# DIFFERENTIAL PROTEOME ANALYSIS IN LIMB GIRDLE MUSCULAR DYSTROPHY 2A

**Project report submitted in partial fulfilment of the degree of**

## Bachelor of Technology

## In

## Biotechnology

**under the supervision of**

**DR. JITENDRAA VASHISTT**

**By**

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**DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS**

**JAYPEE UNIVERSITY OF INFORMATION AND TECHNOLOGY**

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## CERTIFICATE

This is to certify that the work entitled, **"DIFFERENTIAL PROTEOME ANALYSIS IN LIMB GIRDLE MUSCULAR DYSTROPHY TYPE 2A"** submitted by — **Aakriti Singh** and **Sonika Chibh** in partial fulfilment for the award of degree of Bachelor of Technology in Biotechnology of Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Jitendra

**Signature of the Supervisor:**

**Date:** 26th May 2014.

## ACKNOWLEDGEMENT

Any assignment puts to litmus test an individual's knowledge, credibility and experience and thus, sole efforts of an individual are not sufficient to accomplish the desired work. Successful completion of a project involves interest and efforts of many people and so this becomes obligatory on my part to record thanks to them.

Therefore, first of all we would like to thank our guide and mentor **Dr. Jitendraa Vashistt** for his guidance, help and constant encouragement throughout this project. Working under him was an enriching experience.

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# ABSTRACT

**Background & Objectives:** LGMD2A is an autosomal recessive disorder characterized by symmetrical and selective weakness of pelvic, scapular, and trunk muscles, resulting in progressive proximal skeletal muscle wasting. It has been found that LGMD2A is caused by mutation in the gene encoding for calpain-3 resulting in total or partial loss of protein. However, the proteins associated with calpain-3 are not identified. Therefore, our research is focused on identification of proteins which are differentially expressed in LGMD2A.

**Methods:** Gel based proteomic approach is employed with the novel samples of a family suffering from this disorder (3 carriers and 3 affected individuals out of the 6 siblings). We have done SDS-PAGE of serum samples to find differential expressed proteins of samples (normal, carrier and diseased individuals of a single undivided family) and further strengthening the results through mass spectrometry. 2D gel electrophoresis is also used for normal and patient sample comparison to see more clear difference in the serum profiles.

**Results:** The serum SDS-PAGE profiles of the samples showed significant difference in pattern of expression of LAC2_HUMAN, HPT HUMAN, Arylamine N- acetyl transferase proteins. To further determine the differentially expressed protein 2D-PAGE was performed which revealed the incidence of some hypothetical proteins like IGLC HUMAN and APCS.

# 1. INTRODUCTION

Limb girdle muscular dystrophy (LGMD) is a group of disorders affecting voluntary muscles, mainly around the hips and shoulders — the pelvic and shoulder girdles, also known as the limb girdles. Over time, the person with LGMD loses muscle bulk and strength.[ mda.org.,2009,2011]

LGMD2A is a most common form of LGMD, which is an autosomal recessive form. It has been found that limb girdle muscular dystrophy type 2A results from mutations in the gene encoding the calpain 3 protease (15q15.1-15.3), which is a calcium dependent protease. [Saenz et al., 2005].The phenotype shows variability ranging from mild to severe. The phenotypic features involve atrophy of the pelvic, scapular and trunk muscles, but not of cardiac or facial muscles [Pegoraro et al., 2012]. Age at onset is between 8 and 15 years for at least two-thirds of the patients, with a range of about 2 to 40 years. The slowly progressive course of the disease leads to loss of ambulation during adulthood and a near-normal life expectancy. Serum creatine kinase (CK) is markedly elevated and a muscular biopsy shows a dystrophic picture with evidence of necrosis and regeneration. Due to the variability of the phenotype the final diagnosis of LGMD2A relies on protein analysis of the muscle biopsy (Western blot) and mutation detection in the CAPN3 gene. [Schessl et al., 2008]

But calpain 3 mutations and elevated creatine kinase level are also found in some other diseases like Idiopathic eosinophilia (mutations in calpain 3) [Krahn et al., 2006] and Duchenne and Beckers muscular dystrophy (elevated creatine kinase levels) [mda.org, 2011]. There are some proteins which are also found in association with calpain 3 for example titin (giant cytoskeleton protein that spans half life of the sarcomere and is important for both sarcomere assembly and function), which has strong binding with calpain 3 and serve as a stabilizing role for CAPN3, keeping it an inactive state and preventing its rapid hydrolysis. It has also found that if there is any mutation in the titin, it will also affect the function or activity of calpain 3 or vice versa [Kramerova et al., 2007]. Therefore, till date now there is no biomarker specific for LGMD2A .Our main focus is to find differential expressed proteins which we can further use as a diagnostic biomarker.

# 2. REVIEW OF LITERATURE

## 2.1 MUSCULAR DYSTROPHY (MD) AND ITS TYPES

Muscular dystrophy is a group of inherited disorders that are characterized by progressive skeletal muscle weakness, defects in muscle proteins, and the death of muscle cells and tissue. [Brown et al., 2005]

### Signs and symptoms

Progressive muscular wasting, Poor balance, Drooping eyelids, Atrophy, Scoliosis(curvature of the spine and the back), Inability to walk, Frequent falls, Waddling gait, Calf deformation, Limited range of movement, Respiratory difficulty, Joint contractures, Cardiomyopathy, Arrhythmias, Muscle spasms, Gowers´ sign.[ Brown et al.,2005]

Muscular dystrophies, or MD, are a group of inherited conditions, which means they are passed down through families. They may occur in childhood or adulthood. There are many different types of muscular dystrophy. They include:

- Beckers muscular dystrophy
- Duchenne muscular dystrophy
- Emery-Dreifuss muscular dystrophy
- Facioscapulohumeral muscular dystrophy
- Limb-girdle muscular dystrophy
- Myotonia congenital
- Myotonic dystrophy

## 2.2 LIMB GIRDLE MUSCULAR DYSTROPHY

Limb-girdle muscular dystrophy is a group of diseases that cause weakness and wasting of the muscles. The muscles most affected are those closest to the body (proximal muscles), specifically the muscles of the shoulders, upper arms, pelvic area, and thighs. Symptoms of limb-girdle muscular dystrophy vary widely, but most commonly are atrophy, myoglobinuria, myotonia, elevated serum CK, and, in 20% of cases, cardiomyopathy. [Medline Plus]

The severity, age of onset, and features of limb-girdle muscle dystrophy vary among the many subtypes of this condition and may be inconsistent even within the same family. Signs and symptoms may first appear at any age and generally worsen with time, although in some cases they remain mild. [Shields et al., 1986],[Walton et al.,1988]

Weakening of the heart muscle (cardiomyopathy) occurs in some forms of limb-girdle muscular dystrophy. Some affected individuals experience mild to severe breathing problems related to the weakness of muscles needed for breathing.[ Shields et al.,1986],[Walton et al.,1988]

### Limb Girdle Muscular Dystrophy Type2A

LGMD 2A, also known as Calpainopathy, is an autosomal recessive form of limb-girdle muscular dystrophy. It is characterized by weakness of pelvic, scapular, and trunk muscles, resulting in progressive proximal skeletal muscle wasting. It has been found that most mutations lead to the loss of Calpain-3 activity in the muscle [Pathak et al., 2010]. The age of onset can vary from early childhood to the middle age. But on the other hand, there are some diseases which also have mutations of calpain-3 (Idiopathic eosinophilia myositis) [Krahn et al.,2006] and Duchenne and Beckers muscular dystrophy have also elevated creatine kinase level[mda.org 2009,2011]. Calpain 3 role is also associated with other proteins e.g. titin, dysferlin, sarcomeric complex etc [ Kramerova et al.,2007].

#### Calpain-3 and dysferlin association

Dysferlin is a transmembrane protein plays an important role in vesicle trafficking and membrane repair and help in resealing the disrupted sarcolemma by fusing vesicles in muscle membrane [Bansal et al., 2004], [Bansal et al., 2003]. Role of CAPN3 has been elucidated in LGMD2B and Miyoshi myopathy [Anderson et al., 2000]. Recently it was shown that dysferlin and CAPN3 biochemically interact in co-immunoprecipitation assays [Huang et al., 2005]. Thus, the reciprocal loss of CAPN3 and dysferlin in LGMD2A and LGMD2B biopsies and the finding of a biochemical interaction between these proteins leads to the suggestion that dysferlin functions to stabilize CAPN3 at the membrane and vice versa [Lennon et al., 2003].

## Calpain 3 and Titin association

The localization of CAPN3 in skeletal muscle is not clear, while CAPN3 has been localized at the N2-line[Keira et al.,2003], Z disc, M-line, costameres, myotendinous junctions and nuclei in various reports[Keira et al.,2003],[ Baghdiguian et al.,1999],[ Taveau et al.,2003]. Yeast two hybrid assays have identified that titin bind to CAPN3 at N2 line and the M line [Sorimachi, et al., 1995], [Kinbara et al., 1997]. It has been proposed that titin might serve a stabilizing role for CAPN3 keeping it in an inactive state and preventing its rapid autolysis. It is also possible that titin directly controls CAPN3 activity or access to its substrates [Haravuori et al., 2001], [Garvey et al., 2002].Therefore, if any mutation or changes in the titin protein it will affect the role of or activity of Calpain-3 or vice-versa. Future studies will be necessary to confirm if alternative cellular sites of localization exist.

