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Proteomic reference map for sarcopenia research: mass spectrometric identification of key muscle proteins of organelles, cellular signaling, bioenergetic metabolism and molecular chaperoning

Paul Dowling,[1,2] Stephen Gargan,[1,2] Margit Zweyer,[3,4] Michael Henry,[5] Paula Meleady,[5] Dieter Swandulla,[6] Kay Ohlendieck[1,2]

[1]Department of Biology, Maynooth University, National University of Ireland, Maynooth, Co. Kildare, Ireland; [2]Kathleen Lonsdale Institute for Human Health Research, Maynooth University, Maynooth, Co. Kildare, Ireland; [3]Department of Neonatology and Paediatric Intensive Care, Children's Hospital, University of Bonn, Bonn, Germany; [4]German Center for Neurodegenerative Diseases, Bonn, Germany; [5]National Institute for Cellular Biotechnology, Dublin City University, Dublin, Ireland; [6]Institute of Physiology, Medical Faculty, University of Bonn, Bonn, Germany.

Abstract

During the natural aging process, frailty is often associated with abnormal muscular performance. Although inter-individual differences exit, in most elderly the tissue mass and physiological functionality of voluntary muscles drastically decreases. In order to study age-related contractile decline, animal model research is of central importance in the field of biogerontology. Here we have analyzed wild type mouse muscle to establish a proteomic map of crude tissue extracts. Proteomics is an advanced and large-scale biochemical method that attempts to identify all accessible proteins in a given biological sample. It is a technology-driven approach that uses mass spectrometry for the characterization of individual protein species. Total protein extracts were used in this study in order to minimize the potential introduction of artefacts due to excess subcellular fractionation procedures. In this report, the proteomic survey of aged muscles has focused on organellar marker proteins, as well as proteins that are involved in cellular signaling, the regulation of ion homeostasis, bioenergetic metabolism and molecular chaperoning. Hence, this study has establish a proteomic reference map of a highly suitable model system for future aging research.

Key Words: aging, mass spectrometry, muscle proteomics, skeletal muscle.

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Mass spectrometry (MS) based proteomics is a key method of modern protein chemistry,[1] including skeletal muscle biochemistry,[2] that can be employed for both the targeted or untargeted detection and characterization of specific proteins.[3,4] The various molecular forms of a muscle protein product that is encoded by a particular gene and influenced by alternative RNA splicing of its transcript,[5] plus dynamic post-translational modifications,[6] are referred to as proteoforms,[7] which form the basic units of the proteome.[8] Mass spectrometry can be used for comparative studies,[9] or the systematic cataloguing of tissue-specific protein constellations,[10] such as the human skeletal muscle proteome.[11-13] In principle, the main proteomic approaches can be divided into bottom-up *versus* top-down analyses,[14] which differ in their starting material prior to mass spectrometric analysis.[15] Bottom-up proteomics is a gel-free and peptide-focused methodology for protein identification,[16,17] while top-down proteomics specializes on proteoform-centric and often the gel-based detection and characterization of intact protein species.[18,19]

To date, a variety of proteomic studies have focused on the characterization of skeletal muscle tissues using MS-based proteomics.[12] This has included skeletal muscle specimens and its secretome from various species using both top-down and bottom-up proteomics.[9,11,20-23] Systematic protein cataloguing studies,[24-27] have resulted in the identification of a large number of dynamic proteoforms that are characteristic for voluntary striated muscles.[11] The proteomic screening of contractile tissues has revealed crucial information on myofiber types in slow *versus* fast muscles,[28-31] the transformation of muscles due to changed activity patterns,[32-34] the impact of physical activity,[35-38] the pathophysiological

effects of primary muscular disorders,[39-41] dysfunctions of the musculature in the context of co-morbidities,[42-44] and the natural aging process.[45-50]

The underlying objective of this study was to establish a proteomic reference map of a highly suitable model system for aging research of the neuromuscular system. Crude protein extracts from normal tissue specimens of 100 mg wet tissue weight were used to establish a methodological approach for the optimum utilization of small muscle biopsy specimens. In addition, a minimum of preparative steps was employed to simplify the overall experimental bottom-up protocol, accelerate and streamline the proteomic pipeline procedure and avoid the introduction of potential bioanalytical artefacts for the routine detection of key protein species in aged skeletal muscles. This report has focused on the detailed proteomic analysis of aged skeletal muscles with specific emphasis on proteins that are intrinsically involved in cellular signaling mechanisms, the regulation of ion homeostasis, excitation-contraction coupling, bioenergetic metabolism and molecular chaperoning. In addition, proteomic markers of organelles, such as the sarcolemma, caveolae, transverse tubules, sarcoplasmic reticulum, triad junctions, ribosomes, Golgi apparatus, peroxisomes, proteasomes, lysosomes, nucleus, cytosol, the neuromuscular junction, motor neurons and glial cells, were investigated.

Materials and Methods

Materials

Analytical grade chemicals from GE Healthcare (Little Chalfont, Buckinghamshire, UK), Sigma Chemical Company (Dorset, UK) and Bio-Rad Laboratories (Hemel-Hempstead, Hertfordshire, UK) were used for the proteomic analysis of aged mouse hindlimb muscles. MS-grade trypsin, protease inhibitors (cOmplete™, mini protease inhibitor cocktail) and spin filters (Vivacon 500, VN0H22; 30,000 MWCO) were from ThermoFisher Scientific (Dublin, Ireland), Roche (Mannheim, Germany) and Sartorius (Göttingen, Germany), respectively. The determination of protein concentration was carried out with the Pierce 660 nm Protein Assay Reagent from ThermoFisher Scientific (Dublin, Ireland).

Senescent murine hindlimb muscle

Hindlimb muscles were dissected from freshly prepared *post mortem* specimens from 24 months old wild type C57BL6 mice according to institutional regulations. Mouse populations were handled in strict adherence to local governmental and institutional animal care regulations and were approved by the Institutional Animal Care and Use Committee (Amt für Umwelt, Verbraucherschutz und Lokale Agenda der Stadt Bonn, North Rhine-Westphalia, Germany).[27] Mice were kept under specific pathogen-free conditions in isolated, ventilated cages with free access to water and food. A maximum of 4 mice were kept per cage at a temperature of 21-22°C with a 12-hour light/12-hour dark cycle. Tissue samples were quick-frozen in liquid nitrogen and transported on dry ice to Maynooth University in accordance with the regulations of the Department of Agriculture (animal by-product register number 2016/16 to the Department of Biology, National University of Ireland, Maynooth).[51] The mass spectrometric analysis of total skeletal muscle preparations was performed with protein extracts from 6 wild type mice.

