# P.171: Detection of Alpha-Dystroglycan in Muscle Biopsies using a Multiplexed Western Blot Method

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## Introduction

Limb Girdle Muscular Dystrophy Type 2I (LMGD2I) is an autosomal recessive disease of striated muscle caused by mutations in the fukutin-related protein gene (*FKRP*). *FKRP* encodes a glycosyltransferase that is critical for the glycosylation of alpha-dystroglycan (αDG) (Figure-1). The heavily glycosylated αDG is a component of the dystrophin-glycoprotein complex that anchors the intracellular cytoskeleton of muscle cells to the extracellular matrix through interactions of the matriglycan on αDG. In the context of LGMD2I αDG is hypo-glycosylated due to, partial loss of function, mutations in FKRP. This makes muscle cells susceptible to contraction induced injury that results in inflammation, fibrosis, and fatty infiltrate leading to muscle wasting and impaired function.

To support our ongoing clinical trial of a potential therapy, BBP-418, for LGMD2I we sought to identify a biomarker that could describe the glycosylation state of αDG as hypoglycosylation of αDG is at the core of the disease. BBP-418 increases the intracellular concentration of CDP-Ribitol enabling a defective FKRP to be more active allowing αDG to be primed for further glycosylation. Hence, we rationalized that measuring the total αDG and the glycosylated αDG would be a viable strategy. To realize this, we employed a multiplexed western blot approach that could be used to interrogate patient biopsies at baseline and post BBP-418 administration. Here we describe this method and demonstrate its utility to assess biopsy samples from LGMD2I patients.

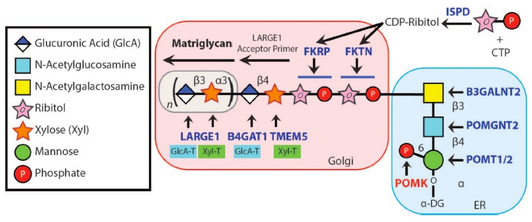



**Figure-1**: FKRP plays a critical role in priming αDG for additional glycosylation (taken from Walimbe et al, 2020)

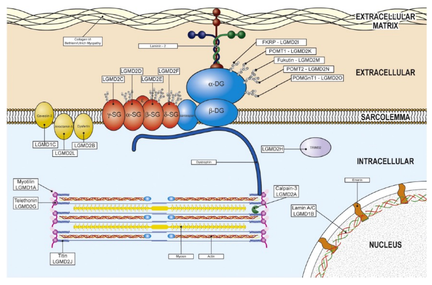



**Figure-2**: αDG is an integral part of the dystrophin-glycoprotein complex (Figure taken from Wicklund et al, 2014).

## Methods

***Muscle Biopsy***

Muscle biopsy samples were collected from tibialis anterior (TA) using a fine needle aspirate. In total three passes were collected. Biopsy samples were snap-frozen immediately after collection and stored at -80°C until analysis.

***Healthy Human Tissue***

Control healthy human muscle tissue (male and female TA) was obtained from BioIVT and stored at -80°C until analysis.

***Tissue Lysis***

Tissue samples were processed using a TissueLyzer II (Qiagen). Approximately 2-40 mgs of tissue was placed in a 2 mL microtube with a steel bead and 1/10 w/v of SDS-Urea lysis buffer supplemented with protease inhibitors. The microtubes were subjected to five cycles of disruption (2 min at 25 Hz) in the TissueLyzer II. Lysates were clarified by centrifugation and the supernatant was transferred to fresh tubes and lysate concentration was determined using 660 nm Protein Assay Kit (Pierce).

***SDS-PAGE***

Polyacrylamide gel electrophoresis was carried out on 4-20% Novex gels (LifeTech). Tissue lysates were diluted to appropriate concentrations in SDS-Urea buffer supplemented with bromophenol blue. Pre-stained protein molecular weight markers were also loaded onto gel.

