



pISSN: 2037-7452 eISSN: 2037-7460
<https://www.pagepressjournals.org/index.php/bam/index>

Publisher's Disclaimer. E-publishing ahead of print is increasingly important for the rapid dissemination of science. The **Early Access** service lets users access peer-reviewed articles well before print / regular issue publication, significantly reducing the time it takes for critical findings to reach the research community.

These articles are searchable and citable by their DOI (Digital Object Identifier).

The **European Journal of Translational Myology** is, therefore, e-publishing PDF files of an early version of manuscripts that undergone a regular peer review and have been accepted for publication, but have not been through the typesetting, pagination and proofreading processes, which may lead to differences between this version and the final one.

The final version of the manuscript will then appear on a regular issue of the journal.

E-publishing of this PDF file has been approved by the authors.

Eur J Transl Myol 2026 [Online ahead of print]

To cite this Article:

Dowling P, Zweyer M, Nebeling F, et al. **Proteomic and bioinformatic profiling of the aging mouse heart-diaphragm system.** *Eur J Transl Myol* doi: 10.4081/ejtm.2026.16061

 ©The Author(s), 2026
Licensee [PAGEPress](#), Italy

Note: The publisher is not responsible for the content or functionality of any supporting information supplied by the authors. Any queries should be directed to the corresponding author for the article.

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article or claim that may be made by its manufacturer is not guaranteed or endorsed by the publisher.



Submitted: 7 August 2026

Accepted: 26 August 2026

Early access: 2 October 2026

Proteomic and bioinformatic profiling of the aging mouse heart-diaphragm system

Paul Dowling,^{1,2} Margit Zweyer,³ Felix Nebeling,⁴ Dieter Swandulla,⁵ Kay Ohlendieck^{1,2}

¹Department of Biology, Maynooth University, National University of Ireland, Maynooth, Co.

Kildare, Ireland; ²Kathleen Lonsdale Institute for Human Health Research, Maynooth University,

Maynooth, Co. Kildare, Ireland; ³German Center for Neurodegenerative Diseases, Bonn, Germany;

⁴Department of Neurooncology, Center for Neurology, University Hospital Bonn, Bonn, Germany;

⁵Institute of Physiology, Medical Faculty, University of Bonn, Bonn, Germany

Abstract

With advancing age, cardiac weakness and a progressive loss of skeletal muscle mass and contractile strength, is observed in most humans. Although considerable inter-individual differences exist in the degree of age-related muscle wasting, progressive cardiac impairment and sarcopenia play a key role during the natural aging process and form an integral part of the frailty syndrome. Here, we have used comparative bottom-up proteomic profiling to study age-related changes in an established murine model of sarcopenia. The simultaneous assessment of heart and diaphragm muscle aging using peptide mass spectrometry showed that senescence is associated with myofiber degeneration, considerable changes in metabolic processes and increased extracellular matrix deposition. A striking reduction in two mitochondrial enzymes, NAD(P) transhydrogenase and hydroxymethylglutaryl-CoA synthase, was identified by proteomics in the senescent heart and diaphragm muscle, respectively. Bioinformatic analyses revealed abundance changes in distinct protein families and potential alterations in protein-protein interaction patterns. The altered protein profile of the heart-diaphragm system, as determined by comparative discovery proteomics, can be helpful to establish an improved biomarker signature for diagnostic, prognostic and therapeutic monitoring of muscle aging.

Key words: aging, biomarker, cardiac proteomics, muscle proteomics, mass spectrometry, senescence

The heart-diaphragm system is characterized by both an anatomical and physiological linkage that forms a crucial cardio-respiratory muscle connection that supports functional and stabilizing organ crosstalk and thereby influences heart rate, blood pressure, circulation and breathing patterns.^{1,2} Of note, this reciprocal organ-organ communication extends to cardiovascular and respiratory pathophysiology, which can cause detrimental interrelationships between heart failure and diaphragm abnormalities in disease and during aging.^{3,4} The aged heart exhibits both structural and functional changes and the senescent cardiovascular system is linked to a weakened supply of oxygen, nutrients and signaling molecules to the musculature.⁵ An exacerbating factor of cardiac dysfunction during aging is the impaired heart-diaphragm system. Importantly, age-related changes in contractile fibers, the most abundant cell type in the body by biomass, are of central importance for the overall aging process in humans.⁶ The frailty syndrome is closely connected to sarcopenia of old age,⁷ which is defined as the progressive loss in muscle mass and contractile strength with advancing age.⁸ A considerable number of elderly suffer from extreme forms of sarcopenia that negatively affects their quality of life,⁹ and has prompted the development of countermeasures to halt muscle wasting in sarcopenic patients.¹⁰⁻¹²

A serious complication of sarcopenia has been established as inspiratory dysfunction due to age-related diaphragm abnormalities.³ Importantly, whole body homeostasis depends on efficient respiration, which is intrinsically linked to the physiological functioning of the thoracic diaphragm muscle,¹³ which represents a specialized type of skeletal muscle with continuous contractile performance that is based on high levels of aerobic oxidative capacity and fatigue resistance.¹ Thus, since bronchoalveolar function and respiratory gas-exchange are fundamentally coupled to the contractile activity of the diaphragm,¹⁴ this essential respiratory muscle can be considered a physiological systems connector.¹⁵

Mass Spectrometry (MS) based proteomics has been established as a key bioanalytical tool to assess systems biological aspects of aging including the establishment of biomolecular signatures of health-span, longevity, mechanisms of senescence and susceptibility to age-related disease.¹⁶ Systematic proteomic methodology is also highly useful to investigate complex changes in the heart-diaphragm system, which is the topic of this report. Previous mass spectrometric studies were shown to be valuable in biogerontology research, including muscle proteomics,¹⁷ to improve our pathobiochemical understanding of the underlying mechanisms involved in the cellular senescence of the neuromuscular system.¹⁸ Here, we have used an Orbitrap Ascend Tribrid mass spectrometer for the comprehensive proteomic profiling of heart and diaphragm muscle aging. As an established model system of cardiac and neuromuscular aging in the field of biogerontology, this study has

focused on the mass spectrometric analysis of young adult versus aged mouse heart,¹⁹ in comparison to diaphragm muscle aging.²⁰

The classification of aging in mouse models is usually based on categorizing 3-6, 10-15 and 18-24 months of age as ‘young adult’, ‘middle-aged’ and ‘old’. Although the middle-aged category can display early signs of aging, more pronounced reductions in muscle mass and contractile function are only seen in the aged group.²¹ Of note, mouse muscle function clearly declines in an age-dependent way after 15 months of age and is reflected by abnormal hindlimb gait and reduced speed and distance for voluntary wheel running.²² Hence, this study used 3 months old mouse diaphragm and heart muscle representing the matured ‘young adult’ group and 24 months old specimens as the ‘senescent’ group with an advanced aging phenotype for comparative proteomic analyses.