Protein extraction from muscle specimens

Total protein extracts were used for the bottom-up proteomic analysis of aged hindlimb muscles from wild type C57BL6 mice.[41] Standardized procedures were employed for optimum muscle tissue preparation, homogenization with help of pulverization of specimens by grinding in liquid nitrogen and subsequent protein extraction.[52] Tissue homogenization was carried out in lysis buffer, consisting of 0.1 M dithiothreitol, 4% (w/v) sodium dodecyl sulfate, 100 mM Tris-Cl, pH 7.6. The solution was supplemented with a protease inhibitor cocktail.[41] The suspensions were briefly treated in a sonicating water bath and then heated for 3 min at 95°C.[53] Samples were then centrifuged at 16,000×*g* for 5 minutes and the protein-containing supernatant extracted for mass spectrometric analysis. Protein determination was carried out with the Pierce 660 nm Protein Assay system.[53] Protein samples were mixed with 8 M urea, 0.1 M Tris, pH 8.9 in Vivacon 500 spin filter units.[54] Following a centrifugation step at 14,000×*g* for 15 min, the further processing of samples, the switching of buffers and peptide generation by trypsin digestion was carried out by the filter-aided sample preparation (FASP) technique.[55]

Proteomic analysis

For the label-free liquid chromatography mass spectrometric analysis of senescent hindlimb muscles from normal mice (n=6 biological repeats; n=2 technical repeats), a Thermo Orbitrap Fusion Tribrid mass spectrometer from Thermo Fisher Scientific (Waltham, MA, USA) was used.[41] The detailed description of all analytical steps, including the listing of buffer composition, FASP protocol, timing of preparative stages, data-dependent acquisition, and bioinformatic data handling, used during the proteomic analysis of muscle proteins, has been described in a recently published methods paper.[56] A Thermo UltiMate 3000 nano system was used for reversed-phase capillary high-pressure liquid chromatography and directly coupled in-line with the Thermo Orbitrap Fusion Tribrid mass spectrometer. The qualitative data analysis of mass spectrometric files was carried out with the UniProtKB-SwissProt database (species: *Mus musculus*) with Proteome Discoverer 2.2 using Sequest HT (Thermo Fisher Scientific) and Percolator. Parameters for the mass spectrometric detection of hindlimb muscle proteins were as follows: i) a value of 0.02 Da for MS/MS mass tolerance, ii) a value of 10 ppm for peptide mass tolerance, iii) variable modification settings for methionine oxidation, iv) fixed modification settings in relation to carbamido-methylation and v) tolerance for the occurrence of up to two missed cleavages. Peptide probability was set to high confidence. A minimum XCorr score of 1.5 for 1, 2.0 for 2, 2.25 for 3 and 2.5 for 4 charge state was employed for the filtering of peptides. The software analysis programme Progenesis QI for Proteomics (version 2.0; Nonlinear Dynamics, a Waters company, Newcastle

upon Tyne, UK) was used to carry out quantitative label-free data analysis. Proteome Discoverer 2.2 using Sequest HT (Thermo Fisher Scientific) and a percolator were employed for the identification of peptides and proteins. Datasets were imported into Progenesis QI software for further analysis. The multi-consensus MS file and listings of proteins in aged murine skeletal muscle that were generated by this proteomic study have been deposited under the title 'Proteomic analysis of senescent mouse hindlimb muscles' with the unique identifier '37txb' to the Open Science Foundation (https://osf.io/37txb/). The standard bioinformatic analysis tools STRING (https://string-db.org) was used for the identification of potential protein-protein interaction patterns.[57]

Results

The mass spectrometric analysis of crude tissue extracts from the aged mouse hindlimb musculature resulted in the identification of a large number of proteins involved in cellular signaling, bioenergetic metabolism and molecular chaperoning. The underlying objective of this investigation was to establish a proteomic reference map of aged skeletal muscles for future sarcopenia research.

Proteomic profiling of proteins involved in the regulation of excitation-contraction coupling and calcium homeostasis in aged skeletal muscle

The temporal and spatial fluxing of calcium ions through subcellular structures in myofibers is tightly regulated and plays a central role in the physiological facilitation of excitation-contraction coupling in skeletal muscles. Members of key protein complexes that are involved in the regulation of calcium homeostasis and second messenger signaling cascades were identified by mass spectrometric screening of aged mouse hindlimb muscles, including i) the voltage-sensing L-type calcium channel complex of the transverse tubules (alpha-1S (voltage sensor), alpha-2/delta-1 and beta-1 subunits of the dihydropyridine receptor), ii) the calcium release channel complex of the triad junctions that is positioned between the transverse tubules and terminal cisternae region of the sarcoplasmic reticulum (ryanodine receptor calcium release channel isoforms RYR1 and RYR2, and the auxiliary proteins triadin, junctophilin JPH1 and JPH2, and the junctional sarcoplasmic reticulum protein JSRP1), iii) luminal high-capacity calcium buffering proteins of the sarcoplasmic reticulum (calsequestrin isoforms fast CSQ1 and slow CSQ2, sarcalumenin), iv) calcium-pumping ATPase complexes involved in the fast re-uptake or removal of calcium from the sarcosol (sarcoplasmic reticulum calcium ATPases of the fast SERCA1 and slow SERCA2 type, as well as the plasma membrane calcium-transporting ATPase PMCA1), and v) additional calcium homeostasis regulators (parvalbumin, protein S100-A1, SH3 and cysteine-rich domain-containing protein STAC3, and stromal interaction molecule STIM1), and are listed in below Table 1. Excitation-contraction uncoupling is one of the mechanisms that is widely discussed to play a central role in the pathophysiological process that may lead to sarcopenia of old age. Findings from previous studies on muscle aging,[45-50,58-65] imply reduced expression patterns and/or shifting to slower isoforms of calcium-handling proteins, such as the voltage sensor complex of transverse tubules, the calcium release channels of triad junctions, luminal and cytosolic calcium-binding proteins and calcium pumps of the sarcoplasmic reticulum.

