Proteomic profiling of the interface between the stomach wall and the pancreas in dystrophinopathy

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Abstract

The neuromuscular disorder Duchenne muscular dystrophy is a multi-systemic disease that is caused by a primary abnormality in the X-chromosomal *Dmd* gene. Although progressive skeletal muscle wasting and cardio-respiratory complications are the most serious symptoms that are directly linked to the almost complete loss of the membrane cytoskeletal protein dystrophin, dystrophic patients also suffer from gastrointestinal dysfunction. In order to determine whether proteome-wide changes potentially occur in the gastrointestinal system due to dystrophin deficiency, total tissue extracts from the interface between the stomach wall and the pancreas of the *mdx-4cv* model of dystrophinopathy were analysed by mass spectrometry. Following the proteomic establishment of both smooth muscle markers of the gastrointestinal system and key enzymes of the pancreas, core members of the dystrophin-glycoprotein complex, including dystrophin, dystroglycans, sarcoglycans, dystrobrevins and syntrophins were identified in this tissue preparation. Comparative proteomics revealed a drastic reduction in dystrophin, sarcoglycan, dystroglycan, laminin, titin and filamin suggesting loss of cytoskeletal integrity in *mdx-4cv* smooth muscles. A concomitant increase in various mitochondrial enzymes is indicative of metabolic disturbances. These findings agree with abnormal gastrointestinal function in dystrophinopathy.

Key Words: Duchenne muscular dystrophy; mdx-4cv; pancreas; proteomics; stomach.

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Duchenne muscular dystrophy is the most common neuromuscular disorder of early childhood and almost exclusively affects boys due to its X-chromosomal pattern of inheritance. The disease is characterized by highly progressive skeletal muscle degeneration in association with reactive myonecrosis and late-onset cardio-respiratory deficiencies.[1] Genetic abnormalities in one of the largest genes in the human genome, the 79-exon spanning *Dmd* gene, trigger the almost complete loss of its full-length protein product, the Dp427-M isoform of dystrophin.[2] However, since several different promoters are involved in the tissue-specific production of a variety of dystrophin isoforms that range in molecular mass from 45 kDa to 427 kDa, the effects of mutations in the *Dmd* gene cause intricate changes in the expression of various dystrophin proteoforms.[3]

This genetic complexity might explain, at least partially, body-wide complications in dystrophinopathies that usually require multi-disciplinary care of Duchenne patients.[4] Besides the striking dystrophic phenotype of muscle-associated necrosis, fibrosis and inflammation that directly impairs muscle strength, cardiac output and respiratory efficiency,[1] additional symptoms include scoliosis, endocrine disturbances, reduced bone mineralization, impaired energy metabolism, gastro-intestinal dysfunction, renal failure and liver disease, as well as cognitive deficits and behavioural abnormalities.[5-7]

In addition to the direct pathophysiological consequences of abnormal expression patterns of dystrophin isoforms in various tissues, pervasive secondary effects on non-muscle systems play a key role in the highly complex dystrophic phenotype. As a result of the systematic usage

of mechanical ventilator support and cardio-protective drug therapy to treat cardio-respiratory insufficiency, the prognosis of Duchenne patients has drastically improved.[8] Unfortunately the increase in life expectancy is associated with a lengthened period of weakened cardiovascular efficiency, which can negatively affect the appropriate circulation of oxygen, hormones and essential metabolites to peripheral tissues. Importantly, enhanced levels of organ crosstalk between the degenerative musculature and the innate immune system causes sterile inflammation in muscular dystrophy. In contrast to the usually positive effects of immune cell infiltration during muscle repair mechanisms following an acute injury, the chronic inflammatory phenotype of dystrophin-deficient muscle fibres appears to be mostly detrimental.[9]