Materials and Methods

Materials

For the comparative proteomic profiling of mouse diaphragm muscle and heart specimens, general chemicals and materials were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK), Bio-Rad Laboratories (Hemel-Hempstead, Hertfordshire, UK) and Sigma Chemical Company (Dorset, UK). Protein concentration was determined with the Pierce 660-nm Protein Assay (ThermoFisher Scientific, Dublin, Ireland). Protein digestion was carried out with trypsin and Lys-C from Promega (Madison, WI, USA). Protease inhibitors were obtained from Roche Diagnostics (Mannheim, Germany).

Animals

Muscle samples for proteomic analysis were harvested from freshly dissected diaphragm and heart specimens from 3 months versus 24 months old C57/BL6 mice.²³ Mice were obtained from the Bioresource Unit of Bonn University where they were kept at a constant room temperature of 22°C on a 12:12-h dark-light cycle with *ad libitum* access to food and water. All protocols and *post mortem* harvesting of muscle specimens were performed under the terms of the German animal protection law and were permitted by local authorities. Tissue samples were quick-frozen in liquid nitrogen and stored at -80°C prior to proteomic analysis in accordance with the Department of Agriculture (animal by-product register number 2016/16 to the Department of Biology, National University of Ireland, Maynooth).

Preparation of mouse muscle extracts

Whole diaphragm and heart specimens from 3 months old (n=6) and 24 months old (n=6) mice were homogenized in lysis solution containing 50 mM Tris-HCl, pH 8.0, 8 M urea and 1 mM EDTA.²⁴ The lysis buffer was supplemented with a freshly prepared protease inhibitor cocktail.²⁵ The muscle tissue extracts were incubated for 1.5 hours at 4°C and then processed for MS-based analysis following centrifugation at 16,000xg for 5 min.²⁶ Following treatment with the Ready Prep 2D clean up kit from Bio-Rad Laboratories (Hemel-Hempstead, Hertfordshire, UK), protein pellets were resuspended in 6 M urea, 2 M thiourea and 10 mM Tris-HCl, pH 8.0.²⁷ Standardized procedures were used for chemical reduction, alkylation and controlled digestion of protein samples, as described previously in detail.²⁶

Mass spectrometry-based proteomic analysis

The proteomic analysis of aged skeletal muscle was carried out with the help of an Orbitrap Ascend Tribrid mass spectrometer from Thermo Fisher Scientific (Waltham, MA, USA). The general bioanalytical approach using peptide MS and the experimental details of the bottom-up proteomic analysis of mouse tissues has been described in detail.²³ Tissue homogenization, protein extraction, protein determination and the filter-assisted sample preparation for buffer exchange and proteolysis to produce peptides for MS analysis was performed by standardized methodology.^{24,25,28} For label-free liquid chromatography (LC) MS analysis, 500 ng of each digested sample was analysed. Peptides were eluted over a 90 minute binary gradient [solvent A: 2% (v/v) acetonitrile (ACN) and 0.1% (v/v) formic acid in LC-MS grade water and solvent B: 80% (v/v) ACN and 0.1% (v/v) formic acid in LC-MS grade water].

Data was acquired with Xcalibur software (Thermo Fisher Scientific). The mass spectrometer was externally calibrated and operated in positive, data-dependent mode. A full survey MS scan was performed in the 300–2000 m/z range. Collision-Induced Dissociation (CID) fragmentation was carried out on the fifteen most intense ions per scan (top 15).²⁶ Data analysis, processing and visualization for protein identification and Label-Free Quantification (LFQ) normalization of MS/MS data was performed using MaxQuant 2.4.9 and Perseus v2.1.6.0 software (www.maxquant.org). Significant proteins were initially identified using MaxQuant software and the Andromeda search engine to explore the detected features against the Uni-ProtKB/SwissProt database for *Mus musculus* (Taxon ID: 10090). The following search parameters were used: i) first search peptide tolerance of 20 ppm, ii) main search peptide tolerance of 4.5 ppm, iii) cysteine carbamidomethylation set as a fixed modification, iv) methionine oxidation set as a variable modification, v) a maximum of two missed cleavage sites and vi) a minimum peptide length of seven amino acids. The False Discovery Rate (FDR) was set to 1% for both peptides and proteins

using a target-decoy approach. Relative quantification was performed using the MaxLFQ algorithm. The “proteinGroups.txt” file produced by MaxQuant was further analysed in Perseus. Proteins that matched to the reverse database or a contaminants database or that were only identified by site were removed. The LFQ intensities were log2 transformed, and proteins with less than 70% valid values were removed from the dataset. Data imputation was performed to replace missing values with values that simulate signals from peptides with low abundance chosen from a normal distribution specified by a downshift of 1.8 times the mean standard deviation of all measured values and a width of 0.3 times this standard deviation. A two-sample *t*-test was performed using $p < 0.05$ on the post-imputed data to identify statistically significant differentially abundant proteins.

Systems bioinformatic analysis

Optimized systems bioinformatic tools were employed for the evaluation of proteomic data,²⁹ including functional enrichment analyses by ShinyGO 0.85 with Gene Ontology (GO) terms,³⁰ and Kyoto Encyclopedia of Genes and Genomes (KEGG),³¹ pathways. In order to group identified muscle proteoforms based on their protein class and to identify potential protein-protein interaction patterns, the PANTHER version 19.0 database of protein families (<http://pantherdb.org/>),³² and the STRING database of known and putative protein interactions that include both direct physical and indirect functional protein associations (<http://string-db.org/>),³³ were used.²⁶

Results

This report outlines the impact of aging on the proteome of the heart-diaphragm system, using the comparative mass spectrometric profiling of 3 months old mouse heart and diaphragm muscle samples versus 24 months old specimens. Following the identification of altered protein species, bioinformatics was employed to visualize trends in the change of protein classes, protein interactions and biological processes.

Comparative proteomic profiling of the aged mouse heart and diaphragm

The bioinformatic PANTHER analysis tool,³² was used to illustrate changes in protein classes in the aged heart-diaphragm system. The systematic proteomic analysis revealed considerable changes in muscle proteins during aging, *i.e.* 158 increased and 201 decreased cardiac proteins, and 338 increased and 413 decreased diaphragm proteins. Figure 1 depicts the graphical presentation of altered protein classes in the aged heart-diaphragm system. Proteome-wide changes during aging affected especially metabolite interconversion enzymes, as well as RNA metabolism proteins, Ca²⁺-binding proteins, cell adhesion molecules, cell junction proteins, molecular chaperones, chromatin

and chromatin-binding proteins, cytoskeletal proteins, defense/immunity proteins, extracellular matrix proteins, gene-specific transcriptional regulators, intercellular signal molecules, membrane traffic proteins, protein modifying enzymes, protein-binding activity modulators, scaffold/adaptor proteins, structural proteins, transfer/carrier proteins, translational proteins, transmembrane signal receptors and transporters.