Proteomic profiling of proteins involved in major bioenergetic pathways in aged skeletal muscle

Since sustained levels of contractile activity require a constant supply of energy in the form of ATP, the regulation and maintenance of bioenergetic pathways is of crucial importance for the proper functioning of the neuromuscular system. The mass spectrometric analysis of aged mouse hindlimb muscles has identified a large number of enzymes involved in skeletal muscle energy metabolism. Table 2 lists major components that are involved in anaerobic glycolysis *versus* oxidative mitochondrial pathways. This includes proteins of the glycolytic pathway, gluconeogenesis and glycogen metabolism, as well as markers of the mitochondrial outer membrane, inner membrane and matrix that are essential components of respiratory complexes I to V. Listed are also key metabolite transporters, such as fatty acid-binding proteins (FABP3, FABP4, FABP5), albumin and the oxygen transporter myoglobin (Table 2). The higher susceptibility of type II fibers to age-related muscular atrophy results in a fast-to-slow transition process, which is clearly reflected by isoform switching of major sarcomeric proteins, as recently reviewed.[66] In analogy, age-associated fiber type shifting was shown to also affect the abundance of bioenergetic enzymes. Previously published reports on skeletal muscle aging,[45-50,58-65] suggest indirect glycolytic-to-oxidative changes in energy metabolism in senescent myofiber populations. Decreased muscle proteins include key enzymes of glycolysis, such as pyruvate kinase and phosphofructokinase. In contrast, increased proteins of oxidative metabolism were identified as mitochondrial succinate dehydrogenase, ATP synthase and NADH dehydrogenase.

Proteomic profiling of molecular chaperones involved in the cellular stress response in aged skeletal muscle

The cellular stress response plays an essential role in the prevention of proteotoxic side effects in skeletal muscles. Both, the various classes of heat shock proteins and associated modulating enzymes are crucial factors that prevent abnormal protein folding and/or facilitate the swift removal of misfolded protein aggregates. Table 3 lists the mass spectrometric identification of key members of heat shock protein (HSP) classes HSPB/HSPE (small HSPs of 10-40 kDa) HSP40 (HSP70 co-chaperones, DNAJ), HSP60 (HSP70 co-chaperone, chaperonin), HSPA (HSP70s of approximately 70 kDa), and HSPC/HSPH (large HSP90/HSP110 of approximately 90 kDa), as well as calcium-binding chaperones of the luminal endoplasmic reticulum (calnexin and calreticulin/calregulin) and protein disulfide isomerase (PDI) in aged mouse hindlimb muscles. The proteomic profiling of the superfamily of peptidyl-prolyl cis-trans isomerases (PPIase) is shown in below Table 4.

Table 1. *Mass spectrometry-based proteomic profiling of proteins involved in the regulation of excitation-contraction coupling and calcium homeostasis in aged mouse hindlimb muscle.*

Accession	Protein name	Gene	Peptides	Coverage (%)	Molecular mass (kDa)
(i) **Transverse tubular voltage-sensing complex**					
Q02789	Voltage-dependent L-type calcium channel, alpha-1S	Cacna1s	18	12.3	210.3
O08532	Voltage-dependent calcium channel, alpha-2/delta-1	Cacna2d1	30	39.6	124.6
Q8R3Z5	Voltage-dependent L-type calcium channel subunit beta-1	Cacnb1	12	25.1	65.5
(ii) **Triad junction calcium release complex**					
E9PZQ0	Ryanodine receptor RYR1	Ryr1	157	46.0	565.0
E9Q401	Ryanodine receptor RYR2	Ryr2	9	2.4	564.8
E9Q9K5	Triadin	Trdn	14	19.6	77.8
Q9ET80	Junctophilin JPH1	Jph1	10	19.7	71.9
Q9ET78	Junctophilin JPH2	Jph2	12	27.2	74.7
Q3MI48	Junctional sarcoplasmic reticulum protein JSRP1	Jsrp1	6	34.3	36.1
(iii) **Luminal calcium buffering in sarcoplasmic reticulum**					
O09165	Calsequestrin CSQ1, fast	Casq1	16	60.7	46.4
O09161	Calsequestrin CSQ2, slow	Casq2	10	42.4	48.2
Q7TQ48	Sarcalumenin	Srl	24	62.5	54.3
(iv) **Calcium removal via sarcoplasmic reticulum and sarcolemma**					
Q8R429	Calcium ATPase SERCA1, fast	Atp2a1	58	55.1	109.4
O55143	Calcium ATPase SERCA2, slow	Atp2a2	47	48.7	114.9
G5E829	Plasma membrane calcium-transporting ATPase PMCA1	Atp2b1	16	18.4	134.8
(v) **Calcium homeostasis regulation**					
P32848	Parvalbumin	Pvalb	17	84.5	11.9
P56565	Protein S100-A1	S100a1	2	29.8	10.5
Q8BZ71	SH3 and cysteine-rich domain-containing protein STAC3	Stac3	4	16.9	41.0
P70302	Stromal interaction molecule 1	Stim1	15	30.4	77.6

Table 2. *Mass spectrometry-based proteomic profiling of key proteins involved in major bioenergetic pathways in aged mouse hindlimb muscle.*