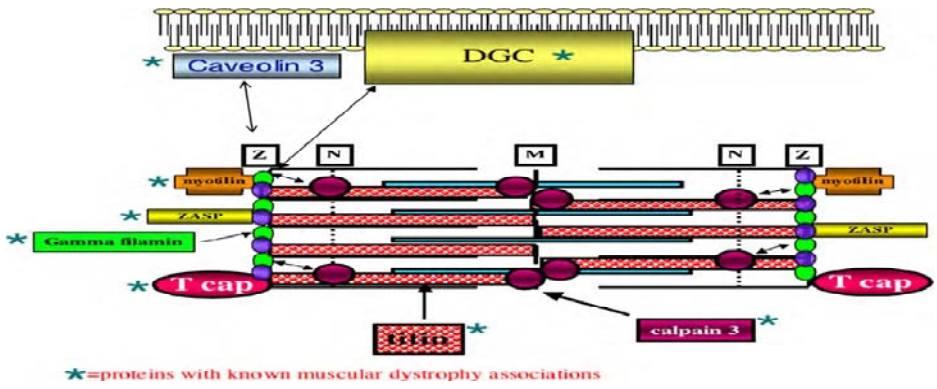



**Figure 1: Calpain 3 in association with other proteins [Kramerova et al., 2007]**

## Calpain 3 and DGC (Dystrophin glycoprotein complex)

Dystrophin glycoprotein complex (DGC), it is a large protein complex that serves both structural and regulatory functions in muscle [Beckmann et al., 1996], [Hoffman et al., 1987]. It has been shown that defects in any one of several members of the DGC or associated proteins can cause any of several muscular dystrophies [Bushby et al., 1999]. It has been found that structure of the DGC in normal and DGC-mutant muscle has also provided convincing evidence that mutation in a single DGC member can have major effects on the ability of non-mutant DGC members to assemble and function [McNally et al., 1996] .Thus, all proteins in this complex serve a common physiological function.

## 2.3 DIAGNOSTICS OF LGMD2A

The diagnosis of LGMD2A is based on medical history, clinical findings, neurophysiological assessments, muscle biopsy. The muscle enzyme CK is usually elevated in LGMD2A [Angelini et al., 2005]. The diagnosis has to be done by identifying the faulty gene (Calpain 3 gene) which is done on a DNA sample from a blood test. It has been also found that molecular diagnosis of LGMD2A is now possible to calpain-3 antibodies, used for protein diagnosis. But LGMD2A cases currently remain undiagnosed, due to either incomplete sensitivity of the methods used for protein analysis [Fanin et al., 2004].

An analysis known as Western blot can sometimes, in LGMD type 2A, show deficient or defective muscle protein, thereby providing valuable information for a targeted DNA analysis [Louise et al., 1998].

### Limitations of diagnosis

There is no particular method for LGMD2A diagnosis because of:

- ✓ Many studies have shown that there is no direct correlation between the amount of calpain-3 detected on Western blots and the severity of the phenotype. A normal amount of CAPN3 on Western blotting may be found in genetically proven calpainopathies, whereas calpain may be reduced in amount or absent in other forms of LGMD as a secondary effect [Pathak et al.,2008].
- ✓ Early in the diagnostic process, doctors often order a blood test for CK level. CK stands for creatine kinase, an enzyme that leaks out of damaged muscle. When elevated CK levels are

found in a blood sample, it usually means muscle is being destroyed by some abnormal process, such as a muscular dystrophy or inflammation [Angelini et al., 2005]. But the increased level of CK is also observed in other forms of limb girdle muscular dystrophy e.g. Duchenne and Beckers muscular dystrophy [mda.org 2009, 2011].

Therefore there is no diagnostic method which tells us that a person is suffering from LGMD2A or not. So our main purpose is to find out differential expressed proteins which can be further used as diagnostic marker.

## 2.4 PROGNOSTICS OF LGMD2A

LGMD2A have initial symptoms of weakness and wasting of muscles in the hip, thigh and shoulder muscles and weakness is usually even on both sides of the body and leg involvement is present [ George Krucik.,2012]. This can result in frequent falls, difficulty in running, climbing stairs and rising from the floor. As the condition progresses, people can have problems with walking. Shoulder and arm weakness can lead to difficulties in raising the arms above the head, and shoulder blade winging may be present. Some people complain of muscle pain, especially in the legs. Joint contractures may be present and more frequently involve the ankles. Facial and neck muscles are not usually involved and therefore swallowing problems are unlikely [K.M.D et al., 2013]

It has been found that there is no involvement of heart problems in this condition. People with LGMD2A are at risk of developing respiratory muscle weakness and experience breathing difficulties with the progression of the condition, but this is usually a very late complication. [K.M.D et al., 2013]

## 2.5 REMEDIES/ TREATMENT

To date there are no specific treatments for LGMD2A, however careful management of the symptoms of the condition can improve a person's quality of life.

Regular physiotherapy is recommended. This can be carried out by a physiotherapist or people can be taught to do this by themselves in their own home. With progression of the muscle weakness, people with LGMD2A are at risk of developing breathing difficulties. Therefore regular monitoring of respiratory function is recommended. Sometimes overnight

studies are indicated. LGMD2A is a variable condition in terms of severity and the weakness is always progressive with time.

Heart and breathing muscles are usually not affected so life expectancy is in normal range. In later stages of the condition, breathing difficulties can occur but are usually less severe than in other muscular dystrophies. These symptoms can include poor sleep, nightmares, tiredness or headaches after waking up in the morning, lack of appetite and falling asleep during the day. [K.M.D et al.,2013]

## 2.6 INCIDENCE OF LGMD2A

### Global Scenario

Calpainopathy is the most common form of LGMD [Bushby & Beckmann 2003], representing approximately 30% of LGMD cases, depending on the geographic region [Chou et al 1999, Zatz et al 2000].

Estimates based on molecular data indicate that the frequency ranges from

- 10% of LGMD cases in a white population from the US [Chou et al 1999,Moore et al 2006]
- 13% in Denmark [Duno et al 2008]
- 21% in the Netherlands [van der Kooi et al 2007]
- 25-28% in Italy [Guglieri et al 2008, Fanin et al 2009b]
- 26% in Japan [Kawai et al 1998]
- 50% in Turkey and India [Dincer et al 1997,Balci et al 2006, Pathak et al 2010]

### Indian Scenario

- ✓ LGMD in India were made in 1975, in a survey of 211 patients of myopathies seen over 25 years, diagnosed almost half the patients to have muscular dystrophy. Out of them 82 were Duchenne Muscular Dystrophy (DMD), 35 LGMD, five fascio-scapulo humeral dystrophies and 14 ocular-oculopharyngeal myopathies.

- ✓ In1998, a paper was published in which a large series of 1950 biopsy-proven myopathies, of which 535 were dystrophies. Among them, 29.2% were labeled as LGMDs, 5.6% severe dystrophies were seen in young girls, resembling DMD and 2.2% had autosomal recessive dystrophies in young boys.

- ✓ Immunocytochemical stains have been used in recent study of Indian patients with LGMDs and information based on immunostaining has emerged. Initial case reports of Adhalinopathy (alpha sarcoglycanopathy) appeared in 2001. This was followed up by case reports of beta and gammasarcoglycanoathy from pediatric centers in **New Delhi**. In 2002, the first series analyzing 25 cases of sarcoglycanopathy came from a neuromuscular center in **Mumbai**, where phenotypic features were studied in detail and some unique features were pointed out.

- ✓ Indeed, sarcoglycanopathy is the best characterized LGMD in India. The second type of LGMD to be immune characterized in India is dysferlinopathy. The initial series of 14 Indian patients with dysferlinopathy from **Mumbai** was published in 2004.

- ✓ Nine cases of LGMD were recently reported from northern India by in 2008. A series comprising 28 patients from south India has been made available in 2008.[ Satish et al.,2008]