Heatmap of proteome-wide changes during aging of the murine heart

A heatmap and listings of decreased versus increased protein species during heart aging is provided in Figure 2. Altered protein species are symbolized by their respective gene names. A noteworthy proteomic change included the drastic age-related reduction in the cardiac enzyme

NAD(P)⁺ transhydrogenase (*NNT* gene). In addition, a diverse array of important cardiac proteins with a lower age-related abundance was identified, including the ubiquitin-conjugating enzyme E2 (*Ube2d3* gene), the ribosomal protein S23 (*RPS23* gene), the mitochondrial acid phosphatase (*ACP6* gene), the mitogen-activated protein kinase (*Mapk14* gene), ankyrin-B (*ANK2* gene), oxysterol-binding protein (*OSBP* gene), histone H4-C1 (*HIST1H4A* gene) and acyl-CoA oxidase (*ACOX1* gene).

Reduced proteins of the acto-myosin apparatus included myosin light chains MYL2 (*Myl2* gene) and MYL3 (*Myl3* gene), myosin-binding protein C (*Mybpc3* gene), the troponins cTnT (*Tnnt2* gene) and cTnI (*Tnni3* gene), and tropomyosin cTM (*Tpm1* gene). Lower expression levels were established for annexin A6 (*Anxa6* gene), dysferlin (*Dysf* gene), cadherin-13 (*Cdh13* gene), α 1-Na⁺/K⁺-ATPase (*Atp1a1* gene), the α ₁-subunit of the dihydropyridine receptor (*Cacna2d1* gene) and calsequestrin (*Casq2* gene), as well as NADH:ubiquinone oxidoreductase subunits A5, AB1, B5, B7, B8, B10, B11, S3, S4, S8, V1 and V2 (numerous *Nduf* genes).

In contrast, proteomics identified increases in α / β -spectrin (*Spta1* and *Sptb* genes), the α -, β - and γ -subunits of fibrinogen (*Fga*, *Fgb* and *Fgg* genes), the plasma corticosteroid-binding globulin (*SERPINA6* gene), myosin light chain kinase (*MYLK3* gene), ankyrin (*ANK1* gene), heavy chain 1 and 4 of the inter-alpha-trypsin inhibitor complex (*Itih1* and *Itih4* genes), the anion exchanger AE1/band 3 protein (*Slc4a1* gene), copper-transporting ceruloplasmin (*Cp* gene), the plasma iron transporter transferrin (*Tf* gene), and carbonic anhydrase isoforms CA1 and CA2 (*Ca1* and *Ca2* genes), and collagen isoform COL-VI (*Col6a1* gene) in the aged heart.

Bioinformatic STRING analysis of potentially altered protein interaction patterns during heart aging

The STRING database of known and putative protein interactions,³³ including both direct physical and indirect functional protein associations, was employed to determine potentially altered protein interaction patterns during cardiac aging. A core protein hub of reduced cardiac components was identified as the contractile and regulatory elements of the heart sarcomere, including myosin light chains, myosin binding protein, tropomyosin and troponin, as well as myosin heavy chain isoform MyHC6. A major protein hub with increased cardiac proteins included the α -, β - and γ -subunits of fibrinogen (Figure 3).

Heatmap of proteome-wide changes during aging of the murine diaphragm

A heatmap and listings of decreased versus increased protein species during diaphragm aging are provided in Figure 4. Altered protein species are symbolized by their respective gene names. The most drastic reduction was shown to occur in mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase HMGCS2 (*Hmgcs2* gene). Other decreased diaphragm proteins included the isoforms 1A1, 1A5 and 2B5 of the detoxifying phase-2 biotransformation enzyme UDP-glucuronosyltransferase (numerous *Ugt* genes), urate oxidase (*Uox* gene), 3-hydroxyanthranilate 3,4-dioxygenase (*Haa* gene), 4-hydroxyphenylpyruvate dioxygenase (*Hpd* gene), the dihydroxyacetone kinase DAK (*Dak* gene), sarcosine dehydrogenase (*Sardh* gene), fructose-bisphosphatase (*Fbp1* gene), arginase (*Arg1* gene), carbamoyl phosphate synthetase (*Cps1* gene), 4-aminobutyrate aminotransferase (*Abat* gene), homogentisate 1,2-dioxygenase (*Hgd* gene), acyl-CoA synthetase (*Acsml* gene), regucalcin (*Rgn* gene), and fatty acid-binding protein L-FABP (*Fabp1* gene)

Increased proteins included the extracellular matrix component collagen, specifically the α -1, α -2 and α -3 chains (*Col6a1*, *Col6a2* and *Col6a3* genes) of isoform COL-VI, in addition to ubiquitin C-terminal hydrolase L1 (*Uchl1* gene), myospryn (*Cmya5* gene), the Na⁺/H⁺-exchanger regulatory factor NHERF2 (*Slc9a3r2* gene), nebulin (*Neb* gene), myozenin isoform MYOZ1 (*Myoz1* gene), myomesin isoforms MYOM1 and MYOM3 (*Myom1* and *Myom3* genes), the β 2-subunit of laminin (*Lamb2* gene), dystrophin (*Dmd* gene), and annexin A1 (*Anxa1* gene). Increased Ca²⁺-handling proteins included the ryanodine receptor (*Ryr1* gene), the α_{1S} , α_2/d_1 and β_1 -subunits of dihydropyridine receptor (*Cacna1s*, *Cacna2d1* and *Cacnb1* genes), the sarcoplasmic reticulum Ca²⁺-ATPase (*Atp2a1* gene), sarcalumenin (*Srl* gene) and calsequestrin CSQ1 (*Casq1* gene). In contrast to aged heart, elements of the NADH:ubiquinone oxidoreductase complex I of the inner mitochondrial membrane were shown to be increased, including subunits A2, A3, A5-A13, C2, B4-B6, B8-B11, S6, S8, V1-V5 and V7 (numerous *Nduf* genes).

Bioinformatic STRING analysis of potentially altered protein interaction patterns during diaphragm aging

In order to determine potential changes in protein-protein interactions in the senescent diaphragm muscle, the STRING analysis tool based on a collection of known and putative protein interactions,³³ was used. A central protein hub of diaphragm proteins with reduced abundance was shown to contain a variety of metabolic enzymes such as betaine-homocysteine S-methyltransferase BHMT, methionine adenosyltransferase MAT1A, carbamoyl phosphate synthetase CPS1 and formimidoyltransferase cyclodeaminase FTCD, as well as transcription factor OCT and the cytosolic Ca²⁺-binding protein regucalcin (Figure 5). Two protein hubs with increased diaphragm proteins included one with myofibrosis-associated proteins, and one with sarcomeric components (Figure 5).

Bioinformatic ShinyGO analysis of alterations in biological processes during aging of the heart-diaphragm system

The bioinformatic ShinyGO analysis program,³⁰ using the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway comparison tool,³¹ was employed to determine changes in biological processes during aging of the heart-diaphragm system. Figure 6 lists increases versus decreases in specific biological processes. In the aged heart, decreased mechanisms included especially myocyte contraction, oxidative phosphorylation and the cytoskeleton. Increased processes comprised of the pentose phosphate pathway and a large number of metabolic reactions. In the senescent diaphragm muscle, decreased processes included a variety of metabolic pathways, as well as the proteasome and biotransformation reactions. Striking increases were identified in oxidative metabolism in the diaphragm, including the citric acid cycle and oxidative phosphorylation, as well as muscle contraction.