Accession	Protein name	Gene	Peptides	Coverage (%)	Molecular mass (kDa)
(i) **Glycolysis, gluconeogenesis and glycogen metabolism**					
P17710	Hexokinase-1	Hk1	19	21.5	108.3
O08528	Hexokinase-2	Hk2	32	43.6	102.5
P47857	Phosphofructokinase, muscle	Pfkm	32	54.6	85.3
P05064	Fructose-bisphosphate aldolase ALDOA	Aldoa	37	89.3	39.4
P17751	Triosephosphate isomerase	Tpi1	13	67.9	26.7
P16858	Glyceraldehyde-3-phosphate dehydrogenase	Gapdh	24	80.8	35.8
P09411	Phosphoglycerate kinase 1	Pgk1	37	84.2	44.6
Q9D0F9	Phosphoglucomutase-1	Pgm1	36	77.4	61.4
P17182	Alpha-enolase	Eno1	28	79.5	47.1
P52480	Pyruvate kinase PKM	Pkm	45	80.2	57.8
Q9R062	Glycogenin-1	Gyg1	11	49.2	37.4
Q9WUB3	Glycogen phosphorylase, muscle	Pygm	53	62.6	97.3
Q9Z1E4	Glycogen synthase, muscle	Gys1	21	40.9	83.9
(ii) **Mitochondria**					
Q60932	Voltage-dependent anion-selective channel protein 1 (mt-VDAC1; outer mitochondrial membrane)	Vdac1	21	76.0	32.4
Q9DCS9	NADH dehydrogenase (inner mitochondrial membrane complex I)	Ndufb10	6	47.7	21.0
Q9CQA3	Succinate dehydrogenase (inner mitochondrial membrane complex II)	Sdhb	16	55.3	31.8
Q9DB77	Cytochrome b-c1 (inner mitochondrial membrane complex III)	Uqcrc2	17	49.4	48.2
P56391	Cytochrome c oxidase (inner mitochondrial membrane complex IV)	Cox6b1	5	61.6	10.1
P56480	ATP synthase subunit beta (inner mitochondrial membrane complex V)	Atp5b	37	79.2	56.3
P54071	Isocitrate dehydrogenase (mitochondrial matrix)	Idh2	26	49.8	50.9
(iii) **Metabolite transportation**					
P11404	Fatty acid-binding protein, heart	Fabp3	8	64.7	14.8
P04117	Fatty acid-binding protein, adipocyte	Fabp4	9	67.4	14.7
Q05816	Fatty acid-binding protein, epidermal	Fabp5	4	30.4	15.1
P07724	Serum albumin	Alb	48	73.7	68.7
P04247	Myoglobin	Mb	10	77.3	17.1

Table 3. *Mass spectrometry-based proteomic profiling of molecular chaperones involved in the cellular stress response in aged mouse hindlimb muscle.*

Accession	Protein name	Gene	Peptides	Coverage (%)	Molecular mass (kDa)
(i) **Small heat shock proteins (10-40 kDa, HSPB, HSPE)**					
P14602	HSPB1 (Hsp27, beta-1)	Hspb1	11	68.9	23.0
Q99PR8	HSPB2 (MKBP, beta-2)	Hspb2	6	56.6	20.4
Q9QZ57	HSPB3 (b-3)	Hspb3	2	26.0	17.2
P23927	HSPB5 (alphaB-Crystallin, alphaBC)	Cryab	8	55.4	20.1
Q5EBG6	HSPB6 (Hsp20, beta-6)	Hspb6	6	61.7	17.5
P35385	HSPB7 (cvHsp, beta-7)	Hspb7	6	48.5	18.6
Q9JK92	HSPB8 (Hsp22; beta-8)	Hspb8	5	25.5	21.5
Q64433	HSPE (10 kDa Hsp, mt)	Hspe1	8	79.4	11.0
(ii) **HSP40 heat shock proteins (HSP70 co-chaperones, DNAJ)**					
P63037	DnaJ A1 HSP	Dnaja1	5	22.9	44.9
Q9QYJ0	DnaJ A2 HSP	Dnaja2	11	40.0	45.8
Q99M87	DnaJ A3 HSP	Dnaja3	9	30.6	52.4
Q9JMC3	DnaJ A4 HSP	Dnaja4	3	12.1	44.9
(iii) **HSP60 heat shock proteins (HSP70 co-chaperone, chaperonin)**					
P63038	HSPD1 (60 kDa Hsp, mt)	Hspd1	55	75.0	61.0
(iv) **HSP70 heat shock proteins (HSPA)**					
Q61696	HSPA1A (70 kDa protein 1A, Hsp72, inducible Hsp70)	Hspa1a	14	27.0	70.1
P17156	HSPA2 (heat shock-related 70 kDa protein 2, Hsp70-2)	Hspa2	28	45.0	69.6
Q61316	HSPA4 (heat shock 70 kDa protein 4, Hsp-110)	Hspa4	28	43.9	94.1
P20029	HspA5 (GRP78, BiP), ER	Hspa5	64	73.4	72.4
P63017	HSPA8 (Hsc70, Hsp73, constitutive Hsp70)	Hspa8	55	83.0	70.9
Q99M31	HSPA14 (heat shock 70 kDa protein 14, Hsp60)	Hspa14	2	5.9	54.7
(v) **HSP90/HSP110 large heat shock proteins (90 kDa, HSPC, HSPH)**					
P07901	HSPC1 (Hsp90AA1, HSP90a)	Hsp90aa1	47	64.7	84.9
P11499	HSPC3 (Hsp90AB1, HSP 90b)	Hsp90ab1	67	80.7	83.3
P08113	HSPC4 (Hsp90B1, GRP94, endoplasmin), ER	Hsp90b1	70	67.0	92.5
Q9CQN1	HSPC5 (75 kDa Hsp, mt)	Trap1	27	36.7	80.2
Q61081	Hsp90 co-chaperone Cdc37	Cdc37	7	19.5	44.6
Q61699	HSPH1 (Hsp110)	Hsph1	19	29.4	96.4

To be continued on next page

Table 3. Continued from previous page.

Accession	Protein name	Gene	Peptides	Coverage (%)	Molecular mass (kDa)
(vi) **Ca^{2+}-binding chaperones of the luminal endoplasmic reticulum**					
P35564	Calnexin (CNX)	Canx	27	45.9	67.3
P14211	Calreticulin (CRT), calregulin	Calr	24	72.4	48.0
(vii) **Protein disulfide isomerase**					
P27773	PDI A3	Pdia3	38	64.0	56.7
P08003	PDI A4	Pdia4	24	36.4	72.0
Q921X9	PDI A5	Pdia5	13	25.5	59.3
Q922R8	PDI A6	Pdia6	10	28.6	48.1

Table 4. Mass spectrometry-based proteomic profiling of the superfamily of peptidyl-prolyl cis-trans isomerases in aged mouse hindlimb muscle.