## TOTAL NUMBER OF MD PATIENTS REGISRETED IN HIMACHAL

**PRADESH**

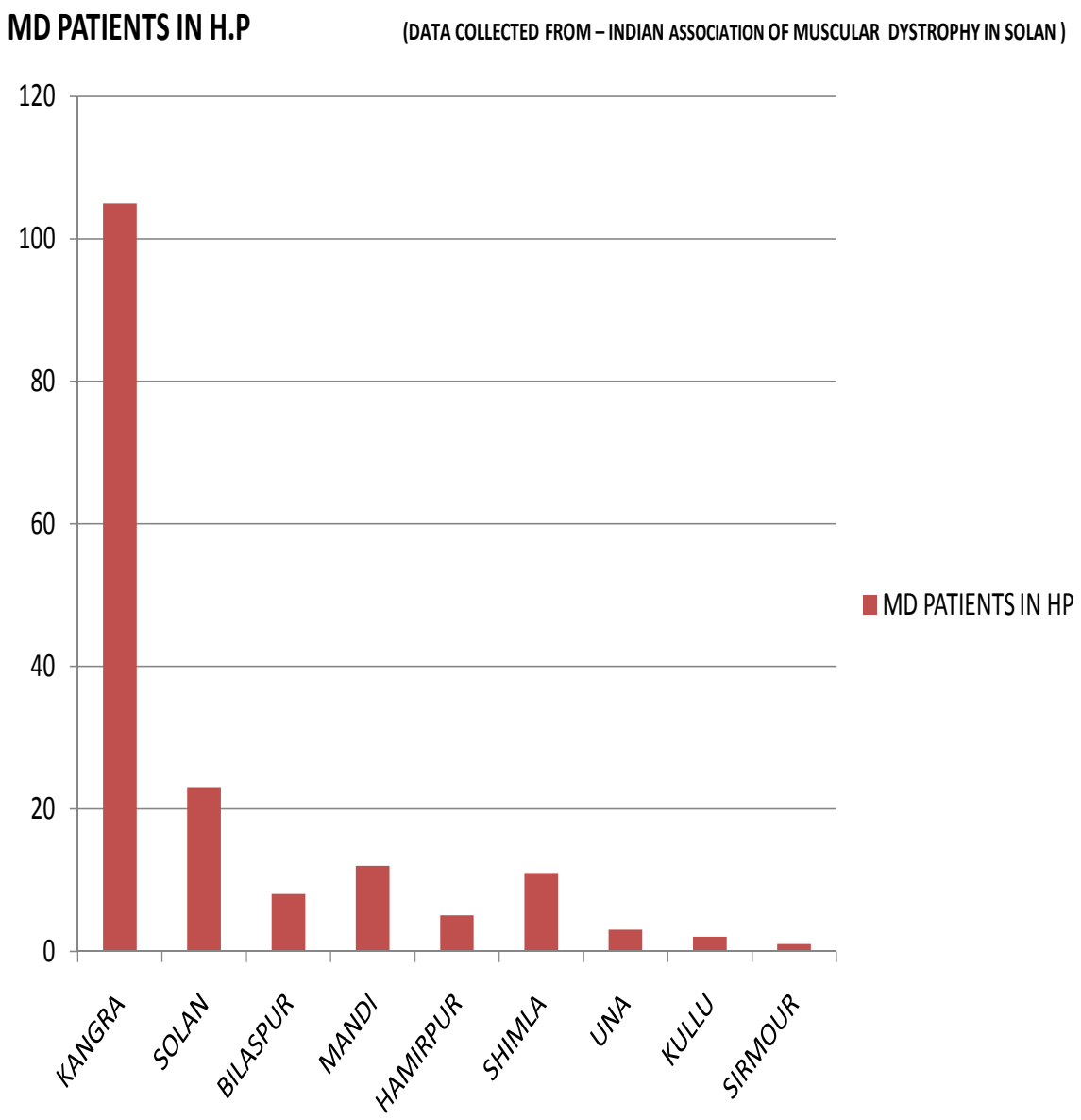



**Figure 2: A large fluctuation was observed in the data reported on total number of Muscular Dystrophy patients in Himachal Pradesh, the exact cause of this fluctuation is not known. (highest number of cases reported on Muscular dystrophy were in Kangra district and lowest were in Sirmour district) (Courtesy: Data collected from Miss Sanjana Goyal, "Head of Indian Association of Muscular Dystrophy" Solan H.P.)**

# 3. LACUNAE

The rationale of the study is to identify novel proteins which are differentially expressed in disease state. Till date, no specific biomarker is available which can precisely be associated with Limb girdle muscular dystrophy type 2A, only elevation of serum creatine kinase and mutations in a protease calpain-3. However, these markers has also a linkage with similar disorders like Idiopathic eosinophilia (mutations in calpain 3) [Krahn et al., 2006], Duchenne and beckers muscular dystrophy (elevated creatine kinase levels)[mda.org.,2009,2011] and reduction of calpain 3 in miyoshi myopathy, therefore no specific biomarker can be associated with LGMD2A.

It has been found that most mutations lead to the loss of calpain-3 activity in muscles by inactivating the enzyme or preventing its expression. However, some missense mutations, predicted or demonstrated not to affect the proteolytic activity, have been shown to disturb the interaction of CAPN3 with titin[Sorimachi, et al.,1995],[Kinbara et al.,1997], affecting one or both of its binding regions. Some mutations may also affect the proposed non-proteolytic functions of CAPN3 at the triads, leading to altered calcium signalling.

Therefore, our main purpose is to investigate differentially expressed protein in LGMD 2A, so that which we can use as a biomarker in future for its diagnosis and prognosis.

# 4. METHODOLOGY

## 4.1 SAMPLE COLLECTION AND SEPARATION OF SERUM

3 ml blood was collected from three diseased and one normal member of the same family (six siblings) and one sample was procured as a control from a normal male belonging to same age group from similar geographical area by the method of venipuncture by a lab technician from Guru Gobind Path lab, Solan Himachal Pradesh.

The blood samples were collected into sterilized eppendorf tubes and allowed to clot for 30 minutes at room temperature which were then centrifuged at 1000G for 10 minutes. The separated serum was collected into 1.5ml eppendorf tube and freezed at –80˚ C.

## 4.2 PREPARATION OF STANDARD AND PROTEIN QUANTIFICATION BY BRADFORD ASSAY

Using standard BSA solution a linear curve was prepared between concentrations of BSA and optical density and the straight line equation y = 0.1162x was obtained from of the graph.

Protein quantification was performed using a 96 well plate in which samples were plated in triplicates (1µL sample + 9µL water per well) and the first column was used as blank (10µL water). 200 µL of Bradford reagent was added to each well and placed in an incubator for 5 minutes maintained at 37˚C. The optical density of the samples were taken at 595nm and the corresponding protein concentration were calculated using the equation y = 0.1162x (the y axis corresponds to optical density and the x axis corresponds to protein concentration)

## 4.3 SDS PAGE

The proteins present in the samples were separated using 10% gels. The resolving gel solution was added in between the plates upto a height of 4 cm and then allowed to polymerize for 30 minutes. A thin layer of water was layered on the top of the resolving gel to prevent oxidation and hence allow polymerization. Water was removed and stacking gel solution was poured on the top of polymerized resolving gel. Combs were inserted and the stacking gel was allowed to polymerize for 30 minutes.

The gel plates containing the polymerized gel were attached to the apparatus and electrophoresis buffer was poured into the electrophoresis tank. 1μL sample was added to each well and then the gel was run at constant current of 50V until the dye reaches the bottom of the gel.

## COOMASSIE BLUE STAINING AND DESTAINING

The gel was stained with 0.1% Coomassie Blue R250 in 10% acetic acid, 50% methanol, and 40% $H_2O$ for about less than one hour, necessary to visualize the bands of interest. To destain the gels were soaked in 10% acetic acid, 50% methanol, and 40% $H_2O$ with multiple changes of this solvent till the dye comes out.

## 4.4 TRYPSINIZATION

**I.** The bands of interest were cut from a SDS PAGE gel.

**II.** The gel pieces were placed in 100µL of 40mM ammonium bicarbonate in 9% acetonitrile solution (The gel piece were cut into small sections of 1–1.5 mm size)

**III.** The gel pieces were incubated with 100µL of 40mM ammonium bicarbonate in 9% acetonitrile at 37 °C for 30 minutes.

**IV.** The solution was discarded from the tube and step III was repeated one more time.

**V.** The gel pieces were dried for 15 minutes by placing then in incubator at 37˚C partially covered with aluminium foil.

**VI.** A vial containing 20 µg of trypsin was reconstituted by adding 100µL of 1mM HCl to this 900µL of 40mM ammonium bicarbonate in 9%ACN was added leading to a final volume of 1ml 20µg per ml.

**VII.** 20µL (0.4 mg of trypsin) of the trypsin solution was added to gel pieces (the gel pieces should be is at the bottom of the tube and covered with liquid).

**VIII.** The whole solution was incubated for 6 hours at 37 °C.

**IX.** After the incubation, the liquid was removed from the gel piece and transferred to a new labelled tube.

X. After digestion, 50µL of 1% formic acid was added, vortexed and shaked for 10 minutes (This wash will contain the more hydrophilic peptides)

XI. This 1% formic acid wash was removed and transferred to a new eppendorf.

XII. To the gel piece 100 µL of 50% acetonitrile containing 1% formic acid was added, vortexed and shaked for 10 minutes (This wash will contain most of the tryptic peptides)

XIII. The 50% acetonitrile containing 1% formic acid wash was removed and transferred to the eppendorf containing the previous 1% formic acid wash.

XIV. 50µL of 100%ACN was added to each of gel fragments, vortexed and removed.

Final 200µL solution contains the extracted peptides which were lyophilized.

## 4.5 TWO-DIMENSIONAL GEL ELETROPHORESIS

**I. Sample preparation-** The serum samples were dissolved in 7 M urea, 2 M thiourea, 2% CHAPS, 60mM DTT, 2% Pharmalyte pH 4–7, 0.002% bromophenol blue.

**II. Rehydration of IPG strips-** The IPG strips were rehydrated with 50µL of serum samples and 200 µL of rehydration buffer. The rehydration process was carried out overnight.

**III. First dimensional 2D-PAGE-** IPG strips were focused at 1000 volts for one hour, at 2000 volts for two hours, at 4000 volts for three hours and then at 8000 volts for one hour giving a total of 25,000 volt-hours.

**IV. Equilibration of IPG strips:** The equilibration step was carried out in two steps for 15minutes each, initially with equilibration buffer I containing DTT and later with equilibration buffer II containing IAA.

**V. Second dimensional 2D-PAGE:** The proteins present in the samples were separated using 10% gels. Electrophoresis was carried out at a constant current of 50V until the dye reaches the bottom of the gel.

**VI. Silver staining:** The gel was placed in a solution containing methanol, acetic acid and water in the ratio of 45:5:50 for 90 minutes. The solution was removed and the gel was washed twice with water for 20 minutes followed by 0.02% sodium thiosulfate for 3 minutes and then rinsed twice with water for 30 seconds. The gel was placed in 0.1% silver nitrate for 30 minutes and then rinsed with water for 30 seconds. The gel was developed using a solution of 2.5% sodium carbonate and 0.02 % formaldehyde for approximately 2-3 minutes. After the developing solution was removed, the gel was quenched with 1% acetic acid for approximately 10 minutes and then rinsed twice with water for 20 minutes.

