



THE MODIFICATIONS OF LONGEVITY REGULATING PATHWAY RESULTING FROM ENDURANCE EFFORT IN ARABIAN HORSES*

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Abstract

Recent studies have shown a highly positive effect of physical exercise on the overall condition of the body. The observed changes occur at the cellular level – proliferation, oxidative respiration, ROS degradation, whole-organism homeostasis improvement and ageing inhibition. The longevity regulating pathway is a broad term encompassing all the biochemical actions that contribute to keeping the organism in good condition and prolonging life duration. The overall aim of the present study was to describe the correlation between intensive physical activity and the expression of genes involved in biochemical processes related to longevity. A group of $n=10$ Arabian horses taking part in an endurance ride for 120 km were included in the study. Blood samples from the horses were taken before and after the ride to perform comparative analyses of the transcriptome profiles. Using high-throughput NGS and bioinformatics tools, the group of 9 genes with the highest up- and downregulation fold change (FC) rates were identified: *PTEN*, *IRS2*, *SESN2*, *CCND1*, *TBC1D1*, *FOXO1*, *KL*, *TP53*, and *SOD2*. The results of this study could lead to the identification of a genetic marker for hot-blooded horse breeding.

Key words: Arabian horse, longevity-regulating pathway, ageing, RNA-seq, transcriptome

The ageing process is often described as the accumulation of changes in an organism over time and includes physical, psychological, and social transitions. Current ageing theories lean toward the damage concept, where the accumulation of damage at the molecular level may cause biological systems to fail, or toward the programmed ageing concept, which inherently describes the internal processes (such as DNA methylation) that may contribute to ageing (Ghosh et al., 2016).

Contemporary research indicates that the salutary effect of physical exercise on the ageing process is an inhibitory factor at the cellular level. The key role of this process is related to mitochondria, where the process of aerobic transformation of energy compounds into ATP takes place. One of the processes regulating the number of mitochondria is mitophagy, selective degradation that often occurs in defective mitochondria following damage or stress. The 5' AMP-activated protein kinase (AMPK) is an enzyme crucial for cellular energy homeostasis that regulates glucose and fatty acid uptake and oxidation when cellular energy is low. The concentration of AMPK in cells is variable and depends on the energetic state of the cell and the ADP/AMP ratio (Laker et al.,

2017). Recent research has shown that the increased level of AMPK in mammalian cells is a response to intense physical activity and plays a role in the regulation of diverse cellular processes, such as mitophagy, the energetic stress response and fatty acid oxidation (Carapeto and Aguayo-Mazzucato, 2021).

Nevertheless, the proliferation and cell division processes controlled by, for example, the p53 (Li et al., 2022) and mTOR signalling pathways (Carosi et al., 2022) significantly affect overall organismal ageing. Cell lifespan has been described as being significantly dependent on an individual's lifestyle, nutritional status, and physical activity, regulating a number of processes at the molecular level. Additionally, the Forkhead Box O family member proteins FOXO (FOXO1, FOXO3, FOXO4 and FOXO6) have been widely described as transcription factors involved in processes such as ageing, cellular metabolism and resistance to oxidative stress. Dysfunction of these genes is often found in the pathogenesis of many age-related diseases (Webb et al., 2014; Du and Zheng, 2021).

The study of Taormina et al. (2019), considering a large group of model organisms such as yeast, invertebrates and vertebrates, including mammals, confirmed

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the presence of some specific genes in the genome implicated in longevity and a probable target of anti-ageing interventions. In mammals, the IGF-1 protein is widely recognized as a key factor in ageing processes. Moreover, the crucial role of IGF-1 has been confirmed by Tyshkovskiy et al. (2023), who conducted RNA-seq analyses across 41 mammalian species. The long-living species were correlated with age-related changes and were enriched for evolutionarily essential genes involved in proteolysis and PI3K-Akt signalling.

The overall aim of the present study was to gain deeper insight into the molecular background of stamina and long-term workload in Arabian horses, considering the mechanisms related to cell longevity and ageing. Arabians have long been known for their racing endurance, including long-distance rides, and are often described as model horses in terms of marathon effort (Ropka-Molik et al., 2019). In this work, based on whole-transcriptome profiling of blood samples before and after the 120 km endurance ride, the longevity regulation pathway and related pathways were reported to be significant, and candidate genes were selected and evaluated.

Material and methods

Animals and high-throughput NGS data

To identify the significant pathways deregulated under endurance conditions, data representing 20 whole-blood transcriptomes were analysed. Blood samples were taken from 10 Arabian horses 1 h before and 1 h after the end of the 120 km endurance ride under the supervision of the president of the veterinary commission (the year of ride – 2018). The exact riding schedule, sample collection and maintenance of the horses were described in detail by Myćka et al. (2023).

Blood samples were taken as a part of veterinary procedures by jugular venipuncture into a Tempus™ Blood RNA Tube (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) and stored at -20°C for further analyses. RNA was isolated from whole blood using the MagMAX™-96 Total RNA Isolation Kit (Invitrogen, Thermo Scientific, Waltham, MA, USA) with a magnetic bead-based purification method according to the manufacturer's protocol. In the last step, using a TapeStation 2200 (RNA ScreenTapes, Agilent Technologies, Santa Clara, CA, USA) and a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), the quality and quantity of the obtained genetic material were validated.

The whole-blood transcriptome data were obtained previously (Myćka et al., 2023), and the raw data were deposited in the GEO database at the accession number GSE226819. The cDNA libraries were prepared with a TruSeq RNA Kit v2 (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. The individual cDNA libraries were ligated with specific indices for pooling during the NGS procedure. The qual-

ity and quantity of the obtained libraries were verified using a Qubit 2.0 (Qubit™ dsDNA BR Assay Kit, Invitrogen, Waltham, MA, USA) and a TapeStation 2200 (D100 ScreenTape, Agilent Technologies, Santa Clara, CA, USA). In the last step, the cDNA libraries were sequenced on a NovaSeq Illumina platform (Illumina) with paired-end 150 bp cycles.

High-throughput data analyses

The quality of the raw data in the first step was tested with FastQC v0.11.7 software, and adapters and low-quality reads were removed. The filtered reads were subsequently mapped to the EquCab3.0 genome with STAR software. Subsequently, the mapped reads were annotated and counted considering the specific gene thresholds with the use of the Ensembl GTF file version 100 (via HTSeq-count software). Whole-transcriptome sequencing yielded an average of approximately 38.3 mln per sample. After filtration, the number of matching reads ranged between 30.6 and 47.1 mln per sample, while the average number of uniquely mapped reads was 32.9 mln per sample. The basic NGS data characteristics were described by Myćka et al. (2023).

