008). The base principle of this technique is the interaction between proteins (target) and antibodies (capture) for detection of protein abundance. The method requires only microliters amount of sample and allows us to study the content of a sample and consequently generate protein profiles. The capture antibodies are coupled to unique color-coded beads, and incubated with the biotinylated sample. After incubation the analysis is performed by the array platform, where each bead is detected and captured targets analysed by a laser using a fluorescently labelled streptavidin(Ellington et al. 2010). Luminex bead array technology can be used to detect several targets depending the capture molecule that is coupled to the beads, chemicals or drugs can also be coupled to beads. Different targets can be chosen to analyse using these technology. It can be used to detect proteins, monoclonal or polyclonal antibodies, DNA from diverse species, bacterial cell wall components.

The aim of this project is to develop an immunoassay with a bead array platform for analysis for interstitial fluid samples from FSHD patients, since currently there is no established and published protocol for this type of samples. The study was performed to develop an optimal protocol for analysis of immunoassay using the suspension bead array platform. The method is based on coupling of the capture molecule to the beads that will bind the target molecule after incubation with the sample . The sample protein content is first first labelled

(biotinylation), and than diluted. After the sample is pre-treated, beads are added to the sample and there is an incubation period, following by an addition of a fluorescent agent that binds to the labelled sample. The beads with the captured proteins are subsequently detected by measuring the fluorescence intensity using the LUMINEX platform.

Materials and Methods

Sample collection

Interstitial fluid (IF) samples were collected from both patients affected by Fascioscapulohumeral Muscular Dystrophy and healthy patients that volunteered to donate their samples. Individuals were recruited based on medical examination and establishment of diagnosis. Samples were collected only from patients consenting on their participation in this study. A needle/catheter was inserted into the muscle and connected to a small hose to drain the IF from the patients. The IF was collected into small tubes at different time points after 24, 72 hours. At each time point 2 samples were retrieved from each patient, one from a lower limb muscle affected by FSHD and one from the not affected muscle. Samples retrieval was approved by the Ethics Committee of the Catholic University School of Medicine, Rome, Italy.

Target and antibody selection and coupling

Targets for the analysis were selected based on: 1. Differential expression estimated by analysis of muscle transcriptome from patients in comparison to controls, 2. Proteins involved in cellular processes affected by FSHD, 3. Biomarker associated to other muscular dystrophies. For each target antibodies were selected from the Human Protein Atlas (www.proteinatlas.org) (Uhlén et al. 2015) based on validation on protein arrays, Western blot and immunohistochemical staining. The antibodies selected were included if their concentration was 0.05 mg/ml and above. The antibodies were collected from freezer and thawed for approximately 30 min at 4°C. The antibodies were diluted to 1.75µg antibody into MES buffer 0.1 M to get a final volume of 100µL. The diluted antibodies remained stored in 4°C until the coupling was executed. Meanwhile, the plates containing the beads (Luminex MagPlex beads) were centrifuged at 2000 rpm for 1 min. A volume of 40µL of 10 beads was transferred into a 96 well plate. The beads were washed in 80µL AB (Activation Buffer- Sodium Hydrogen Phosphate 0.1 M) on magnet, following by the addition of 50µL AB into the wells. An activation solution was arranged by preparation of a 1:20 w/v NHS solution (N-hydroxy succhimide, Sigma Aldrich) and a EDC solution (N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, Sigma Aldrich) by the addition of AB . A volume of 140µL of each solution was taken and mixed with 420µL buffer to make a final concentration of 10mg/mL. An addition of 50µL of this final solution was added into each well containing the beads, followed by incubation at R.T. on plate shaker at 630 rpm. After 20 min the beads were washed in 2x100µL MES buffed on Magnet. In the meantime the diluted antibodies were centrifuged at 2000 rpm for 1 min, followed by the addition of 100µL of the diluted antibodies to the beads. A negative control was prepared by incubating beads with a single unique color code with only 100µL of MES buffer. The plate was sealed and vortexed before incubated for 2 hrs. at R.T. at 650 rpm in dark. The wells

containing beads coupled with antibodies were washed in 2x100µL PBST (PBS, 0.05% Tween 20) followed by the addition of 50µL storage buffer (90% dH_2O, 5% 10xBRE, .1% ProClin) to each well. The plates were sealed, vortexed and incubated for 2 hrs at R.T. For determining the efficiency of the coupled beads a detection antibody solution was made by diluting anti-rabbit IgG coupled to R-PE 1:200. A bead stock was made by mixing equal ratios of each antibody coupled bead. A dispense of 5 µL bead stock, was done for each well in addition of 50µL of the diluted detection antibody. The solution was incubated at R.T. dark, at 650 rpm, after 20 min the wells were washed with 3x100µL PBST 0.05%. A final addition of 100µL was made into each well before running the assay on FlexMap 3D; it was necessary to create a protocol that contains the specific beads that were taken from the Mother plates (Luminex MagPlex beads). This protocol was used in further experiments where the bead stock was from this cluster of beads.

Two bead stocks were made containing the 9 respectively 78 coupled antibodies to the beads plus a bare bead for negative control and the α-Human Albumin coupled to another bead acting as a positive control. The bead stock was performed calculating the volume needed for each bead where the final bead-count is in the range of 50 to 150 beads per 5µL bead stock.

Sample collection and pre-treatment

The IF samples were collected from freezer at -80°C together with a positive control serum sample from a LGMD patient, letting them thaw at 4°C. Protein concentration was measured using the NanoDrop platform following the standard protocol with a volume of 2µl of each sample with the parameters: Protein A280, Sample type Lysozyme, λ280. The sample pre-treatment started with a dilution of the sample with sterile and filtered in 0.45µm PBS buffer (1:7) that were placed in a well of a 96-well plate. The samples were labeled with a solution of biotin (NHS-PEG4-Biotin, Thermo Scientific) dissolved in DMSO (Dimethyl Sulfoxide, Sigma-Aldrich)(0.01 mg/µl of biotin) and diluted in filtered PBS (for every 1µL of DMSO-biotin solution, diluted in 1.65µL of PBF buffer). The volume needed for different biotin amounts was calculated with a protein concentration of 2 mg/ml and an average protein size of 70kDa. The FSHD samples were labeled with a molar excess of biotin with a 20-fold, 30-fold, 50-fold, 60-fold, 100-fold, 250-fold, 300-fold, 350-fold, 400-fold, 450-fold. The positive control samples, were labeled with a 30-fold molar excess of biotin (approximated to 20 mg/ml of protein concentration with an average protein size to 70kDa). The labeled samples were incubated for 2 h at 4°C, with a short and careful vortex and centrifugation of 2000 rpm for 1 min. The reaction was stopped by the addition of 0.5 M Tris-HCL pH 8 (4.1 µL for every 1µL of sample) and letting it react for 20 min on a 650-rpm shaker.

Sample analysis

After the samples have been labeled and the reaction stopped, the sample plate was centrifuged at 2000 rpm for 1 min. An assay buffer containing 90% of PVXCasein (0.1% casein Sigma Aldrich, 0.5% PVA Sigma Aldrich dissolved in PBS), 10% rabbit IgG and 1% ProClin was prepared. 50µL of the buffer was mixed with 1µL of labeled sample. The

diluted samples were heat-treated at 56°C for 30 min followed by a cooling at 23 °C for 10 min. A distribution of 5µL of bead stock was performed into each well containing diluted samples. The plate was sealed, vortex and incubated overnight, R.T., 650 rpm dark.

After the overnight incubation the assay plate was washed with 3x60µL with PBST 0.05% on magnet followed by the addition of the Streptavidin R-phycoerythrin (SAPE) solution. The SAPE solution was prepared by mixing it with PBST 0.05% with a concentration of 0.0013µg/ml The plate was sealed, vortexed and incubated for 20 min R.T. 650 rpm, dark. After the incubation time the plate was washed with 3x60µL with PBST 0.05% on magnet followed by an addition of 60 µL PBST 0.05%. After this treatment the samples were ready and the plate was set for the assay run on the Luminex platform selecting the desired protocol for the beads to analyze. The laser in the Luminex platform detects the fluorescence intensity of the each of 50 to 100 beads; each of them passes through a capillary tube where the fluorescence intensity of each bead is detected by a laser. The results obtained from the platform are the Mean Fluorescence Intensities, which is the mean obtained from all the detected signals of the beads. This data was the one taken in consideration for the results analysis. The data analysis has been performed by plotting for each analyzed target MFI.

