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Matrisome, innervation and oxidative metabolism affected in older compared with younger males with similar physical activity

Bart Lagerwaard[1,2], Arie G. Nieuwenhuizen[1], Annelies Bunschoten[1], Vincent C.J. de Boer[1] & Jaap Keijer[1]*

[1]*Human and Animal Physiology, Wageningen University and Research, Wageningen, The Netherlands;* [2]*TI Food and Nutrition, Wageningen, The Netherlands*

Abstract

Background Due to the interaction between skeletal muscle ageing and lifestyle factors, it is often challenging to attribute the decline in muscle mass and quality to either changes in lifestyle or to advancing age itself. Because many of the physiological factors affecting muscle mass and quality are modulated by physical activity and physical activity declines with age, the aim of this study is to better understand the effects of early ageing on muscle function by comparing a population of healthy older and young males with similar physical activity patterns.

Methods Eighteen older (69 ± 2.0 years) and 20 young (22 ± 2.0 years) males were recruited based on similar self-reported physical activity, which was verified using accelerometry measurements. Gene expression profiles of *vastus lateralis* biopsies obtained by RNA sequencing were compared, and key results were validated using quantitative polymerase chain reaction and western blot.

Results Total physical activity energy expenditure was similar between the young and old group (404 ± 215 vs. 411 ± 189 kcal/day, $P = 0.11$). Three thousand seven hundred ninety-seven differentially expressed coding genes (DEGs) were identified (adjusted *P*-value cut-off of <0.05), of which 1891 were higher and 1906 were lower expressed in the older muscle. The matrisome, innervation and inflammation were the main upregulated processes, and oxidative metabolism was the main downregulated process in old compared with young muscle. Lower protein levels of mitochondrial transcription factor A (*TFAM*, $P = 0.030$) and mitochondrial respiratory Complexes IV and II ($P = 0.011$ and $P = 0.0009$, respectively) were observed, whereas a trend was observed for Complex I ($P = 0.062$), in older compared with young muscle. Protein expression of Complexes I and IV was significantly correlated to mitochondrial capacity in the *vastus lateralis* as measured *in vivo* ($P = 0.017$, $R^2 = 0.42$ and $P = 0.030$, $R^2 = 0.36$). A trend for higher muscle-specific receptor kinase (MUSK) protein levels in the older group was observed ($P = 0.08$).

Conclusions There are clear differences in the transcriptome signatures of the *vastus lateralis* muscle of healthy older and young males with similar physical activity levels, including significant differences at the protein level. By disentangling physical activity and ageing, we appoint early skeletal muscle ageing processes that occur despite similar physical activity. Improved understanding of these processes will be key to design targeted anti-ageing therapies.

Keywords Physical activity; Muscle ageing; Matrisome; Mitochondrial capacity

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**Correspondence to: Jaap Keijer, Human and Animal Physiology, Wageningen University and Research, PO Box 338, 6700AH Wageningen, The Netherlands. Email: jaap.keijer@wur.nl*
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Introduction

Ageing is associated with a decline in skeletal muscle mass and strength, also known as sarcopenia.[1] Sarcopenia is linked to an increased risk of developing disabilities, negatively impacting self-reliance and quality of life.[2,3] The loss of muscle strength is disproportional to the loss in muscle mass, suggesting that intrinsic changes in the muscle could explain the loss in muscle functionality, often referred to as muscle quality.[4,5] The underlying causes of loss of muscle mass and quality with age have been suggested to be related to, among others, a decline in skeletal muscle mitochondrial capacity,[6] decreased innervation,[7] decreased protein synthesis,[8] decreased circulation of anabolic hormones,[9] low-grade inflammation,[10] decreased muscle repair[11] or a combination of these phenomena.

Many of the factors affecting muscle mass and quality are modulated by physical activity. However, many studies examining age-related changes in skeletal muscle do not explicitly take the effect of physical activity levels into account, although levels of physical activity decrease with advancing age.[12,13] The effect of physical activity is especially apparent for mitochondrial capacity, because, although the decline in skeletal muscle mitochondrial capacity is likely due to inherent age-related alterations in mitochondrial synthesis and degradation, it has been reported that active older individuals have a comparable mitochondrial capacity to similarly active younger individuals.[14,15] This exemplifies the challenge to disentangle the effect of age and physical activity, making it difficult to attribute age-related changes to either of these two factors.