Accession	Protein name	Gene	Peptides	Coverage (%)	Molecular mass (kDa)
P17742	PPIase PPIA	Ppia	13	77.4	18.0
P24369	PPIase PPIB	Ppib	14	55.1	23.7
P30412	PPIase PPIC	Ppic	2	11.8	22.8
Q9CR16	PPIase PPID	Ppid	16	42.2	40.7
Q9QZH3	PPIase PPIE	Ppie	3	14.6	33.4
Q99KR7	PPIase PPIF, mt	Ppif	7	39.3	21.7
A2AR02	PPIase PPIG	Ppig	5	9.0	88.3
Q9D868	PPIase PPIH	Ppih	3	14.9	20.5
P26883	PPIase FKBP1A	Fkbp1a	9	79.6	11.9
P45878	PPIase FKBP2	Fkbp2	5	31.4	15.3
Q62446	PPIase FKBP3	Fkbp3	11	48.2	25.2
P30416	PPIase FKBP4	Fkbp4	17	38.0	51.6
O54998	PPIase FKBP7	Fkbp7	6	25.7	24.9
O35465	PPIase FKBP8	Fkbp8	6	20.4	43.5
Q9Z247	PPIase FKBP9	Fkbp9	14	27.5	63.0
Q61576	PPIase FKBP10	Fkbp10	11	30.3	64.7
Q9D1M7	PPIase FKBP11	Fkbp11	2	14.4	22.1
P59024	PPIase FKBP14	Fkbp14	3	16.1	24.3
Q9QUR7	PPIase NIMA-interacting PIN1	Pin1	3	29.7	18.4

Since skeletal muscle aging is associated with impaired microcirculation, imbalanced proteostasis, mitochondrial dysfunction, abnormal ion handling and stem cell exhaustion, a high degree of cellular and oxidative stress occurs in senescent fibers. Although the hierarchy within these different degenerating pathways has not yet been determined, it is clear from the characterization of aged muscles that a variety of molecular chaperones are upregulated during sarcopenia.[45-50,58-65] This includes a considerable number of large heat shock proteins, but especially drastically elevated levels of small heat shock proteins, such as alphaB-crystallin/HspB5 and the muscle-specific cardiovascular cvHsp/HspB7.[65]

Importantly, PPIases are intrinsically involved in the cellular stress response in skeletal muscles. The PPIases mediate a rate-limiting step during protein folding by catalyzing the cis/trans-isomerization of the peptidyl-prolyl peptide bond. This is a crucial step of peptide synthesis that generates a properly folded and functional protein species. The members of this superfamily of immunophilins, that was identified by the mass spectrometric screening of aged mouse hindlimb muscles, are listed in Table 4. This detailed table of identified PPIases is included in this report to demonstrate the biochemical sensitivity of the streamlined bottom-up proteomic approach used to generate a reference map of proteins from crude extracts of senescent mouse muscles.

Proteomic profiling of organellar and subcellular markers in aged skeletal muscle

Robust marker proteins that are enriched in subcellular structures are extremely important for the swift cell biological characterization of changes in senescent skeletal muscles. Table 5 lists identified marker proteins of the sarcolemma, caveolae, ribosomes, the Golgi apparatus, peroxisomes, proteasomes, lysosomes, the nucleus and cytosol in aged muscles. In addition, proteomic markers of the neuromuscular junction, motor neurons and Schwann cells are listed in Table 5. Above tables have already covered proteomic markers of the transverse tubular membrane system, triad junctions and the sarcoplasmic reticulum.

Bioinformatic analysis of potential protein-protein interactions within protein clusters in aged skeletal muscle

Following mass spectrometric identification, muscle proteins were analyzed for potential protein interaction patterns. As shown in Figure 1, bioinformatic STRING analyses,[57] indicates interaction patterns within protein clusters that are involved the regulation of excitation-contraction coupling, calcium homeostasis and major bioenergetic pathways of aerobic and anaerobic metabolism, as well as the cellular stress response. Due to the complexity of functional and physical protein clustering and the large number of interacting protein species, the illustrations depicted in Figure 1 are not presented to give detailed information on individual protein interactions, but instead are shown to provide a general overview of complex formation of identified protein groupings in aged skeletal muscles.

Discussion

Skeletal muscles contribute to approximately half of the biomass in the average human body.[67] Voluntary muscles are involved in locomotion, posture, balance, bodily protection, respiration, heat homeostasis and communication.[68-70] The range of muscular disorders ranges from genetic diseases of early childhood,[71] to progressive muscle wasting syndromes in the elderly.[72-76] It is therefore crucial to establish proteomic reference maps of skeletal muscles at different age.[77-82] This report focused on the establishment of the proteomic profile of the senescent mouse hindlimb musculature. The proteomic databank can now be used as the scientific basis for detailed future studies into age-associated alterations in distinct muscle protein families.

The proteomic analysis has covered important markers of organelles, such as the nucleus, Golgi apparatus, sarcolemma, caveolae, transverse tubules, triad junctions, sarcosol, sarcoplasmic reticulum, mitochondria, lysosomes, proteasomes and peroxisomes. Metabolic adaptations of aged muscles can be studied at the level of both aerobic and anaerobic bioenergetic pathways. The proteomic strategy presented here has identified key markers for such studies, including enzymes that are present in major mitochondrial substructures.[83] This is important, since mitochondrial abnormalities and oxidative stress have been implicated in sarcopenia of old age.[84,85] The identified mitochondrial markers encompass the voltage-dependent anion-selective channel VDAC1, NADH dehydrogenase, succinate dehydrogenase, cytochrome b-c1, cytochrome c oxidase, ATP synthase subunit beta and isocitrate dehydrogenase of the outer membrane, inner membrane complexes I to V and the mitochondrial matrix, respectively.[86] The presence of glycolytic enzymes and other components involved in glucose metabolism were clearly revealed by proteomics.[87] Especially interesting was the identification of both hexokinase isoforms, HKI and HKII, the enzymes that mediate the initial priming step of muscle glycolysis and are differentially affected by hormonal regulation.[88]