Results

Every human has a volume of ~ 20% of bodyweight of Insterstitial Fluid (IF) (Wiig and Swartz 2012). Due to this high abundance in the body as well as its location through the body it is an optimal samples to investigate tissues without retrieving biopsies. Being in direct contact with cells IF is a samples that can provide local information regarding tissue secretion, health status or cell damage. The interstitial fluid is a complex biological sample that contains water, solutes and structural molecules of the interstitial matrix as well as secreted proteins and proteins leaking from cells as a consequence of cell damage. It is an important sample since it contains nutrients and waste products from the surrounding cells. It may also transport antigens and cytokines for immune regulation depending the organs or tissues where it is located. IF as a biological sample has not yet thoroughly characterized and remains largely unknown, resulting in no published protocols for analysis of biomolecules content of IF. This sample gives a lot of information of the tissue and is less complex compared to serum and plasma, it might not contain cells and it contains a lower protein concentration. IF compared to serum and plasma can be retrieved in only small quantities (µL) compared to the larger quantities that can be obtained of serum and plasma (ml).

The suspension bead array technology has been selected for the analysis due to he high throughput it has regarding targets and sample analysis, the allowance of performing the test with few sample, and the principle of immunocapture between proteins and antibodies that allow to detect proteins in low abundance in the sample. This technology represents a viable and suitable platform for testing the IF samples because it can detect proteins that are in low concentration and presents a high throughput considering the targets per sample and the samples per run, it can be analyzed up to 96 targets in a single sample and up o 96 samples in one run. It also allows the use of minimal sample (1µL) to perform the experiment.

Study design

Based on former established protocols for plasma, serum and cerebrospinal fluid analysis (Häggmark et al., 2013) several steps were modified and tested with the objective of developing an optimized protocol of the immunoassay and perform protein profiling of IF using the suspension bead array platform (Schwenk and Nilsson 2011). The protocol includes the following steps direct sample labeling with biotin, bead- antibody coupling, heat-treatment of the biotinylated sample, immunocapture of the proteins with the antibodies coupled to the beads and analysis using a LUMINEX instrument (Figure 1). Protein concentration of IF samples was measured prior the analysis to evaluate the feasibility in analyzing the protein content using immunoassays. Based on protein concentration measurements several steps in the analysis were identified to be suboptimal. The protein

abundances in the samples were estimated as mean fluorescent intensity to assess the viability of creating the protein profiles utilizing the multiplex antibody-based technique.

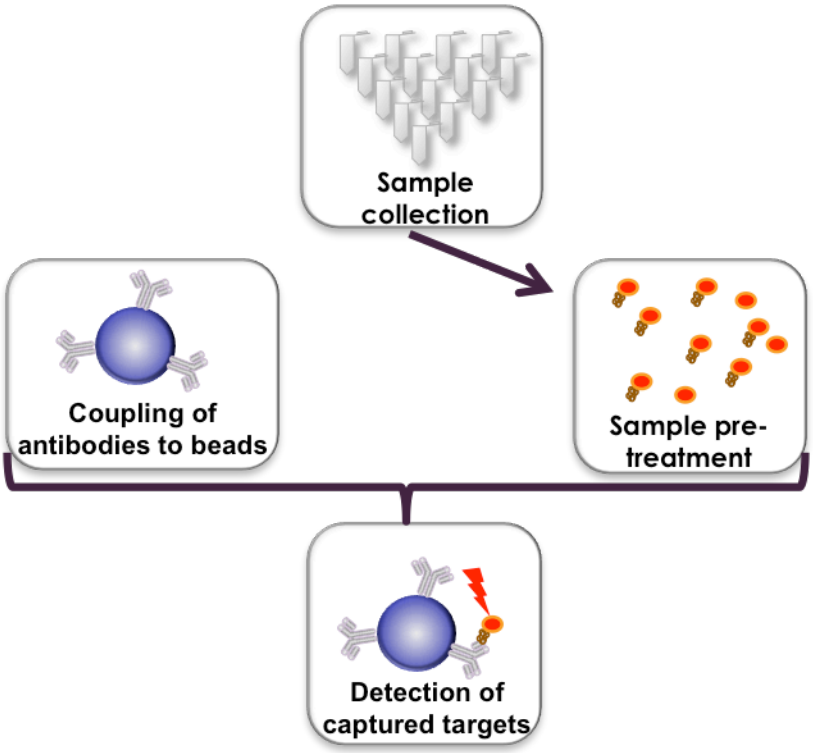

Figure 1. Assay protocol of the study used in this project. Steps involved in the protocol are the sample collection, the biotinylation of the sample, the antibody-coupling to the beads, and the detection of the captured targets once the immunocapture has happened between the labeled sample and the coupled- antibodies to the beads.

The tests were first made for a small set of protein targets, ten to optimized the protocol, followed by a second set of protein targets containing 80 different antibody coupled beads.

Protein Concentration

In comparison to serum and plasma IF is a less complex sample, devoid of certain components like blood cells. The protein concentration was measured for all the FSHD samples as well as for the controls prior to initiation of the study to ensure that protein concentration is high enough to enable detection of proteins (Table 1). The protein concentrations were considerably lower; ten fold lower, than plasma and serum protein concentration previously measured (≈ 20 mg/ml). This indicates the need of minimizing dilution of the sample and optimization of the biotinylation step. Even though there were twelve available samples to test including controls, the main samples used in the experiments were the positive and negative samples with a time after insertion of 24.28 hours, and the controls with the similar time after insertions. The samples used were from Patient 1, p7-G, p7-7; the samples from

patient 2, p12-7, p12-G, the controls of Patients 4 and 5, p13-8 and p14-8. These samples were analyzed through the whole project testing different parameters of the steps involved in the protocol.

FSHD affected muscle				FSHD not affected mucle				Healthy Controls			
Patient	**Sample**	**Protein Contentration (mg/ml)**	**Time After Insertion (hrs)**	**Patient**	**Sample**	**Protein Contentration (mg/ml)**	**Time After Insertion (hrs)**	**Patient**	**Sample**	**Protein Contentration (mg/ml)**	**Time After Insertion (hrs)**
P1	p7-G	1.5	24-28	**P1**	p7-7	1.5	24-28	**P4**	p13-8	1.6	24-28
P1	p7-H	1.26	28-36	**P1**	p7-8	1.2	28-36	**P4**	p13-9	1.49	28-33
P2	p12-6	2.02	24-28	**P2**	p12-F	2.22	24-28	**P5**	p14-8	1.7	23-27
P2	p12-7	2	28-35	**P2**	p12-G	1.9	28-35	**P5**	p14-9	2.05	27-35
				P3	p16-AE	1.6	96-100				
				P3	p16-AF	1.73	100-108				

Table 1: FSHD samples from patients affected and healthy patients. Samples from affected and non affected muscles were obtained from the same patients. Different samples were recovered at varying times referring at the Time After Insertion. Samples from healthy volunteers were recovered at different hours.

Based on the protein content of IF samples, main steps to be modified and tested were identified: (1) the sample labeling, (2) the dilution factors and (3) the incubation for the immune-capture of targeted proteins. In the labeling step, biotinylation was performed in the presence of different concentrations of biotin to ensure labeling of the proteins. Assay volumes were minimized to avoid unnecessary dilution of the sample and several incubation times were tested for capturing the protein targets on antibody labeled beads. There were no changes in the antibody-bead coupling protocol.

Protein Targets and Antibodies Selection

To select protein targets to be analyzed, different strategies were considered. Protein targets were selected based on the differential gene expression in affected patients in comparison to healthy individuals, proteins expressed in muscle tissue and proteins involved in FSHD relevant processes like necrosis and inflammation. The first approach

was to analyze already existing RNA sequencing data for FSHD patient samples and healthy individual in order to screen for differentially expressed genes. The genes considered for the final target list were genes with differential expression of at least 2 fold change and p-value over 0.5. The highest 40 expressed genes in FSHD and the 40 less expressed genes in patients were considered and revised in a genome browsing online database, Ensembl (www.ensembl.org) that would help validate the genes found in the RNA sequencing. Targets that had no information in the database were removed from the final list. Another strategy was to perform a literature study from where 20 proteins were chosen taking in consideration proteins involved in inflammation processes and proteins expressed from certain genes involved in FSHD. In addition a list of proteins of interest selected for clinical and diagnosis purposes (communicated by our collaborator Giorio Tasca). After compiling the gene list proteins with transmembrane regions were excluded to ensure detection of soluble proteins. In total 165 genes were included in the list.

Availability of the antibodies for the analysis was investigated to select validated and target specific antibodies for the analysis from the Human Protein Atlas repository (Uhlén et al. 2015). For this, several aspects were taken into consideration; that the antibody passed a protein array test, the ability to recognize the specific target on Western Blot and the antibody concentration is higher than 0.05mg/ml. A final list of 79 targets and corresponding 84 antibodies were used in the experiment (Supplementary Table 1).