Dystrophin was previously established to be present in all muscle cell types,[10] including smooth muscles,[11] and dystrophinopathies were shown to be associated with gastrointestinal dysfunction.[4,5,12-15] Thus, to investigate whether the smooth muscle protein constituents of the gastrointestinal system are majorly affected by dystrophin deficiency, we carried out a mass spectrometry-based proteomic analysis of the established genetic *mdx-4cv* mouse model of Duchenne muscular dystrophy, which also displays considerable abnormalities in the gastrointestinal system.[16,17] Since the stomach wall exists in close anatomical connection to the pancreas, we performed the biochemical survey of potential proteome-wide changes using total tissue extracts from the interface between these two essential organs of the gastrointestinal system. Initially the members of the dystrophin-glycoprotein complex were identified in smooth muscle[18-20], followed by the establishment of smooth muscle and pancreatic marker proteins. The comparative mass spectrometric analysis exposed that the drastic reduction in dystrophin is accompanied by a decrease in other members of the dystrophin-associated complex, as well as structural proteins and the actin-crosslinker filamin. Increases were identified in a variety of mitochondrial components. These findings suggest that the disintegration of cytoskeletal integrity due to the loss of dystrophin and accompanying metabolic disturbances are associated with abnormal gastrointestinal function in X-linked muscular dystrophy.

Materials and Methods

Materials

For the proteomic profiling of the interface between the gastrointestinal system and the pancreas in dystrophinopathy, analytical grade reagents and general materials were purchased from Sigma Chemical Company (Dorset, UK), GE Healthcare (Little Chalfont, Buckinghamshire, UK) and Bio-Rad Laboratories (Hemel-Hempstead, Hertfordshire, UK). Extraction buffers were supplemented with a protease inhibitor cocktail from Roche Diagnostics (Mannheim, Germany). Sequencing grade-modified trypsin and the enzyme Lys-C, as well as Protease Max Surfactant Trypsin Enhancer, were obtained from Promega (Madison, WI, USA). Protein concentration was determined with the Pierce 660-nm Protein Assay from ThermoFisher Scientific (Dublin, Ireland). For filter-aided sample preparation, Vivacon 500 (Product number: VN0H22) filter units were purchased from Sartorius (Göttingen, Germany).

Ethical approval, animal license and animal maintenance

Male wild type C57/BL6 mice and male *mdx-4cv* mice were obtained from the Bioresource Unit of the University of Bonn.[21] All procedures adhered to German legislation on the use of animals in experimental research (Amt für Umwelt, Verbraucherschutz und Lokale Agenda der Stadt Bonn, North Rhine-Westphalia, Germany). Mice were kept under standard conditions.[22] For tissue dissection, protein extraction and subsequent proteomic analyses, 12-months old mice were used. Freshly dissected tissue specimens were quick-frozen in liquid nitrogen and transported to Maynooth University in accordance with the Department of Agriculture (animal by-product register number 2016/16 to the Department of Biology, National University of Ireland, Maynooth) on dry ice and stored at -80ºC prior to proteomic analysis.[23]

Preparation of mouse tissue extracts for proteomic analysis

Tissue samples representing the interface between the stomach wall and the pancreas (25 mg weight) from wild type (n=8) versus dystrophic *mdx-4cv* (n=8) mice were lysed by homogenisation with 200µl of lysis solution containing 100mM Tris-Cl pH 7.6, 4% (w/v) sodium dodecyl sulfate and 0.1M dithiothreitol. Lysis buffer was supplemented with a protease inhibitor cocktail.[21] The suspension was incubated at 95°C for 3 minutes and then sonicated for 30 seconds.[24] Following centrifugation at 16,000x*g* for 5 minutes, tissue extracts were processed for mass spectrometry-based proteomic analysis.[22] Protein digestion and further processing of the generated peptides was carried out by filter-aided sample preparation (FASP), as recently described in detail.[25]

Label-free liquid chromatography mass spectrometry and proteomic data analysis

Protein identification and comparative proteomics was carried out by an optimized label-free liquid chromatography mass spectrometry procedure. Details of the proteomic workflow describing all preparative steps and analytical procedures using data-dependent acquisition, as well as bioinformatic data handling, were recently outlined in detail.[25] A Thermo UltiMate 3000 nano system was used for reverse-phased capillary high-pressure liquid chromatography and directly coupled in-line with a Thermo Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The qualitative data analysis of mass

Table 1. *Proteomic identification of smooth muscle protein markers at the interface between the stomach wall and the pancreas*