## 5. RESULTS AND DISCUSSIONS

### 5.1 LGMD2A ANALYSIS IN A FAMILY SUFFERING FROM THIS DISORDER

**SERUM SAMPLES:-**

**1S:- DISEASED (55 YRS), MALE**

**2S:- DISEASED (53 YRS), MALE**

**3S:- CARRIER (49 YRS), FEMALE**

**4S:- DISEASED (48 YRS), FEMALE**

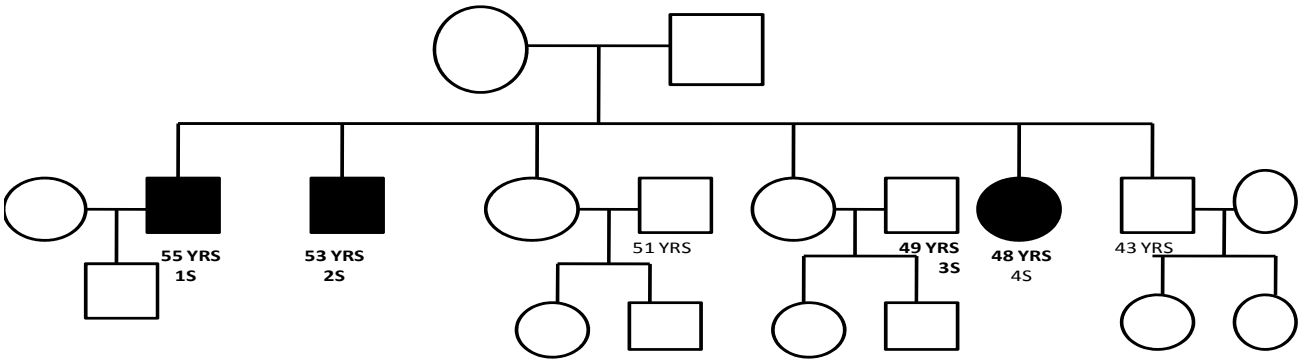



**Figure 3: Family pedigree: Squares indicate males and circles indicate females, open symbols represent unaffected individuals and solid symbols represent affected individuals. Pedigree analysis of a family in which out of six siblings three of them were affected by LGMD2A.**

### 5.2 CALCULATION OF PROTEIN CONCENTRATION USING BRADFORD ASSAY

| S.NO | BLANK | 1S | 2S | 3S | 4S |
|---|---|---|---|---|---|
| 1 | 0.420 | 1.336 | 1.353 | 1.250 | 1.377 |
| 2 | 0.228 | 1.383 | 1.327 | 1.317 | 1.373 |
| 3 | 0.243 | 1.356 | 1.354 | 1.397 | 1.429 |
| MEAN | 0.297 | 1.362 | 1.344 | 1.321 | 1.393 |
| Subtracting samples mean value from blank value | | | | | |
| Abs 595nm | - | 1.065 | 1.047 | 1.024 | 1.096 |
| Calculating protein concentration from equation y = 0.1162x | | | | | |
| Protein (ug/uL) | - | 9.165 | 9.010 | 8.8812 | 9.432 |

## 5.3 SDS-PAGE PROFILE

Serum proteins profiling of all the diseased, carrier and normal healthy individual was carried out using 10% SDS PAGE and gels are shown in Figure 4 to 7. In these gel figures 1S, 2S and 4S are diseased person serum proteins whereas 3S and N are serum proteins of carrier and normal healthy individual. The proteins of serum show similar banding pattern on SDS-PAGE (Figure 4 to 7). However, they are several distinct protein bands which show a markedly over expression in diseased persons serum. Therefore, the study focused on the identification of these three proteins for comparison purpose with clinical isolates. For the purpose of reproducibility of the data, the SDS-PAGE analysis was carried out for approximately four times (analytical replicates) and representative gels are incorporated in the results (Figure 4 to 7). It was not possible to get more samples of blood therefore the biological replicates were not done.

## SDS PAGE GELS

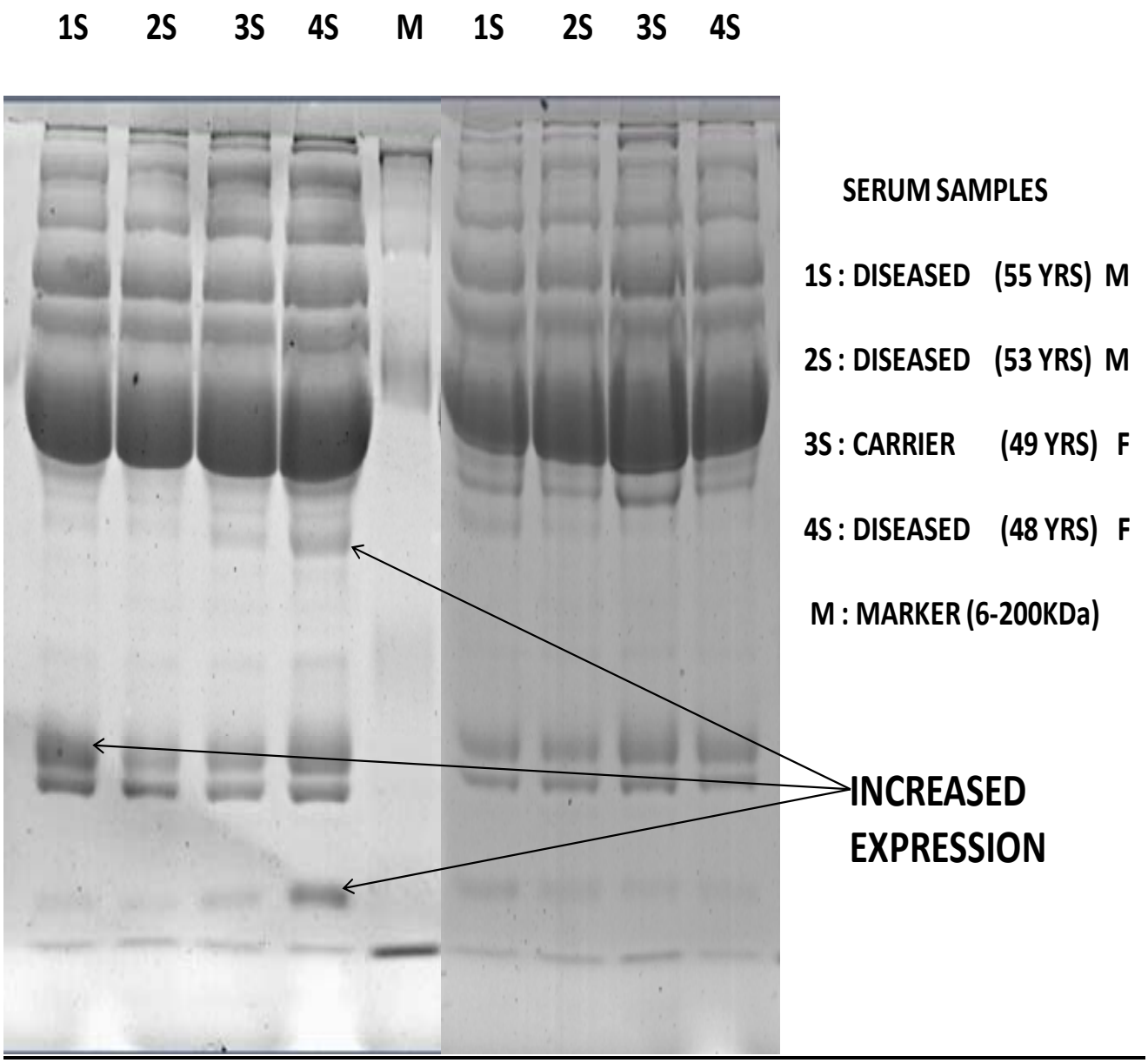



**Figure 4: SDS-PAGE profile of Serum of all the diseased and carrier and normal healthy individual. The sample was heat denatured at 100 ºC in sample buffer (6.25mM Tris-HCl pH 6.8, 10% glycerol, 1% β-mercaptoethanol, 1% SDS). Protein bands were separated on 12% gel and detected by coomassie blue staining. Increased expression of bands was seen in lane 1 and 4 containing diseased samples**