Antibody Coupling

Two sets of antibodies were coupled to different sets of beads one for initial optimization and one for analysis of protein abundance. A coupling efficiency test (Fig. 2) was performed to evaluate the efficacy of both sets of beads to ensure successful immobilization of antibodies on the magnetic beads. This can be tested due to the fact that the antibodies coupled to the magnetic beads were purified polyclonal antibodies raised in rabbit; for this reason, a solution containing fluorescent anti-rabbit IgG is added to the beadstock with a dilution of the beadstock of 1:5 to the solution of IgG. With these procedure detects the quantity of antibodies that have been coupled to each of the beads. The test showed an optimal MFI (Mean Fluorescence intensity) with signals above 1000 MFI and the negative control (bare bead) close to zero. The second set of antibodies coupled had similar coupling efficiency with high MFI results (Supplementary Figure 1). All bead with coupled antibodies had signal intensities above noise level.

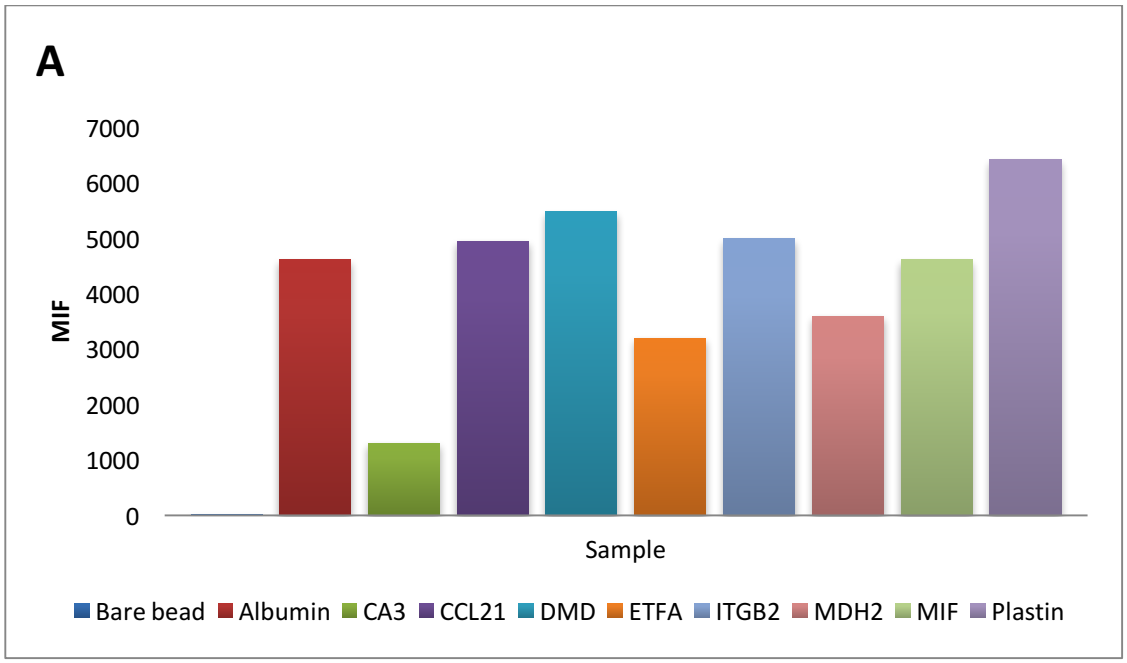

Fig. 2: Results obtained from the CEF (Coupling Efficiency Test) perfomed to the first set of antibody-bead coupling.

Optimization of the protocol

Immunoassays for detection of proteins using the LUMINEX instrument have been developed for serum, plasma, cerebrospinal fluids e.t.c. The IF samples contain approximately 2mg/ml protein amounts and resembles more the cerebrospinal fluid samples. To ensure that the proteins are detectable, the protocol for analysis of CSF was used and further modified. For each with modified parameters, test samples were incubated with beads and mean fluorescent intensities measured. Along with the Interstitial Fluid samples, there were a positive controls tested, in some of the tests a LGMD sample and a DMD were added to the test. In some of the cases only the sample from LGMD disease was used as a positive control. Mean fluorescent intensities were plotted for each protein in each sample. To facilitate comparison of obtained signals all following graphs have the Mean Fluorescent Intensity scale set to 0-2000.

The first parameter to be modified is the dilution of the sample prior to and during the analysis. The samples are diluted prior to the addition of biotin, biotinylated and subsequently the labeling reaction stopped by adding Tris-HCL. Prior to the detection of protein abundance the samples are again diluted due to addition of fluorescently labeled streptavidin. The sample dilution used was changed to 1:2 from 1:7 in comparison to in the initial protocol (Häggmark et al., 2013). The tests were performed first with the small set of antibodies and signals detected for 9 protein targets, Albumin, Plastin, Carbonic anhydrase 3 , Electron transfer flavor protein A, Malate dehydrogenase 2, Macrophage migration inhibitory factor, C-C motif chemokine 21, Dystrophin and Integrin beta-2. In Figure 3 it is shown that the intensities of Plastin were higher compared to the negative control (Bare Bead) in the samples belonging to Patient 1- and Patient 2+. There are peaks also for Human Albumin present in both control sample and a smaller peak for Patient 2+. There is also higher intensity for CCL21 for P2+ and CNTRL1. Although there are

signals detected for some of the proteins when the dilution is decreased the signals are not as high as expected which raises a question if the labeling of the sample is optimal. Only adjusting the dilution in the labeling step is not sufficient and modifications in the further steps need to be considered.

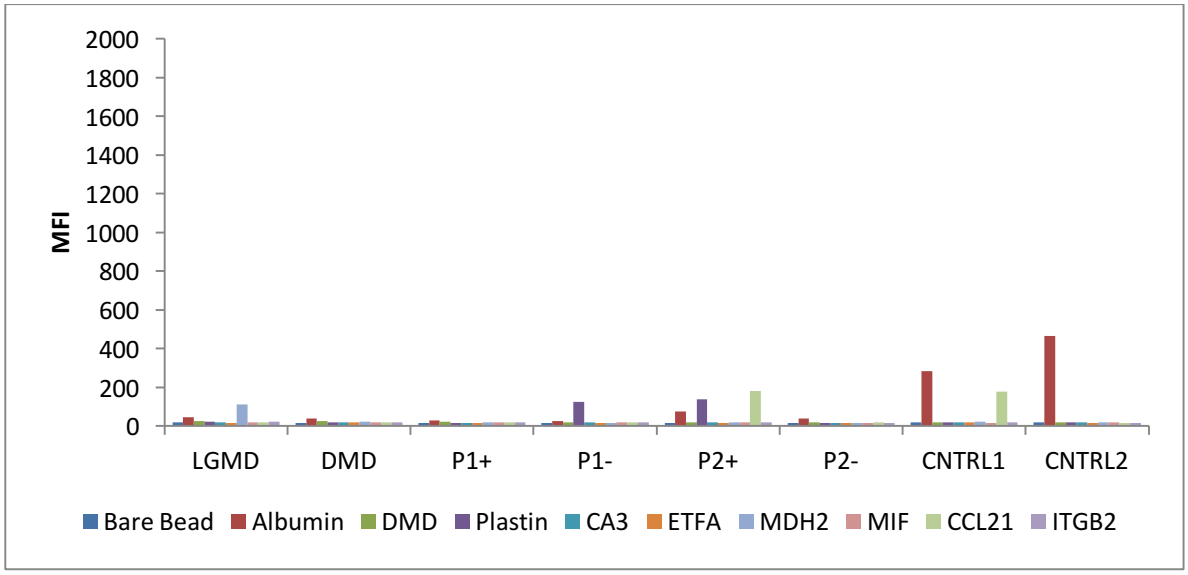

Figure 3: Results obtained when the dilution factor was modified by 1:2 during the labeling step for the first set of antibodies coupled to the beads. The samples tested were from LGMD and DMD patients acting as positive controls, and FSHD samples from two patients of both healthy and affected muscle as well as for two control patients active as negative control.

Biotin Fold

The original protocol developed by Schwenk et al. 2006 was optimized for biotin labeling of serum and plasma (Schwenk et al. 2008). The excess of biotin over the content of protein of the samples were adjusted and tested to assess the suitable fold for interstitial fluid samples. The folds tested were 20, 30, 40, 50, 60, 100, 300, 350, and 400. The biotin fold was tested on the first set of antibodies coupled to the beads. Figure 4 shows that fold 60 suits the best for this sample where intensity over 2000 was obtained on CCL21. As seen in the figure with a fold of 30 and 100 no high intensities were obtained from the samples. Similar results were obtained with the folds 20, 40, 300 , 200 and 400, where the intensities were not as high as the obtained with the 60-fold.