Accession	Protein	Gene	Coverage %	Peptides	Molecular mass (kDa)
O08638	Myosin heavy chain smMyHC, Myosin-11, smooth muscle	Myh11	56	132	226.9
Q3THE2	Myosin regulatory light chain 12B, smooth muscle	Myl12b	62	9	19.8
Q6PDN3	Myosin light chain kinase, smMLCK, smooth muscle	Mylk	20	35	212.8
P62737	Actin-2, alpha, smooth muscle	Acta2	77	24	42.0
Q921U8	Smoothelin (Smsmo)	Smtn	33	20	100.2
Q99LM3	Smoothelin-like protein 1	Smtn11	34	10	49.5
Q8CI12	Smoothelin-like protein 2	Smtn12	22	7	49.5
P37804	Transgelin (smooth muscle protein 22-alpha)	Tagln	71	16	22.6
Q08091	Calponin-1 (smooth muscle Calponin H1)	Cnn1	60	13	33.3
Q8BTM8	Filamin-A	Flna	62	121	281.0
Q80X90	Filamin-B	Flnb	49	90	277.7
Q8VHX6	Filamin-C	Flnc	59	124	290.9
A2ASS6	Titin	Ttn	56	1586	3904.1
A2AAJ9	Obscurin	Obscn	30	146	966.0
P26039	Talin-1	Tln1	44	83	269.7
Q71LX4	Talin-2	Tln2	32	55	253.5
Q9QXS1	Plectin	Plec	49	213	533.9
E9Q557	Desmoplakin	Dsp	43	119	332.7

spectrometric files was carried out with the help of the UniProtKB-SwissProt *Mus musculus* database with Proteome Discoverer 2.2 using Sequest HT (Thermo Fisher Scientific) and Percolator.[24,25] For protein identification, the following crucial search parameters were employed: (i) peptide mass tolerance set to 10 ppm, (ii) MS/MS mass tolerance set to 0.02 Da, (iii) an allowance of up to two missed cleavages, (iv) carbamidomethylation set as a fixed modification and (v) methionine oxidation set as a variable modification.[24,25] Peptides were filtered using a minimum XCorr score of 1.5 for 1, 2.0 for 2, 2.25 for 3 and 2.5 for 4 charge states, with peptide probability set to high confidence. Quantitative label-free data analysis was performed using Progenesis QI for Proteomics (version 2.0; Nonlinear Dynamics, a Waters company, Newcastle upon Tyne, UK). Peptide and protein identification were achieved with Proteome Discoverer 2.2 using Sequest HT (Thermo Fisher Scientific) and Percolator, and were then imported into Progenesis QI software for further analysis.[25] Protein identifications were reviewed, and only those which passed the following criteria were considered differentially expressed between experimental groups with high confidence and statistical significance: (i) an ANOVA p-value of ≤0.01 between experimental groups; (ii) proteins with ≥2 unique peptides contributing to the identification.[21,24,25] To calculate the maximum fold change for a protein, Progenesis QI calculates the mean abundance for that protein in each experimental condition. These mean values are then placed in a condition-vs-condition matrix to find the maximum fold change between any two condition's mean protein abundances.[25]

Results

Mass spectrometric analysis of the interface between the stomach wall and the pancreas

The proteomic analysis of the interface between the stomach wall and the pancreas, using tissue preparations from 12-months old wild type versus dystrophic *mdx-4cv* mice, resulted in the mass spectrometric identification of 5,541 individual protein species. This information on proteins was generated from the combined findings from 16 separate mass spectrometric sample runs. The protein listing of the multi-consensus file was employed to search for protein markers of both smooth muscle cells and pancreatic cells (Tables 1-3). Reliable markers of both organ systems have previously been established by extensive body-wide proteomic surveys as part of the Human Proteome Project[26,27] and been validated by large-scale antibody-based investigations as part of the establishment of the Human Protein Atlas.[28] The below sections list the identification of tissue markers found in this study.

Table 2. *Proteomic identification of members of the core dystrophin-glycoprotein complex at the interface between the stomach wall and the pancreas*

Accession	Protein	Gene	Coverage %	Peptides	Molecular mass (kDa)
P11531	Dystrophin	Dmd	33	95	425.6
Q62165	Dystroglycan	Dag1	15	12	96.8
P82350	Alpha-sarcoglycan	Sgca	35	10	43.3
P82349	Beta-sarcoglycan	Sgcb	42	9	34.9
P82347	Delta-sarcoglycan	Sgcd	23	5	32.1
P82348	Gamma-sarcoglycan	Sgcg	22	5	32.1
O70258	Epsilon-sarcoglycan	SGce	9	2	49.7
Q9D2N4	Alpha-dystrobrevin	Dtna	23	11	84.0
O70585	Beta-dystrobrevin	Dtnb	17	6	74.4
Q61234	Alpha-1-syntrophin	Snta1	22	9	53.6
Q99L88	Beta-1-syntrophin	Sntb1	45	19	58.0
Q61235	Beta-2-syntrophin	Sntb2	25	10	56.3