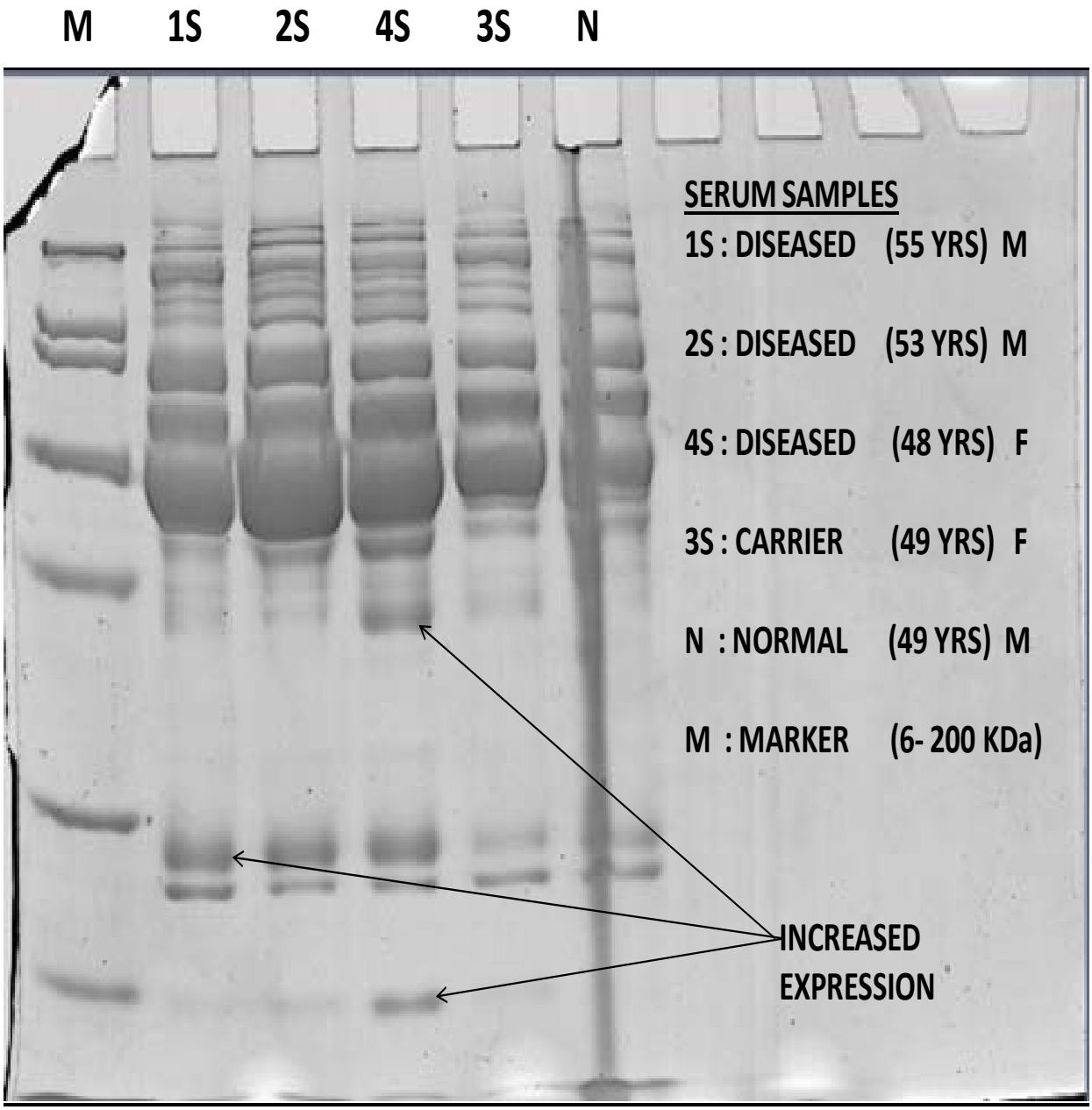



**Figure 5: SDS-PAGE profile of Serum of all the diseased and carrier and normal healthy individual, increased expression of bands was seen in lane 1 and 3 containing diseased samples**

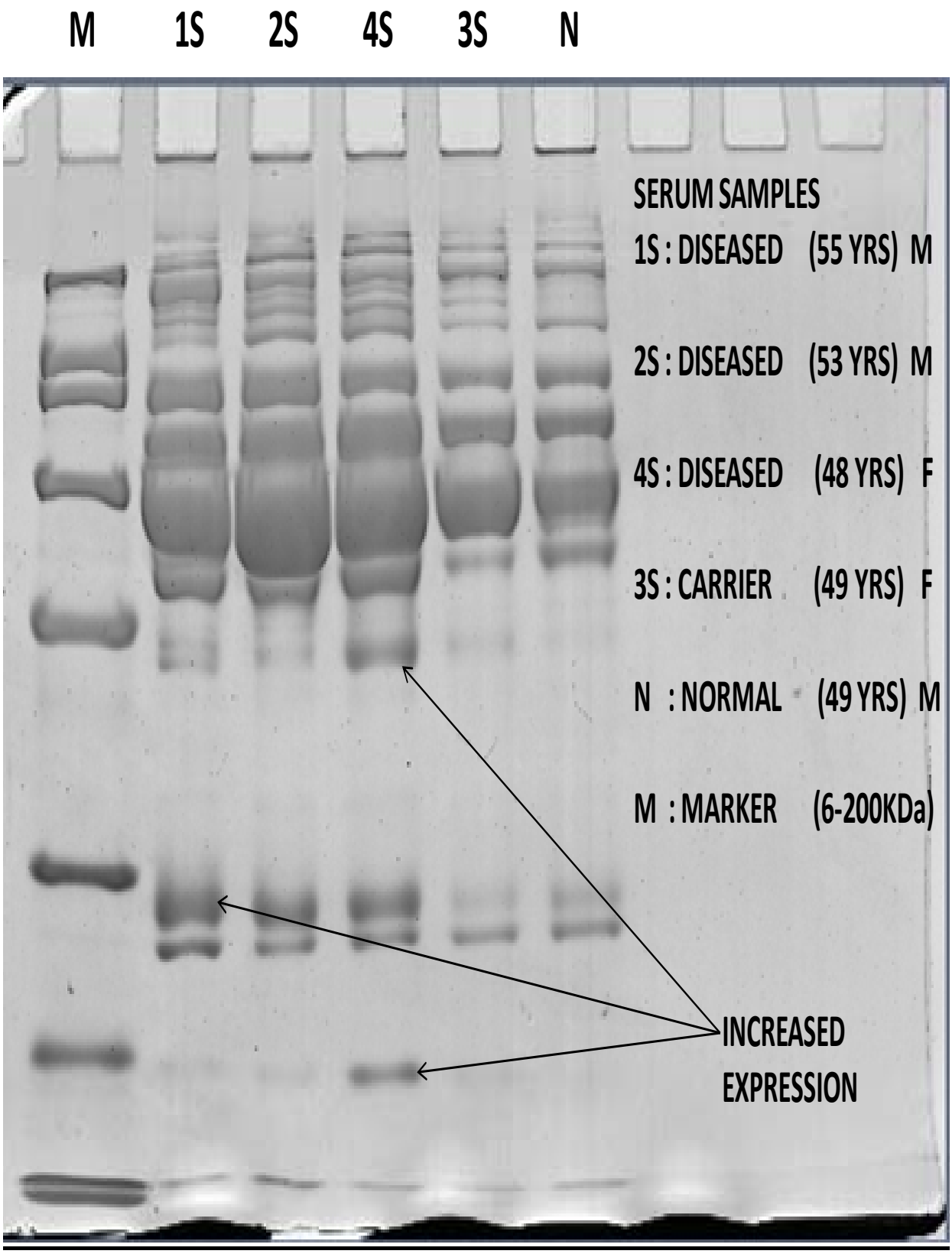



**Figure 6: Increased expression of bands was seen in lane 1 and 3 containing diseased samples**

## 5.4 TRYPSINIZATION

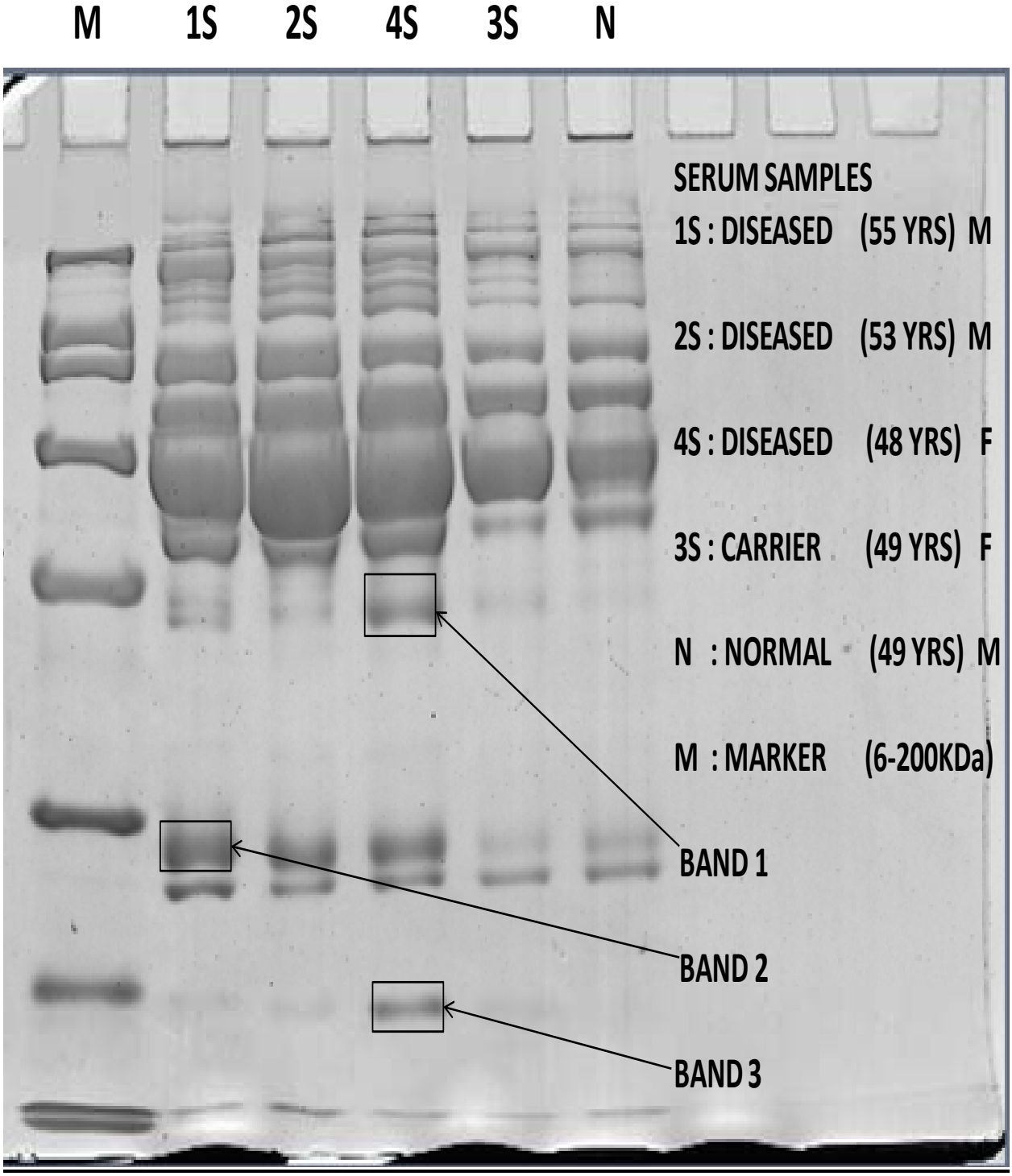



**Figure 7: Differentially expressed proteins identified on SDS PAGE gel in band 1, 2 and 3 were taken forward for 2D PAGE analysis**

## 5.5 MASS SPECTROMETRY RESULTS

The protein spots of the coomassie stained gels were utilized for mass spectrometry.