Discussion

This study evaluated the impact of aging on the proteome of the heart-diaphragm system. One of the most striking proteomic findings was the MS-based identification of reduced levels of NAD(P)⁺ trans-hydrogenase (NNT) in the aged heart. This crucial enzyme is intrinsically involved in the supply of mitochondrial NADPH,³⁴ and its reduced expression is implicated in bioenergetic dysfunction and mitochondrial redox imbalance.³⁵ Thus, the drastically reduced expression levels of NNT might be associated with the impaired oxidative capacity and mitochondrial abnormalities during cardiac aging.³⁶ The large number of cardiac proteins with a lower abundance in the aged phenotype may affect diverse biological processes, including the ubiquitin-conjugating enzyme E2,

the ribosomal protein S23 of the 40S ribosomal subunit, the mitochondrial acid phosphatase involved in the regulation of lipid metabolism and lipid balance, the mitogen-activated protein kinase which functions in pathway integration of inflammatory and stress signals, the scaffolding protein ankyrin-B, the oxysterol-binding protein that functions as an intracellular lipid carrier, the replication-dependent and DNA-packaging histone H4-C1 and acyl-CoA oxidase ACOX1 that utilizes very long-chain fatty acids and mediates the first step in fatty acid β -oxidation.

Major changes in the contractile apparatus of the senescent heart are indicated by the reduced abundance of the ventricular regulatory MYL2 and essential MYL3 myosin light chains and cardiac myosin-binding protein C of the thick filament, as well as cardiac troponin cTnT, cardiac troponin cTnI and cardiac tropomyosin cTM of the thin filament. These alterations in key components of the acto-myosin apparatus might be associated with one of the key features of cardiac aging, *i.e.* dysregulation of the sarcomeric contraction-relaxation cycle and accompanying weakened contractile function in the aged heart.^{5,37-39}

Noteworthy reductions in crucial muscle membrane repair proteins included the Ca^{2+} -dependent and phospholipid-binding protein annexin isoform ANXA6 and dysferlin. A lack of dysferlin as essential Ca^{2+} -sensor and trigger of internal repair vesicle fusion could severely impair the protective membrane patching following cardiac damage. In addition, reduction in a marker of vascular protection was identified as cadherin-13/T-cadherin. Of note, sarcolemmal excitability, excitation-contraction-coupling at transverse tubules, and luminal calcium buffering in the sarcoplasmic reticulum might be negatively affected by reduced expression of the essential $\alpha 1$ -subunit of the Na^+/K^+ -ATPase, the auxiliary $\alpha_2\delta_1$ -subunit of the voltage-sensing L-type calcium channel and the Ca^{2+} -binding cardiac calsequestrin isoform CSQ2, respectively. Interestingly, a large number of components of complex I of the inner mitochondrial membrane were identified by MS-based analysis, including reduced subunits of NADH:ubiquinone oxidoreductase.^{5,37-39}

Interesting heart-associated proteoforms of increased abundance included especially erythrocyte proteins, such as the membrane cytoskeletal α/β -spectrin, the α -, β - and γ -subunits of fibrinogen, and the plasma corticosteroid-binding globulin SERPINA6. Elevated levels of the cardiac-specific myosin light chain kinase MYLK3 suggest altered degrees of phosphorylation of regulatory myosin light chains. Additional new potential biomarker candidates of cardiac remodelling during aging include increases in the membrane cytoskeletal adaptor ankyrin ANK1, heavy chain 1 and 4 of the inter-alpha-trypsin inhibitor complex, the anion exchanger AE1/band 3 protein, copper-transporting ceruloplasmin, the plasma iron transporter transferrin, and carbonic anhydrase isoforms CA1 and CA2. Importantly, increases in the general fibrosis marker collagen isoform COL-VI, specifically

the α -1 chain, was observed in the senescent murine heart, indicating age-related collagen deposition.^{5,37-39}

The findings from bioinformatic STRING analysis of potentially altered protein-protein interaction patterns in the senescent heart suggest that age-associated changes/adaptations occur within the acto-myosin apparatus that may influence cardiac contractility and the physiological fine regulation of senescent cardiomyocytes. Increased protein hubs are represented by the α -, β - and γ -subunits of fibrinogen in combination with heavy chains of the inter-alpha-trypsin inhibitor complex and ceruloplasmin. The anion exchanger AE1 appears to form interaction patterns with α/β -spectrin, ankyrin and the carbonic anhydrase isoforms CA1 and CA2.

In the aged diaphragm, the most reduced protein was identified as mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase HMGCS2, a key metabolic enzyme that is involved the conversion of fatty acids into ketone bodies.⁴⁰ Its lower concentration indicates dysfunction of the metabolic hub that regulates ketogenesis and possibly induces glucose-related cellular damage.⁴¹ Overall, a considerable number of diaphragm proteins was identified with reduced expression level in aged muscle tissue that could negatively influence important biochemical and cell biological mechanisms. These reduced protein species included the detoxifying phase-2 biotransformation enzyme UDP-glucuronosyl-transferase, urate oxidase that converts uric acid into allantoin, 3-hydroxyanthranilate 3,4-dioxygenase of the tryptophan-kynurenine pathway, 4-hydroxy-phenylpyruvate dioxygenase involved in tyrosine metabolism, the dihydroxyacetone kinase DAK that mediates phosphorylation of dihydroxyacetone and the regulation of the innate immune response, sarcosine dehydrogenase providing the conversion of the non-proteinogenic amino acid sarcosine into glycine, the gluconeogenic enzyme fructose-bisphosphatase that catalyses the rate-limiting conversion of fructose 1,6-bisphosphate to fructose 6-phosphate, the urea cycle enzymes arginase and carbamoyl phosphate synthetase, 4-aminobutyrate aminotransferase providing γ -aminobutyric acid destruction, the enzyme named homogentisate 1,2-dioxygenase that is involved in metabolizing phenylalanine and tyrosine, mitochondrial lipid-processing acyl-CoA synthetase ACSM1, the Ca^{2+} -binding senescence marker protein SMP-30 named regucalcin, and the long-chain fatty acid-binding protein FABP1/L-FABP.^{3,4,20,42-44}

Major proteomic changes that demonstrated increases in specific diaphragm proteins included the core matrisomal heterotrimeric collagen isoform COL-VI with its α -1, α -2 and α -3 chains indicating age-related myofibrosis in the diaphragm muscle. Elevated levels of ubiquitin C-terminal hydrolase Uchl1, the scaffold protein myospryn, the Na^+/H^+ -exchanger regulatory factor NHERF2, the giant actin-associated sarcomeric protein nebulin, myozenin isoform MYOZ1 of the sarcomeric Z-disc and myomesin isoforms MYOM1 and MYOM3 of the sarcomeric M-band, the β 2-subunit of

the basal lamina glycoprotein laminin, the membrane cytoskeletal protein dystrophin, the Ca^{2+} -dependent phospholipid-binding protein annexin ANXA1, also represent interesting biomarker candidates of muscle aging.