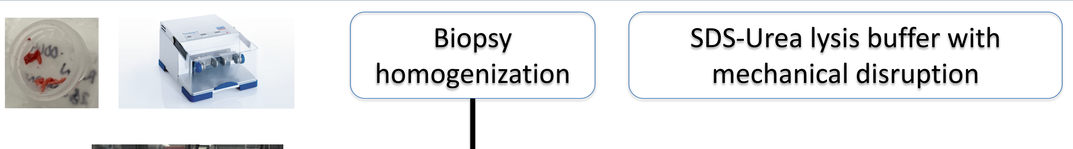
***Western Blotting***

Proteins were transferred to a nitrocellulose membrane (NC) using an iBlot2 Gel Transfer Device (Invitrogen). The membrane was dried then rinsed with water. The membrane was blocked with Intercept blocking buffer (LiCor) and probed with AF6868 alpha Dystroglycan antibody (R&D Systems) and IIH6C4 alpha Dystroglycan antibody (Millipore) overnight at 4°C. The primary antibody solution was discarded and the washed four times with TBST. The blot was then probed with IR800CW Mouse Anti-Sheep and IR680 Goat Anti-Mouse secondary antibodies (LiCor) for one hour at room temperature. The secondary antibody solution was discarded, and the membrane was wash four times with TBST. The membrane was visualized on an Odyssey CLx imager (LiCor) and intensities of the 700 and 800 nm channels were recorded.

***Data Analysis***

Analysis was carried on GraphPad Prism (version 9). Signal intensities were normalized to total protein and the ratio of 700 nm normalized signal/800 nm normalized signal was determined. Linear fits were assessed using the linear regression function of Prism. Statistical significance was determined using an unpaired t-test using Prism.

## Western Blot Workflow




## Antibody Specificity

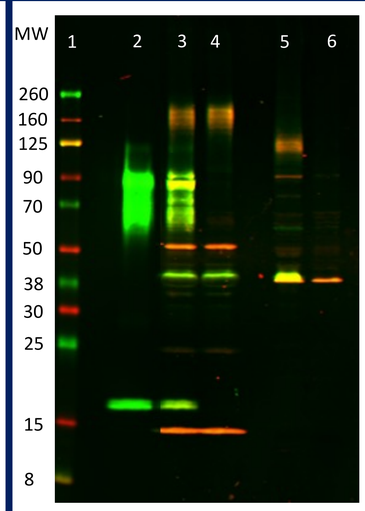



**Figure-3** Western blot merged image showing the specificity of both primary antibodies. Also shown is the staining of rhDG.

| Lane | Description |
|---|---|
| 1 | Molecular weight markers |
| 2 | rhDG |
| 3 | rhDG + TA lysate |
| 4 | TA lysate only |
| 5 | WT HEK293 lysate |
| 6 | DAG1 KO HEK293 lysate |

The specificity of the primary antibodies was assessed using recombinant protein and lysates. Lane 2 is recombinant human αDG (rhDG) only showing staining for AF6868, Lane 3 & 4 are healthy donor tibialis anterior (TA) lysate spiked with recombinant human αDG and only TA lysate show staining with both the AF6868 and IIH6C4 antibodies. Lane 5 & 6 are a lysates from HEK293 and an engineered *DAG1* knockout HEK293 cells. The lack of signal in both channels in the 125 kDa for lane 6 demonstrates both antibodies react with αDG as the knockout lacks αDG while the parental HEK293 shows staining by both primary antibodies.

## Signal Linearity

The linear range of the method was determined using a dilution series of healthy control TA. The experiment was performed from lysate generated from three different masses of healthy TA from the same donor (Figure-4)

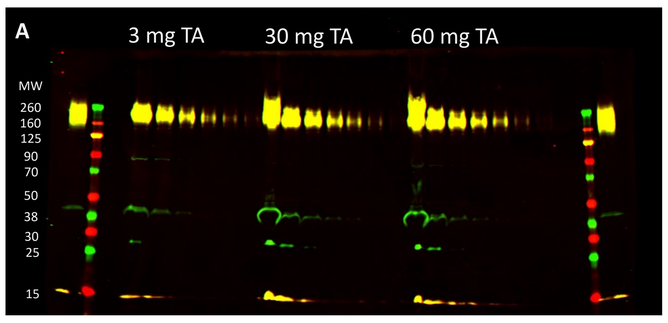



**Figure-4 A**. Western blot merged image showing dilution series of TA lysate starting from 60 µgs to 1 µg. The 3 mg TA tissue prep had insufficient material to include a 60 µg point. **B**. Linear plot of the 700 and 800 nm signals from the analysis for the western blot.