In total, 6458 differentially expressed genes (DEGs) were used in the study. The analysis was performed using DESeq2 software v3.16. The calculation model included 3 factors – horse ID, condition (before or after the ride), and sex (gelding or mare) – and the following criteria were used: fold change ≥ 1.0 and adjusted P value < 0.05 (Benjamini-Hochberg correction, 2000).

The DAVID software combined with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to perform the gene enrichment analysis of the obtained DEGs (false discovery rate) based on the Fisher exact test after multiple testing (Bonferroni and Hochberg correction, 2000). String V 11.5 software with the *Equus caballus* reference was used to identify interactions between the detected DEGs.

qPCR validation

For qPCR validation, 7 DEGs were selected – *CCND1*, *IRS2*, *SES2*, *FOXO1*, *KL*, *TP53*, and *SOD2*. Total RNA (250 ng) from each of the 20 samples was used to synthesise cDNA using the High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. The primers used were designed using Primer3 Input v.4.1.0 software and based on dedicated reference sequences (Supplementary Table 1). Subsequently, real-time PCR was performed in triplicate for each sample with the RT HS-PCR Mix SYBR® (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's protocol and using the QuantStudio7Flex platform (Applied Biosystems). The expression was calculated using the delta-delta CT method and based on the endogenous control gene *GAPDH*. The comparison of relative quantity (RQ) and RNA-seq data was performed using Pearson correlation (R package).

Results

Differentially expressed genes, deregulation of longevity-regulating pathways and related pathways

The obtained DEGs (fold change (FC) = 1.0; adjusted P value <0.05) were used to identify the most significant pathways (false discovery rate (FDR) value based on Fisher's exact test after multiple testing; Bonferroni and Hochberg) for the study considering longevity and ageing processes (Supplementary Figure 1). The longevity-regulating pathway (FDR<0.001), as well as the co-operating pathways – the mTOR (FDR<0.001), FOXO (FDR<0.001), and AMPK (FDR<0.001) signalling pathways – were generally upregulated (Table 1; Figure 1). A comparison of the DEG sets belonging to each of the 4 identified pathways revealed 30 common DEGs, including 5 downregulated and 25 upregulated genes (Table 2; Figure 2). The highest FC deviations were observed for the downregulated genes, followed by the *FOXO1* gene (FC = 1.94) and *SESN2* (FC = 5.11) for the upregulated genes. Considering the longevity pathway itself, 30 DEGs were characterised, including 8 downregulated and 22 upregulated genes under endurance conditions (Table 3). The highest FC deviation noticed for down-regulated genes was the *KL* gene (FC = 4.54) and *ADCY4* (FC = 7.67) for upregulated genes.

The network between selected DEGs and candidate gene selection

To highlight the genes associated with the most important processes related to adaptation to endurance, an analysis of the molecular network was performed. Two sets of DE genes were used independently (DEGs common to 4 selected pathways and DEGs belonging to the longevity pathway; Figures 3 and 4, respectively). We identified DEGs related to the regulation of apoptotic processes (GO0042981), reactive oxygen species metabolic processes (GO0072593) and cellular responses to stress (GO0033554) (Figures 3 and 4). Some genes were found to be involved in the longevity regulation pathway and metabolic processes, such as the response to starvation (GO0042594) (*SESN2*, *PRKAG1*, *PRKAG2*, *PRKAA1*, *FOXO1*, *FOXO3*, and *PCK2*) and the regulation of lipolysis in adipocytes (ecb04923) (*IRS2*, *PIK3CB*, *PIK3CD*, *KRAS*, *TP53*, *AKT2*, *ADCY4*, and *ADCY6*) (Figures 3 and 4).

The *PTEN*, *IRS2*, *SESN2*, *CCND1*, *TBC1D1*, *FOXO1*, *KL*, *TP53*, and *SOD2* genes were chosen according to the highest up- and downregulation FC rates, their biological function and their involvement in few molecular pathways at once (Figure 1).

Table 1. The significant enrichment in molecular pathways based on a comparative analysis of DEG sets basing on Kyoto Encyclopedia of Genes and Genomes (KEGG) database