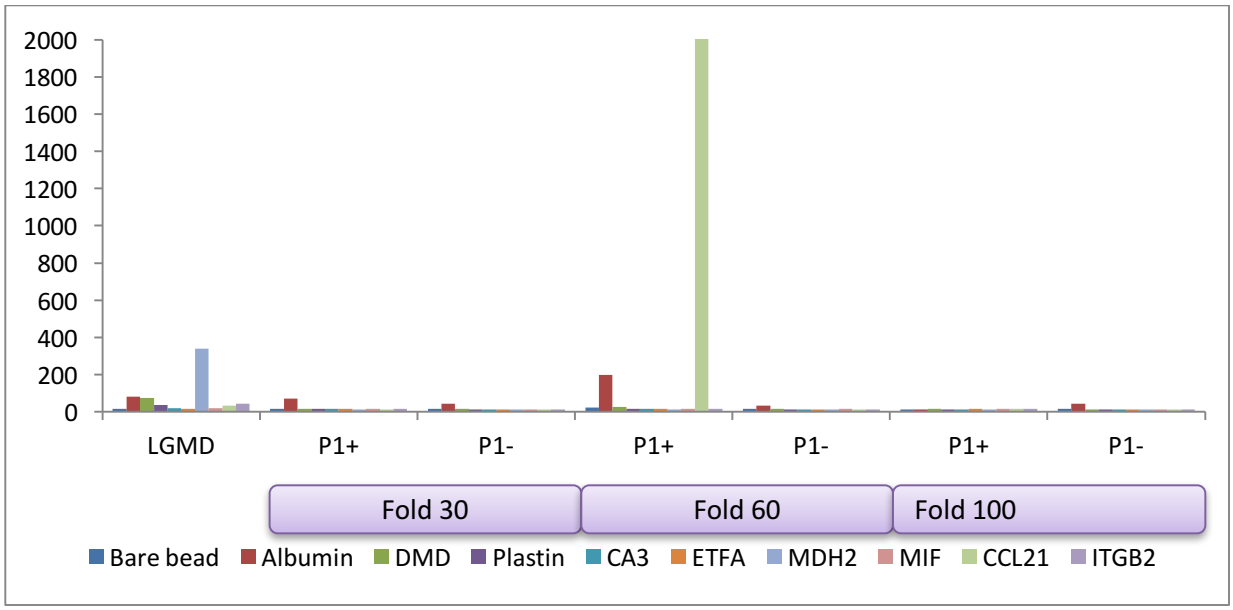

Figure 4. Results obtained from the test modifying the biotin fold. The test was performed with the first set of beads and the two different samples from the same patient, from the healthy and the affected muscle.

Sample dilution and heat-treatment

To effectively use the labeled sample dilution with assay buffer (PVX casein buffer + 10% rabbit IgG + .1% ProClin) after labeling and prior to the detection step was modified. The protocol stated that for each microliter of labeled sample an addition of 50µL of the assay buffer should be added for detection of captured proteins on antibody labeled beads. The dilution of labeled samples was modified since the protein concentration is low. Addition of different volumes of the labeled samples, with decreasing dilution rate, were tested The volumes tested were 1µL, 2µL, 4µL, 5µL, 8µL and 10µL. On the other, hand the heat-treatment remained unchanged, non the temperature or time were modified. Figure 5 shows the results obtained when testing 1µL and 10µL of labeled sample. As the figure shows, the signals obtained with subsequent the labeled sample with a total dilution rate of 1:50 were higher than the ones obtained when testing dilution rate of 1:5 . Other tests performed with different volumes of labeled samples showed that there is a decrease in the signals while testing larger volume of the sample with the assay buffer. This can be due to the fact that there are residual buffers from the biotinylation step or unbound biotin itself that interfere with the immunoassay. This could lead to a difficulty in detection of the captured biotinylated protein and decreased signals. The high signals obtained from the test and shown in the figure are in Patient 1 in the positive sample, internexin neuronal intermediate filament protein (INA), ret finger protein-like 4B (RFPL4B), Zic family member 3 (ZIC3) and lin-28 homolog A (LIN28A) while in the patient 2 there is no signal detected in the positive sample. While in the negative sample. Comparison of signals detected shows that ret finger protein-like 4B (RFPL4B) is detected in both patient samples from on-affected muscle. RFPL4b is also detected in Control 2. In control 1 there are no signals detected.

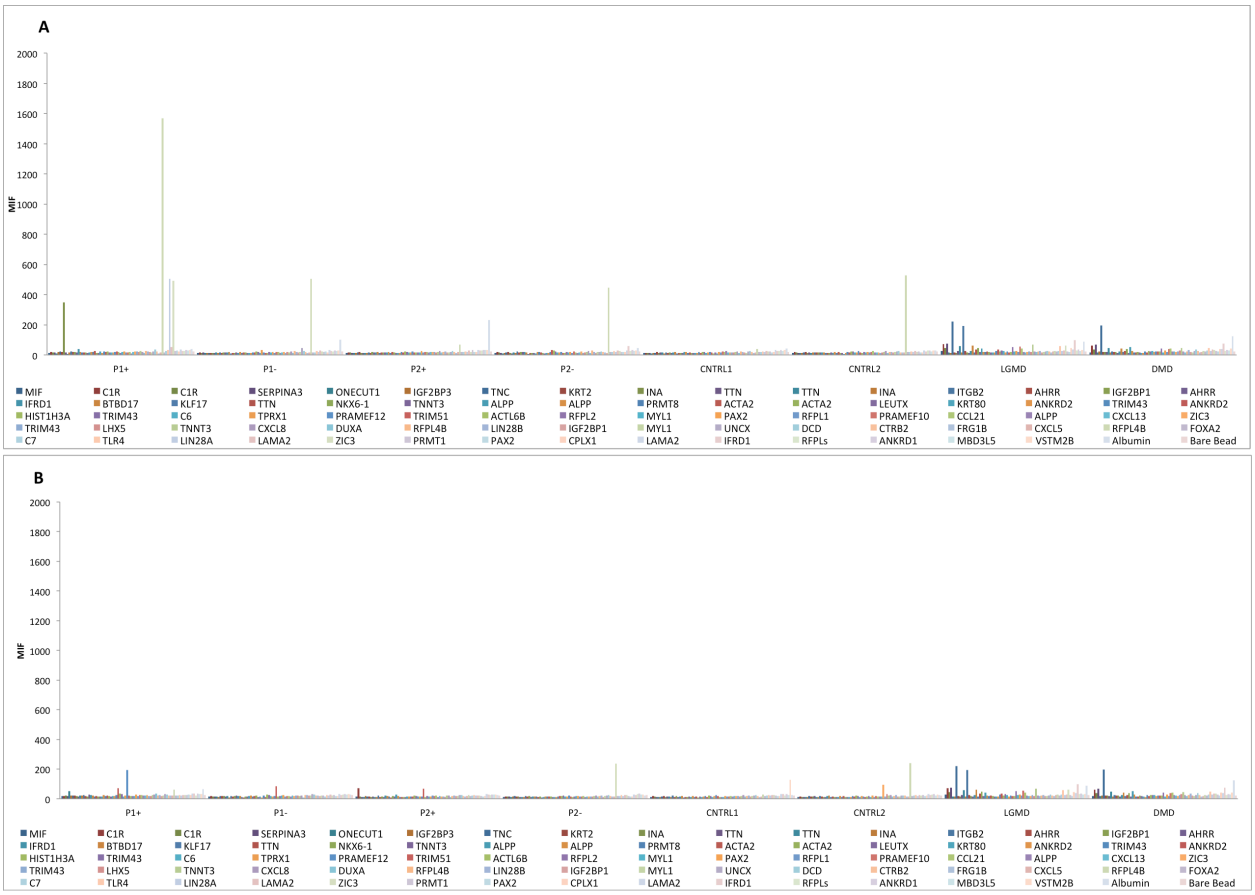

Figure 5. MFI obtained from the test where the dilution with the assay buffer was modified. (A) Results obtained from the established dilution factor of 1µL for every 50µL of assay buffer. The samples of four patients were tested, two different patients from both, healthy and affected muscle, in addition of two control patients. (B) Results obtained from testing 10µL of labeled sample with a final volume of 50µL. The test was performed with the second set of antibodies coupled to the beads and for two patients from both, healthy and affected muscle, plus two control patients.