**Protein band 1 (45kDa):** Protein band 1 is overexpressed in diseased female sample 4S and also present in carrier as well as in normal individual but its expression is very less in carrier and normal (Figure 5). The mass spectrometry analysis of band 3 reveals a following peptide sequences

'KQLVEIEKV'

'RILGGHLDAKG'

'KQKVSVNERV'

'RVGYVSGWGRN'

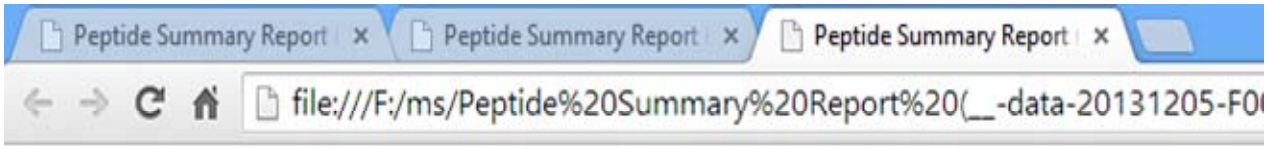
'RHYEGSTVPEKKT'

The peptide sequence is matched with 'Haptoglobin'of OS=Homosapiens'

(Accession no HPT_HUMAN).

Haptoglobin human HPT (45 KDa) is expressed in liver showed increased local arterial haptoglobin expression and found that haptoglobin is involved in cell migration and arterial restructuring probably Since cell migration and matrix turnover are important features in the pathology of arthritis and cancer.

## MASS SPECTROMETRY RESULTS

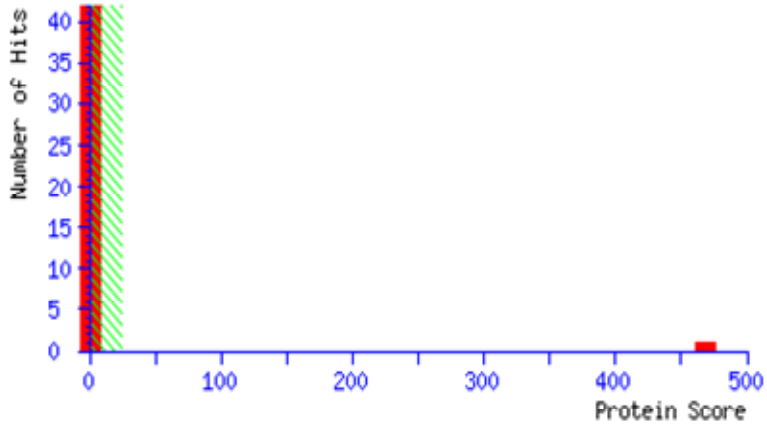



# {MATRIX SCIENCE} Mascot Search Results

```
User            : Jitendra
Email           : jvashist@gmail.com
Search title    :
MS data file    : DATA.TXT
Database        : SwissProt 2012_03 (535248 sequences; 189901164 residues)
Taxonomy        : Homo sapiens (human) (20255 sequences)
Timestamp       : 5 Dec 2013 at 11:00:49 GMT
Protein hits    : HPT_HUMAN Haptoglobin OS=Homo sapiens GN=HP PE=1 SV=1
```

## Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 24 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.




## Peptide Summary Report

**Figure 8 : Mascot results of LC MS of Band 1 showing identified peptide sequences and matched with HPT HUMAN PROTEIN [Homo sapiens]**

**Protein band 2 (16kDa):**

Protein band 2 is overexpressed in all diseased sample1S, 2S and 4S and clearly seen down-regulated in carrier as well as in normal individual (Figure 5).

The mass spectrometry analysis of band 2 reveals a two long peptide sequences

'RGGWCLQVNQLLYWALTTIGFQTTMLGGYFYIPPVNKY'

'RGGWCLQVNQLLYWALTTIGFQTTMLGGYFYIPPVNKYSTGMVHLLLQVTIDGR'

The peptide sequence is matched with 'arylamine N-acetyltransferase 2' of OS=Homo sapiens' (Accession no gi|73759739).

Arylamine N acetyl transferase-2 (16 KDa) involved in metabolism of various compounds and their role have been elicited in adverse drug reactions and as a risk factors in different kinds of cancer (Butcher et al., 2002). An NAT2 altered expression levels have been seen in colorectal cancer, where they might have an important role in protein acetylation/ deacetylation homeostasis by which they promote cancer cell survival (Brockton et al., 1999).

.

# MASCOT Search Results

## Protein View: gi|73759739

### arylamine N-acetyltransferase 2 [Homo sapiens]

| | |
|---|---|
| **Database:** | NCBInr |
| **Score:** | 26 |
| **Expect:** | 6.6e+002 |
| **Nominal mass ($M_r$):** | 16869 |
| **Calculated pI:** | 5.47 |
| **Taxonomy:** | Homo sapiens |

This protein sequence matches the following other entries:

- gi|73759741 from Homo sapiens

Sequence similarity is available as an NCBI BLAST search of gi|73759739 against nr.

### Search parameters

| | |
|---|---|
| **MS data file:** | DATA.TXT |
| **Enzyme:** | Trypsin: cuts C-term side of KR unless next residue is P. |
| **Fixed modifications:** | Carbamidomethyl (C) |
| **Variable modifications:** | Oxidation (M) |

### Protein sequence coverage: 36%

Matched peptides shown in ***bold red***.

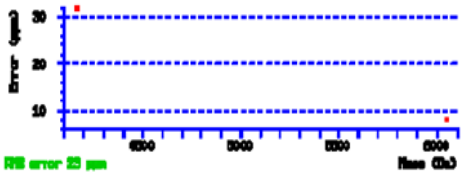
```
  1 MDIEAYFERI GYKNSRNKLD LETLTDILEH QIRAVPFENL NMHCGQAMEL
 51 GLEAIFDHIV RRNRGGWCLQ VNQLLYWALT TIGFQTTMLG GYFYIPPVNK
101 YSTGMVHLLL QVTIDGRNYI VDAGSGSSSQ MWQPLELISG KDQPQVP
```

Unformatted sequence string: 147 residues (for pasting into other applications).

Sort peptides by ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Show predicted peptides also

| Query | Start – End | Observed | Mr(expt) | Mr(calc) | ppm | M | Score | Peptide |
|---|---|---|---|---|---|---|---|---|
| r150 | 65 – 100 | 596.0383 | 4165.2173 | 4165.0846 | 31.9 | 0 | | R.GGWCLQVNQLLYWALTTIGFQTT |
| r152 | 65 – 117 | 865.1681 | 6049.1256 | 6049.0773 | 7.97 | 1 | | R.GGWCLQVNQLLYWALTTIGFQTT |



Mascot: http://www.matrixscience.com/

**Figure 9 : Mascot results of LC MS of Band 2 showing identified peptide sequences and matched with arylamine N-acetyl transferase 2 [Homo sapiens]**

**iii) Protein band 3 (11kDa):**

Although, the protein **band 3** is not present in carrier as well as in normal individual but its expression is high in the only in diseased female sample 4S (Figure 5).

The nano LC-MS/MS of **band 3** reveals a sequence of twelve amino acids '**KAGVETTTPSKQ**'.

The peptide sequence is matched with 'Ig lambda-2 chain C regions of OS=Homo sapiens' of Acinetobacter sp. (Accession no LAC2_HUMAN).

LAC2_HUMAN (11 KDa) also known as immunoglobin lambda 2 chains have implicated fold changes of expression levels in the neuromuscular disorder Facioscapulohumeral muscular dystrophy (FSHD) (Winokur et al., 2003).

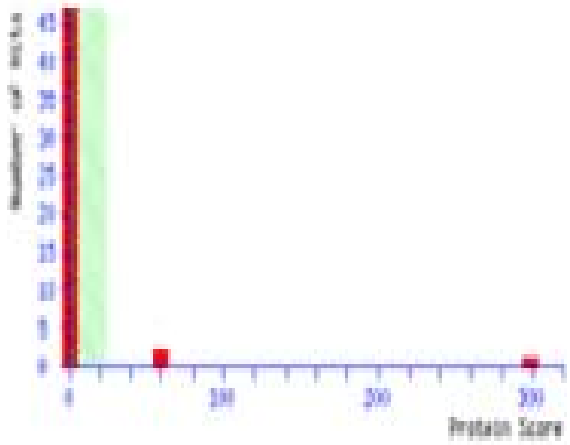
Variable expression of kappa and lambda chains of immunoglobin has indication in blood cell dyscrasia, such as kidney disease and B-cell lymphoma. So the altered expression of LAC2_HUMAN may have a role in disease progression of LGMD-2A

# Mascot Search Results

| | |
|---|---|
| User | : Jitendra |
| Email | : jvashist@gmail.com |
| Search title | : |
| MS data file | : DATA.TXT |
| Database | : SwissProt 2012_03 (535348 sequences; 189901164 residues) |
| Taxonomy | : Homo sapiens (human) (20355 sequences) |
| Timestamp | : 5 Dec 2013 at 10:56:51 GMT |
| Protein hits | : LAC2_HUMAN Ig lambda-2 chain C regions OS=Homo sapiens GN=IGLC2 PE=1 SV=1<br>IGKC_HUMAN Ig kappa chain C region OS=Homo sapiens GN=IGKC PE=1 SV=1<br>LAC1_HUMAN Ig lambda-1 chain C regions OS=Homo sapiens GN=IGLC1 PE=1 SV=1 |

## Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 24 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.