Here, we aimed to better understand the effects of early ageing on skeletal muscle function using a population of healthy older and young males in a narrow age range (65–71 and 19–27 years, respectively). Furthermore, in efforts to disentangle the effect of age and physical activity, both age groups were selected for similar levels of physical activity based on self-reported physical activity, verified using accelerometry. The age range was chosen because it can be anticipated that changes occurring in earlier phases of the ageing process are the most amenable to intervention and will most likely offer targets for intervention. In addition, many studies investigating the effect of age on muscle function employed a wide range of ages for subject recruitment, which further blurs the effect of age because the loss of muscle mass with age is thought not to be linear.[16] Furthermore, the ageing trajectory is different between males and females, demanding separate studies for each of the sexes.[17,18] Gene expression profiles of *vastus lateralis* biopsies, obtained by RNA sequencing, were compared between the groups, and key results were validated using quantitative polymerase chain reaction (qPCR) and western blot. Our results show that the cell adhesion, the matrisome, innervation, inflammation and oxidative metabolism are the most significantly regulated processes. Additionally, we generate novel hypotheses on muscle ageing that will be of substantial value to test and aid the design of anti-ageing interventions for a population that is still amendable for improvements in skeletal muscle quality.

Materials and methods

Compliance with ethical standards

The study was approved by the medical ethical committee of Wageningen University and conducted in accordance with the principles of the Declaration of Helsinki (Fortaleza, Brazil 2013) and according to national law (WMO, The Hague, 1998). The study is registered in the Dutch trial register (NL7695). Subjects were in writing and verbally informed on all experimental procedures, including possible risks and discomforts. All subjects provided written informed consent before testing.

Study population

Low to moderately physical active healthy males between the age of 19–25 (young) and 65–71 (older) years were recruited using a self-reported exercise frequency of 1–2 h of structured exercise per week or a Baecke habitual physical activity score between 7 and 10 points.[19] Primary exercise modalities in the young males were running (5), squash (3), weightlifting (4), badminton (1), table tennis (1), football (1), swimming (1), tennis (1), basketball (1), volleyball (1) and climbing (1). Primary exercise modalities in the older males were running (6), walking (4), weightlifting (3), cycling (3) and tennis (2). All subjects reported no functional impairments that limited them in engaging in exercise/physical activities, and all older males reached a minimum score of 11 on the short performance battery test (SPPB), indicating no physical impairments in this group.[20] None of the subjects were identified as a regular smoker ($>$5 cigarettes per week), used recreational drugs during the study or reported recent use of performance enhancing drugs or supplements. Subjects were non-anaemic (haemoglobin concentration $>$8.0 mmol/L), verified using HemoCue Hb 201 microcuvette (HemoCue AB, Sweden). None of the subjects had health concerns regarding respiratory or metabolic disease. One older subject used cholesterol-lowering medication, one used diuretic medication, and one used both cholesterol-lowering and diuretic medication. An overview of subject characteristics can be found in *Table* 1. *In vivo* measurements of mitochondrial capacity and physical activity using accelerometry, but not of gene or protein expression, were reported earlier.[21] Here, information was used only from those individuals of which high-quality RNA was obtained from the *vastus lateralis* (i.e. two elderly subjects were excluded).

Table 1 Physical characteristics of the subjects

	Young (N = 20)	Older (N = 18)	P-value
Age (years)	22 ± 2.0	69 ± 2.0	**<0.0001**
BMI (kg/m^2)	22.6 ± 1.9	25.4 ± 1.8	<0.0001
Body fat (%)	15.3 ± 3.1	25.0 ± 4.1	<0.0001
Fat-free mass (kg)	63.5 ± 6.9	61.1 ± 5.3	0.25
MVC dominant arm (kg)	54 ± 7	50 ± 8	0.17
Skinfold *vastus lateralis* (mm)	10.4 ± 2.7	9.2 ± 2.8	0.19
Haemoglobin (mmol/L)	9.4 ± 0.4	9.1 ± 0.7	0.19
SPPB score		11.65 ± 0.5	
Physical activity			
Baecke Questionnaire score	8.3 ± 0.8	8.6 ± 0.9	0.26
Accelerometry			
SPA (%)	79.1 ± 7.3	75.2 ± 6.0	0.0778
LPA (%)	15.5 ± 3.4	19.7 ± 5.6	**0.0074**
MVPA (%)	4.4 ± 2.2	5.1 ± 2.3	0.32
MET (kcal/kg/h)	1.37 ± 0.12	1.45 ± 0.11	0.037
TEE (kcal/day)	2,478 ± 375	2,851 ± 343	**0.003**
PAEE (kcal/day)	404 ± 215	411 ± 189	0.11
PAL	1.4 ± 0.1	1.4 ± 0.1	0.51
Wear time (h/day)	14.6 ± 1.1	15.5 ± 0.8	**0.0078**
Mitochondrial capacity *vastus lateralis*	2.13 ± 0.53 (n = 9)	1.58 ± 0.49 (n = 6)	*0.0606*