Capturing of biotinylated proteins on beads

After the heat-treatment of the diluted labeled samples the antibodies coupled to the beads are allowed to the proteins in the samples. This capturing step was also modified and incubation times 2 hours, 4 hours, 6 hours and an overnight incubation (O/N) were tested. Figure 6 shows the experiments performed with protein capturing incubation times of 6 hours (Figure A) and overnight Incubation (Figure B), the results show that six hours incubation the signals are higher than a overnight incubation. The results from the two and four hours incubation showed lower signals that the ones obtained from the six hours incubation. The proteins with high signals in both FSHD samples from affected muscle are RFPL4B and Albumin. Signals for RFPL4B are detected in both negative samples but are not as high as in the positive samples in patient (P1) and higher in the second patient (P2). Albumin is more abundant in positive samples than in the negative samples of both patients and the controles. Histone H3.1 is detected (HIST1H3A) in both patient P1 and P2 in not-affected samples but not detected in the positive samples. In Patient 1 of the negative sample there is a signal of alpha-1-antichymotrypsin (SERPINA3), while in Patient 2 there is no signal of these protein but a presence of ret finger protein-like 4B (RFPL4B). In the negative control samples there are also variations of the proteins present in both samples, in Control 1 there are

signals of ankyrin repeat domain 2 (ANKRD2), ret finger protein-like 4B (RFPL4B) and Albumin while in the Control Patient 2 there is a presence of serpin peptidase inhibitor (SERPINA3), and Albumin.

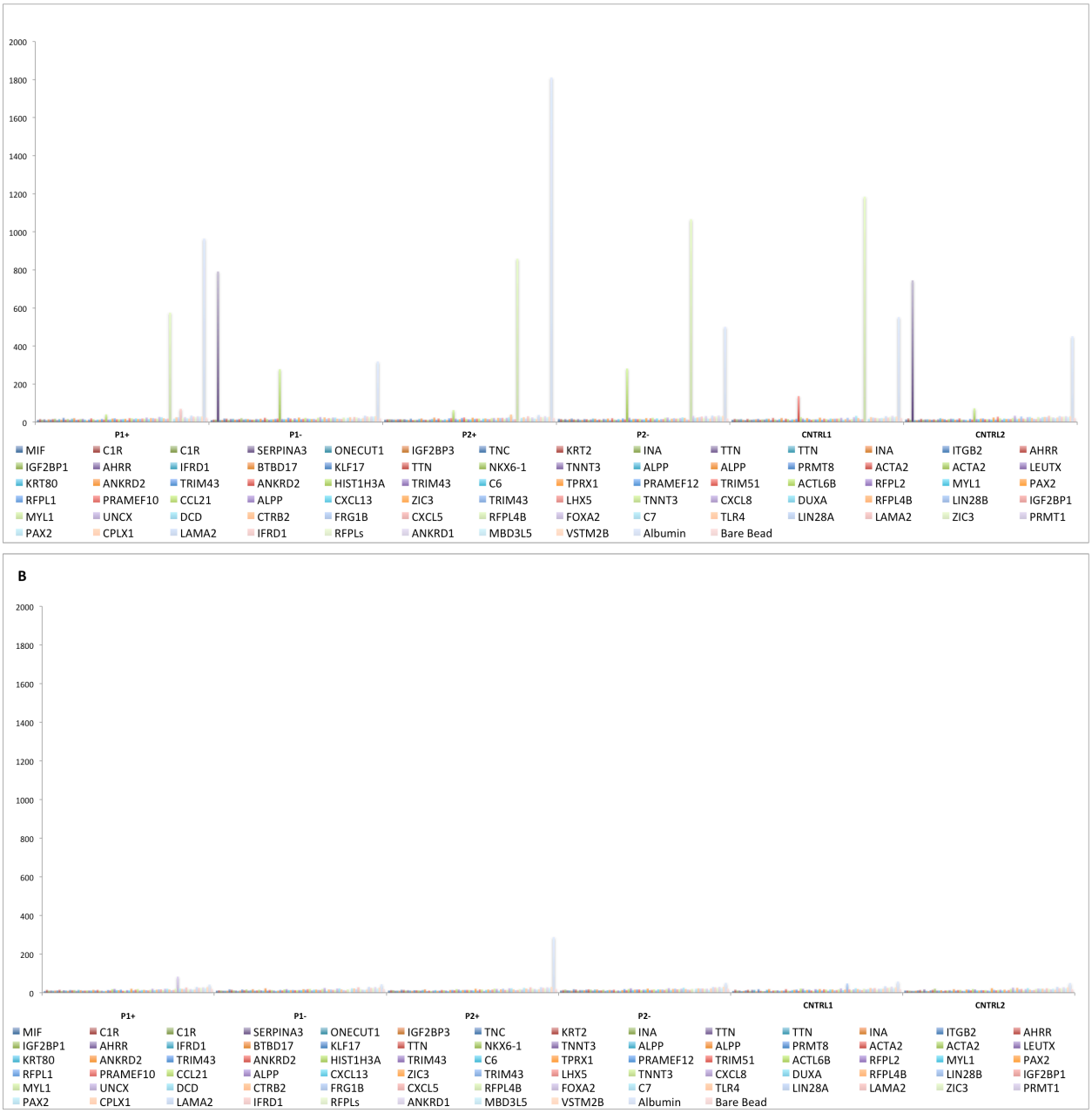

Figure 6: Results obtained from the tests performed to evaluate different incubation times for the immunocapture step. The tests were performed with six FSHD samples from 2 different patients and two healthy individuals. (A)Results obtain for six hours of incubation time. (B) Results obtained for O/N incubation.

Discussion

The aim of this project was to develop and optimize an immunoassay to test Interstitial Fluid Samples from patients affected by Fascioscapulohumeral Muscular Dystrophy for protein profiling. IF is a diluted sample with protein concentration with 1-2 mg/ml, 10 times lower than the cerebrospinal fluid samples with protein profiles already analyzed. A protocol was developed based on the modifications of immunoassay procedure used for analysis of cerebrospinal. Several parameters, that dependent on the protein concentration, were optimized to ensure the detections of the proteins in the sample. The biotinylation procedure with respect to biotin to protein ratio and the sample dilution throughout the experiment were modified due. Another parameter that was altered was the amount of sample used for the assay, due to the assumption that larger amount of sample in the assay would improve detection of low abundant proteins if captured on beads. The incubation time of the sample with the beads was changed since the incubation is performed at Room Temperature and the sample might contain proteases, that will degrade protein targets upron prolonged incubation time. The incubation time was reduced from Over Night to sis hours. This achievement and protocol is a novel development since there are no current published protocols for the analysis of Interstitial Fluid Samples.

The IF sample is obtained in small quantities (µL), and development of a protocol using just 1µL of sample was made in order to optimize usage of sample and minimize wasting it. The protein concentration in the sample was much lower compared to the serum and plasma; adjustments were made starting from the sample labeling. The biotinylation was the first step modified and tested, resulting in an excess of 60 of the biotin as the optimal for these samples (Altin, J.G., et al. (1995). A one-step procedure for biotinylation and chemical cross-linking of lymphocyte surface and intracellular membrane-associated molecules. Anal. Biochem.224:38 2-9). The optimal fold change of biotin for the samples recommended by the manufacturers would be of 20-fold excess for protein concentration less than 20 mg/ml. However, when tested several fold changes including the manufacturer recommendation, the optimal fold excess shown in the results was 60-fold change compared to the other fold changes, this may be due to the fact that when added more biotin to the sample the remaining biotin interfere with the coupling and the signal is not as high as expected. Another reason is that when added more biotin there could be aggregates formed and the signal would not be as high because it could interfere with the detection. And when added less biotin it is not sufficient to bind efficiently to the sample.

Following with the sample dilution, this adjustment was made throughout the experiment; the dilution ratio was changed in the sample labeling and the dilution with the labeled sample and the assay buffer. The dilution rate in the sample-labeling step was changed to 1: 4 and the dilution rate with the assay buffer was changed to 1:8. The results obtained from this modification showed higher signals than having a more diluted sample. The other

parameter that was optimized was the incubation time with the coupled antibodies with the beads with the sample, it was modified and tested showing that six hours incubation is sufficient so the immunocapture occurs.

This new protocol would help perform efficient immunoassays with Interstitial Fluid Sample. This could help analyze the protein content of both, the affected muscle and the non-affected muscle to create a protein profile of the disease. To have a better understanding of the mechanisms involved in the disease and in the muscles. It could help find viable biomarkers for detecting the disease in an early stage as well as for tracking the disease progression. These processes can be done by creating the protein profile of the disease, so there would be a better understanding of the mechanism happening in the muscles.