## Peptide Summary Report

**Figure 10 : Mascot results of LC MS of Band 3 showing identified peptide sequences (score 300) and matched with LAC2 HUMAN, IGKC HUMAN**

## 5.6 2D PAGE RESULTS

### TWO DIMENSIONAL GEL ELECTROPHORESIS:

The proteomic study was also done with the normal (N) and diseased (1S) samples using two dimensional gel electrophoresis (4-7 pH range) (Figure 11 to 13). The protein amount that loaded on IPG strip for analytical preparations was 50 µg and it gave good visualization, after silver staining.

2DE gel serum protein profiles of normal (N) and diseased (1S) were also run in 3-10 pH range (data not shown). 3-10 pH range IPG strips were used to see the all the proteins, however most of the proteins were found towards anodic region and placed in between 4-7 pH range therefore for better resolution and visualization IPG strips with pH 4-7 range were also used. 2DE data obtained using 4-7 pH range was considered best for comparison purpose.

Silver stained 2D gel profiles of normal (N) and diseased (1S) are also compared with their respective SDS-PAGE profiles and clearly shows that there are more protein spots in the region of 25 to 14kDa. 2D gel profiles of normal (N) and diseased (1S) also give an indication of differences in the protein expression. This is a good agreement of the proposed hypothesis of differential protein expression in normal (N) and diseased (1S) persons. To prove these proteins as a biomarker, other high end studies like DIGE and iTARQ are essential. However, the present study gives a clue of specific proteins which can be easily identified using SD-PAGE and act as a diagnostic tool in the patients of LMGD2A.

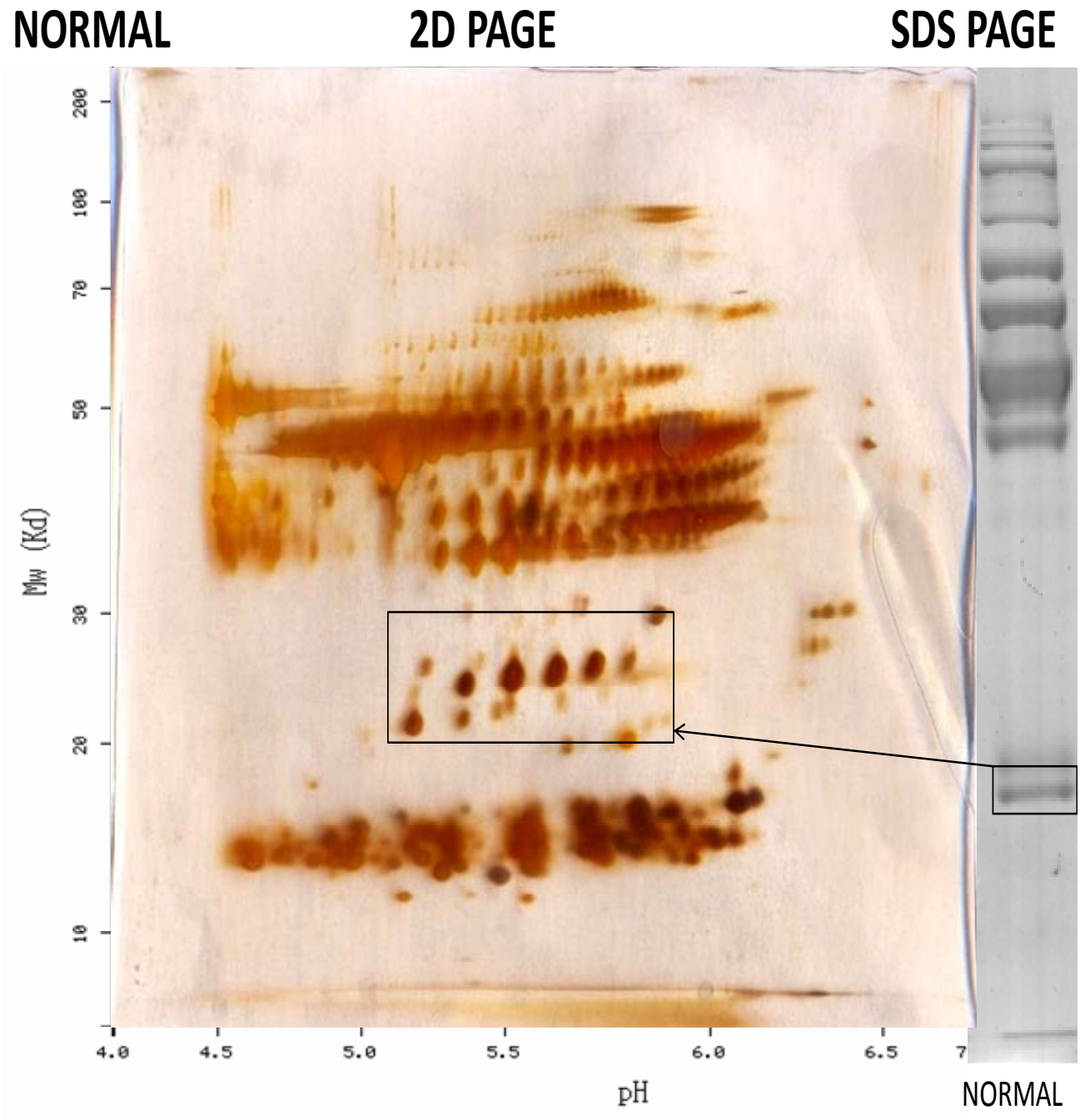



**Figure 11: Comparison of Silver stained 2D gel profile normal (N) with coomassie stained SDS-PAGE profile of same individual.**

## 2D PAGE RESULTS

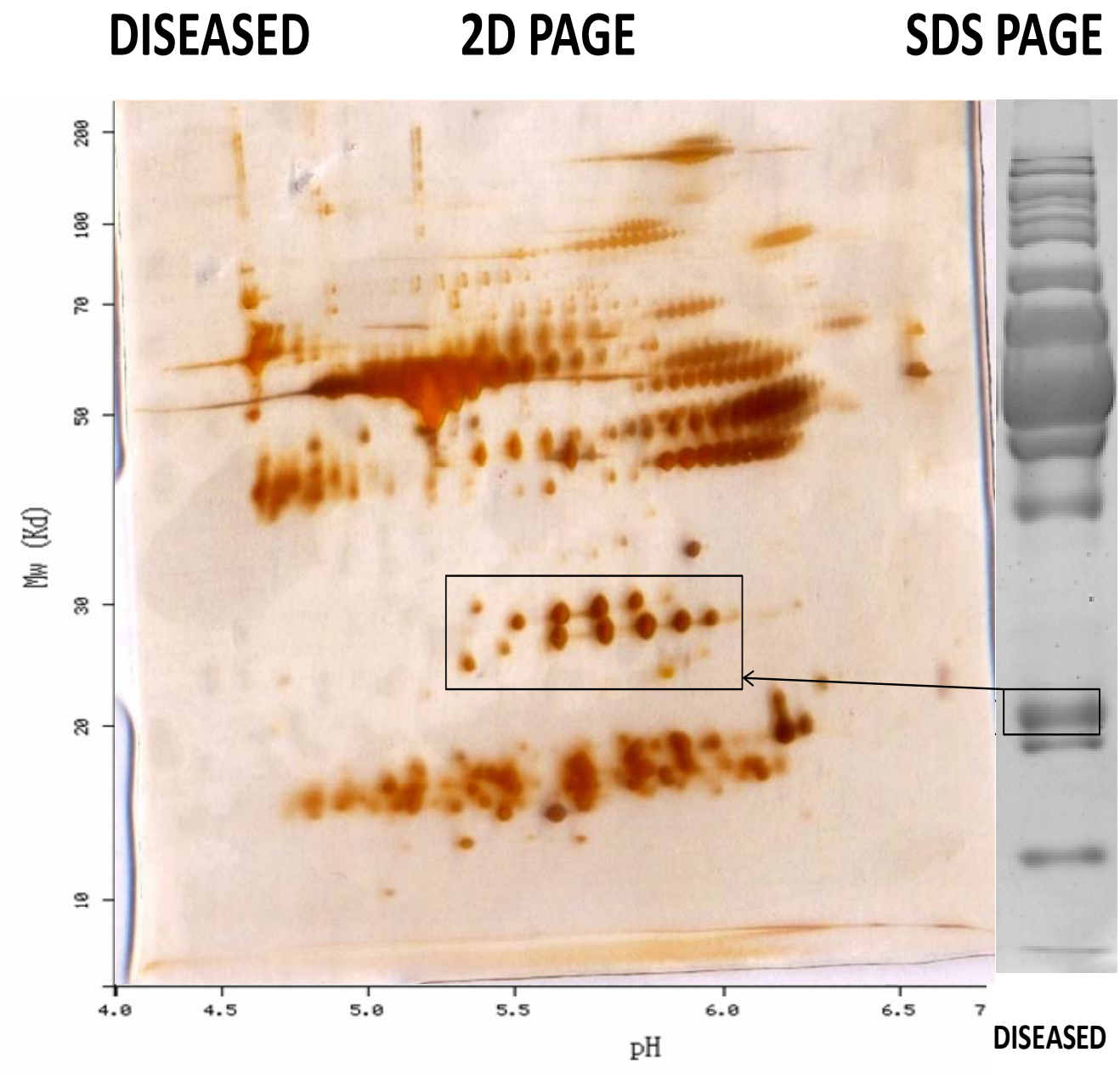



**Figure 12 : Comparison of Silver stained 2D gel profile diseased (1S) with coomassie stained SDS-PAGE profile of same individual**