Pathway	N	Upregulated DEGs	Downregulated DEGs
Longevity regulating pathway (KEGG: ecb04211)	30	<i>AKT2</i> , <i>CREB3L4</i> , <i>NRAS</i> , <i>PRKAG1</i> , <i>PIK3CA</i> , <i>ADCY6</i> , <i>RELA</i> , <i>CAMKK2</i> , <i>PRKAG2</i> , <i>PRKAA1</i> , <i>ADIPOR1</i> , <i>IGF1R</i> , <i>KRAS</i> , <i>FOXO3</i> , <i>RB1CC1</i> , <i>PPARG</i> , <i>PIK3CD</i> , <i>PIK3CB</i> , <i>SOD2</i> , <i>IRS2</i> , <i>SESN2</i> , <i>ADCY4</i>	<i>KL</i> , <i>CAMK4</i> , <i>ADCY3</i> , <i>FOXO1</i> , <i>EIF4EBP1</i> , <i>EHMT2</i> , <i>TP53</i> , <i>EIFE2</i>
mTOR signalling pathway (KEGG: ecb04150)	54	<i>AKT2</i> , <i>ATP6V1A</i> , <i>ATP6V1B2</i> , <i>ATP6V1C1</i> , <i>ATP6V1C1</i> , <i>ATP6V1D</i> , <i>ATP6V1E1</i> , <i>ATP6V1G1</i> , <i>BRAF</i> , <i>CLIP1</i> , <i>DEPTOR</i> , <i>DDIT4</i> , <i>KRAS</i> , <i>NRAS</i> , <i>RAF1</i> , <i>SKP2</i> , <i>SOS2</i> , <i>TNFRSF1A</i> , <i>WDR59</i> , <i>WNT4</i> , <i>WNT9A</i> , <i>CAB39</i> , <i>FNIP1</i> , <i>FZD4</i> , <i>FZD8</i> , <i>GSK3B</i> , <i>GRB10</i> , <i>IKBKB</i> , <i>IGF1R</i> , <i>LAMTOR3</i> , <i>MAPK3</i> , <i>MAP2K2</i> , <i>PTEN</i> , <i>PIK3CA</i> , <i>PIK3CB</i> , <i>PIK3CD</i> , <i>PRKAA1</i> , <i>RHOA</i> , <i>RPS6KA1</i> , <i>RPS6KA2</i> , <i>RPS6KA3</i> , <i>SESN2</i>	<i>RRAGB</i> , <i>WNT2B</i> , <i>WNT5B</i> , <i>WNT7A</i> , <i>EIF4EBP1</i> , <i>EIF4E2</i> , <i>GRB2</i> , <i>LPIN1</i> , <i>LPIN3</i> , <i>PRR5</i> , <i>PRKCA</i> , <i>RPS6</i>
FOXO signalling pathway (KEGG: ecb04068)	59	<i>AKT2</i> , <i>AGAP2</i> , <i>BRAF</i> , <i>BCL6</i> , <i>CREBBP</i> , <i>EP300</i> , <i>GABARAP</i> , <i>KRAS</i> , <i>MDM2</i> , <i>NRAS</i> , <i>RAF1</i> , <i>SKP2</i> , <i>SOS2</i> , <i>CCNG2</i> , <i>CDKN1A</i> , <i>CDKN2D</i> , <i>FOXO3</i> , <i>FOXO4</i> , <i>G6PC3</i> , <i>GADD45A</i> , <i>GRB2</i> , <i>IKBKB</i> , <i>IGF1R</i> , <i>IRS2</i> , <i>IL10</i> , <i>MAPK13</i> , <i>MAPK14</i> , <i>MAPK3</i> , <i>MAPK8</i> , <i>MAP2K2</i> , <i>PTEN</i> , <i>PIK3CA</i> , <i>PIK3CB</i> , <i>PIK3CD</i> , <i>PLK3</i> , <i>PRKAA1</i> , <i>PRKAG1</i> , <i>PRKAG2</i> , <i>STAT3</i> , <i>SIPR4</i> , <i>SOD2</i> , <i>TGFBR1</i>	<i>ATM</i> , <i>FASLG</i> , <i>RBL2</i> , <i>SMAD3</i> , <i>EGF</i> , <i>FOXO1</i> , <i>HOMER3</i> , <i>IL7R</i> , <i>MAPK11</i> , <i>MAPK12</i> , <i>NLK</i> , <i>PCK2</i> , <i>PLK4</i> , <i>TGFBR2</i> , <i>CCNB1</i> , <i>CCNB3</i> , <i>CCND1</i>
AMPK signalling pathway (KEGG: ecb04152)	42	<i>PFKFB2</i> , <i>PFKFB3</i> , <i>PFKFB4</i> , <i>AKT2</i> , <i>CRTC2</i> , <i>RAB10</i> , <i>RAB2A</i> , <i>TBC1D1</i> , <i>ADIPOR1</i> , <i>CREB3L4</i> , <i>CAB39</i> , <i>CAMKK2</i> , <i>CPT1A</i> , <i>FOXO3</i> , <i>G6PC3</i> , <i>GYS1</i> , <i>GYS2</i> , <i>IGF1R</i> , <i>IRS2</i> , <i>LIPE</i> , <i>PPARG</i> , <i>PIK3CA</i> , <i>PIK3CB</i> , <i>PIK3CD</i> , <i>PRKAA1</i> , <i>PRKAG1</i> , <i>PRKAG2</i> , <i>PPP2CB</i> , <i>PPP2R3C</i> , <i>PPP2R5A</i> , <i>PPP2R5B</i> , <i>PPP2R2A</i>	<i>ACACA</i> , <i>ACACB</i> , <i>CCND1</i> , <i>EEF2K</i> , <i>EIF4EBP1</i> , <i>FOXO1</i> , <i>FBP1</i> , <i>PCK2</i> , <i>PFKL</i> , <i>SCD5</i>

N – number of identified DEGs.

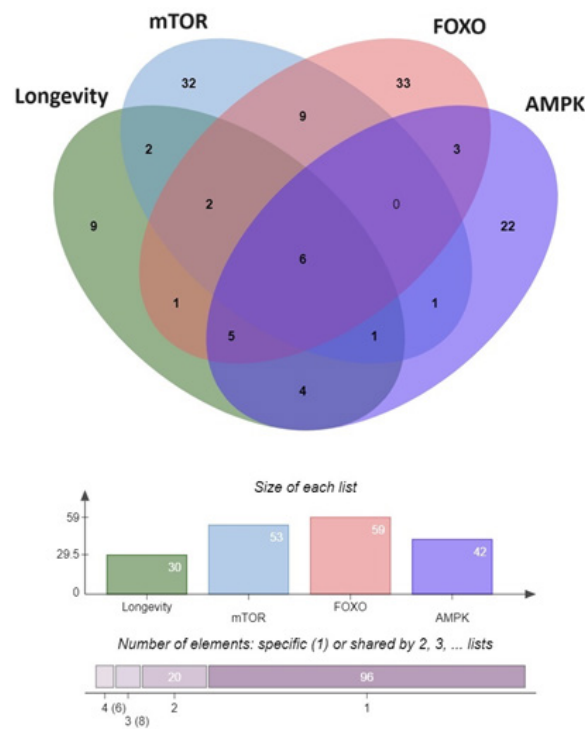


Figure 1. The correlation between four pathways mentioned in the study with the common DEG sets of the identified genes (Venny 2.1.0.)

Table 2. The genes common for the longevity, mTOR, FOXO and AMPK signalling pathways

Gene	Gene name	FC	
<i>FOXO1</i>	Forkhead box O1	1.94	Downregulated
<i>CCND1</i>	Cyclin D1	1.88	
<i>EIF4EBP1</i>	Eukaryotic translation initiation factor 4E binding protein 1	1.84	
<i>PCK2</i>	Phosphoenolpyruvate carboxykinase 2	1.61	
<i>EIF4E2</i>	Eukaryotic translation initiation factor 4E family member 2	1.55	
<i>AKT2</i>	AKT serine/threonine kinase 2	1.51	Upregulated
<i>NRAS</i>	NRAS proto-oncogene, GTPase	1.54	
<i>PRKAG1</i>	Protein kinase AMP-activated non-catalytic subunit gamma 1	1.57	
<i>IKBKB</i>	Inhibitor of nuclear factor kappa B kinase subunit beta	1.59	
<i>GRB2</i>	Growth factor receptor bound protein 2	1.64	
<i>PIK3CA</i>	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha	1.67	
<i>G6PC3</i>	Glucose-6-phosphatase catalytic subunit 3	1.67	
<i>CAB39</i>	Calcium binding protein 39	1.69	
<i>PTEN</i>	Phosphatase and tensin homolog	1.77	
<i>PRKAG2</i>	Protein kinase AMP-activated non-catalytic subunit gamma 2	1.82	
<i>PRKAA1</i>	Protein kinase AMP-activated catalytic subunit alpha 1	1.86	
<i>BRAF</i>	B-Raf proto-oncogene, serine/threonine kinase	2.04	
<i>IGF1R</i>	Insulin like growth factor 1 receptor	2.08	
<i>KRAS</i>	KRAS proto-oncogene, GTPase	2.11	
<i>MAP2K2</i>	Mitogen-activated protein kinase kinase 2	2.17	
<i>FOXO3</i>	Forkhead box O3	2.19	
<i>RAF1</i>	Raf-1 proto-oncogene, serine/threonine kinase	2.21	
<i>SKP2</i>	S-phase kinase associated protein 2	2.51	
<i>PIK3CD</i>	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta	2.82	
<i>PIK3CB</i>	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta	2.90	
<i>MAPK3</i>	Mitogen-activated protein kinase 3	2.95	
<i>SOD2</i>	Superoxide dismutase 2	3.30	
<i>IRS2</i>	Insulin receptor substrate 2	3.54	
<i>SOS2</i>	SOS Ras/Rho guanine nucleotide exchange factor 2	3.78	
<i>SESN2</i>	Sestrin 2	5.11	

FC – Fold Change.

