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HAL Authorization

Serum and urine proteomic profiling reveals biomarkers suitable for monitoring the outcome of therapeutic interventions in muscular dystrophies

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Therapy-responsive biomarkers are an important need in the muscular dystrophy field where new treatments are currently in clinical trials. In the present study, serum samples from Duchenne muscular dystrophy (DMD) patients and their respective age-matched controls (77 individuals) collected in the frame of the ADNA program (Advanced Diagnostic for New Therapeutic Approaches) were analyzed by a comprehensive high-resolution mass spectrometry approach. Several proteins and protein fragments were found to be abnormally present in sera and urines of DMD patients, limb-girdle muscular dystrophy type 2D (LGMD2D) and their respective animal models. Levels of one of the found biomarkers, fragments of the myofibrillar structural protein myomesin-3 (MYOM3) were assayed in therapeutic model systems where stable restoration of α -sarcoglycan expression in KO-SGCA mice was achieved by systemic injection of a viral vector. Following administration of the therapeutic agents MYOM3 was restored toward wild-type levels in a dose-dependent manner. MYOM3 fragments showed lower inter-individual variability compared with the commonly used creatine kinase assay, and correlated better with the restoration of the dystrophin-associated protein complex and muscle force. These data suggest that the MYOM3 fragments hold promise for minimally invasive assessment of experimental therapies for DMD and other neuromuscular disorders.

Sample organization

In order to decrease the number of analyses, samples from the cohort (103 individuals) were organized into 3 groups: **G1**: DMD, 39 individuals, **G2**: G1 DMD after 6 months of corticosteroid treatment, 26 individuals, **G3**: healthy controls, 38 individuals. In each group samples were further organized into sub-group according to the subject age. Numbers below each pool indicate the interval of age and the number of patients (in brackets)

DMD	G1-1	G1-2	G1-3	G1-4	G1-5	G1-6
Age (number)	3-4 (7)	4-6 (11)	6-7 (4)	7-8 (4)	14-16 (6)	16-20 (7)
DMD-cortico	G2-1	G2-2	G2-3	G2-4		
Age (number)	3-4 (7)	4-6 (11)	6-7 (4)	7-8 (4)		
Healthy controls	G3-1	G3-2	G3-3	G3-4	G3-5	G3-6
Age (number)	3-4 (5)	4-6 (6)	6-7 (5)	7-8 (5)	12-16 (10)	16-20 (7)

Examples of proteins overexpressed in urine and serum from young DMD versus age matched healthy controls