Note: All significant P-values have been made bold.
Data are presented as mean ± standard deviation. MVC is maximum voluntary contraction. Time spent in sedentary physical activity (SPA), light physical activity (LPA) and moderate-to-vigorous physical activity (MVPA) are expressed as a percentage of total wear time measured using 5-day accelerometer data. MET is metabolic equivalent of task. TEE is total energy expenditure. PAEE is physical activity energy expenditure. PAL is physical activity level. SPPB is short physical performance battery. Mitochondrial capacity is defined as the rate of recovery of muscle oxygen consumption after exercise determined by near-infrared spectroscopy.[21]

Accelerometry

Subjects were instructed to wear a triaxial accelerometer (wGT3X-BT, Actigraph, USA) for 7 consecutive days using an elastic band at the waist of the non-dominant leg. The accelerometer was worn during all activities, excluding showering, swimming and sleeping. Wear time was manually verified using daily diaries on shower and bedtimes. Counts were sampled at 30 Hz and stored in 60-s epochs to determine counts per minute (CPM). Two weekend days and three weekdays were used for data analysis. The percentage of wear time in sedentary physical activity (SPA), light physical activity (LPA) and moderate-to-vigorous physical activity (MVPA) was determined using the cut-offs provided by Troiano et al.,[12] being 0–99, 100–2019 and 2020–∞, respectively. Activity kcals per day were calculated using the counts from all axis according to the Freedom VM3 equation.[22]

Sample collection and RNA extraction

Subjects were fasted and refrained from exercise and alcohol 24 h prior to testing. Biopsies from *vastus lateralis* were collected using the suction modified Bergström technique[23] from the non-dominant leg under local anaesthesia using a using a 5-mm Bergström needle. Collected tissue was snap-frozen in liquid nitrogen, grinded and stored at −80°C until further analysis. Total RNA was extracted by dissolving approximately 30 mg grinded frozen muscle tissue in 1 mL Trizol reagent in TissueLyser II (Qiagen) for 2 min at 30 Hz. A 240 μL of cold chloroform was added to the lysates, shaken thoroughly for 30 s and incubated on ice for 5 min before centrifugation at 12 000 RPM for 15 min at 4°C (Mikro 200R, Hettich, Germany). The aqueous layer was transferred to a new tube, and 700 μL of ice-cold isopropanol was added, mixed with RLT buffer (Qiagen) and loaded onto RNAeasy spin columns (Qiagen). RNA purification was done according to manufacturers' protocol. RNA concentration was measured using NanoDrop spectrophotometer (Thermo Fisher). RNA integrity was measured using RNA ScreenTape on the 2200 Tapestation (Agilent). All RNA samples had an RNA integrity value (RIN) > 7.0 and 260/280 ratio > 1.8.

RNA sequencing and differential gene expression analysis

RNA preparation, library construction and sequencing on BGISEQ-500 were performed at Beijing Genomics Institute. Clean reads were obtained in FASTQ format, and quality check was performed using FASTQC.[24] Reads were aligned to the human genome (GRch38.p13) using STAR,[25] and counts were quantified using HTSeq.[26] Average sequencing depth was 23 M paired end reads, of which at least 92% were uniquely mapped.