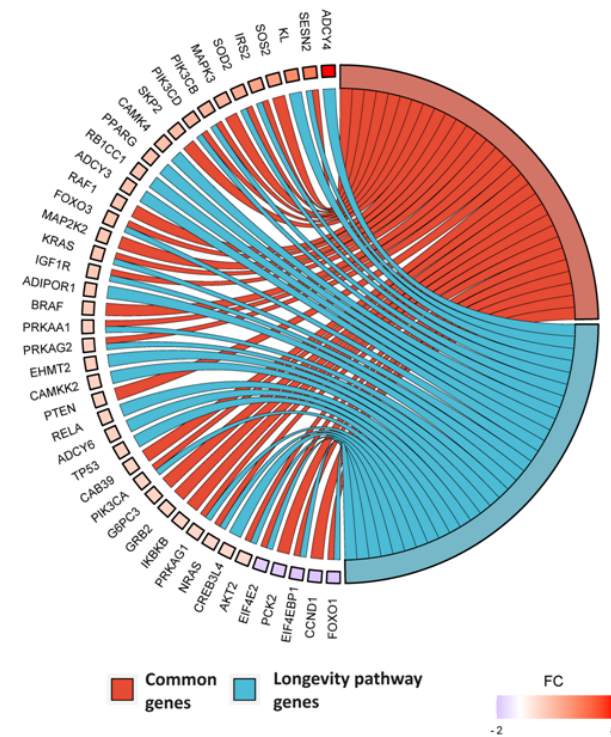


Figure 2. The correlation between longevity pathway genes itself and genes common for the mTOR, FOXO, AMPK and longevity pathways with FC values included

Table 3. The DEGs identified as significant under endurance condition and belonging to longevity pathway

Gene	Gene name	FC	
<i>KL</i>	Klotho	4.54	Downregulated
<i>CAMK4</i>	Calcium/calmodulin dependent protein kinase IV	2.42	
<i>ADCY3</i>	Adenylate cyclase 3	2.25	
<i>FOXO1</i>	Forkhead box O1	1.94	
<i>EIF4EBP1</i>	Eukaryotic translation initiation factor 4E binding protein 1	1.83	
<i>EHMT2</i>	Euchromatic histone lysine methyltransferase 2	1.81	
<i>TP53</i>	Tumor protein p53	1.74	
<i>EIF4E2</i>	Eukaryotic translation initiation factor 4E family member 2	1.54	
<i>AKT2</i>	AKT serine/threonine kinase 2	1.51	
<i>CREB3L4</i>	cAMP responsive element binding protein 3 like 4	1.53	Upregulated
<i>NRAS</i>	NRAS proto-oncogene, GTPase	1.54	
<i>PRKAG1</i>	Protein kinase AMP-activated non-catalytic subunit gamma 1	1.57	
<i>PIK3CA</i>	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha	1.66	
<i>ADCY6</i>	Adenylate cyclase 6	1.75	
<i>RELA</i>	RELA proto-oncogene, NF-kB subunit	1.76	
<i>CAMKK2</i>	Calcium/calmodulin dependent protein kinase kinase 2	1.78	
<i>PRKAG2</i>	Protein kinase AMP-activated non-catalytic subunit gamma 2	1.82	
<i>PRKAA1</i>	Protein kinase AMP-activated catalytic subunit alpha 1	1.85	
<i>ADIPOR1</i>	Adiponectin receptor 1	2.07	
<i>IGF1R</i>	Insulin like growth factor 1 receptor	2.08	
<i>KRAS</i>	KRAS proto-oncogene, GTPase	2.10	
<i>FOXO3</i>	Forkhead box O3	2.19	
<i>RB1CC1</i>	RB1 inducible coiled-coil 1	2.29	
<i>PPARG</i>	Peroxisome proliferator activated receptor gamma	2.39	
<i>PIK3CD</i>	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta	2.82	
<i>PIK3CB</i>	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta	2.90	
<i>SOD2</i>	Superoxide dismutase 2	3.30	
<i>IRS2</i>	Insulin receptor substrate 2	3.53	
<i>SESN2</i>	Sestrin 2	5.11	
<i>ADCY4</i>	Adenylate cyclase 4	7.67	

FC – Fold Change.

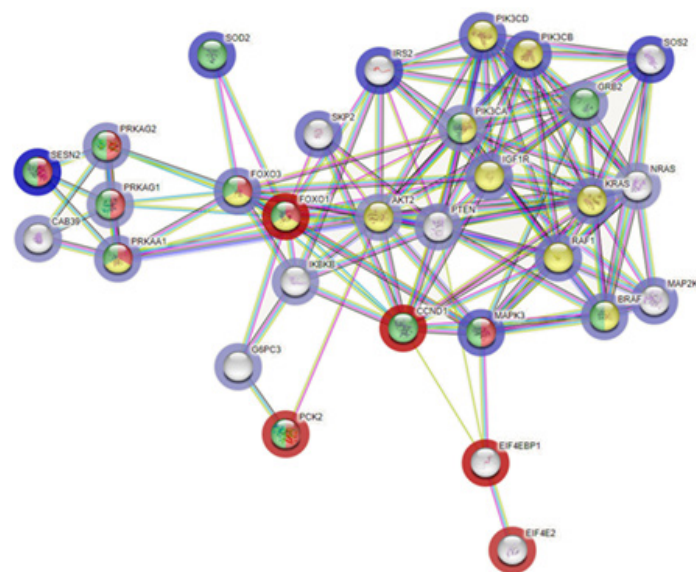


Figure 3. The correlation between genes common for the for the longevity, mTOR, FOXO and AMPK signalling pathways, respectively: Red – response to starvation (GO0042594), Yellow – regulation of apoptotic process (GO0042981), Green – response to stress (GO0006950), Upregulation (blue halo) – downregulation (red halo)