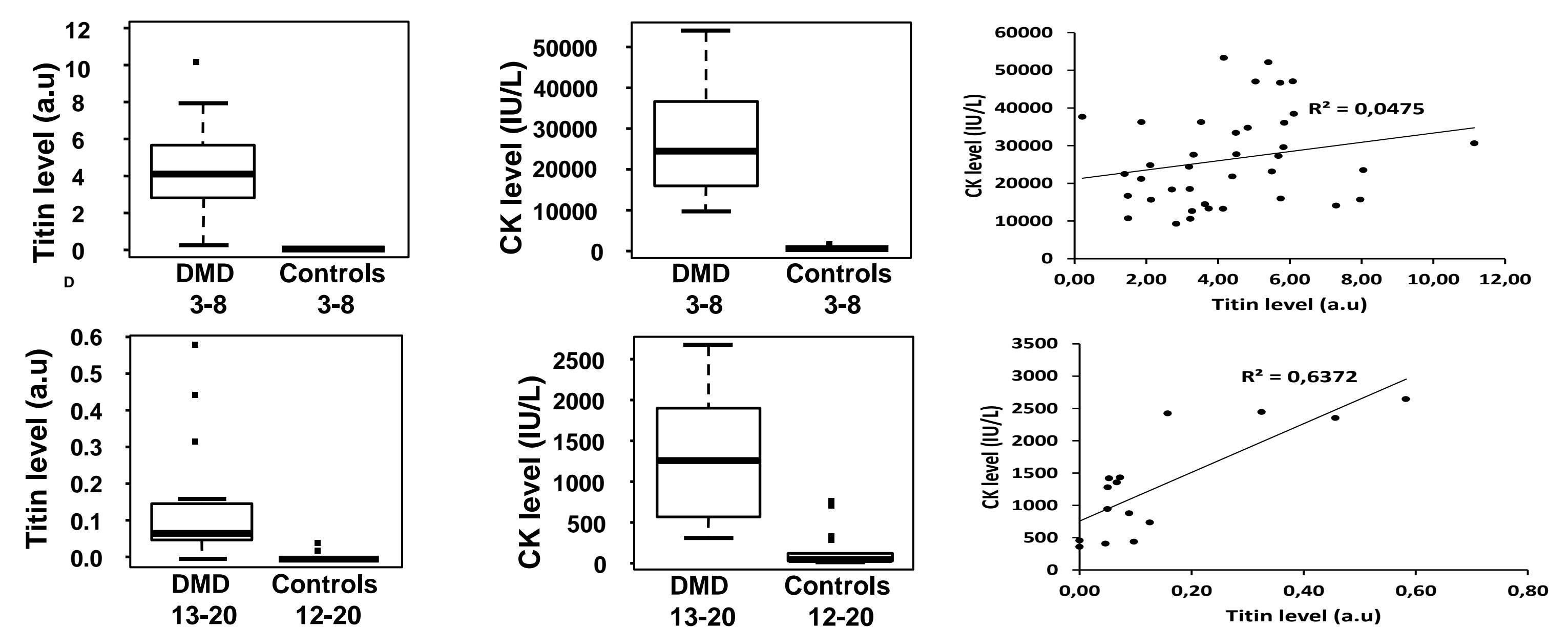
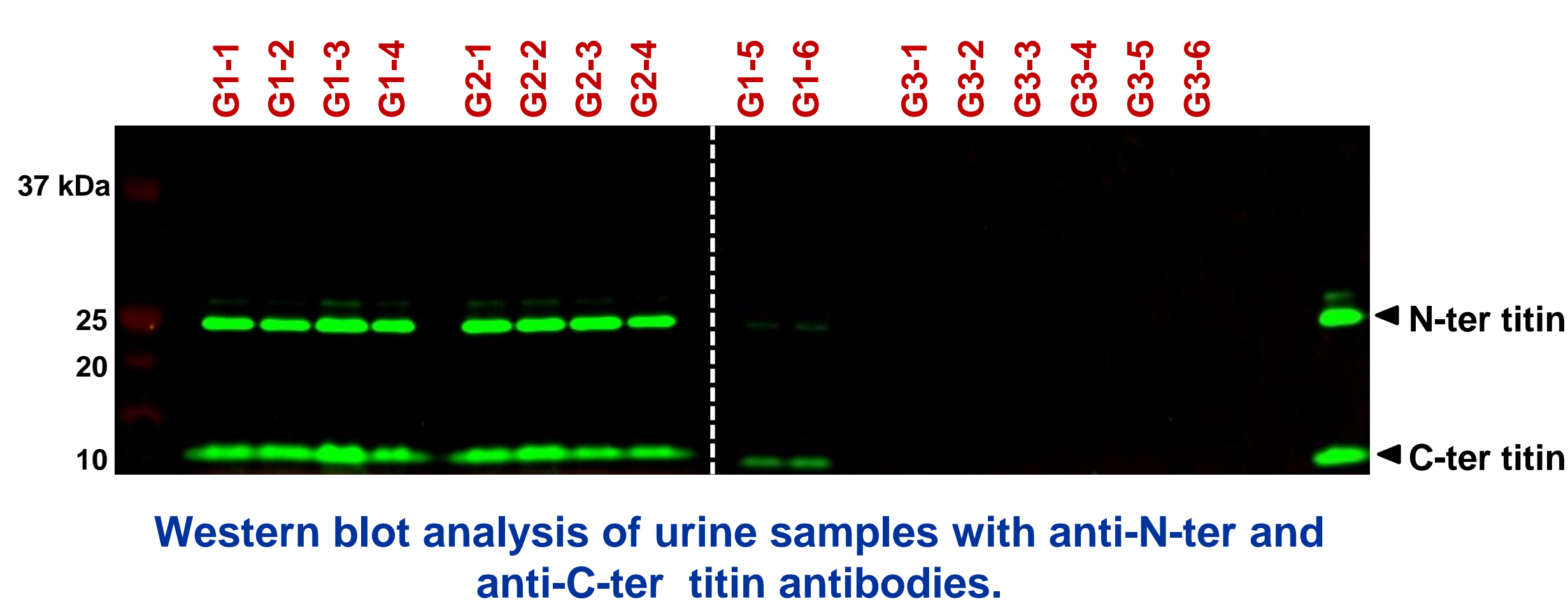
Urine	ANOVA (p-value)	Fold change	Serum	ANOVA (p-value)	Fold change
Titin	1.72E-06	20	MYOM2	9.8e-05	100.1
Myosin-7	1.57E-05	44	MYOM3	1.5e-05	49.7
Filamin-C	3.54E-05	5	CK-M	2.9e-05	39.8
Myosin-2	4.25E-05	8	Myosin-7	2.2e-05	38.3
Cytosolic 5'-nucleotidase 1A	4.75E-05	26	β -enolase	7.4e-05	34.8
Obscurin	5.03E-05	38	Carbonic anhydrase 3	8.6e-05	23.9
Myc box-dependent-interacting protein	2.44E-04	11	Alanine aminotransferase 1	3.0e-05	15.6
Haptoglobin	7.31E-04	5	Fructose-bisphosphate aldolase A	9.3e-05	14.2
Myosin-binding protein C, slow-type	8.55E-04	14	Pyruvate kinase PKM	1.1e-05	12.8
Serine protease HTRA3	1.56E-03	6	Vinculin	7.2e-05	10.3
14 kDa phosphohistidine phosphatase	5.09E-03	8	L-lactate dehydrogenase B	2.4e-05	5.4