| Sample | 700 nm slope | 800 nm slope | 700 nm intercept | 800 nm intercept | 700 nm $r^2$ | 800 nm $r^2$ |
|---|---|---|---|---|---|---|
| 3 mg TA | 1168 | 938 | -1371 | -946 | 0.9961 | 0.9963 |
| 30 mg TA | 1597 | 1146 | -5561 | -3271 | 0.9649 | 0.9759 |
| 60 mg TA | 1623 | 1204 | -4970 | -3273 | 0.9808 | 0.9851 |

## Analysis of Patient Samples

A subset of biopsies from patients enrolled in our natural history study were analyzed using this western blot method to determine the extent of αDG glycosylation. Since patient samples are expected to have reduced αDG glycosylation we interrogated two concentrations of patient lysate to ensure that signals would fall in the linear range of the assay. We included more compound pathogenic heterozygous patients in our testing as we expect these patients to have reduced glycosylation relative to L276I homozygous patients. Shown in Figure-5 there is reduced staining for the glycosylated form of αDG and present is a significant amount of αDG that is not glycosylated.

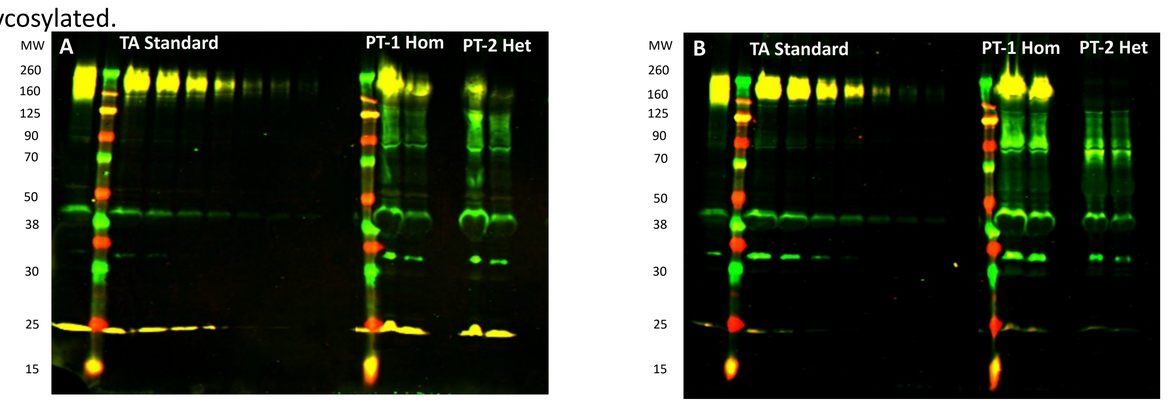



**Figure-5 A,B.** Western blot merged examples containing patient samples. The TA standard span 25 µg to 0 µg. Patient samples were run at two concentrations of lysate and results of ratio were averaged. **C.** Plot of the normalized ratio determined by interpolation of signal intensities and using the raw signal intensities from integration.(700 nm/800 nm) **D**. Plot of the interpolated amounts of total αDG (aDG) and glycosylated αDG (aDG-gly) for the homozygous and compound heterozygous patients relative to healthy tissue. Heterozygous *CP denotes compound pathogenic heterozygous mutation.

## Conclusions

- A multiplexed western blot method has been developed to detect the extent of αDG glycosylation relative to total αDG from muscle biopsy samples and presented as the αDG ratio.
- The two primary antibodies used showed good specificity for αDG and glycosylated αDG.
- The detection signals show good linearity with lysate concentration.
- LGMD2I patient samples from an ongoing longitudinal natural history study were analyzed and a significant reduction in αDG glycosylation was measured.
- As expected, the compound pathogenic heterozygous patients showed a greater loss in glycosylation content than homozygotes. This finding aligns with known differences in disease onset and progression for compound pathogenic heterozygotes who generally exhibit an earlier disease onset and more rapid progression than homozygotes.
- This method may prove useful in further clinical trials in LGMD2I where status of glycosylation could serve as a primary disease biomarker.

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