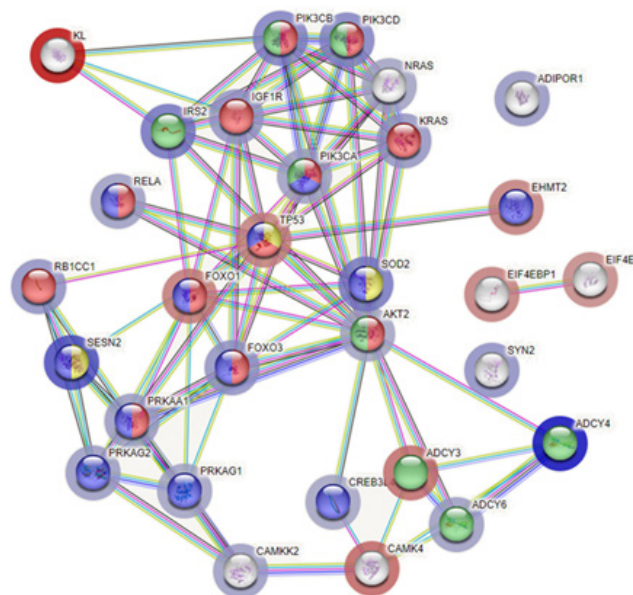


Figure 4. The correlation between genes involved in the longevity regulating pathway, respectively: Red – regulation of apoptotic process (GO0042981), Yellow – reactive oxygen species metabolic process (GO0072593), Green – regulation of lipolysis in adipocytes (ecb04923), Blue – cellular response to stress (GO0033554), Upregulation (blue halo) – downregulation (red halo)

Validation results

Real-time PCR analysis of the 7 selected DEGs enabled us to obtain a positive correlation coefficient between the RNA-seq and real-time PCR data for all the analysed genes. The highest and most significant (P value <0.05) correlations were shown for the *CCND1* (0.593), *TP53* (0.453), *SOD2* (0.443), *KL* (0.402), and *SESN2* (0.396) genes. Nevertheless, for the *FOXO1* gene, which was downregulated according to the NGS data ($FC = 1.88$), qPCR confirmed that this gene was downregulated under endurance conditions,

but the difference between the groups was greater ($FC = 4.23$), and the correlation coefficient was low and not significant. For the majority of the tested genes, the FC values were greater for qPCR than for NGS (Figure 5). Moreover, we did not observe a correlation between *IRS2* gene expression and either method used. The differences might result from database annotation, in which 9 splicing variants are available (EquCab3.0 [GCA_002863925.1]), and the designed amplicon may not have included the total expression of this gene.

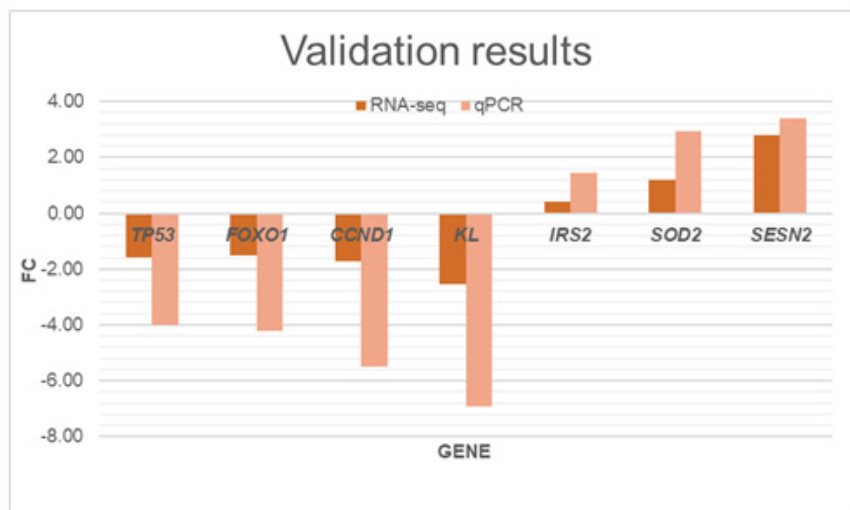


Figure 5. The validation results, the FC values obtained from RNA-seq and qPCR method compared for each of the selected genes

Discussion

The endurance predisposition for running long distances is a unique feature. Arabians are the only horses capable of successfully and safely competing at a distance of approximately 160 km within one day. Such a high workload seems to mobilise the organism to induce several molecular mechanisms that determine survival and activate repair mechanisms in cells. Each of those processes must be grounded in the genome and could be described as an individual's genetic predisposition. In the present study, significant up- and downregulations of gene expression were found, which may help to elucidate the role of some biochemical pathways in maintaining overall organism homeostasis. In the Arabian horses included in this study, a highly significant deregulation of the longevity pathway and 3 related pathways – mTOR, AMPK and FOXO – was observed after the long-distance ride. All of these modifications play crucial biochemical roles in cellular metabolism, and the described modifications must be considered a response to the extraordinary conditions to which the horses were exposed.

The mTOR signalling pathway is a regulator of cell growth, cell proliferation, cell motility, cell survival, protein synthesis, autophagy, and transcription (Carosi et al., 2022). Based on the obtained results, the most important genes included in the pathway were upregulated after long-distance delivery – *PTEN* (FC = 1.77), *IRS2* (FC = 3.54), and *SESN2* (FC = 5.11). The *PTEN* gene encodes a phosphatase and tensin homologue and is involved in the regulation of the cell cycle, preventing rapid cell growth and division (Xu et al., 2022). Several connections between physical exercise and *PTEN* expression have been shown in previous studies (Liu et al., 2012; Dashtiyan et al., 2017). Upregulation of that gene may be considered to confer protection at the molecular level. The *IRS2* gene encodes insulin receptor substrate 2,

a cytoplasmic signalling molecule that mediates the effects of insulin, insulin-like growth factor 1, and other cytokines by acting as a molecular adaptor between diverse receptor tyrosine kinases and downstream effectors (Krause et al., 2020). As described by Hanyuda et al. (2016), physical exercise promoted a significant increase in the expression level of the *IRS2* gene, which led to our conclusion regarding its importance in maintaining glucose homeostasis. The highest upregulated gene, *SESN2*, encodes Sestrin2, a highly evolutionarily conserved protein critically involved in cellular responses to various stresses (Lenhare et al., 2017). Sestrins constitute a family of evolutionarily conserved stress-inducible proteins that suppress oxidative stress and regulate adenosine monophosphate-dependent protein kinase (AMPK)-mammalian target of rapamycin (mTOR) signalling. Through these crucial actions, sestrins serve as important regulators of metabolic homeostasis. Accordingly, inactivation of Sestrin genes in invertebrates results in diverse metabolic pathologies, including oxidative damage, fat accumulation, mitochondrial dysfunction and muscle degeneration, which resemble accelerated tissue ageing (Yu et al., 2023). Considering the biochemical mechanism of cellular respiration, any disorder of *SESN2* gene expression may lead to serious consequences, such as hypoxia, in whole organisms. Moreover, according to the most recent studies, *SESN2* could be considered a genetic marker for glioma diagnosis and prognosis due to the influence of sestrins on the formation of reactive oxygen species (ROS) (Xu et al., 2023). It is commonly accepted that an increase in ROS content as a result of exercise and above a certain threshold can cause toxic effects and lead to DNA damage (Wang et al., 2021). *SESN2* transcription is activated by oxidative stress, and its antioxidant activity upon increased ROS production protects against mitochondrial damage and apoptotic cell death (Sánchez-Álvarez et al., 2019). The results show-

ing highly upregulated *SESN2* gene expression in endurance horses after physical effort may suggest strong correlation between physical exertion and activating the organisms' anti-damage and longevity mechanisms via modulation of ROS metabolism.