Mass-spectrometry analysis

20 mg of total protein, for serum, and 300 μ g, for urine, were assembled for each sub-group with equal presentation of each individual. For serum, after depletion of the 12 most abundant serum proteins, samples were analyzed by a LTQ Velos Orbitrap mass spectrometer. For urine, a volume corresponding to 10 μ g of proteins of each subgroup were used for the mass spectrometry analysis. Data were processed with an in house Mascot search server using SwissProt database followed by label-free analysis of protein expression. The comparison of young DMD patients with their age matched controls revealed 24 proteins more abundant in DMD and 13 in sera of healthy subjects. Twenty proteins more abundant in DMD patients and 12 more abundant in healthy controls were identified by this approach in urines. The myomesin-3 (MYOM3) protein was chosen for further analysis as serum biomarker and titin protein as urinary biomarker.

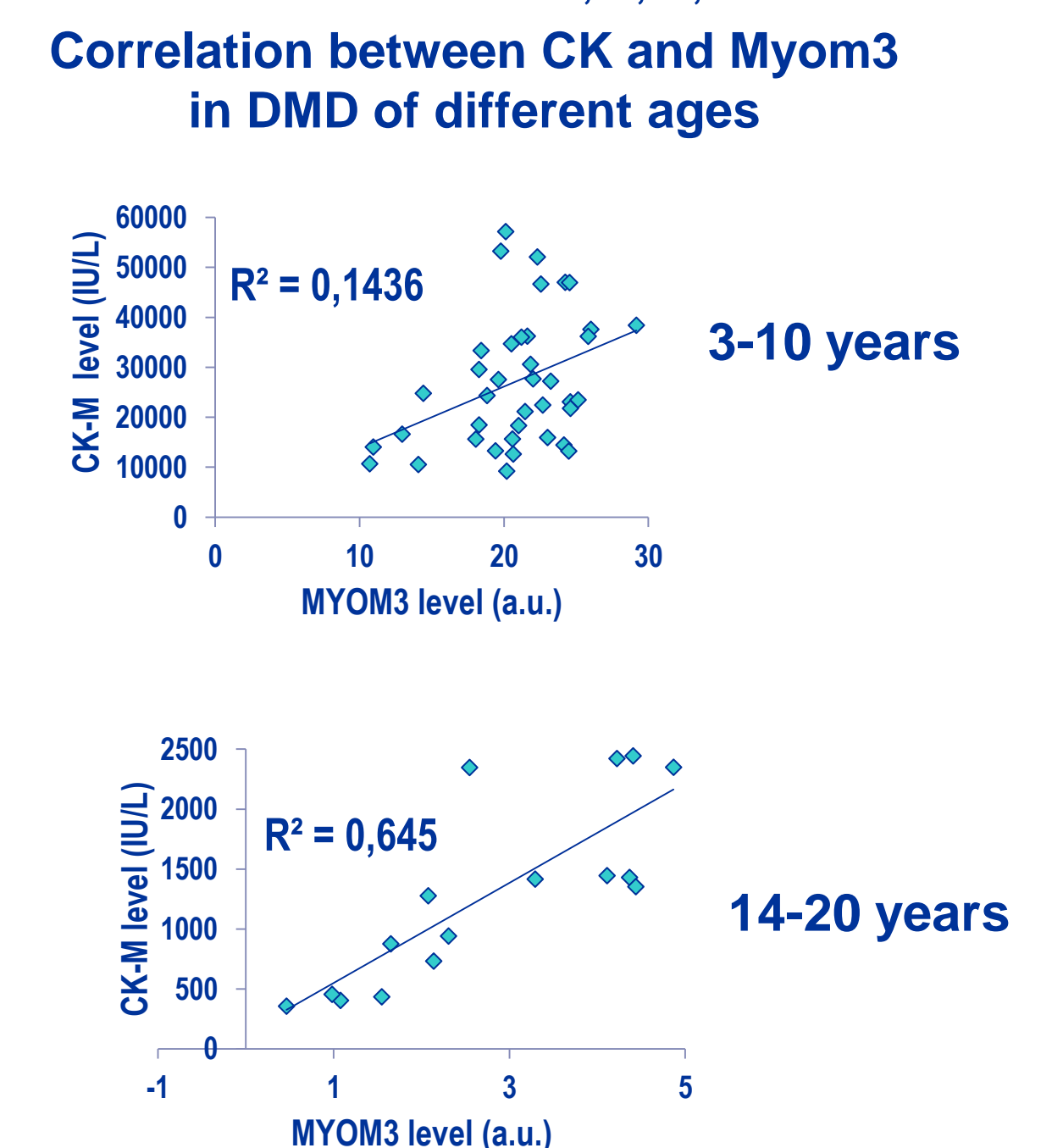
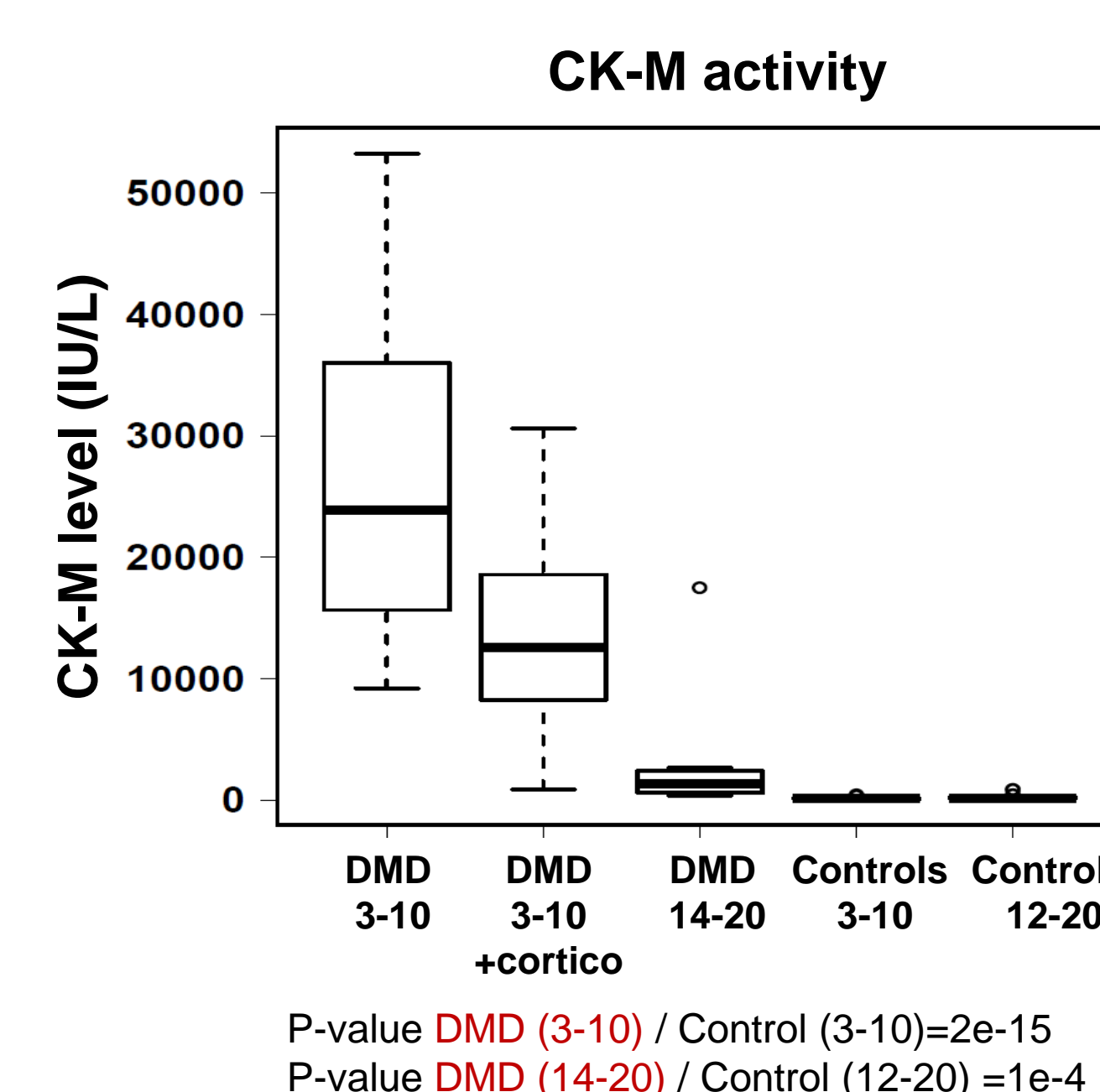
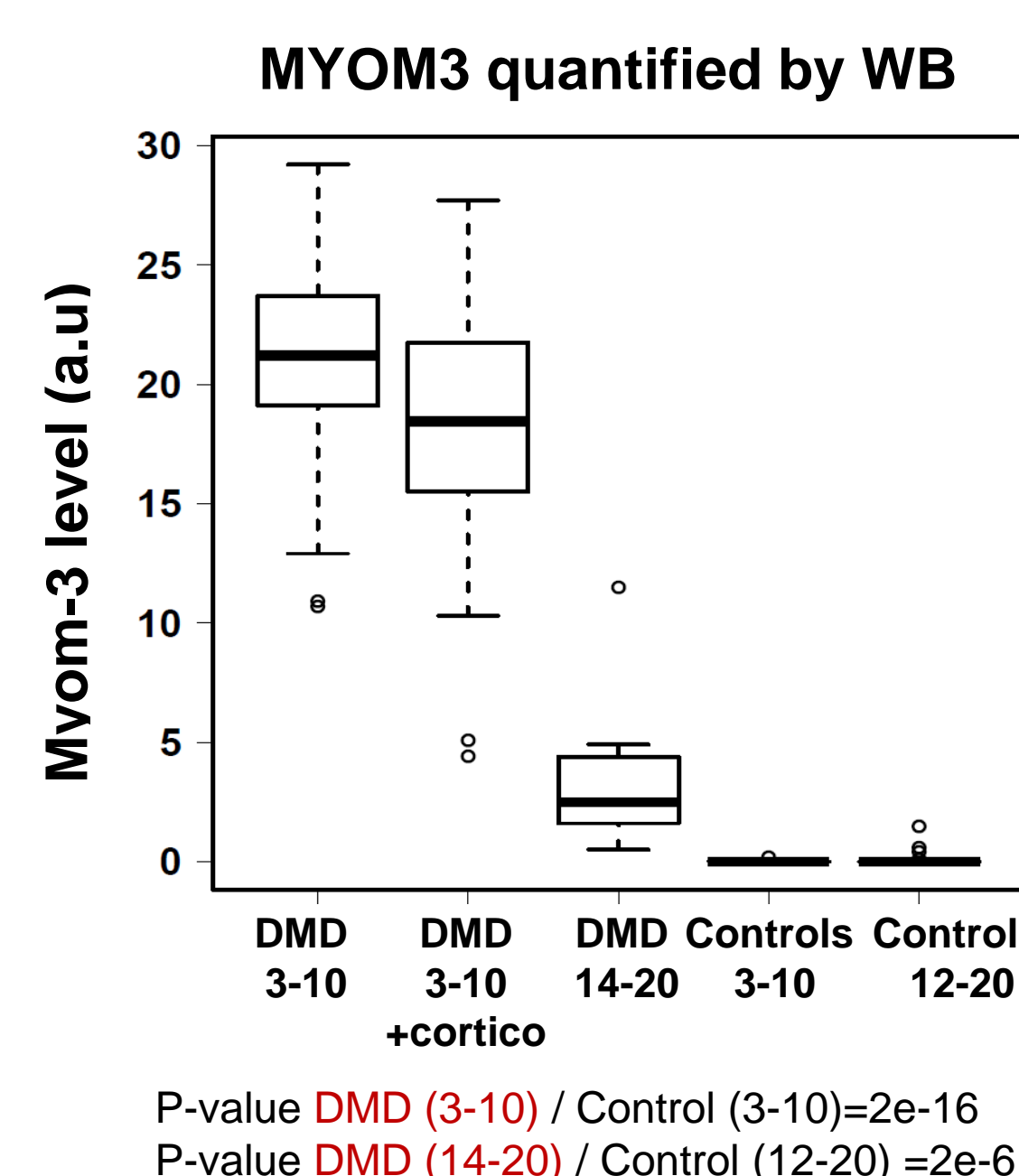
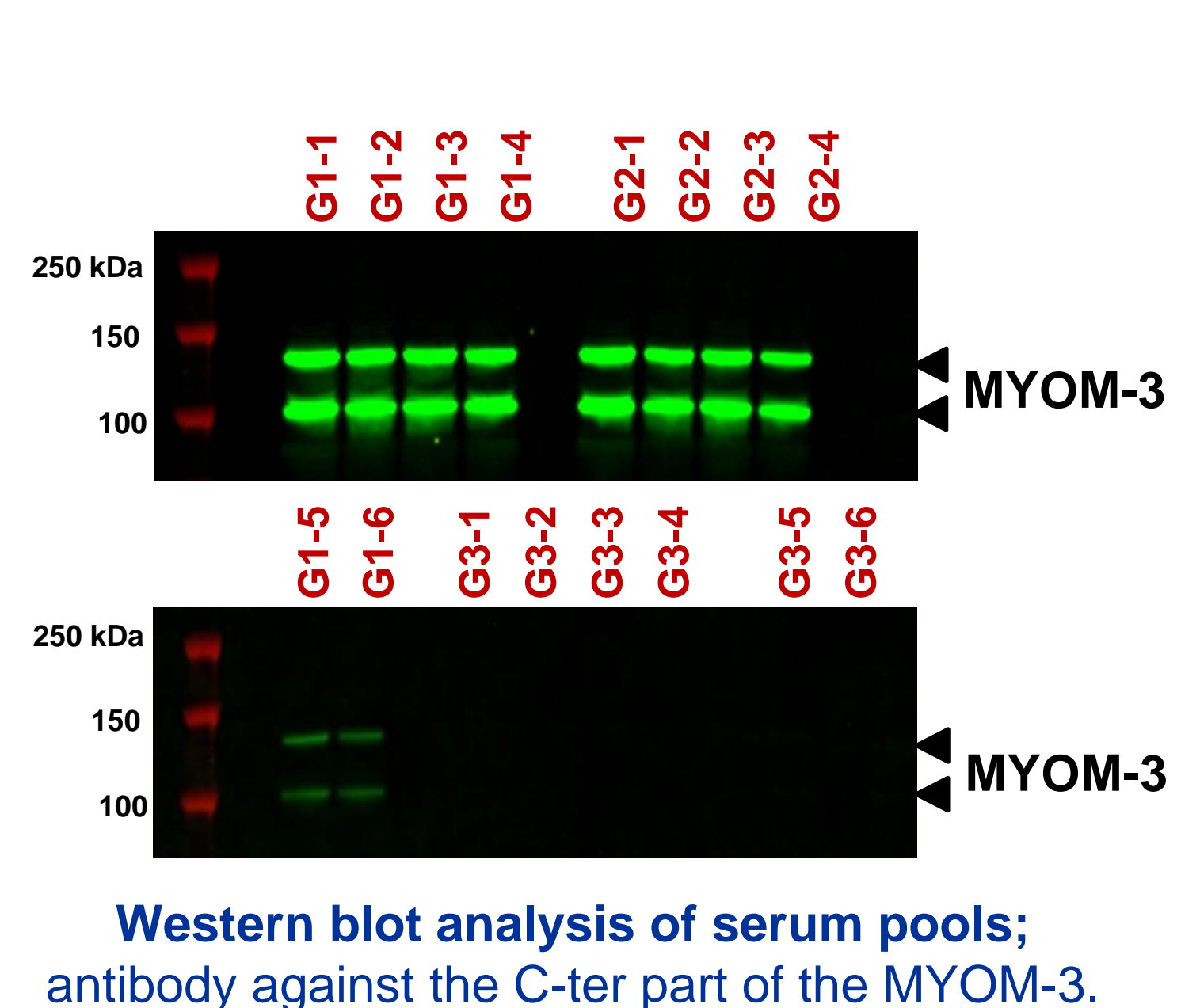
Titin as biomarker