Excitation-contraction coupling and calcium handling were previously shown to be impaired in aged skeletal muscles.[89-91] The proteomic catalogue presented here lists all major players of this crucial signaling mechanism, including the voltage-sensing L-type calcium channel of the transverse tubules, the ryanodine receptor calcium release channel of the sarcoplasmic reticulum and its auxiliary complex at the triad junctions, as well as crucial regulatory proteins and luminal calcium binding proteins. This will enable future systems biological investigations to better determine the mechanisms that underlie excitation-contraction uncoupling and associated muscular weakness in aged organisms.[91]

The expression levels of the protein constituents of the cellular stress response are highly abundant and diverse in skeletal muscles,[92] and considerably affected during muscle adaptations and neuromuscular disease.[93] The proteomic cataloguing of aged skeletal muscles reported in this article has listed a large number of molecular chaperones and heat shock proteins. This included small heat shock proteins (HSPB/HSPE), which are present at high

Table 5. *Mass spectrometry-based proteomic profiling of organellar and subcellular markers in aged mouse hind-limb muscle.*

Accession	Protein name	Gene	Peptides	Coverage (%)	Molecular mass (kDa)
(i) **Sarcolemma**					
Q8VDN2	Na^+/K^+-ATPase, alpha-1	Atp1a1	34	38.7	113.0
Q6PIE5	Na^+/K^+-ATPase, alpha-2	Atp1a2	30	36.8	112.2
Q9WV27	Na^+/K^+-ATPase, alpha-4	Atp1a4	7	7.8	114.9
P14094	Na^+/K^+-ATPase, beta-1	Atp1b1	6	27.6	35.2
P14231	Na^+/K^+-ATPase, beta-2	Atp1b2	8	32.8	33.3
P97370	Na^+/K^+-ATPase, beta-3	Atp1b3	2	9.7	31.8
Q9ESD7	Dysferlin	Dysf	27	18	237.9
Q69ZN7	Myoferlin	Myof	34	21	233.3
P82348	Sarcoglycan, gamma	Sgcg	2	11.3	32.1
(ii) **Caveolae**					
P49817	Caveolin-1	Cav1	12	68	20.5
Q9WVC3	Caveolin-2	Cav2	4	40.7	18.2
P51637	Caveolin-3	Cav3	3	25.8	17.4
(iIi) **Organellar markers (ribosome, Golgi apparatus, peroxisome, proteasome, lysosome)**					
P14206	40S ribosomal protein SA (ribosome)	Rpsa	18	63.1	32.8
P55937	Golgin-160 (Golgi apparatus)	Golga3	6	6.0	167.2
P24270	Catalase (peroxisome)	Cat	30	63.9	59.8
P61089	Ubiquitin-conjugating enzyme E2 (proteasome)	Ube2n	8	55.3	17.1
P11438	Lysosome-associated membrane glycoprotein 1 (lysomsome)	Lamp1	3	7.6	43.9
(iv) **Nucleus**					
O08579	Emerin (inner nuclear membrane)	Emd	5	28.2	29.435
P48678	Lamin-A/C (nuclear lamina)	Lmna	60	73.7	74.2
P21619	Lamin-B2 (nuclear lamina)	Lmnb2	15	24.8	67.3
P10922	Histone H1.0	H1f0	5	27.3	20.9
Q64522	Histone H2A	Hist2h2ab	8	66.2	14.0
P02301	Histone H3.3	H3f3c	7	47.1	15.3
P62806	Histone H4	Hist1h4a	12	60.2	11.4

To be continued on next page

Table 5. *Continued from previous page.*

Accession	Protein name	Gene	Peptides	Coverage (%)	Molecular mass (kDa)
(v) **Cytosol**					
P13634	Carbonic anhydrase CA1	Ca1	2	10	28.3
P00920	Carbonic anhydrase CA2	Ca2	3	22.7	29.0
P16015	Carbonic anhydrase CA3	Ca3	17	85	29.4
P06151	Lactate dehydrogenase	Ldha	23	74.4	36.5
P09528	Ferritin heavy chain	Fth1	10	62.1	21.1
P29391	Ferritin light chain 1	Ftl1	6	48.6	20.8
(vi) **Neuromuscular junction, motor neuron and Schwann cells**					
P13595	Neural cell adhesion molecule NCAM1	Ncam1	10	12.4	119.4
P27573	Myelin protein P0	Mpz	5	20.2	27.6
P04370	Myelin basic protein	Mbp	4	17.6	27.2
Q9CQW1	Synaptobrevin	Ykt6	3	15.2	22.3
O09117	Synaptophysin	Sypl1	2	13	28.9

density in myofibers,[65,94,95] the group of co-chaperoning Hsp40 and Hsp60 proteins, the highly diverse Hsp70 (HSPA) family of constitutive and inducible chaperoning proteins,[96] the Hsp90 (HSPC/HSPH) class of large heat shock proteins,[97] protein disulfide isomerases, calcium-binding chaperones and the superfamily of PPIases that catalyze the cis/trans-isomerization of the peptidyl-prolyl peptide bond during protein folding.[98,99]

The newly presented proteomic map of muscle-associated proteins in senescent mice can now be employed for the systematic evaluation of complex changes and adaptations during age-related muscle degeneration. This could have significant implications for the establishment of new proteomic biomarker signatures of aging.[78,79,100,101] Hence, the new MS-based reference map could be useful for detailed future evaluations of (i) muscle fiber type shifting,[33,66,102] (ii) metabolic disturbances and alterations of the gut-muscle axis,[103] (iii) mitochondrial dysfunction, inflammation and impaired cellular signaling,[84] and (iv) protein modifications,[6] as well as the potential influence of (v) pharmacological therapies and related treatments,[74,75,104] (vi) physical activity levels,[105,106] (vii) nutritional interventions,[104,107-109] and (viii) gender specific differences,[110] on the development of different forms of sarcopenia.[71-73] Hence, novel proteomic biomarkers could be beneficial for better defining and differentiating the pre-sarcopenic loss of skeletal muscle mass and decline in contractile function,[111,112] from acute *versus* chronic forms of sarcopenia of old age.[113,114]

The quality of life can be severely impacted by the progressive loss of muscle mass and accompanying dysfunction of the skeletal musculature in the elderly. In order to better understand the multi-factorial etiology of age-related muscle wasting, it is imperative to determine in more detail the molecular mechanisms that trigger myofiber degeneration in the senescent organism. Animal models of aging play a critical role in sarcopenia research.[100,115,116] This report has outlined the mass spectrometric analysis of total protein extracts from crude muscle tissue extracts, which has resulted in the establishment of a proteomic reference map of the aged mouse hindlimb musculature. Figure 2 gives an overview of identified protein groupings in senescent skeletal muscles using bottom-up proteomics.