The AMPK signalling pathway plays a role in activating glucose and fatty acid uptake when cellular energy is low; it belongs to a highly conserved eukaryotic protein family and is expressed in a number of tissues, including the liver, brain, and skeletal muscles. In response to binding AMP and ADP, the net effect of AMPK activation results in the stimulation of hepatic fatty acid oxidation, ketogenesis, the stimulation of skeletal muscle fatty acid oxidation and glucose uptake, the inhibition of cholesterol synthesis, lipogenesis, and triglyceride synthesis (Mihaylova and Shaw, 2011). In the present study, there were both significantly deregulated genes belonging to that pathway, considered downregulated (FC = 1.88), and upregulated *TBC1D1* (FC = 1.52). The *CCND1* gene encodes cyclin D1, a protein that belongs to the highly conserved cyclin family and is characterised by a dramatic periodicity in protein abundance throughout the cell cycle. The cyclin D1 protein has been shown to interact with the tumour suppressor protein Rb, and the expression of this gene is positively regulated by Rb. Mutations, amplifications and overexpression of this gene, which alters cell cycle progression, are frequently observed in a variety of tumours and may contribute to tumorigenesis (Figueiredo et al., 2016). The *TBC1D1* gene encodes TBC1 domain family member 1, which is presumed to play a role in regulating cell growth and cell differentiation. A higher expression of this gene was related to an improvement in cardiometabolic parameters in exercising animals (Coqueiro et al., 2019). Moreover, as previously described by Myčka et al. (2023), long-lasting physical exertion may trigger metabolism to switch the main energy source from carbohydrates to lipids due to a higher caloric content. At the molecular level, increased expression of genes related to fatty acid degradation was observed, while the transcription of genes associated with the glycolysis/gluconeogenesis and insulin signalling pathways was silenced. These results lead to the conclusion that demanding conditions motivate organisms to take action to survive as long as possible, and they also serve to improve future responses to challenging circumstances.

The FOXO pathway, which includes Forkhead Box O family member proteins, has been widely described as a crucial biochemical mechanism involved in processes such as ageing, cellular metabolism and resistance to oxidative stress (Du and Zheng, 2021). However, in the present study, some downregulated genes included in that pathway were revealed: *FOXO1* (FC = 1.94) and *CCND1* (FC = 1.88). *FOXO1* is a primary protein in the FOXO pathway and is described as a transcription factor that plays important roles in the regulation of gluconeogenesis and glycogenolysis by insulin signalling and is also central to the decision of a preadipocyte to commit

to adipogenesis (Ren et al., 2023). An organism cannot activate every mechanism at once, which indicates that a biochemical hierarchy considering energy consumption occurs. When the lipid-carbohydrate metabolism switch is activated, the main energy resources are used for fat consumption. Glucose metabolism, when characterised by all of its features, becomes a secondary issue that explains the downregulation of the *FOXO1* gene.

The longevity regulating pathway is a broad term that includes all the biochemical actions aimed at keeping the organism in good condition and prolonging its life duration. The regulation of longevity depends on genetic and environmental factors. The overall response of these pathways is believed to promote cellular fitness and ultimately longevity via activation of autophagy, stress defence mechanisms, and survival pathways while attenuating proinflammatory mediators and cellular growth. The mTOR, AMPK and FOXO pathways are crucial for longevity regulation, while certain genes are strictly involved in this process. In the present study, there were both down- and upregulated genes involved in the longevity-regulating pathway, as indicated by the following results: downregulated *KL* (FC = 4.54) and *TP53* (FC = 1.54) and upregulated *SOD2* (FC = 3.30) and *SESN2* (FC = 5.11).

The *KL* gene encodes the klotho transmembrane protein, which, in addition to other effects, provides some control over the sensitivity of the organism to insulin and appears to be involved in ageing. As an ageing suppressor, klotho is an important molecule in ageing processes, and its overexpression results in longevity (Kim et al., 2015). Interestingly, our results showed that the *KL* gene was one of the genes most downregulated in response to long-term exposure in Arabian horses. Despite its involvement in lipid metabolism, the klotho gene improves skeletal muscle regenerative capacity through the activation of stem cell occurrence and proliferation as well as angiogenesis (Liu et al., 2007). In turn, research performed on a *KL*-deficient mouse model during endurance running strongly indicated that this factor could be a key modulator of muscle performance (Phelps et al., 2013). The authors showed that *KL*-deficient (hypomorphic) mice were inclined to run at the same speed, but the amount of time spent voluntarily running, which can result in repeated bouts of exhaustion, was significantly lower (Phelps et al., 2013). This observation led to the hypothesis that klotho deficiency is strongly associated with muscle performance. Moreover, the analysis of klotho gene silencing in muscular dystrophy confirmed the loss of regenerative capacity in muscle tissue (Wehling-Henricks et al., 2016). On the one hand, the downregulation of the *KL* gene during endurance running detected in the present study suggested significant exhaustion of the body, which was likely high enough to inhibit all regenerative processes in muscle tissue and to activate other more direct homeostatic processes. On the other hand, the findings of previous studies performed on klotho mice and wild-type mice (Phelps et al., 2013)

may indicate that the blood *KL* gene can be considered an overtraining and exhaustion biomarker. Based on the present results, we hypothesised that lower *KL* gene expression is preferable and indicative of better endurance in horses.

In turn, the *TP53* gene encodes the tumour protein P53, a regulatory protein that is often mutated in cancers and is described as the major tumour suppressor protein (Bartlett et al., 2014). p53 plays a role in the regulation or progression of the cell cycle, apoptosis, and genomic stability through several mechanisms, which makes its role in overall longevity unquestionable. Several studies have shown that p53 plays a key role in modulating aerobic exercise capacity and improving training (Wang et al., 2012; Bartlett et al., 2014). However, the study of Saleem et al. (2013) revealed decreased expression of the *TP53* gene after exposure to intensive physical exertion in mice. After the experimental exercises, the animals had significantly lower levels of *TP53* expression than did the animals at rest. We found that a similar decrease in expression occurred. These findings lead to the conclusion that the role of the p53 protein depends on exercise intensity and is important during resting conditions, while during intensive exercise, it becomes a secondary issue.