Proteomic identification of smooth muscle protein markers

Marker proteins of smooth muscle cells that were identified with a high degree of sequence coverage by unique peptides included major types of contractile components, such as myosin heavy chain isoform smMyHC, myosin regulatory light chain isoform MLC-12B, myosin light chain kinase isoform smMLCK and α-actin-2 (Tables 1). Marker proteins were determined by comparison to the data repository of the Human Proteome Map displaying the expressed products of the approximately 20,000 protein-coding genes[26,27] and the tissue-based map of the proteome in the Human Protein Atlas.[28] An additional set of excellent protein markers of smooth muscle cells was identified as smoothelin, the smooth muscle protein 22-α transgelin, smooth muscle calponin-1 and filamin-A (Table 1). More general

Table 3. *Proteomic identification of pancreatic protein markers at the interface between the stomach wall and the pancreas*

Accession	Protein	Gene	Coverage %	Peptides	Molecular mass (kDa)
P00688	Pancreatic alpha-amylase	Amy2	78	34	57.3
Q6P8U6	Pancreatic triacylglycerol lipase	Pnlip	57	20	51.4
Q5BKQ4	Pancreatic lipase-related protein 1	Pnliprp1	72	28	52.7
P17892	Pancreatic lipase-related protein 2	Pnliprp2	46	20	54.0
P00683	Pancreatic ribonuclease	Rnase1	66	7	16.8
Q9D733	Pancreatic secretory granule membrane major glycoprotein GP2	Gp2	37	14	59.1
Q64285	Bile salt-activated lipase	Cel	54	25	65.8
Q9Z0Y2	Phospholipase A2	Pla2g1b	67	7	16.3
Q8VCK7	Syncollin	Sycn	27	4	14.6
Q7TPZ8	Carboxypeptidase A1	Cpa1	88	24	47.4
Q9CQC2	Colipase	Clps	50	4	12.4
Q9CR35	Chymotrypsinogen B	Ctrb1	77	13	27.8
Q3SYP2	Chymotrypsin-C	Ctrc	26	5	29.5
Q91X79	Chymotrypsin-like elastase family member 1	Cela1	79	11	28.9
P05208	Chymotrypsin-like elastase family member 2A	Cela2a	85	12	28.9
Q9CQ52	Chymotrypsin-like elastase family member 3B	Cela3b	71	12	28.9

Table 4. *Mass spectrometric identification of proteins with a decreased abundance at the interface between the stomach wall and the pancreas in the mdx-4cv model of Duchenne muscular dystrophy.*

Accession	Protein	Gene	Peptides	Anova (p)	Fold change
Q9JMH9	Unconventional myosin-XVIIIa	Myo18a	2	0.01375	44
P97347	Repetin	Rptn	2	0.042085	31.1
Q66K08	Cartilage intermediate layer protein 1	Cilp	2	0.028968	29.1
Q19LI2	Alpha-1B-glycoprotein	A1bg	2	0.004887	13.5
P11531	Dystrophin	Dmd	21	0.004186	12.2
Q61292	Laminin subunit beta-2	Lamb2	2	0.004332	11.1
P82350	Alpha-sarcoglycan	Sgca	2	0.012255	8.8
P03987	Ig gamma-3 chain C region	-	4	0.005865	7.9
Q9WUB3	Glycogen phosphorylase, muscle form	Pygm	3	0.007123	5.6
P21550	Beta-enolase	Eno3	2	0.040089	5.2
O55143	SERCA2 calcium ATPase	Atp2a2	2	0.00994	4.2
A2AAJ9	Obscurin	Obscn	4	0.02586	3.5
P97447	Four and a half LIM domains protein 1	Fhl1	2	0.014399	3.4
P13542	Myosin-8	Myh8	4	0.007965	3
E9PZQ0	Ryanodine receptor 1	Ryr1	3	0.04258	3
A2ASS6	Titin	Ttn	32	0.027786	2.8
P70670	Nascent polypeptide-associated complex subunit alpha, muscle-specific form	Naca	3	0.024156	2.8
Q921I1	Serotransferrin	Tf	2	0.010739	2.8
Q8BTM8	Filamin-A	Flna	2	0.004813	2.4
P58252	Elongation factor 2	Eef2	3	0.007363	2.3
Q60675	Laminin subunit alpha-2	Lama2	3	0.019277	2.3
Q9JI91	Alpha-actinin-2	Actn2	2	0.02299	2.3
Q5SX40	Myosin-1	Myh1	8	0.023716	2.2
Q11011	Puromycin-sensitive aminopeptidase	Npepps	4	0.012667	1.5
Q62165	Dystroglycan	Dag1	2	0.012359	1.5
Q99KI0	Aconitate hydratase, mitochondrial	Aco2	2	0.024421	1.5