Interestingly, a variety of key proteins of excitation-contraction coupling and muscle relaxation were shown to be increased during diaphragm aging, including the ryanodine receptor Ca^{2+} -release channel, the α_{1S} , α_2/δ_1 and β_1 -subunits of the voltage-sensing L-type Ca^{2+} -channel dihydropyridine receptor of the transverse tubules, the fast SERCA1 isoform of the sarcoplasmic reticulum Ca^{2+} -ATPase ATP2A1 and the luminal Ca^{2+} -binding proteins sarcalumenin and fast calsequestrin CSQ1 of the sarcoplasmic reticulum. This suggests considerable remodeling of the regulatory apparatus of Ca^{2+} -homeostasis and the excitation-contraction-relaxation cycle. In contrast to the aged heart, a large number of subunits of the NADH:ubiquinone oxidoreductase complex I of the inner mitochondrial membrane were shown to be increased.^{3,4,20,42-44}

The analysis of direct physical and/or indirect functional protein associations of proteins with an increased concentration identified two hubs in the aged diaphragm. Of note, fibrosis-related proteins were found in the apparent collagen deposition cluster including fibrillin FBN1, laminin and collagen isoform COL-VI, with a link to the membrane cytoskeletal protein dystrophin. The other protein hub consists of mostly sarcomeric components, such as the thin filament actin-binding protein nebulin, myozenin isoform MYOZ1 and α -actinin-2 protein of the Z-disc, myomesin isoform MYOM3 of the M-band, the scaffold proteins myospryn and obscurin, and the muscle-specific Kelch-like family members KLHL40 and KLHL41 that are involved in sarcomeric stabilization, plus a link to the essential cytoskeletal protein dystrophin.^{3,20,21}

The bioinformatic ShinyGO analysis presented in this report, which is based on comprehensive MS-based proteomic data, summarizes important alterations in biological processes during aging of the heart-diaphragm system. This includes decreased cellular and biochemical mechanisms that are involved in cardiomyocyte contraction, oxidative phosphorylation and the cytoskeletal network. Increased processes comprised of the pentose phosphate pathway and a large number of metabolic reactions in the heart. In the senescent diaphragm muscle, decreased processes included a variety of metabolic pathways, as well as the proteasome and biotransformation reactions. Striking increases were identified in oxidative metabolism in the diaphragm, including the citric acid cycle and oxidative phosphorylation, as well as muscle contraction.

In conclusion, building on previous large-scale studies of murine models of aging,⁴⁵⁻⁴⁸ the altered protein profile of the heart-diaphragm system, as determined by comparative discovery proteomics in combination with systems bioinformatic analysis, provides a new set of potential indicators of muscle aging. The proteome-wide changes agree with previous study into age-related muscle

wasting and potential fast-to-slow transitions.¹⁷ In the long-term, orthogonal verification analyses and extrapolation to aspects of human aging,¹⁸ should be helpful to establish a more comprehensive biomarker signature for improved diagnostic, prognostic and therapeutic monitoring of the heart-diaphragm system.

List of Abbreviations

ANXA, Annexin A

ACN, Acetonitrile

CID, Collision-induced dissociation

CSQ, Calsequestrin

EDTA, Ethylenediaminetetraacetic acid

FDR, False discovery rate

GO, Gene ontology

HMGCS, Hydroxy-methylglutaryl-CoA synthase

KEGG, Kyoto encyclopedia of genes and genomes

LC, Liquid chromatography

LFQ, Label-free quantification

MS, Mass spectrometry

MYL, Myosin light chain

m/z, Mass-to-charge ratio

NNT, NAD(P)⁺ trans-hydrogenase

TM, Tropomyosin

TnI, Troponin subunit I

TnT, Troponin subunit T

Corresponding Author

Kay Ohlendieck, Department of Biology, Maynooth University, National University of Ireland, Maynooth W23F2H6, Co. Kildare, Ireland.

E-mail: kay.ohlendieck@mu.ie

0000-0002-6266-4510

Paul Dowling

E-mail: paul.dowling@mu.ie

ORCID iD: 0000-0002-9290-9267

Margit Zweyer:

E-mail: margit.zweyer@dzne.de

ORCID iD: 0000-0001-5615-1869

Felix Nebeling

E-mail: felix.nebeling@dzne.de

ORCID iD: 0000-0002-2749-7163

Dieter Swandulla

E-mail: swandulla@uni-bonn.de

ORCID iD: 0000-0003-0923-7090

Contributions

Paul Dowling, Dieter Swandulla and Kay Ohlendieck were involved in the conceptualization and initiation of this project, as well as the design of the research strategy. Margit Zweyer, Felix Nebeling and Paul Dowling were involved in the preparation of muscle tissues and performed the biochemical experiments and analyzed the data. Paul Dowling and Kay Ohlendieck performed the mass spectrometric and bioinformatic analysis. All authors were involved in the writing and final editing of the manuscript.

Conflict of interest

The authors declare no competing interests.

Ethical publication statement

The German Center for Neurodegenerative Diseases (DZNE) committee approved the present study (AZ-2024-254, 26.4.2024). For the German animal welfare protocol, the veterinary office carries

out routine monthly inspections of the DZNE in Bonn. Animal facilities are monitored every 3 months according to the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA).

Availability of data and materials

All data generated or analyzed during this study are included in the article. Further inquiries can be directed to the corresponding author.

Generative AI statement

The authors declare that no Generative AI was used in the creation of this manuscript.

Funding

This work was supported by a project grant from the Kathleen Lonsdale Institute for Human Health Research, Maynooth University, and equipment funding was provided under research infrastructure calls by Science Foundation Ireland (SFI-12/RI/2346/3) and Research Ireland (23/RI/1203).

References

1. Sieck GC, Fogarty MJ. Diaphragm muscle: a pump that can not fail. *Physiol Rev* 2025;105:2589–2656.
2. Anderson RH, Razavi R, Taylor AM. Cardiac anatomy revisited. *J Anat* 2004;205:159–77.
3. Kelley RC, Ferreira LF. Diaphragm abnormalities in heart failure and aging: mechanisms and integration of cardiovascular and respiratory pathophysiology. *Heart Fail Rev* 2017;22:191–207.
4. Salah HM, Goldberg LR, Molinger J, et al. Diaphragmatic function in cardiovascular disease: JACC review topic of the week. *J Am Coll Cardiol* 2022;80:1647–59.
5. Castro C, Delwarde C, Shi Y, Roh J. Geroscience in heart failure: the search for therapeutic targets in the shared pathobiology of human aging and heart failure. *J Cardiovasc Aging* 2025;5:10.20517/jca.2024.15.
6. Sayer AA, Cooper R, Arai H, et al. Sarcopenia. *Nat Rev Dis Primers* 2024;10:68.
7. Picca A, Coelho-Junior HJ, Calvani R, et al. Biomarkers shared by frailty and sarcopenia in older adults: A systematic review and meta-analysis. *Ageing Res Rev* 2022;73:101530.