The *SOD2* gene encodes superoxide dismutase 2, a member of the iron/manganese superoxide dismutase family. The SOD2 protein transforms toxic superoxide, a byproduct of the mitochondrial electron transport chain, into hydrogen peroxide and diatomic oxygen. This activity allows SOD2 to clear mitochondrial reactive oxygen species (ROS) and, in turn, confers protection against cell death. As a result, this protein plays an antiapoptotic role against oxidative stress, ionising radiation, and pro-inflammatory cytokines (Minig et al., 2009). Due to its functions in cellular respiration, the *SOD2* gene is considered a genetic marker of long-term exercise endurance in athletes (Ogino et al., 2021). As described in previous studies, increased expression of *SOD2* occurs in response to physical exercise and is necessary to remove excess ROS and protect cells (Baghaiee et al., 2016). The SOD concentration in blood has been recently proposed as a biomarker of the risk of elimination in endurance horses (Bollinger et al., 2023). It was previously found that, after a long-distance ride, the horses that did not complete the race due to lameness had higher SOD levels in their blood than did the finishers. The authors concluded that higher SOD levels indicate exposure to oxidative stress of unknown origin, including overtraining or insufficient rest, which worsens during competitions and can result in lameness. Our results confirmed the significantly elevated *SOD2* gene expression in endurance horses after a ride, which, together with the upregulation of *SES2*, can be regarded as one of the main molecular mechanisms activated during long-term efforts to remove ROS.

Long-term physical effort motivates organisms to enact several mechanisms that enable survival. According to the changes in gene expression described in this study, the top priority in such circumstances is the nutrition and

respiration of the cells. Longevity pathway upregulation can lead to long-term effects through cell cycle regulation, glucose homeostasis and proper oxygenation. Additionally, the widely described ROS neutralisation mechanisms constitute one of the main ways to prevent DNA and cell damage. Based on the results obtained in the present study, we propose 3 genes for use as biochemical markers of long-term exercise endurance. The *KL* gene can be considered an overtraining and exhaustion biomarker whose expression is downregulated. The *SOD2* gene upregulation is a signal of a proper oxygenation rate and efficient cellular respiration. The high *SES2* gene expression allows us to consider effective oxidative damage prevention and ROS neutralisation.

Ethics approval

The Local Ethics Committee of the National Research Institute of Animal Production, Kraków, confirmed that ethical committee oversight was not needed for this research. The II Local Ethics Committee for Animal Experiments decision number 18.10.2021.

Data and model availability statement

The authors declare all the data available.

Author contributions

Conceptualisation: Grzegorz Myćka and Katarzyna Ropka-Molik; analysis and interpretation of the data: Katarzyna Ropka-Molik, Grzegorz Myćka, Anna Cywińska, Tomasz Szmatoła and Monika Stefaniuk-Szmukier; performance of the experiments and preparation of results: Anna Cywińska, Tomasz Szmatoła, Monika Stefaniuk-Szmukier and Katarzyna Ropka-Molik; writing – draft: Grzegorz Myćka and Katarzyna Ropka-Molik; writing – review and editing: Tomasz Szmatoła, Monika Stefaniuk-Szmukier and Anna Cywińska; supervision and funding acquisition: Katarzyna Ropka-Molik; and graphic and photographic documentation: Tomasz Szmatoła and Grzegorz Myćka. Data integrity and accuracy: Grzegorz Myćka.

References

- Baghaiee B., Botelho Teixeira A.M., Tartibian B. (2016). Moderate aerobic exercise increases SOD-2 gene expression and decreases leptin and malondialdehyde in middle-aged men. *Sci. Sports*, 31.
- Bartlett J.D., Close G.L., Drust B., Morton J.P. (2014). The emerging role of p53 in exercise metabolism. *Sports Med.* Mar., 44: 303–309.
- Benjamini Y., Hochberg Y. (2000). On the adaptive control of the false discovery rate in multiple testing with independent statistics. *J. Educ. Behav. Stat.*, 25: 60–83.
- Bollinger L., Bartel A., Weber C., Gehlen C. (2023). Pre-ride biomarkers and endurance horse welfare: analyzing the impact of the elimination of superoxide dismutase, δ -aminolevulinic-dehydratase, thiobarbituric acid reactive substances, iron, and serum amyloid A levels in elite 160 km endurance rides. *Animals*, 13: 1670–1680.
- Carapeto P.V., Aguayo-Mazzucato C. (2021). Effects of exercise on cellular and tissue aging. *Aging (Albany NY)*, 13: 14522–14543.