Listed are proteoforms that were recognized by at least 2 unique peptides.

muscle-associated markers included obscurin, talin, plectin, desmoplakin and the giant protein titin.

Proteomic identification of members of the core dystrophin-glycoprotein complex

All major components of the muscle dystrophin-glycoprotein complex,[26-28] with the exception of the highly hydrophobic and low-molecular-mass protein sarcospan, were identified in smooth muscle cells. Table 2 lists the mass spectrometric identification of dystrophin, dystroglycan, α-sarcoglycan, β-sarcoglycan, δ-sarcoglycan, γ-sarcoglycan, ε-sarcoglycan, α-dystrobrevin, β-dystrobrevin, α-1-syntrophin, β-1-syntrophin and β-2-syntrophin.

Proteomic identification of pancreatic protein markers

Pancreatic marker proteins that were identified with a high degree of sequence coverage by unique peptides included major types of pancreatic lipases involved in fat digestion, such as pancreatic triacylglycerol lipase PNLIP, bile salt-activated lipase CEL, phospholipase A2/PLA2G1B, colipase CLPS and pancreatic lipase-related protein PNLIPRP2 of 54 kDa (Table 3),[26-28] as well as pancreatic lipase-related protein PNLIPRP1 that was previously demonstrated by the human proteome mapping initiative to exhibit the highest protein expression level in pancreas.[27] Digestive proteinases included carboxypeptidase, chymotrypsin CTRC,

Table 5, a. *Mass spectrometric identification of proteins with an increased abundance at the interface between pancreas and the stomach wall in the mdx-4cv model of Duchenne muscular dystrophy. Listed are proteoforms that were recognized by more than 2 unique peptides.*

Accession	Protein	Gene	Peptides	Anova (p)	Fold change
O08601	Microsomal triglyceride transfer protein large subunit	Mttp	4	0.046044	57.9
P35441	Thrombospondin-1	Thbs1	7	0.002387	10.7
P01878	Ig alpha chain C region	-	4	0.009506	9
Q9WUR9	Adenylate kinase 4, mitochondrial	Ak4	3	0.015851	8.5
P47738	Aldehyde dehydrogenase, mitochondrial	Aldh2	3	0.02759	8.4
Q9WU79	Proline dehydrogenase 1, mitochondrial	Prodh	3	0.028809	7.4
P15105	Glutamine synthetase	Glul	3	0.035918	6.9
Q8C196	Carbamoyl-phosphate synthase, mitochondrial	Cps1	3	0.048286	5.3
Q9QYF1	Retinol dehydrogenase 11	Rdh11	3	0.009304	4.8
O35381	Acidic leucine-rich nuclear phosphoprotein 32 family member A	Anp32a	3	0.000147	4.6
P97742	Carnitine O-palmitoyl-transferase 1	Cpt1a	6	0.007153	4.3
P16675	Lysosomal protective protein	Ctsa	3	0.017105	4.1
Q8K1B8	Fermitin 3	Fermt3	3	0.003082	3.6
Q05920	Pyruvate carboxylase, mitochondrial	Pc	3	0.021666	3.4
Q99LP6	GrpE protein 1, mitochondrial	Grpel1	3	0.00354	3.3
E9Q4Z2	Acetyl-CoA carboxylase 2	Acacb	3	0.022647	3.2
P51855	Glutathione synthetase	Gss	3	0.008555	3
Q8CC88	von Willebrand factor A domain-containing protein 8	Vwa8	9	0.002283	2.8
Q9JII6	Aldo-keto reductase family 1 member A1	Akr1a1	4	0.017949	2.8
Q8CGK3	Lon protease, mitochondrial	Lonp1	7	0.000701	2.7
P01942	Hemoglobin subunit alpha	Hba	3	0.000471	2.5
Q9JLJ2	4-trimethylamino-butyraldehyde dehydrogenase	Aldh9a1	3	0.035947	2.5
P29351	Tyrosine-protein phosphatase non-receptor type 6	Ptpn6	4	0.023282	2.4