8. Cruz-Jentoft AJ, Sayer AA. Sarcopenia. *Lancet* 2019;393:2636–46.
9. Petermann-Rocha F, Balntzi V, Gray SR, et al. Global prevalence of sarcopenia and severe sarcopenia: a systematic review and meta-analysis. *J Cachexia Sarcopenia Muscle* 2022;13:86–99.
10. Maccarone MC, Caregnato A, Regazzo G, et al. Effects of the Full-Body in-Bed Gym program on quality of life, pain and risk of sarcopenia in elderly sedentary individuals: preliminary positive results of a Padua prospective observational study. *Eur J Transl Myol* 2023;33:11780.
11. Hassani M, Renzini A, Nguyen L, Coletti D. Changes in physical performance with aging in master athletes and in the general population: an update. *Eur J Transl Myol* 2026;36:14884.
12. Coletti C, Acosta GF, Keslacy S, Coletti D. Exercise-mediated reinnervation of skeletal muscle in elderly people: An update. *Eur J Transl Myol* 2022;32:10416.
13. Bordoni B, Zanier E. Anatomic connections of the diaphragm: influence of respiration on the body system. *J Multidiscip Healthc* 2013;6:281–91.
14. Kocjan J, Adamek M, Gzik-Zroska B, et al. Network of breathing. Multifunctional role of the diaphragm: a review. *Adv Respir Med* 2017;85:224–232.
15. Bordoni B, Morabito B, Escher AR. Diaphragm's role as a systems-connector muscle: a narrative review. *Cureus* 2025;17:e94679.
16. Argentieri MA, Xiao S, Bennett D, et al. Proteomic aging clock predicts mortality and risk of common age-related diseases in diverse populations. *Nat Med* 2024;30:2450–60.
17. Dowling P, Gargan S, Swandulla D, Ohlendieck K. Fiber-type shifting in sarcopenia of old age: proteomic profiling of the contractile apparatus of skeletal muscles. *Int J Mol Sci* 2023;24:2415.
18. Kedlian VR, Wang Y, Liu T, et al. Human skeletal muscle aging atlas. *Nat Aging* 2024;4:727–744.
19. Basilicata MG, Malavolta M, Marozzi S, et al. Electron microscopy and multi-omics reveal mitochondrial dysfunction and structural remodeling in the hearts of elderly mice. *Aging Cell* 2025;24:e70286.
20. Shavlakadze T, Xiong K, Mishra S, et al. Age-related gene expression signatures from limb skeletal muscles and the diaphragm in mice and rats reveal common and species-specific changes. *Skelet Muscle* 2023;13:11.
21. Ersoy U, Kanakis I, Alameddine M, et al. Lifelong dietary protein restriction accelerates skeletal muscle loss and reduces muscle fibre size by impairing proteostasis and mitochondrial homeostasis. *Redox Biol* 2024;69:102980.

22. Soffe Z, Radley-Crabb HG, McMahon C, et al. Effects of loaded voluntary wheel exercise on performance and muscle hypertrophy in young and old male C57Bl/6J mice. *Scand J Med Sci Sports* 2016;26:172–88.
23. Dowling P, Gargan S, Zweyer M, et al. Proteomic reference map for sarcopenia research: mass spectrometric identification of key muscle proteins located in the sarcomere, cytoskeleton and the extracellular matrix. *Eur J Transl Myol* 2024;34:12564.
24. Gargan S, Dowling P, Zweyer M, et al. Proteomic identification of markers of membrane repair, regeneration and fibrosis in the aged and dystrophic diaphragm. *Life (Basel)* 2022;12:1679.
25. Murphy S, Zweyer M, Henry M, et al. Proteomic analysis of the sarcolemma-enriched fraction from dystrophic mdx-4cv skeletal muscle. *J Proteomics* 2019;191:212–27.
26. Dowling P, Gargan S, Zweyer M, et al. Protocol for the bottom-up proteomic analysis of mouse spleen. *STAR Protoc* 2020;1:100196.
27. Dowling P, Gargan S, Zweyer M, et al. Proteomic reference map for sarcopenia research: mass spectrometric identification of key muscle proteins of organelles, cellular signaling, bioenergetic metabolism and molecular chaperoning. *Eur J Transl Myol* 2024;34:12565.
28. Gargan S, Ohlendieck K. Sample preparation and protein determination for 2D-DIGE proteomics. *Methods Mol Biol* 2023;2596:325–37.
29. Murphy S, Zweyer M, Swandulla D, Ohlendieck K. Bioinformatic analysis of the subproteomic profile of cardiomyopathic tissue. *Methods Mol Biol* 2023;2596:377–95.
30. Ge SX, Jung D, Yao R. ShinyGO: a graphical gene-set enrichment tool for animals and plants. *Bioinformatics* 2020;36:2628-9.
31. Kanehisa M, Furumichi M, Sato Y, et al. KEGG for taxonomy-based analysis of pathways and genomes. *Nucleic Acids Res* 2023;51:D587–92.
32. Thomas PD, Ebert D, Muruganujan A, et al. PANTHER: Making genome-scale phylogenetics accessible to all. *Protein Sci* 2022;3:8–22.
33. Szklarczyk D, Kirsch R, Koutrouli M, et al. The STRING database in 2023: protein-protein association networks and functional enrichment analyses for any sequenced genome of interest. *Nucleic Acids Res* 2023;51:D638–46.
34. Francisco A, Figueira TR, Castilho RF. Mitochondrial NAD(P)⁺ transhydrogenase: from molecular features to physiology and disease. *Antioxid Redox Signal* 2022;36:864–84.
35. Navarro CDC, Francisco A, Costa EFD, et al. Aging-dependent mitochondrial bioenergetic impairment in the skeletal muscle of NNT-deficient mice. *Exp Gerontol* 2024;193:112465.