The established protein clusters that are associated with the contractile apparatus in sarcomeres, various organelles, the excitation-contraction coupling apparatus, the extra-sarcomeric cytoskeleton, the extracellular matrix, bioenergetic pathways, the molecular chaperoning system and the nerve-muscle connection, are crucial entities for normal physiological functioning and efficient adaptability of the neuromuscular system. Many of these protein families are affected in sarcopenia of old age and are therefore crucial targets for studying the cellular mechanisms that cause the age-related loss in skeletal muscle

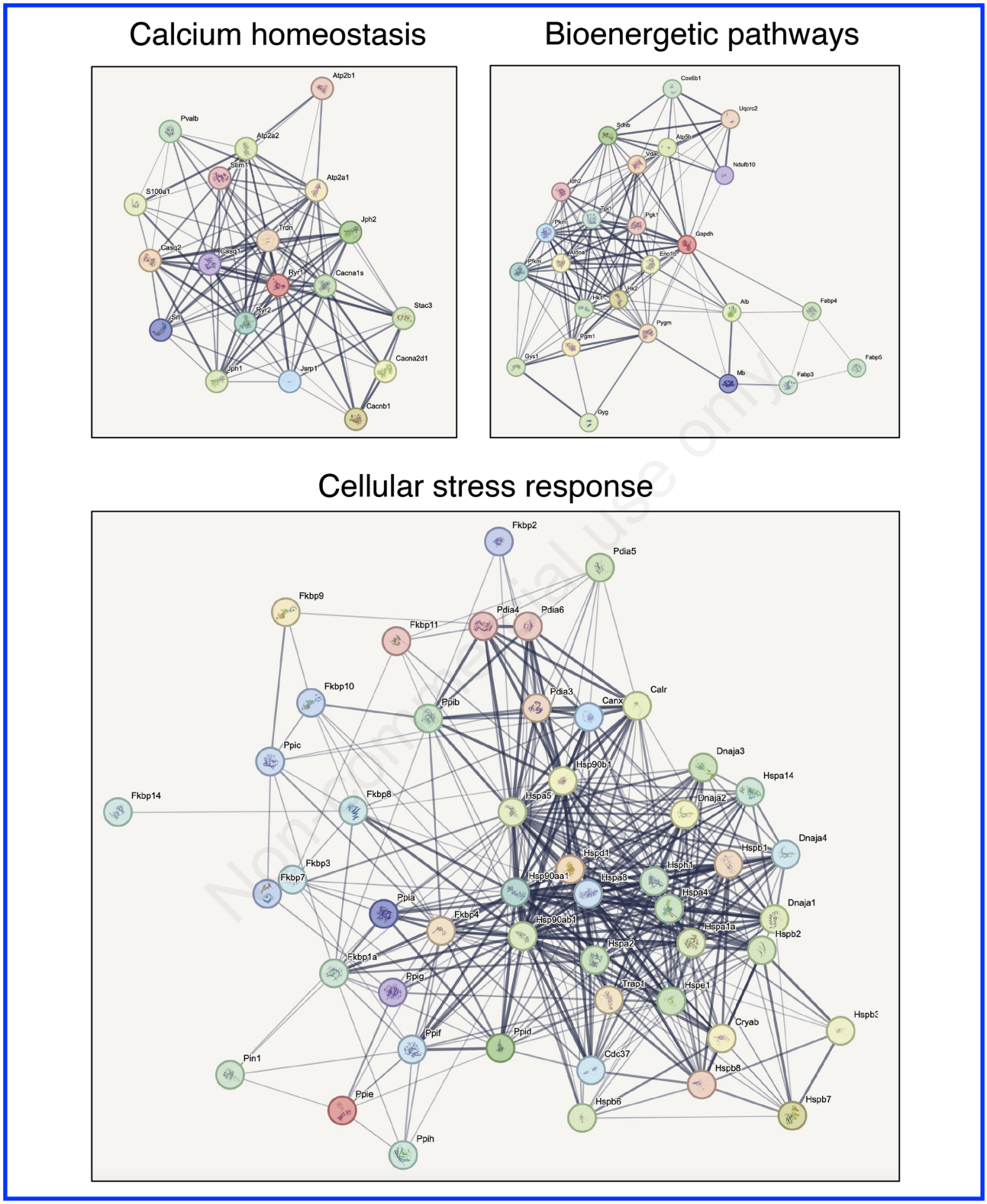

Figure 1. *Overview of potential protein-protein interactions within major protein complexes that were detected by bottom-up proteomics of total extracts from aged mouse skeletal muscle. The data used to generate the individual images are the listings of identified protein families that are linked to calcium homeostasis and the regulation of excitation-contraction coupling (Table 1), bioenergetic pathways and metabolite transportation (Table 2), and molecular chaperones involved in the cellular stress response (Tables 3 and 4). The publicly available bioinformatic analysis tool STRING (https://string-db.org) was used for the analysis of protein interaction patterns.*[57]