- Carosi J.M., Fourrier C., Bensalem J., Sargeant T.J. (2022). The mTOR–lysosome axis at the centre of ageing. *FEBS Open Biol.*, 12: 739–757.
- Coqueiro R.D.S., Soares T.J., Pereira R., Correia T.M.L., Coqueiro D.S.O., Oliveira M.V., Marques L.M., de Sá C.K.C., de Magalhães A.C.M. (2019). Therapeutic and preventive effects of exercise on cardiometabolic parameters in aging and obese rats. *Clin Nutr ESPEN*, 29: 203–212.
- Dashtiyan A.A., Sepehrimanesh M., Tanideh N., Esmail Afzalpour M. (2017). The effect of endurance training with and without vitamin E on expression of p53 and PTEN tumor suppressing genes in prostate glands of male rats. *Biochimie Open*, 4: 112–118.
- Du S., Zheng H. (2021). Role of FoxO transcription factors in aging and age-related metabolic and neurodegenerative diseases. *Cell Biosci.*, 11.
- Figueiredo V.C., Roberts L.A., Markworth J.F., Barnett M.P., Coombes J.S., Raastad T., Cameron-Smith D. (2016). Impact of resistance exercise on ribosome biogenesis is acutely regulated by post-exercise recovery strategies. *Physiol. Rep.*, 4.
- Ghosh S., Sinha J.K., Raghunath M. (2016). Epigenomic maintenance through dietary intervention can facilitate DNA repair process to slow down the progress of premature aging. *IUBMB Life*, 68: 717–721.
- Hanyuda A., Kim S.A., Martinez-Fernandez A., Qian Z.R., Yamauchi M., Nishihara R., Morikawa T., Liao X., Inamura K., Mima K., Cao Y., Zhang X., Wu K., Chan A.T., Giovannucci E.L., Meyerhardt J.A., Fuchs C.S., Shivdasani R.A., Ogino S. (2016). Survival benefit of exercise differs by tumor IRS1 expression status in colorectal cancer. *Ann. Surg. Oncol.*, 3: 908–917.
- Kim J.H., Hwang K.H., Park K.S., Kong I.D., Cha S.K. (2015). Biological role of anti-aging protein klotho. *J. Lifestyle Med. Mar.*, 5: 1–6.
- Krause C., Geißler C., Tackenberg H., El Gammal A.T., Wolter S., Spranger J., Mann O., Lehnert H., Kirchner H. (2020). Multi-layered epigenetic regulation of IRS2 expression in the liver of obese individuals with type 2 diabetes. *Diabetologia*, 10: 2182–2193.
- Laker R.C., Drake J.C., Wilson R.J. (2017). Ampk phosphorylation of Ulk1 is required for targeting of mitochondria to lysosomes in exercise-induced mitophagy. *Nat. Commun.*, 8: 548.
- Lenhare L., Crisol B.M., Silva V.R.R., Katashima C.K., Cordeiro A.V., Pereira K.D., Luchessi A.D., da Silva A.S.R., Cintra D.E., Moura L.P., Pauli J.R., Ropelle E.R. (2017). Physical exercise increases Sestrin 2 protein levels and induces autophagy in the skeletal muscle of old mice. *Exp. Gerontol.*, 97: 17–21.
- Li L., Hao Z., Beibei Ch., Bingke Xi., Ruyuan Z., Yage L., Xuan D., Zimengwei Y., Dandan Z., Fangfang M., Sihua G., Orekhov A.N., Prentki M., Lili W., Shuzhen G., Dongwei Z. (2022). BaZiBuShen alleviates cognitive deficits and regulates Sirt6/NRF2/HO-1 and Sirt6/P53-PGC-1 α -TERT signaling pathways in aging mice. *J. Ethnopharmacol.*, 282: 114653.
- Liu G., Detloff M.R., Miller K.N., Santi L., Houlé J.D. (2011). Exercise modulates microRNAs that affect the PTEN/mTOR pathway in rats after spinal cord injury. *Exp. Neurol. Jan.*, 233: 447–456.
- Liu H., Fergusson M.M., Castilho R.M., Liu J., Cao L., Chen J. (2007). Augmented Wnt signaling in a mammalian model of accelerated aging. *Science*, 317: 803–806.
- Mihaylova M., Shaw R. (2011). The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. *Nat. Cell Biol.*, 13: 1016–1023.
- Minig V., Kattan Z., Beeumen J., Brunner E., Becuwe P. (2009). Identification of DDB2 protein as a transcriptional regulator of constitutive SOD2 gene expression in human breast cancer cells. *J. Biol. Chem.*, 12.
- Myćka G., Ropka-Molik K., Cywinska A., Szmatoła T., Stefaniuk-Szmukier M. (2023). Molecular insights into the lipid-carbohydrates metabolism switch under the endurance effort in Arabian horses. *Equine Vet. J.*, 56.
- Ogino T., Matsunaga N., Tanaka T., Tanihara T., Terajima H., Yoshitane H., Fukada Y., Tsuruta A., Koyanagi S., Ohdo S. (2021). Post-transcriptional repression of circadian component CLOCK regulates cancer-stemness in murine breast cancer cells. *Elife*, 10.
- Phelps M., Pettan-Brewer C., Ladiges W., Yablonka-Reuveni Z. (2013). Decline in muscle strength and running endurance in Klotho deficient C57BL/6 mice. *Biogerontology*, 14: 729–739.
- Ren H., Chen Y., Zhu Z., Xia J., Liu S., Hu Y., Qin X., Zhang L., Ding Y., Xia S., Wang J. (2023). FOXO1 regulates Th17 cell-mediated hepatocellular carcinoma recurrence after hepatic ischemia-reperfusion injury. *Cell Death Dis.*, 14: 367.
- Ropka-Molik K., Stefaniuk-Szmukier M., Musiał A.D., Velie B.D. (2019). The genetics of racing performance in Arabian horses. *Int. J. Genom.*, 2019: 9013239.
- Saleem A., Hood D.A. (2013). Acute exercise induces tumour suppressor protein p53 translocation to the mitochondria and promotes a p53-Tfam-mitochondrial DNA complex in skeletal muscle. *J. Physiol.*, 591: 3625–3636.
- Sánchez-Álvarez M., Strippoli R., Donadelli M., Bazhin A.V., Cordani M. (2019). Sestrins as a therapeutic bridge between ROS and autophagy in cancer. *Cancers (Basel)*, 11: 1415.
- Taormina G., Ferrante F., Vieni S., Grassi N., Russo A., Mirisola M.G. (2019). Longevity: lesson from model organisms. *Genes*, 10.
- Tyshkovskiy A., Ma S., Shindyapina A.V., Tikhonov S., Lee S.G., Bozaykut P., Castro J.P., Seluanov A., Schork N.J., Gorbunova V., Dmitriev S.E., Miller R.A., Gladyshev V.N. (2023). Distinct longevity mechanisms across and within species and their association with aging. *Cell*, 186.
- Wang F., Wang X., Liu Y., Zhang Z. (2021). Effects of exercise-induced ROS on the pathophysiological functions of skeletal muscle. *Oxid. Med. Cell Longev.*, 2021: 3846122.
- Wang P.Y., Zhuang J., Hwang P.M. (2012). p53: exercise capacity and metabolism. *Curr. Opin. Oncol.*, 24: 76–82.
- Webb A.E., Brunet A. (2014). FOXO transcription factors: key regulators of cellular quality control. *Trends Biochem. Sci.*, 39: 159–169.
- Wehling-Henricks M., Li Z., Lindsey C., Wang Y., Wele S.S., Ramos J.N., Khanlou N., Kuro O.M., Tidball J.G. (2016). Klotho gene silencing promotes pathology in the mdx mouse model of Duchenne muscular dystrophy. *Hum. Mol. Genet.*, 25.
- Xu H., Lu X., Wang C., Ning J., Chen M., Wang Y., Yuan K. (2022). Potential roles of PTEN on longevity in two closely related argopecten scallops with distinct lifespans. *Front. Physiol.*, 13: 872562.
- Xu L., Liu Z., Wang H., Lu J., Xu J., Meng Y., Huang K., Liu B. (2023). SESN2 could be a potential marker for diagnosis and prognosis in glioma. *Genes*, 14.
- Yu Ch., Zhang P., Liu S. Niu Y., Fu L. (2023). SESN2 ablation weakens exercise benefits on resilience of gut microbiota following high-fat diet consumption in mice, *Food Sci. Hum. Well.*, 12: 1961–1968.

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