36. Rosa FLL, de Souza IIA, Monnerat G, et al. Aging triggers mitochondrial dysfunction in mice. *Int J Mol Sci* 2023;24:10591.
37. Chen MS, Lee RT, Garbern JC. Senescence mechanisms and targets in the heart. *Cardiovasc Res* 2022;118:1173-87.
38. Zheng P, Yan W, Ding Y, et al. Cardiovascular ageing: hallmarks, signaling pathways, diseases and therapeutic targets. *Signal Transduct Target Ther* 2026;11:142.
39. Song Y, Spurlock B, Liu J, Qian L. Cardiac aging in the multi-omics era: high-throughput sequencing insights. *Cells* 2024;13:1683.
40. Grünert SC, Baumgartner MR, Bouchereau J, et al. Mitochondrial 3-hydroxy-3-methylglutaryl-coenzyme A synthase deficiency: From metabolism to clinical implications. *Genet Med* 2025;27:101484.
41. Wang Y, Ping LF, Bai FY, et al. Hmgcs2 is the hub gene in diabetic cardiomyopathy and is negatively regulated by Hmgcs2, promoting high glucose-induced cardiomyocyte injury. *Immun Inflamm Dis* 2024;12:e1191.
42. Herranz N, Gil J. Mechanisms and functions of cellular senescence. *J Clin Invest* 2018;128:1238–46.
43. Hernandez-Segura A, Nehme J, Demaria M. Hallmarks of cellular senescence. *Trends Cell Biol* 2018;28:436–453.
44. López-Otín C, Blasco MA, Partridge L, et al. Hallmarks of aging: an expanding universe. *Cell* 2023;186:243–78.
45. Takasugi M, Nonaka Y, Takemura K, et al. An atlas of the aging mouse proteome reveals the features of age-related post-transcriptional dysregulation. *Nat Commun* 2024;15:8520.
46. Scifo E, Morsy S, Liu T, et al. Proteomic aging signatures across mouse organs and life stages. *EMBO J* 2025;44:4631–60.
47. Morsy S, Scifo E, Xie K, et al. Deciphering the transcriptomic signatures of aging across organs in mice. *Aging Cell* 2026;25:e70357.
48. Wang Q, Xu Z, Ding X, et al. Ten mouse organs proteome and metabolome atlas from adult to aging. *Genome Med* 2025;17:116.

Panther analysis of alterations in protein class during heart-diaphragm aging

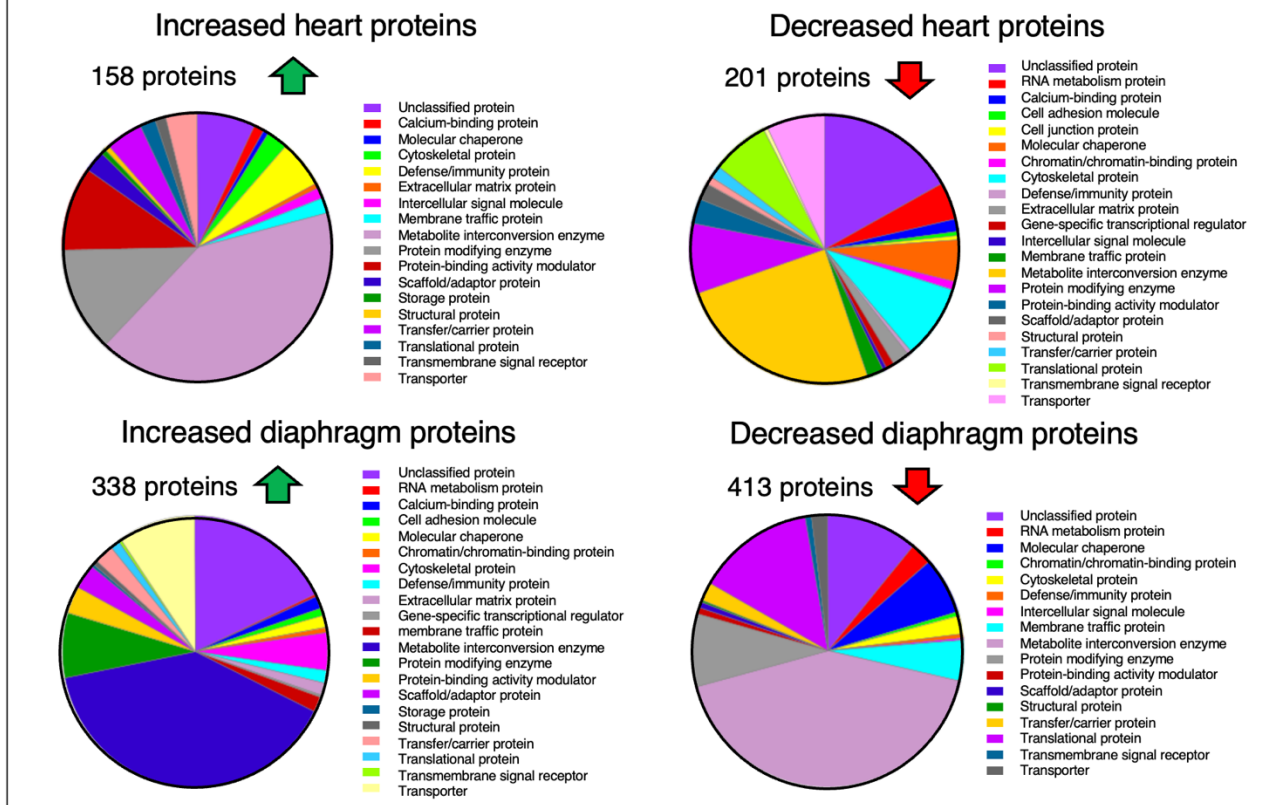


Figure 1. Comparative bioinformatic analysis of protein classes with an increased versus decreased abundance in the aged mouse heart-diaphragm system. The publicly available bioinformatic analysis tool PANTHER,³² was used for the identification of protein classes.

putative protein interactions that include both direct physical and indirect functional protein associations was used for the identification of cardiac protein interaction patterns.