Titin is one of the largest known protein (34,350 amino acids) of ~ 4,000 kDa. Most of the titin peptides identified in urines corresponded to the N- and C- terminal part of the protein. Western blot analysis confirmed the presence of 2 fragments of well defined sizes in DMD patients. Fragments of the same size are also present in urines from GRMD dog and *mdx* mice (data not shown).



MYOM3 fragments as biomarkers for DMD diagnosis and follow up of gene therapy treatment

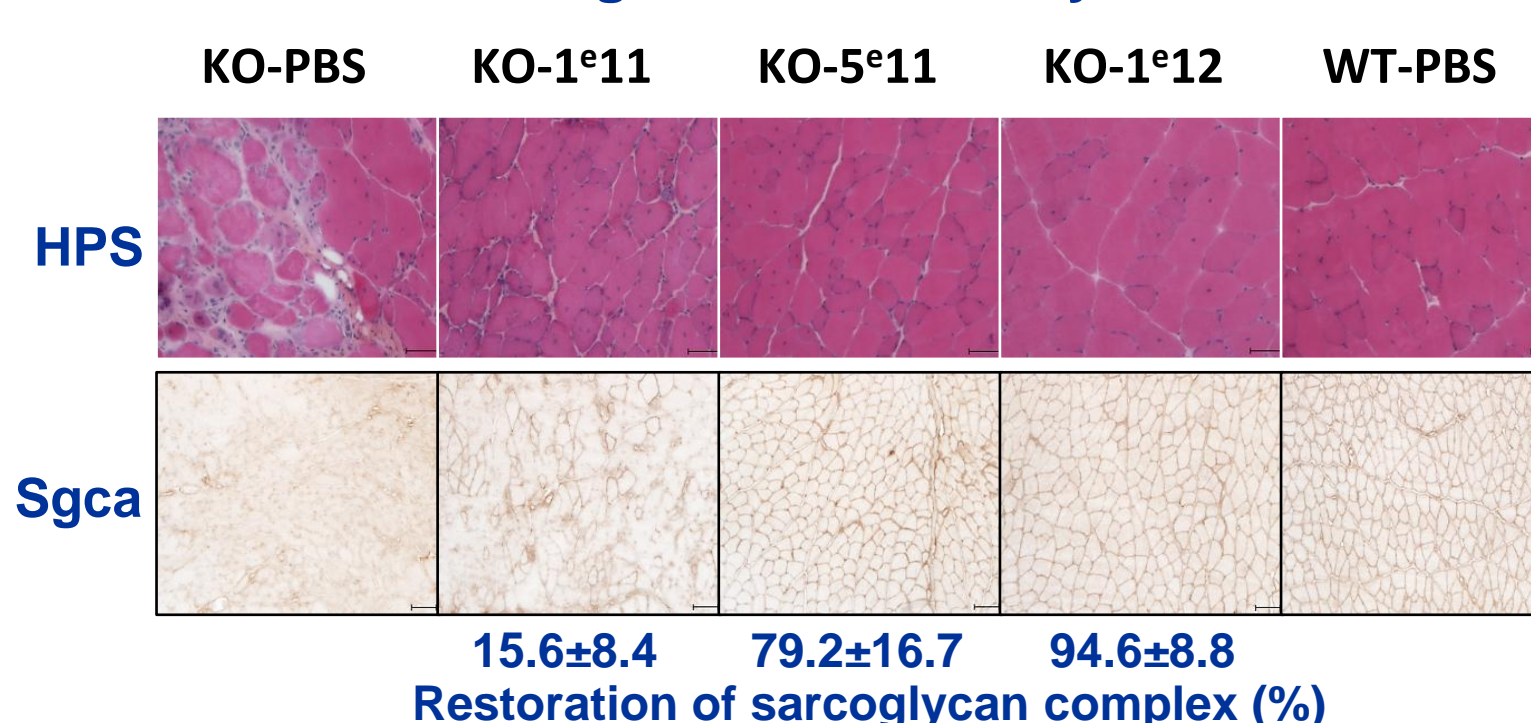
MYOM3 (UniProtKB accession no. Q5VTT5), a protein of 1,437 amino acids (162.2 kDa), is a member of closely related structural proteins detected at the M-band of the sarcomere in striated skeletal muscles (mainly in in intermediate speed fibres type IIa) including also MYOM2 (fast fibres) and MYOM1 (ubiquitous). These proteins are composed of a unique head domain followed by a conserved sequence of either fibronectin- or immunoglobulin-like domains. They may be involved in sarcomere stability and resistance during intense or sustained stretching. Western blot analysis confirmed that MYOM3 was represented in serum of DMD patients by C-terminal fragments of well defined size. Fragments of the same size are present in sera from GRMD dog, *mdx* mice and LGMD 2A,B,C,D mice.



Comparison of serum CK-M and MYOM3 (142 individuals). In all DMD groups, the expression levels of the CK-M and MYOM3 fragments were higher in DMD as compared to healthy controls. Absence of correlation between these biomarkers in young patients (3-10 years old) indicates different mechanisms of protein release from muscle fibers.