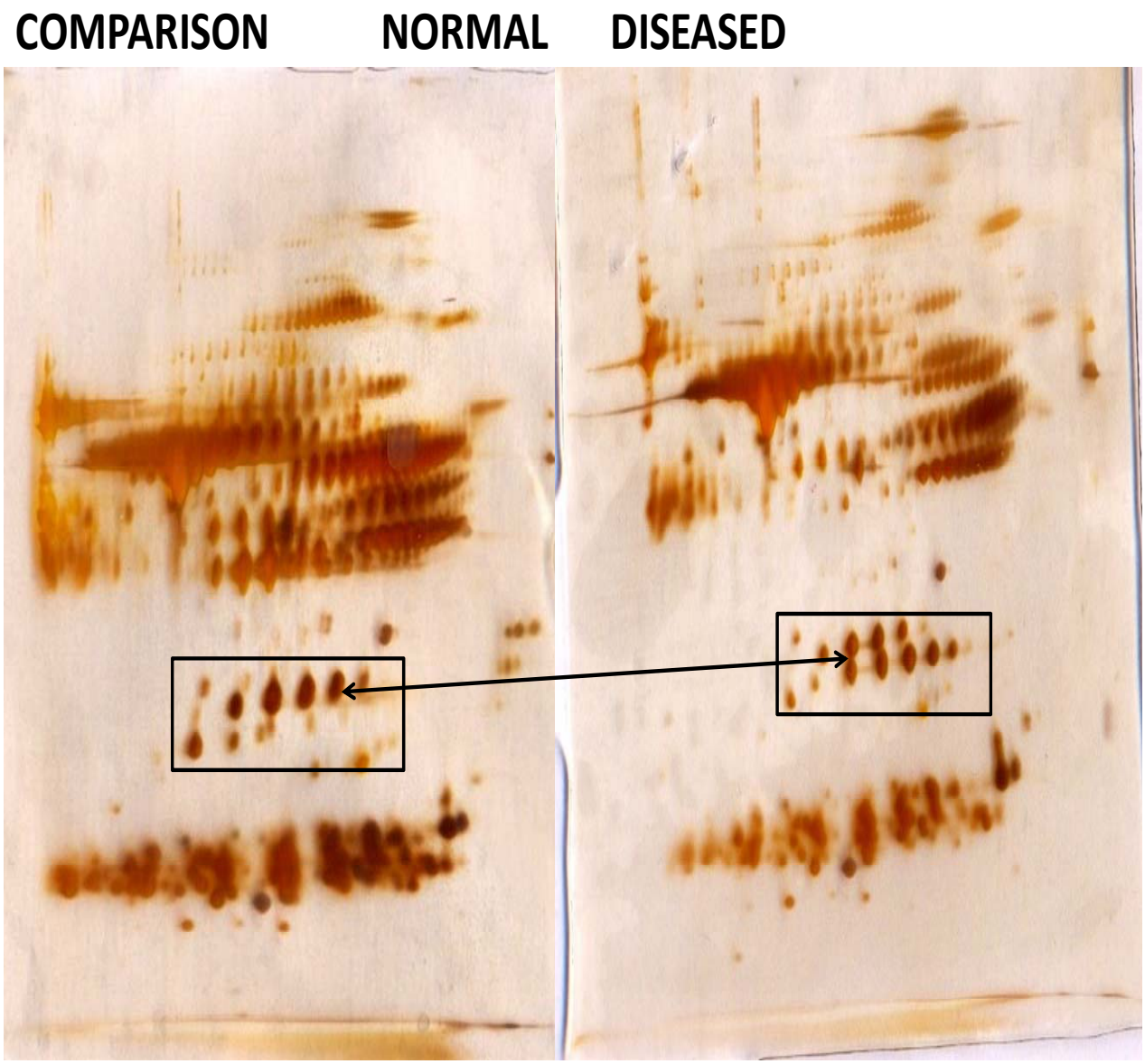



**Figure 13: Comparison of Silver stained 2D gel profiles of normal (N) and diseased (1S) using 13 cm, pH 4-7 strip.**

## 6. CONCLUSION

Present study was undertaken to identify protein biomarkers as a diagnostic tool for LGMD-2A. By differential proteome expression profile of proteins, three altered proteins namely LAC2_HUMAN, HPT HUMAN, Arylamine N- acetyl transferase have been identified, which can be further explored as biomarker for LGMD-2A

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# 8. APPENDIX

## 8.1 SAMPLE COLLECTION AND SEPARATION OF SERUM

Needles, syringes, blood collection tubes, antiseptic, cotton balls, bandages, gel packs blood samples, centrifuge, eppendorf tubes, pipettes (1mL, 100µL), freezer.

## 8.2 PREPARATION OF STANDARD FOR BRADFORD ASSAY FOR PROTEIN QUANTIFICATION

Stock solution of BSA (0-1 mg/ml), water, cuvettes, spectrophotometer, test tubes, stand

## 8.3 PROTEIN QUANTIFICATION

96- well ELISA plates, Bradford reagent, serum samples, water, pipettes, microplate reader machine, incubator.

## 8.4 SDS PAGE

**CHEMICALS REQUIRED:-** Acrylamide solutions (for resolving & stacking gels), distilled water, Gel loading buffer, Running buffers, Staining and destaining solutions, serum samples, molecular weight markers.

**EQUIPMENS USED:-** An electrophoresis chamber and power supply, glass plates (a short and a top plate), casting stand, combs

**REAGENTS:-**

I. **30% Polyacrylamide solution** (29.2g acrylamide+0.8g bisacrylamide in 50 ml of water, dissolve completely using a magnetic stirrer, make the volume upto 100ml).
II. **1.5 M Tris**, pH 8.8 (18.16gm in 100ml water)
III. **1 M Tris**, pH 6.8 (12.11gm in 100 ml water)
IV. **10% SDS** (10gm SDS in 100mL distilled water).

V. **10% ammonium persulfate** (20mg in 200ml water)- freshly prepared.

VI. **10x SDS running buffer** 3.027gm Tris base, 14.4gm glycine (6ml of 10% SDS was prepared in separate beakers). When properly dissolved mix three of them and make upto 100ml.(working standard is 1X buffer)

**Resolving gel (10%) 10ml**

| | |
|---|---|
| $dH_20$ | 3.175ml |
| 30% acrylamide mix | 4.0 ml |
| 1.5M Tris pH8.8 | 2.5 ml |
| 10% SDS | 0.1 ml |
| 10% APS | 0.1 ml |
| TEMED | 0.004ml |

**Stacking gel (5%) 10 ml**

| | |
|---|---|
| $dH_20$ | 3.175ml |
| 30% acrylamide mix | 4.0 ml |
| 1.5M Tris pH8.8 | 2.5 ml |
| 10% SDS | 0.1 ml |
| 10% APS | 0.1 ml |
| TEMED | 0.004ml |

**GEL LOADING BUFFER:-** (10ml solution)

**I.** 1.25 ml 1M Tris-HCl pH 6.8

II. 0.3 g per 3ml of 10% SDS

III. 0.2 ml of 20% glycerol

IV. 0.5 ml of 5% β-mercaptoethanol

V. 0.02% of bromophenol Blue

**LOADING DYE:-** 190ul loading dye + 10ul B-Mercaptoethanol + 1ul sample.

**STAINING SOLUTION:-** 0.25g of Coomassie Brilliant Blue R250 was added to 50ml of the staining solution 90 ml methanol: water (50ml: 40ml) was added first and then 10ml of Glacial acetic acid was added very slowly. It was properly mixed using a magnetic stirrer.

**DESTAINING SOLUTION:-** 50ml of methanol: 10ml acetic acid: 40ml water

**8.5 TRYPSINIZATION:-** extraction of peptides from SDS PAGE gel pieces [19]

20ml of 1% formic acid, 10ml of 9% acetonitrile, 40mM ammonium bicarbonate, trypsin, 50% acetonitrile, 100%ACN**,** eppendorf, pipette

.

**8.6 LYPHOLIZATION**

Lyophilizer, overnight frozen samples, bottleneck flask, rubber bands

**8.7 2-D PAGE**

I. **Lyophilized serum samples**
II. **Rehydration buffer** containing 10ml of 8M urea, 2% CHAPS, 50mM DTT, 0.2% biolyte ampholytes, bromophenol blue.
III. **Nanopure water**
IV. **Equilibration buffer I** containing 20 ml of 6M urea, 2% SDS, 0.375 M Tris HCl (pH 8.8), 20% glycerol and 2% DTT.
V. **Equilibration buffer II** containing 20ml of 6M urea, 2% SDS, 0.375M Tris HCl (pH 8.8) and 20% glycerol.
VI. **30% Glycerol solution:** 70 ml of 30% glycerol solution
VII. **Iodoacetamide:** 0.5 gm of iodoacetamide
VIII. **IEF Cell and IEF focusing tray with lid**
IX. **IPG Strips pH 4-7, 11cm**
X. **Electrode wicks (precut)**
XI. **Blotting filter papers**
XII. **Mineral oil**
XIII. **Forceps, pipette, stirplate, plastic wrap, power supply**
XIV. **Tris/glycine/SDS running buffer/silver staining reagents.**