Proteomics of stomach wall and pancreas in dystrophinopathy
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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.

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

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. 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.

Dystrophin was previously established to be present in all muscle cell types, including smooth muscles, and dystrophinopathies were shown to be associated with gastrointestinal dysfunction. 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. 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, 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. 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. 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 -80°C prior to proteomic analysis.

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. The suspension was incubated at 95°C for 3 minutes and then sonicated for 30 seconds. Following centrifugation at 16,000xg for 5 minutes, tissue extracts were processed for mass spectrometry-based proteomic analysis. Protein digestion and further processing of the generated peptides was carried out by filter-aided sample preparation (FASP), as recently described in detail.

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. 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
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. 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. 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. 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. 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.

### 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 and been validated by large-scale antibody-based investigations as part of the establishment of the Human Protein Atlas. The below sections list the identification of tissue markers found in this study.

| Accession | Protein Description | 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 (Smso) | Smtn | 33         | 20       | 100.2               |
| Q99LM3    | Smoothelin-like protein 1 | Smtnl1 | 34         | 10       | 49.5                |
| Q5CI12    | Smoothelin-like protein 2 | Smtnl2 | 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               |
Proteome 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\textsuperscript{26,27} and the tissue-based map of the proteome in the Human Protein Atlas.\textsuperscript{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 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      | Sgcβ | 42         | 9        | 34.9                 |
| P82347    | Delta-sarcoglycan     | Sgcd | 23         | 5        | 32.1                 |
| P82348    | Gamma-sarcoglycan     | Sgcg | 22         | 5        | 32.1                 |
| 070258    | Epsilon-sarcoglycan   | Sgcε | 9          | 2        | 49.7                 |
| 070585    | Alpha-dystrobrevin    | Dtna | 23         | 11       | 84.0                 |
| Q9D2N4    | Beta-dystrobrevin     | Dtnb | 17         | 6        | 74.4                 |
| Q61234    | Alpha-1-syntrophin    | Snta1| 22         | 9        | 53.6                 |
| Q9L88     | Beta-1-syntrophin     | Sntb1| 45         | 19       | 58.0                 |
| Q61235    | Beta-2-syntrophin     | Sntb2| 25         | 10       | 56.3                 |

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                 |
| 09D733    | 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    | Syncolin                               | 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                 |
Proteomics of stomach wall and pancreas in dystrophinopathy
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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.

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 |
| Q19L12    | 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 | 0.024136 | 2.8 |
| Q9211I    | 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         |
| Q9JJ91    | 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         |
| Q99K10    | Aconitase hydratase, mitochondrial | Aco2 | 2 | 0.024421 | 1.5 |

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

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,
Proteomics of stomach wall and pancreas in dystrophinopathy
Eur J Transl Myol 31 (1): 9627, 2021 doi: 10.4081/ejtm.2021.9627

chymotrypsin-like elastases CELA1, CELA2A and CELA3B. In addition, highly enriched pancreatic markers were identified as pancreatic α-amylase AMY2, syncoolin, 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, 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 | Mtp   | 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     | Grpe1 | 3        | 0.00354   | 3.3         |
| E9Q4Z2    | Acetyl-CoA carboxylase 2         | Acaeb | 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         |
| Q9JH6     | 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         |
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 hyrdatase, 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.²⁹ Besides late-onset cardio-respiratory

| 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 | Ctnmd1 | 5 | 0.024074 | 2.2 |
| O89053 | Corin-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 | 0.008079 | 2 |
| P56480 | ATP synthase subunit beta, mitochondrial | Atp5fb1 | 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 | Nuclearbindin-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 | 3 | 0.004151 | 1.6 |
| P61979 | Heterogeneous nuclear ribonucleoprotein K | Hnmpk | 4 | 0.006035 | 1.6 |
| Q3U0V1 | Far upstream element-binding protein 2 | Khsrp | 3 | 0.017072 | 1.6 |
| Q9DOE1 | Heterogeneous nuclear ribonucleoprotein M | Hn rpm | 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 |

**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.
Proteomics of stomach wall and pancreas in dystrophinopathy

insufficiency, many other organ systems are impaired in Duchenne patients, including the gastrointestinal tract. 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. 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, 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 Ca2+-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 synclinin, as well as the pancreatic lipases PNLP, PNLP RP1, PNLP RP2, CEL and PLA2G1B. 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 and dystrophin-associated proteins 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. 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-sarcoclemmal 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 dysprobrin 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/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 dystrophinopathy. Reduction in myosin isoform XVIIα, repentin, the cartilage intermediate layer protein, serotransferrin, the ryanodine receptor and the Ca2+-ATPase agree with the pathophysiological concept of changes in cytoskeletal maintenance, extracellular matrix organisation, cartilage scaffolding and Ca2+-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 and pancreatic α-amylase in mdx-4cv urine, 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, ion proteases, stress-70 protein, GrpE protein). This could relate to a shift in metabolic pathways and the need for increased energy production in dystrophinopathy.
processes due to dystrophin deficiency. Disturbed energy metabolism has also been observed in the diaphragm, liver and kidney 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.

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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