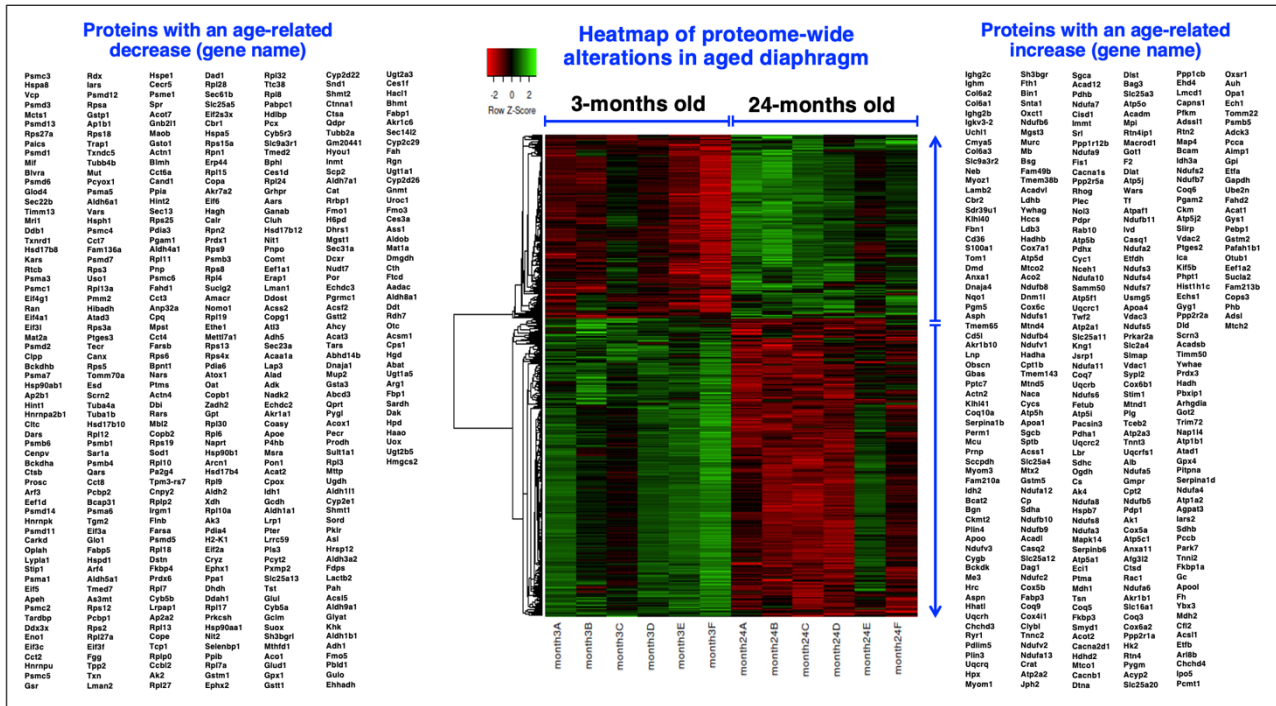


Figure 4. Heatmap and listing of proteome-wide changes during mouse diaphragm aging.

STRING analysis of alterations in protein interactions during diaphragm aging

Decreased diaphragm proteins ↓

Increased diaphragm proteins 

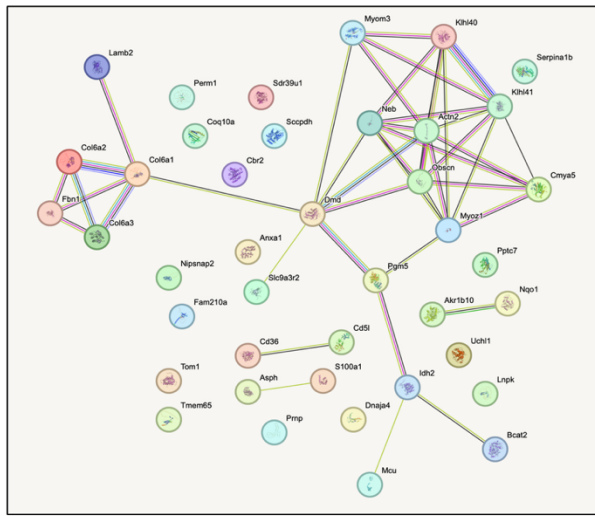
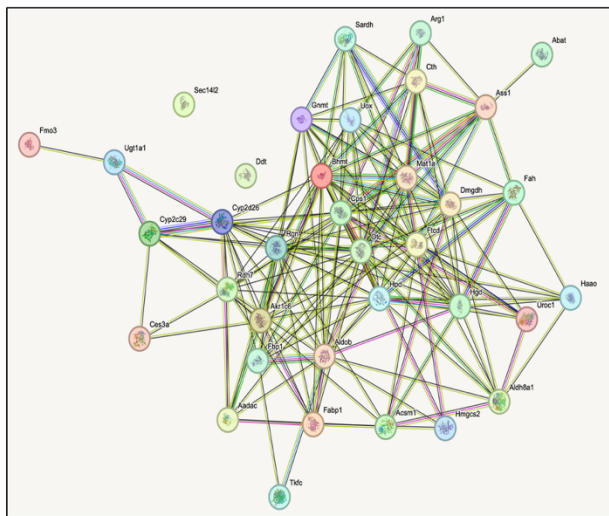
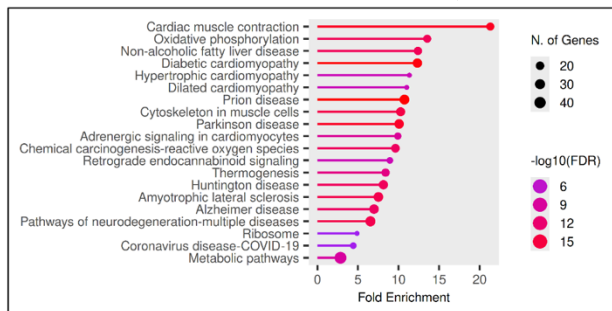


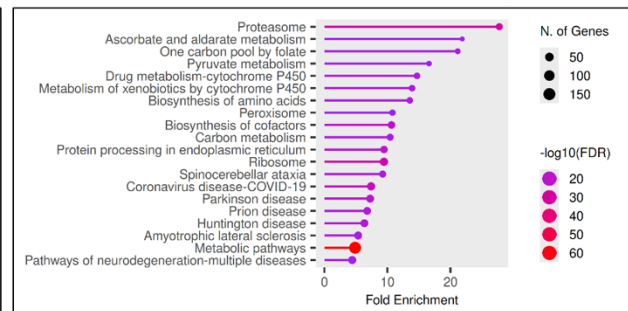
Figure 5. Bioinformatic STRING analysis of alterations in protein-protein interactions during mouse diaphragm aging. The publicly available bioinformatic STRING database,³³ of known and putative protein interactions that include both direct physical and indirect functional protein associations was used for the identification of diaphragm muscle protein interaction patterns.

ShinyGO analysis of alterations in biological processes during heart/diaphragm aging

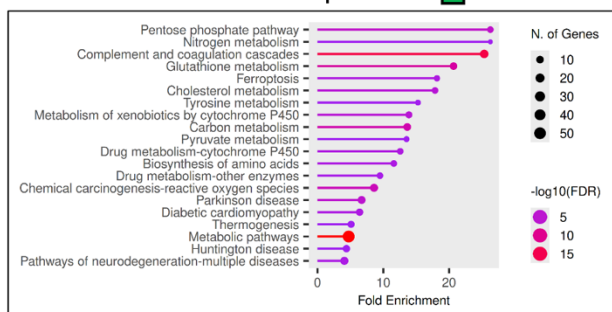
Decreased heart proteins



Decreased diaphragm proteins



Increased heart proteins



Increased diaphragm proteins

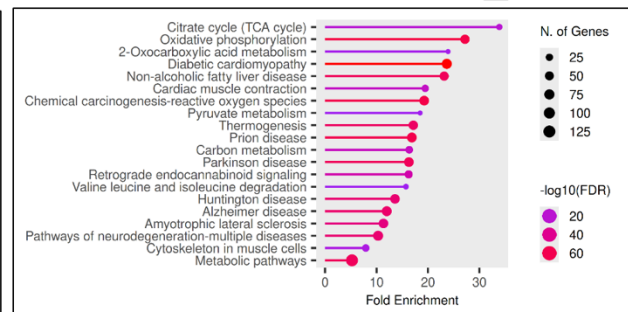


Figure 6. ShinyGO analysis of alterations in biological processes during aging of the mouse heart-diaphragm system. Functional enrichment analyses were carried with ShinyGO 0.85 with Gene Ontology (GO) terms,³⁰ and Kyoto Encyclopedia of Genes and Genomes (KEGG),³¹ pathways.