chymotrypsin-like elastases CELA1, CELA2A and CELA3B. In addition, highly enriched pancreatic markers were identified as pancreatic α-amylase AMY2, syncollin, pancreatic ribonuclease RNase1 and pancreatic secretory granule membrane major glycoprotein GP2 (Table 3).[26-28]

Proteins with a decreased abundance at the interface between the stomach wall and the pancreas in the mdx-4cv model of dystrophinopathy

The most reduced protein species in the tissue specimens under investigation were identified as myosin-XVIIIa, repetin, cartilage intermediate layer protein 1 and α-1B-glycoprotein (Table 4). Of note, the expression levels of core members of the dystrophin-glycoprotein complex including dystrophin, laminin, α-sarcoglycan and dystroglycan, were confirmed to be decreased in the dystrophic phenotype. Additional protein species with a reduced concentration were identified as the smooth muscle marker filamin-A and the cytoskeletal proteins obscurin and titin.

Proteins with an increased abundance at the interface between the stomach wall and the pancreas in the mdx-4cv model of dystrophinopathy

A large number of diverse proteins was identified with elevated expression levels in the *mdx-4cv* mouse preparation (Table 5, a, b). The most increased protein

Table 5, b. *Mass spectrometric identification of proteins with an increased abundance at the interface between pancreas and the stomach wall in the mdx-4cv model of Duchenne muscular dystrophy. Listed are proteoforms that were recognized by more than 2 unique peptides.*

Q61233	Plastin-2	Lcp1	3	0.015693	2.4
Q9JLZ3	Methylglutaconyl-CoA hydratase, mitochondrial	Auh	3	0.04646	2.3
P26039	Talin-1	Tln1	3	0.00156	2.3
Q9CZN7	Serine hydroxymethyl-transferase, mitochondrial	Shmt2	3	0.021521	2.3
P30999	Catenin delta-1	Ctnnd1	5	0.024074	2.2
O89053	Coronin-1A	Coro1a	3	0.01593	2.2
P50171	Estradiol 17-beta-dehydrogenase 8	Hsd17b8	3	0.012644	2.2
P52825	Carnitine O-palmitoyl-transferase 2, mitochondrial	Cpt2	6	0.013181	2.1
P63038	60 kDa heat shock protein, mitochondrial	Hspd1	5	0.007267	2.1
Q8BGQ7	Alanine--tRNA ligase, cytoplasmic	Aars	3	0.007629	2.1
P38647	Stress-70 protein, mitochondrial	Hspa9	7	0.000879	2
P56480	ATP synthase subunit beta, mitochondrial	Atp5f1b	3	0.02928	2
P02088	Hemoglobin subunit beta-1	Hbb-b1	5	0.008807	1.9
Q8VDD5	Myosin-9	Myh9	18	0.006118	1.8
P26231	Catenin alpha-1	Ctnna1	3	0.0094	1.8
Q02819	Nucleobindin-1	Nucb1	5	0.002401	1.7
P11983	T-complex protein 1 subunit alpha	Tcp1	4	0.000647	1.7
Q8QZT1	Acetyl-CoA acetyltransferase, mitochondrial	Acat1	3	0.030345	1.7
P80313	T-complex protein 1 subunit eta	Cct7	3	0.002462	1.7
Q68FD5	Clathrin heavy chain 1	Cltc	4	0.004151	1.6
P61979	Heterogeneous nuclear ribonucleoprotein K	Hnrnpk	3	0.006035	1.6
Q3U0V1	Far upstream element-binding protein 2	Khsrp	3	0.017072	1.6
Q9D0E1	Heterogeneous nuclear ribonucleoprotein M	Hnrnpm	3	0.006943	1.6
O35643	AP-1 complex subunit beta-1	Ap1b1	5	0.013862	1.5
Q9DBG3	AP-2 complex subunit beta	Ap2b1	4	0.004566	1.5
P40142	Transketolase	Tkt	3	0.013685	1.5
Q93092	Transaldolase	Taldo1	3	0.004619	1.5