The proteins detected with the optimization of the protocol in both positive samples were ret finger protein-like 4B (RFPL4B) that is a transcription factor and Albumin that is a positive control in the sample, while in both negative sample there are not high but present also peaks of a histone cluster (HIST1H3A) which is a member of the histone family (H3) and play a mayor role in transcription regulation, DNA repair and regulation as well as chromosomal stability. In Patient 1 of the negative sample there is a signal of serpin peptidase inhibitor (SERPINA3) that is a protein that inhibit the activity of some serine proteases, while in Patient 2 there is no signal of these protein but a presence of the transcription factor ret finger protein-like 4B (RFPL4B). In the negative Control 1 there are signals of ankyrin repeat domain 2 (ANKRD2) which is a stretch responsible muscle, ret finger protein-like 4B (RFPL4B) and Albumin while in the Control Patient 2 there is a presence of the inhibitor of serine proteases alpha-1-antichymotrypsin (SERPINA). The protein abundance pattern not entirely identical for patient 1 and 2. While albumin is higher in the positive samples in comparison to the negative samples, HIST31A is present only in the negative samples. RFPL4B does not have the same abundance pattern in patient 1 and 2 but rather the opposite. This indicates that protein profiling procedure developed requires additional optimization experiments.

Furthermore, the findings shown in the results are not sufficient to ensure conclusive characterization of protein profiles in FSHD affected and not affected muscle. For that, a larger number of samples have to be tested so there would be a statistical significant number to support the findings. There should also be other sets of targets so there would be a more complete protein profile of the disease. For this, hundreds of proteins should be tested that have direct significance to the disease and to assumed mechanisms involved. There have to be more tests with several antibodies per target to ensure the findings of an specific target.

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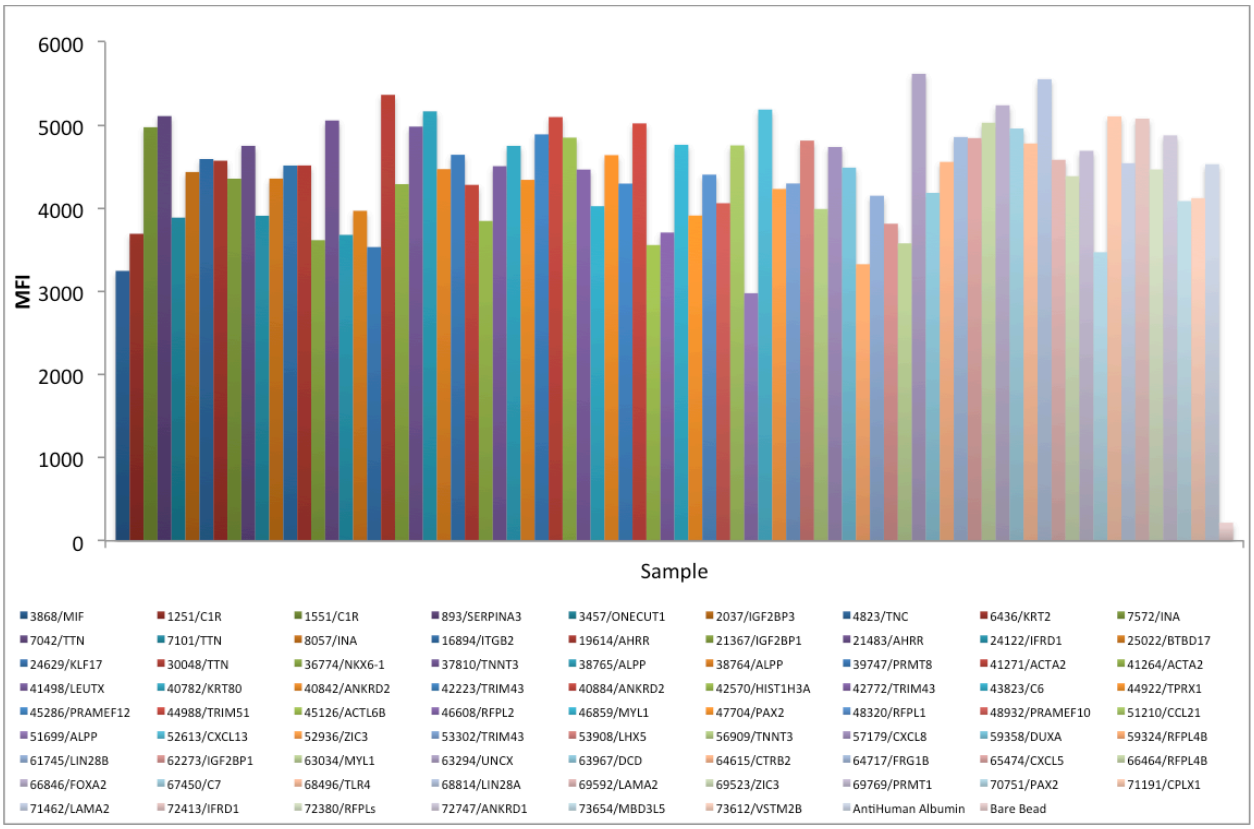
Supplementary Information

Antibody	Gene	Gene Description	Antibody	Gene	Gene Description
HPA041271	ACTA2	actin, alpha 2, smooth muscle	**HPA071462**	LAMA2	laminin, alpha 2
HPA041264	ACTA2	actin, alpha 2, smooth muscle	**HPA041498**	LEUTX	leucine twenty homeobox
HPA045126	ACTL6B	actin-like 6B	**HPA053908**	LHX5	LIM homeobox 5
HPA021483	AHRR	aryl-hydrocarbon receptor repressor	**HPA068814**	LIN28A	lin-28 homolog A (C. elegans)
HPA019614	AHRR	aryl-hydrocarbon receptor repressor	**HPA061745**	LIN28B	lin-28 homolog B (C. elegans)
HPA051699	ALPP	alkaline phosphatase	**HPA073654**	MBD3L5	methyl-CpG binding domain protein 3-like 5protein
HPA038764	ALPP	alkaline phosphatase, placental	**HPA003868***	MIF	macrophage migration inhibitory factor
HPA038765	ALPP	alkaline phosphatase, placental	**HPA063034**	MYL1	myosin, light chain 1, skeletal, fast, smooth muscle
HPA072747	ANKRD1	ankyrin repeat domain 1 (cardiac muscle)	**HPA046859**	MYL1	myosin, light chain 1, skeletal, fast, smooth muscle
HPA040884	ANKRD2	ankyrin repeat domain 2 (stretch responsive	**HPA036774**	NKX6-1	NK6 homeobox 1
HPA040842	ANKRD2	ankyrin repeat domain 2 (stretch responsive	**HPA003457**	ONECUT1	one cut homeobox 1
HPA025022	BTBD17	BTB (POZ) domain containing 17	**HPA047704**	PAX2	paired box 2
HPA001251	C1R	complement component 1, r subcomponent	**HPA070751**	PAX2	paired box 2
HPA001551	C1R	complement component 1, r subcomponent	**HPA048932**	PRAMEF10	PRAME family member 10;PRAME family member
HPA043823	C6	complement component 6	**HPA045286**	PRAMEF12	PRAME family member 12
HPA067450	C7	complement component 7	**HPA069769**	PRMT1	protein arginine methyltransferase 1
HPA026700*	CA3	carbonic anhydrase III, muscle specific	**HPA039747**	PRMT8	protein arginine methyltransferase 8
HPA051210*	CCL21	chemokine (C-C motif) ligand 21	**HPA048320**	RFPL1	ret finger protein-like 1
HPA071191	CPLX1	complexin 1;complexin 2	**HPA072380**	RFPL1	ret finger protein-like 1
HPA064615	CTRB2	chymotrypsinogen B2;chymotrypsinogen B1	**HPA046608**	RFPL2	ret finger protein-like 2
HPA052613	CXCL13	chemokine (C-X-C motif) ligand 13	**HPA059324**	RFPL4B	ret finger protein-like 4B
HPA065474	CXCL5	chemokine (C-X-C motif) ligand 5	**HPA066464**	RFPL4B	ret finger protein-like 4B
HPA057179	CXCL8	chemokine (C-X-C motif) ligand 8	**HPA000893**	SERPINA3	serpin peptidase inhibitor
HPA063967	DCD	dermcidin	**HPA068496**	TLR4	toll-like receptor 4
HPA059358	DUXA	double homeobox A	**HPA004823**	TNC	tenascin C
HPA066846	FOXA2	forkhead box A2	**HPA037810**	TNNT3	troponin T type 3 (skeletal, fast)
HPA064717	FRG1B	FSHD region gene 1 family	**HPA056909**	TNNT3	troponin T type 3 (skeletal, fast)
HPA042570	HIST1H3A	histone cluster 1	**HPA044922**	TPRX1	tetra-peptide repeat homeobox 1
HPA024122	IFRD1	interferon-related developmental regulator 1	**HPA053302**	TRIM43	tripartite motif containing 43
HPA072413	IFRD1	interferon-related developmental regulator 1	**HPA042223**	TRIM43	tripartite motif containing 43
HPA021367	IGF2BP1	insulin-like growth factor 2 mRNA binding	**HPA042772**	TRIM43	tripartite motif containing 43
HPA062273	IGF2BP1	insulin-like growth factor 2 mRNA binding	**HPA044988**	TRIM51	tripartite motif-containing 51
HPA002037	IGF2BP3	insulin-like growth factor 2 mRNA binding	**HPA007042**	TTN	titin
HPA007572	INA	internexin neuronal intermediate filament	**HPA007101**	TTN	titin
HPA008057	INA	internexin neuronal intermediate filament	**HPA030048**	TTN	titin
HPA016894*	ITGB2	integrin, beta 2 (complement component 3	**HPA063294**	UNCX	UNC homeobox
HPA024629	KLF17	Kruppel-like factor 17	**HPA073612**	VSTM2B	V-set and transmembrane domain containing 2B
HPA006436	KRT2	keratin 2	**HPA052936**	ZIC3	Zic family member 3
HPA040782	KRT80	keratin 80	**HPA069523**	ZIC3	Zic family member 3
HPA069592	LAMA2	laminin, alpha 2			