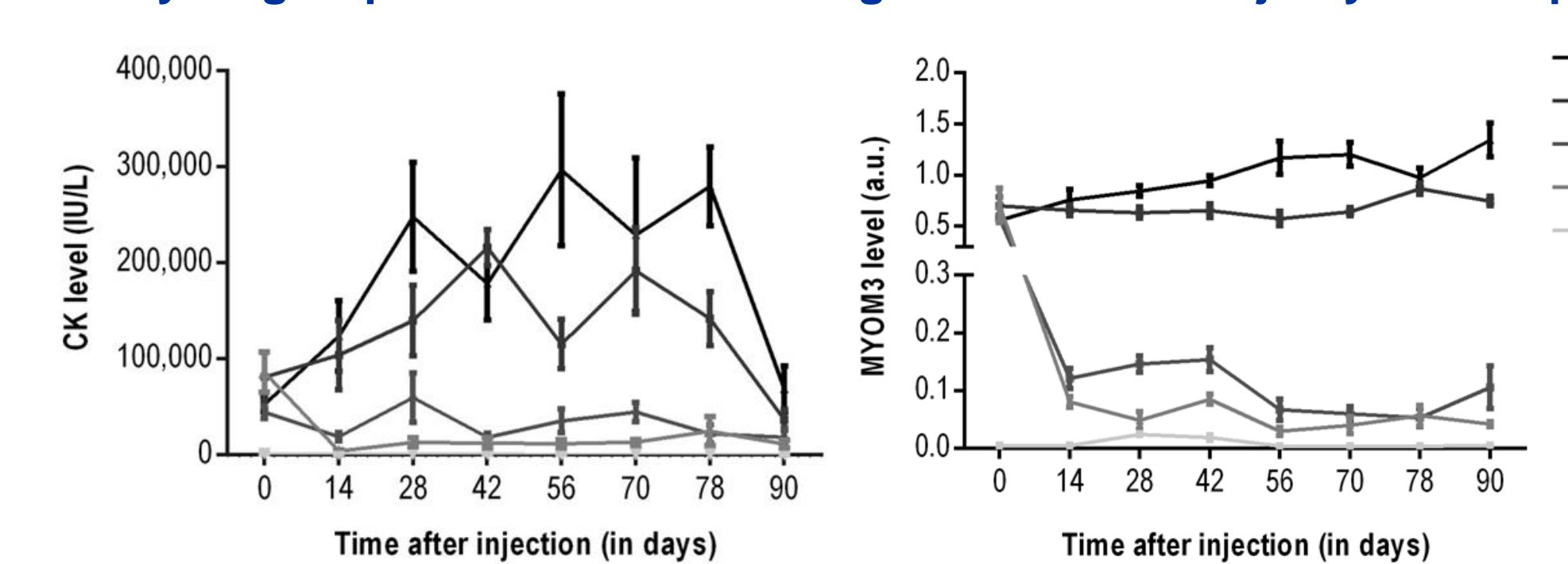
MYOM3 fragments enable monitoring of gene therapy treatment efficacy in KO α -sarcoglycan mice (KO-Sgca) treated with different doses of rAAV2/8 vector

Immunostaining of α -sarcoglycan permits to distinguish the KO-Sgca, low, medium and WT mice. However, no statistically significant difference was found between medium and high rAAV doses by this method.



Comparison of CK and MYOM3 serum levels as biomarkers for monitoring gene therapy efficacy

A threshold 3000 IU/L of CK separates KO-Sgca mice from all other experimental groups. Nevertheless, when applying the Student's test (P-value threshold < 0.01), differences only between few time points/injection doses appeared as statistically significant (Table below). Due to the low inter-individual variability, measurement of the MYOM3 fragments enabled nearly all groups of mice to be distinguished at the majority of time points.



Capacity of CK and MYOM3 biomarkers to distinguish different experimental conditions of the treatment

	KO_PBS / KO_1e11	KO_PBS / KO_5e11	KO_PBS / KO_1e12	KO_PBS / WT	KO_1e11 / KO_5e11	KO_1e11 / KO_1e12	KO_1e11 / WT	KO_5e11 / KO_1e12	KO_5e11 / WT	KO_1e12 / WT
J0	0.193	0.338	0.618	0.496	0.459	0.210	0.000	0.003	0.253	0.229
J14	0.409	0.709	0.003	0.022	0.003	0.031	0.002	0.028	0.000	0.046
J28	0.023	0.147	0.000	0.025	0.000	0.008	0.000	0.012	0.001	0.116
J42	0.009	0.394	0.000	0.013	0.000	0.009	0.001	0.000	0.001	0.000
J56	0.010	0.062	0.002	0.029	0.002	0.022	0.002	0.020	0.001	0.032
J70	0.002	0.695	0.001	0.082	0.000	0.054	0.000	0.046	0.000	0.029
J79	0.352	0.025	0.000	0.003	0.000	0.002	0.000	0.002	0.000	0.010
J90	0.021	0.272	0.001	0.085	0.001	0.073	0.001	0.046	0.000	0.231

Due to the low inter-individual variability, measurement of the MYOM3 fragments enabled nearly all groups of mice to be distinguished with either of the thresholds (P-values < 0.01 or 0.05) at the majority of time points, while differences in CK levels between only few time points/injection doses appeared as statistically significant. Red: P<0.01; yellow: p<0.05

Conclusions

Analysis of proteins and protein fragments differentially expressed in urine and serum of DMD and healthy controls allowed to find new biomarkers for diagnosis and therapeutic monitoring. Profound analysis of other than MYOM3 proteins found in the present study could provide more complete information concerning tiny process occurring in muscle fibers in muscular dystrophies