species in the tissue specimen under investigation were shown to be the microsomal triglyceride transfer protein and thrombospondin. Interestingly, the expression of a large number of mitochondrial proteins was found to be significantly increased. This included the mitochondrial isoforms of adenylate kinase, aldehyde dehydrogenase, proline dehydrogenase, carbamoyl-phosphate synthase, pyruvate carboxylase, GrpE protein, lon protease, methylglutaconyl-CoA hydratase, serine hydroxymethyl-transferase, carnitine O-palmitoyl-transferase, 60kDa heat shock protein, stress-70 protein, ATP synthase and acetyl-CoA acetyltransferase (Tables 5, a, b).

Discussion

Duchenne muscular dystrophy is a highly progressive neuromuscular disorder that initially affects the skeletal musculature, which is characterized by fibre degeneration, reactive myofibrosis and sterile inflammation.[2,9] Besides late-onset cardio-respiratory

insufficiency,[4] many other organ systems are impaired in Duchenne patients,[5-7] including the gastrointestinal tract.[12-15] In analogy to Duchenne patients, dystrophic *mdx*-type animal models of X-linked muscular dystrophy, which were used in this investigation in the form of the *mdx-4cv* mouse, also exhibit impairments of the gastrointestinal system.[16,17] This report describes the characterization of a crucial tissue junction within the gastrointestinal system using mass spectrometry. The proteomic profiling of the interface between the stomach wall with its extensive layers of smooth muscle cells and the pancreas with its key exocrine function in digestion revealed interesting changes in various protein families in dystrophinopathy.

Importantly, the large-scale proteomic analysis of the tissue preparation under investigation clearly identified typical protein markers that are enriched in smooth muscles of the gastrointestinal tract,[26-28] including smooth muscle myosin heavy chain (smMyHC, myosin-11), smooth muscle myosin light chain (MLC-12B), smooth muscle myosin light chain kinase (smMLCK) and smooth muscle actin (α-actin-2), as well as the cytoskeletal smooth muscle protein smoothelin, the Ca^{2+}-binding protein calponin-1 and the actin-crosslinking protein filamin-A. In relation to pancreatic protein markers, a large number of key pancreas components were identified by mass spectrometry with a considerable coverage of their peptide sequences. The pancreas acts as a heterocrine gland with exocrine functions based on the secretion of digestive enzymes to the gastrointestinal tract and endocrine functions that involve the release of essential hormones. Distinct pancreatic markers were detected in tissue extracts from the tissue preparation including pancreatic α-amylase and the pancreatic acinar cell protein syncollin, as well as the pancreatic lipases PNLIP, PNLIPRP1, PNLIPRP2, CEL and PLA2G1B.[26-28] This established the presence of both the stomach wall with its extensive smooth muscle layers and the anatomically connected pancreatic cells in this mouse tissue preparation.

The detailed proteomic analysis of the interface between the stomach wall and the pancreas confirmed previous biochemical and cell biological studies that have shown the presence of both dystrophin[10,11] and dystrophin-associated proteins[18-20] in smooth muscle cells. Dystrophin exists in various tissue-specific isoforms with the largest proteoforms of apparent 427 kDa being mostly expressed in muscle cells and the nervous system.[10] In skeletal muscles, the dystrophin isoform Dp427-M is tightly linked to the integral glycoprotein β-dystroglycan which in turn binds via α-dystroglycan to laminin-211 and the wider collagen network of the extracellular matrix. Since full-length dystrophin acts as an actin-binding protein through its amino-terminal and rod domains, this interaction provides a strengthening trans-sarcolemmal linkage between the intracellular cytoskeleton and the extracellular basal lamina. Other members of the core dystrophin complex are represented by the integral sarcoglycans and the cytosolic components dystrobrevin and syntrophin. The mass spectrometric findings presented here clearly identified the main members of the dystrophin-glycoprotein complex in the stomach wall/pancreas tissue preparation, including dystrophin, α/β-dystroglycan, α/β/δ/γ/ε-sarcoglycan, α/β-dystrobrevin, α1/β1/β2-syntrophin and laminin.