Supplementary Table 1. List of the Target Proteins selected to use in the project, the antibodies for each gene and the gene description. * Targets used in the first set of antibodies coupled to the beads.



Supplementary Figure 1. The Couple Efficiency Test performed to the second set of antibodies coupled to the beads. The graph shows that the antibodies coupled to the beads and have mean fluorescent intensities above 1000.

Indira Lopez
21/03/2016

Project Plan

Development of immunoassays for muscle dialysis samples from patients affected by Muscular Dystrophyp

Objectives

The aim of this project is to develop a method to analyse protein expression profiles and identify proteins that have different abundance in muscle fluid. Protein profiles will be analysed in muscle interstitial fluid from Facioscapulohumeral dystrophy (FSHD) affected tissue and tissue using immunoassays and compared. The objects of the project are:

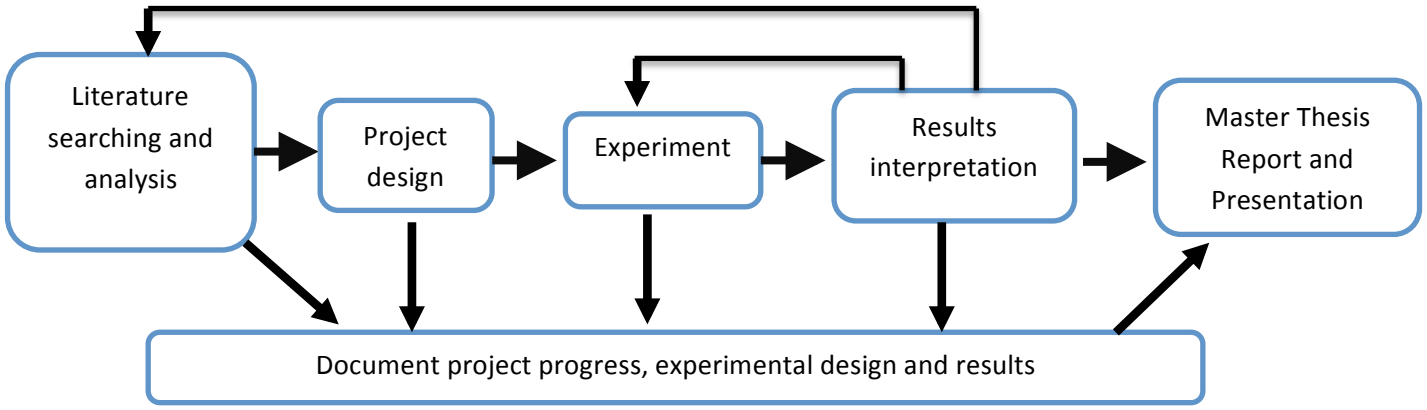
- Acquire relevant information and knowledge regarding the project e.g antibody-based proteomics, suspension bead array analysis and FSHD by searching for literature.
- Selection of experimental protocol used to conduct protein based on the literature
- Designing the project with the experimental conditions needed for each step.
- Perform the sample preparation taking in consideration the steps needed such as biotinylation, heat treatment and sample dilution.
- Optimize immunocapture of targeted proteins on beads.
- Analyze the mean fluorescent intensity signals from the Luminex platform.
- Compare the protein levels in healthy and diseased samples.

Background

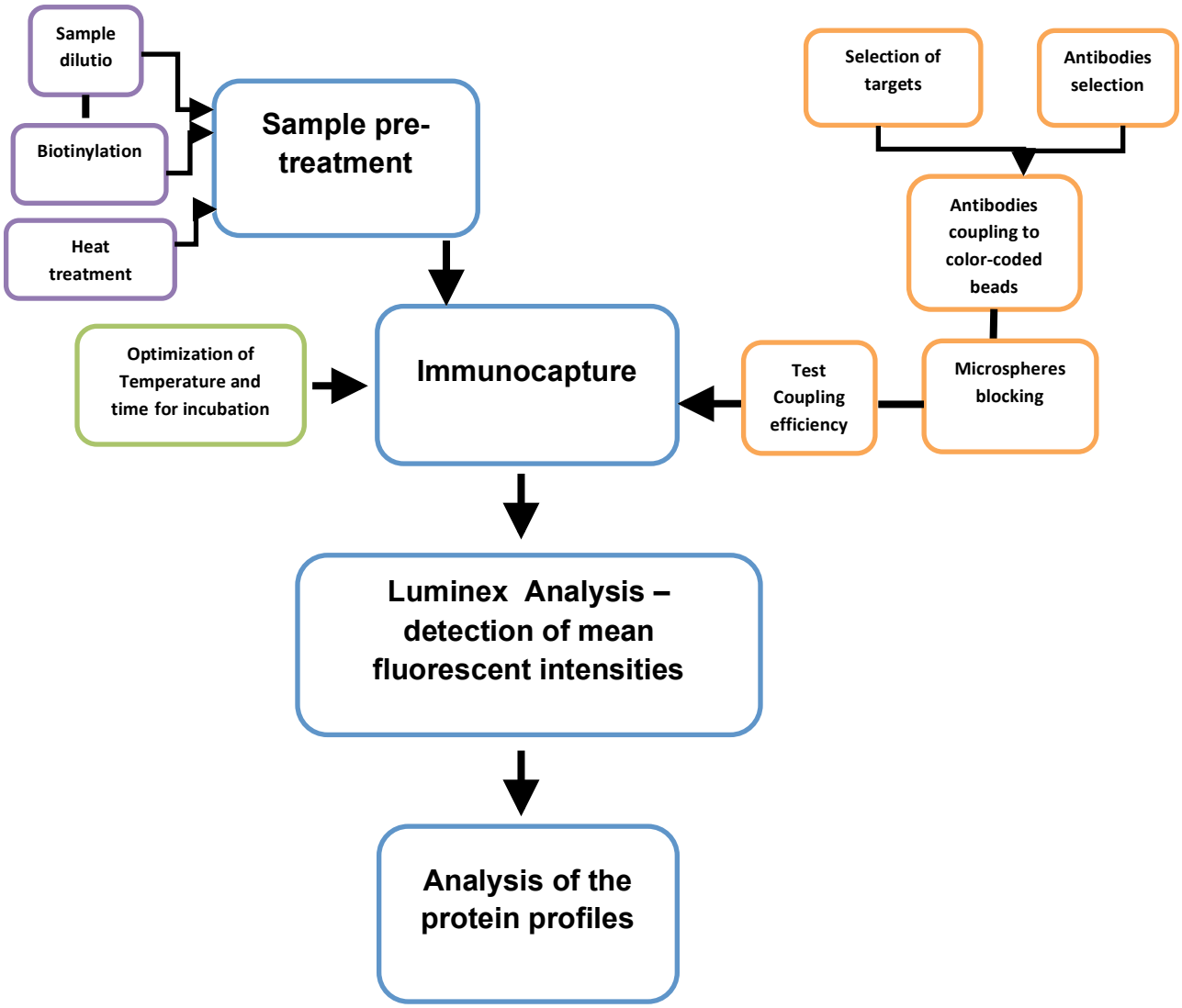
Facioscapulohumeral muscular dystrophy (FSHD) is a condition affecting 1 in 20 000 people. Symptoms of FSHD can be classified in 2 groups, the adult-onset and the infantile onset. Diagnosis is sometimes difficult to assess in particular when unknown mutations are causing the disease. The mutations cause muscle degeneration and muscle wasting. The most common symptoms involve the degeneration of muscles of the face, shoulders and upper arms. By understanding the difference between the proteome of healthy and diseased muscle we aim to identify differentially expressed proteins that can potentially serve as biomarker candidates. For this there are several techniques that are available to analyze the protein profiles of samples, such as affinity-based proteomics.