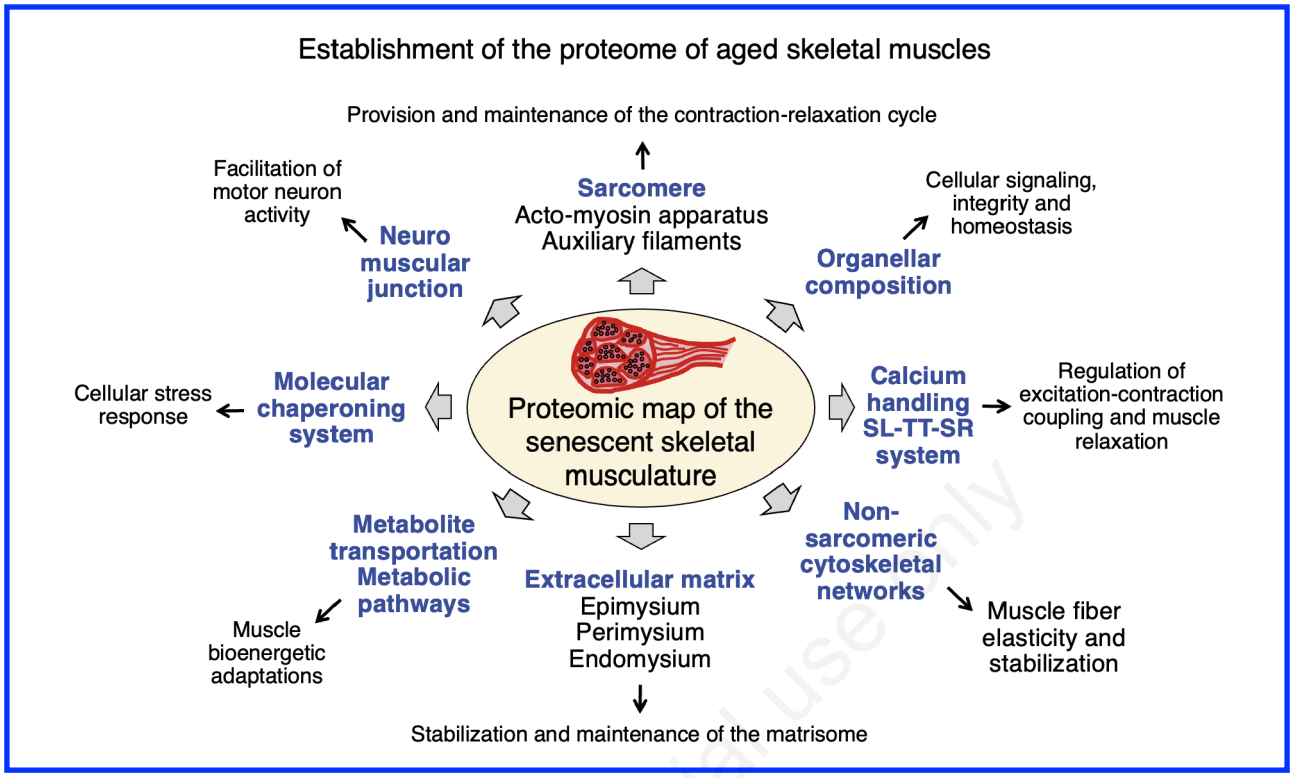

Figure 2. *Overview of mass spectrometrically identified protein groupings in aged mouse hindlimb muscles.*

mass and contractile strength.[45-50,58-65] The proteomic reference map presented in this article covers the main constituents involved in the regulation and maintenance of the excitation-contraction-relaxation cycle and its structural embedding in the intracellular cytoskeleton and provision of lateral force transmission through costameres and the extracellular matrix.

Building on these findings, tissue proteomics can now be used to routinely study small amounts of starting material for the in-depth screening of age-associated processes, such as excitation-contraction uncoupling, myonecrosis, cytoskeletal collapse, an altered cellular stress response, myofiber type shifting, abnormal calcium homeostasis, impaired bioenergetics, contractile weakness and reactive myofibrosis. Hence, the establishment of the proteomic profile of senescent muscles can be utilized as the basis of biomedical knowledge for detailed future investigations into age-related changes and/or adaptations of distinct protein families.

List of abbreviations

BiP, Binding immunoglobulin protein
CA, Carbonic anhydrase
CAM, Neural cell adhesion molecule
cv, cardiovascular
CSQ, Calsequestrin
ER, Endoplasmic reticulum
FABP, Fatty acid binding protein
FASP, Filter assisted sample preparation
FKBP, FK506 binding protein
GRP, Glucose-regulated protein
Hsc, Heat shock cognate
HSP, Heat shock protein
JPH, Junctophilin
JSRP, Junctional sarcoplasmic reticulum protein
MKBP, Myotonic dystrophy protein kinase binding protein
MS, Mass spectrometry
mt, mitochondrial
NIMA, 'never in mitosis gene a' kinase
OSF, Open Science Foundation
PDI, Protein disulfide isomerase.
PMCA, Plasma membrane calcium-transporting ATPase
PPIase, Peptidyl-prolyl cis/trans isomerases
RYR, Ryanodine receptor
SERCA, Sarcoplasmic or endoplasmic reticulum calcium ATPase
SL, Sarcolemma
SR, Sarcoplasmic reticulum
STAC3, SH3 and cysteine-rich domain-containing protein
TT, Transverse tubules
VDAC, voltage-dependent anion-selective channel

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, MZ and PD were involved in the preparation of muscle tissues and performed the biochemical experiments and analyzed the data. MH and PM per-

formed 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 competing interests.

Ethics approval

Local governmental and institutional animal care regulations were followed and approved by the Institutional Animal Care and Use Committee (Amt für Umwelt, Verbraucherschutz und Lokale Agenda der Stadt Bonn, North Rhine-Westphalia, Germany). Transportation and usage of tissue specimens was in accordance with the regulations of the Department of Agriculture (animal by-product register number 2016/16 to the Department of Biology, National University of Ireland, Maynooth). The study conforms with the Helsinki Declaration of 1964, as revised in 2013, concerning human and animal rights.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Corresponding Author

Kay Ohlendieck, Department of Biology, Maynooth University, National University of Ireland, Maynooth W23F2H6, Co. Kildare, Ireland.
Tel.: 353.1.7083842.
ORCID ID: 0000-0002-6266-4510
E-mail: kay.ohlendieck@mu.ie

Paul Dowling
ORCID ID: 0000-0002-9290-9267
paul.dowling@mu.ie

Stephen Gargan
ORCID ID: 0000-0003-1005-8372
stephen.gargan@mu.ie

Margit Zweyer
ORCID ID: 0000-0001-5615-1869
margit.zweyer@dzne.de

Michael Henry
ORCID ID: 0000-0001-5312-4961
michael.henry@dcu.ie

Paula Meleady
ORCID ID: 0000-0001-5306-310X
paula.meleady@dcu.ie

Dieter Swandulla
ORCID ID: 0000-0003-0923-7090
swandulla@uni-bonn.de

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