The comparison of wild type versus *mdx-4cv* tissue preparations demonstrated a drastic reduction in dystrophin and concomitant reduction in sarcoglycan, dystroglycan and laminin. Thus, in analogy to both skeletal muscles and the heart, the dystrophin-glycoprotein complex appears to be also majorly affected in smooth muscles of the stomach wall. Of note, the expression of the smooth muscle marker filamin-A, in addition to obscurin and titin, was also found to be decreased in the *mdx-4cv* phenotype. This suggests that the collapse of the cytoskeletal support system may weaken contractile smooth muscle function and thereby facilitate gastrointestinal dysfunction in dystrophinoapthy.[12-17] Reduction in myosin isoform XVIIIa, repetin, the cartilage intermediate layer protein, serotransferrin, the ryanodine receptor and the Ca^{2+}-ATPase agree with the pathophysiological concept of changes in cytoskeletal maintenance, extracellular matrix organisation, cartilage scaffolding and Ca^{2+}-cycling through the sarcoplasmic reticulum, respectively.

The drastic increase of the large subunit of microsomal triglyceride transfer protein is probably due to alterations in the *mdx-4cv* pancreas, since this protein facilitates the transport of fat molecules between phospholipid surfaces. Previous proteomic surveys revealed decreased pancreatic marker proteins in biofluids, including reduced levels of chymotrypsin-like elastase family member 2A in *mdx-4cv* serum[23] and pancreatic alpha-amylase in *mdx-4cv* urine,[22] indicating decreased secretion of these enzymes into circulation. However, no altered abundance of these pancreatic proteins was observed in this report. Higher levels of the adhesive glycoprotein named thrombospondin-1, a protein that is highly expressed in both muscle and pancreas, indicates potential adaptations in the mediation of cell-to-cell-to-matrix interactions due to dystrophin deficiency. Increased protein levels in the *mdx-4cv* preparation including a large number of mitochondrial proteins, such as enzymes involved in oxidative phosphorylation (ATP synthase), nucleotide homeostasis (adenylate kinase), alcohol metabolism (aldehyde dehydrogenase), amino acid degradation (proline dehydrogenase), ammonia removal (carbamoyl-phosphate synthase), pyruvate conversion (pyruvate carboxylase), detoxification (methylglutaconyl-CoA hydratase), purine biosynthesis (serine hydroxymethyl-transferase), fatty acid oxidation (carnitine O-palmitoyl-transferase, acetyl-CoA acetyltransferase) and the cellular stress response (60kDa heat shock protein, lon protesae, stress-70 protein, GrpE protein).[26-28] This could relate to a shift in metabolic

processes due to dystrophin deficiency. Disturbed energy metabolism has also been observed in the diaphragm,[21] liver[29] and kidney[30] of the *mdx-4cv* mouse model of X-linked muscular dystrophy. Loss of dystrophin appears to cause severe primary and secondary abnormalities in a variety of tissue systems and organ crosstalk seems to play a major role in the cellular pathogenesis of dystrophinopathy.[24]

Conclusions

The proteomic profiling of the interface between the stomach wall and the pancreas in the *mdx-4cv* model of dystrophinopathy has confirmed the multi-systemic character of the dystrophic phenotype. The systematic and large-scale survey of this tissue preparation by mass spectrometry revealed the presence of the core members of the dystrophin-glycoprotein complex in the gastrointestinal tract.

Comparative proteomics identified a reduced concentration of dystrophin and its associated proteins sarcoglycan and dystroglycan, as well as laminin, obscurin, titin and filamin.

This agrees with the pathophysiological concept of a loss of cytoskeletal and plasmalemmal integrity in smooth muscle cells. A change in mitochondrial proteins suggests that metabolic disturbances and/or adaptations play a role in abnormal gastrointestinal function. The newly described proteomic changes can now be used to establish novel biomarker candidates for studying the gastrointestinal system in X-linked muscular dystrophy and potentially improve differential diagnosis, prognosis and therapy monitoring.

Authors contributions

PD, DS, and KO were involved in the conceptualization and initiation of this project, as well as the design of the research strategy. SG and PD performed the biochemical experiments and analysed the data. MZ and HS were involved in the preparation of tissue samples. MH and PM performed the mass spectrometric and bioinformatic analysis. All authors were involved in the writing and final editing of the manuscript.

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Conflict of Interest

The authors declare no conflicts of interests

Ethical Publication Statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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