Affinity-based proteomics is an approach being used for the analysis of samples such as using body fluids for diagnosis, prognosis and surveillance that target specific biomarkers. An example of such tool is the bead array like the Luminex platform. This method uses microspheres and a reporter dye as the base technique, where antibodies are coupled with color-coded beads and mixed to create an antibody array in suspension and the samples are biotinylated to create protein profiles. In this project we will use muscle dialysis samples from diseases individuals and healthy individuals to create and compare protein profiles and find biomarkers candidates.

Project Work Breakdown Structure



Experiment Work Breakdown Structure



Milestones

1. Searching for the literature regarding the research field and the project and selecting relevant articles,
2. Analyzing relevant information and acquiring knowledge about the project such as antibody-based proteomics, suspension bead array analysis and FSHD from the literature,
3. Sample preparation,
    a. Biotinylation of the sample,
    b. Dilution of the sample,
    c. Heating treatment of the sample,
4. Selection of targets and corresponding antibodies for the analysis,
    a. Select 50 targets for the analysis regarding expression, disease and related processes,
    b. Selection of the antibodies,
    c. Coupling the antibodies to color-beads and test their efficiency,
    d. Blocking of the microspheres,
5. Immunocapture of targeted proteins on the beads,
    a. Optimization of sample dilution,
    b. Optimization of temperature and time for incubation,
6. Analysis of mean fluorescent intensity signals generated by the measurement of protein abundance,
7. Compare protein levels in healthy and diseased samples.

MoSCoW Analysis

Must

1. Identify key words and search articles relevant for the project, such as muscular dystrophies, Facioscapulohumeral muscular dystrophy, bead array, Luminex, Immunoassay, to acquire knowledge regarding the disease, the technique, samples, reagents like antibodies and parameters for conduction of experiments.

2. Plan the overall project and the experiment. For this step I must know prior to the search the overall project and the main objectives. Once I have planned the project and the steps needed for the experiment I will start with the necessary steps for performing the experiment.

3. Learn how to conduct the experiment e.g. couple the antibodies to the beads and use the Luminex instrument.

4. Select the protein targets that are expected to be present in interstitial muscle fluid. Select for each target antibodies that are validated and quality assured.

5. Couple the antibodies to color-coded beads and test their coupling efficiency.

6. Prepare the sample for the immunocapture step, biotinylate, dilute the sample and apply heat treatment before proceeding to the immunocapture.
7. Optimize samples working dilutions.
8. Optimize the temperature for the incubation of the sample with the beads.
9. Analyze the signals from the Luminex platform and analyze the protein profiles. So, for the final test, test the parameters to improve and choose the best conditions for the experiment get an optimal result at the end without wasting time and resourcing.
10. Revise the report to modify any aspects if needed.
11. Perform more tests to assure the results.

Should

1. Reade the literature to acquire more information about other parameters relevant for the project, beside the temperature and incubation time, that can improve analysis of proteins in body fluids.
2. Revise the Project Plan while performing the experiments, to modify the requirements if needed.
3. Optimize the steps in the experiments, such as immunocapture, biotinylation and sample dilution.

Could

1. Analyze several samples for a wider comparison.
2. I could increase the number of proteins to analyze to have more options of immunocapture.
3. Increase the number of antibodies to have more options to capture the target.

Would

1. Validate the findings with a sandwich assay.

Stakeholder Analysis

Primary Stakeholders

Indira Lopez- I am a major stakeholder since I am directly responsible for developing the project and obtaining final results. My interests from this project include acquiring knowledge of how a research laboratory functions, get to work directly with experiments; learn how to operate instruments, how to handle samples. In addition, I aim to fulfill the course and get the credits for the Degree Project in Biotechnology.

Cristina Al-Khalili Szigyarto- As a direct supervisor, she has an invested interest since she is in charge of creating and developing the central idea of the project. Her primary goals are to obtain measurable measurable, reproducible and accurate results at the end of the project.

Researchers and collaborators- When this project gets results, researchers working with rare muscle dystrophies will get more information useful to continue their projects and increasing their understanding of affected muscle pathology, function and characteristics. Furthermore, they will be able to develop treatment for this disease.

School of Biotechnology in AlbaNova - This School is responsible for financing and supervising the project course; their primary goal is to achieve optimal results to continue supporting and fostering future Master's in Biotechnology graduates and developing more projects and courses in the future.

Secondary Stakeholders

Clinician/Physician- They would be interested in this project development, so they could find new ways of treating FSHD in a more effective way since nowadays there is no optimal treatment for this disease.

Affected Patients- They will get a direct benefit from this project since there will be more information and knowledge about the disease, in consequence, treatment will be closer to find; resulting in a direct benefit to them.

Business Case

Societal- Since FSDH is a condition affecting 1 in 2000 people, it is a rare disease, and there are no many tests for monitoring disease progression in a clinical setup. This also complicated finding new and more effective treatments. People suffering for this muscular dystrophy struggles with the disease in addition to psychological problems. The family directly surrounding a person affected also modifies their lifestyle in order to accommodate the patient necessities. The disease has an indirect impact on a large number of individuals.

Financial- Since this is a progressive disease, affecting arms and face muscles, patients cannot support themselves, so the burden of the financial aspects of the treatments falls on to the society and the families. Compared to common diseases, treatment of these types of diseases are very expensive. A study which the main objective was to estimate the cost of a similar illness DMD (Duchenne muscular dystrophy) assessed that the direct cost of the disease ranges goes from $23,920 and $54,270 annually. With indirect cost this number increases to $80,120 and $120,910 per patient a year.

Ethical – Since it is a rare disease, usually governmental bodies and pharmaceutical companies do not invest money in finding new treatments since they patients affected are so few, they don't represent a viable business. A project like this would be helpful in finding new clinical tests and treatments without big pharmaceutical companies being involved. Even so, patients affected now may not be directly benefited from this project because results are no immediate, but if no one invests time in researching rare diseases further generations will be equally affected as nowadays.

Environmental Aspects- This project involves an improvement to communities that have a diseased family member affected by this muscle dystrophy. However, no direct effect on the environment can be estimated.

SWOT ANALYSIS

Strengths

1. There is a large amount of information available for public reading regarding the specifications necessary of the project and the parameters of the experiment and functionality of the platform.
2. This approach allows the analysis of samples such as body fluids
3. With this project we can create and compare protein profiles
4. There a lot of resources available for performing the experiment, such as buffers, samples, and laboratory equipment.

Weaknesses

1. The amount of samples and quantity are limited, so there are a limited number of experiments that can be performed.
2. The Luminex platform may display failures, or break down, stopping the process of the experiment.
3. The prioritizing of the experiment parameters could be wrong that could lead to incorrect results.

Opportunities

1. By creating and compare the protein profiles, we could find biomarker candidates.
2. Generate more information and knowledge regarding FSHD.

Threats

1. The analysis from the experiment could be interpreted wrongly leading to mistaken results.

Timeplan

Gantt Schedule

		W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11	W12	W13	W14	W15	W16	W17	W18	W19	W20	W
	Activities/ dates	01/18-01/22	01/25-01/29	02/01-02/05	02/08-02/12	02/15-02/19	02/22-02/26	02/29-03/04	03/07-03/11	03/14-03/18	03/21-03/25	03/28-04/01	04/04-04/08	04/11-04/15	04/18-04/22	04/25-04/29	05/02-05/06	05/09-05/13	05/16-05/20	05/23-05/27	05/30-06/3	06/
Initiation of the master thesis	Start up meeting																					
	Searching for literature																					
	Selecting relevant information for the project																					
Project planing	Designing the project																					
	Developing the Project Plan																					
	Prioritization																					
Project implementation	Selection of the target proteins																					
	Selection of the antibodies																					
	Sample preparation																					
	Couple antibodies to color-coded beads																					
	Dilution of the sample																					
	Biotinylation of the sample																					
	Immunocapture of targeted proteins																					
	Optimization of the time of incubation																					
	Optimization of incubation temperature																					
	Optimization of buffers																					
	Analysis from the Luminex signals																					
	Analysis of the protein levels																					
Project management	Half-time meeting																					
	Revision of the project plan																					
Reporting	Write the draft master tesis																					
	Make the presentation																					
Project completion	Write the oposition task																					
	Oral presentation and opposition																					
	Revise final master tesis																					