Data analysis and statistical testing were performed in R version 3.6 and using appropriate Bioconductor packages. Differentially expressed genes (DEGs) between the groups were identified using DESeq2.[27] Genes with less than 10 reads per row were removed, and Benjamini–Hochberg

multiple testing correction was used to obtain adjusted *P*-values; a false discovery rate (FDR) of 5% was accepted. Principal component analysis was done using variance stabilizing transformed (VST) data. Associations between gene expression and the continuous variable MVPA were done using the Limma-Voom.[28] Normalization factors were calculated using calcNormfactors using EdgeR,[29] and low-expressed genes were filtered. Voom was used to calculate the mean–variance relationship of the log counts, and linear model was fitted using weighted least squares for each gene. Empirical Bayes was used for smoothing of standard errors.[30]

Gene set enrichment analyses

Gene set enrichment analysis was done using clusterProfiler[31] for the "biological process" gene ontology gene sets. Gene sets were considered enriched with a Benjamini–Hochberg adjusted *P*-value < 0.05, and GO terms were filtered based on 75% overlap in genes. Protein interaction networks were made using STRING v10[32] with *P*-value for enrichment < 1.0 E-16. Edges represent molecular action, and nodes were coloured manually according to protein function. Nodes with no connection to the network were deleted. The human MitoCarta gene set was used as a reference inventory for mitochondrial genes,[33] and the matrisome gene set as a reference inventory for extracellular matrix (ECM) genes.[34]

Semi-quantitative real-time polymerase chain reaction

cDNA was synthesized from 300 ng total RNA with ISCRIPT cDNA synthesis kit (Bio-Rad) (5′ 25°C, 30′ 42°C, 5′ 85°C, cooled to 10°C and stored at −20°C). Lowly expressed genes were preamplified using the SsoAdvanced PreAmp Supermix (Bio-rad). Transcripts were quantified using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) and SYBR green master mix (Bio-Rad). The cycling programme was set as 95°C for 30s and 60°C for 30 s in 40 cycles. Primers were designed using NCBI primer blast; an overview of primer sequences can be found in *Table* 2. Normalized expression was calculated according to the ΔΔCq method by making use of geometric averaging of multiple reference genes using CFX Maestro software (Bio-Rad). Data are presented as fold change of expression, which was set to one for the young group.

Protein isolation and western blotting

Protein extraction was done by adding 500 μL of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.25% NaDeoxylate and protease and deacylase inhibitors trichostatin A (1 μM) and nicotinamide (20 mM)) to ±25 mg frozen grinded muscle tissue and lysed using Qiagen TissueLyser II for 1 min at 30 Hz. Lysates were centrifuged for 7 min at 10 000 *g* at 4°C, and protein concentration of supernatants were determined using BCA protein assay kit (Pierce). A 25 μg protein was loaded on NuPAGE 4%–12% BIS-TRIS and TRIS-acetate gels (Invitrogen), transferred to nitrocellulose or polyvinylidene fluoride (PVDF) membrane, blocked in 3% BSA in PBS with 0.1% Tween-20 at room temperature and incubated overnight with antibodies in the same buffer at 4°C. Primary antibodies used are anti-OXPHOS cocktail (1:1000, #ab110411 Abcam), anti-MUSK (1:1000, #ab92950, Abcam) and anti-TFAM (1:2000, #ab131607, Abcam). IR-dye-based secondary antibodies (LI-COR) were used to detect antibody signals using an Odyssey scanner (LI-COR). Quantification of

Table 2 Primer sequences

Symbol	RefSeq	Forward primer[a]	Reverse primer[a]	bp
ITGA7	NM_002206.3	GAACAGCACCTTTCTGGAGGAGTA	CACTGTGGAGGCATCTCGGA	118
CDH15	NM_004933.3	CTGTTGGCCCAGAGCCT	TGTGGTTCTCGGATACGCTG	145
BDNF	NM_001709.5	GCCAGAATCGGAACCACGAT	CTCACCTGGTGGAACTCGG	79
MUSK	NM_005592.4	ACCTAAAATAACTCGTCCTCCC	TTCGGGAATTTTCCCTGAGAG	140
MYH8	NM_002472.3	TGACCTCAGCAGTAACGCAG	AGGGCGTTCTTGGCTTTAGT	300
NTRK2	NM_001007097.3	ACAGCAACCTGCAGCACATC	ATTGCCCACCAGGATCAGTTC	104
NR4A1	NM_173157.3	TCGGGGACTGGATTGACAGTA	CCCATGCCGGTCGGTGAT	116
NR4A3	NM_173200.2	ATCCAGAAGCTGGGCAGAAAA	TCAGCAGTGTTTGACCTGATGG	129
PRKAG3	NM_017431.3	GATGACGAACTGCGGAAACC	GGCCTTCTTGATCTCCAGC	129
PPARGC1A	NM_013261.5	ACCCCAGAGTCACCAAATGAC	GCAGTTCCAGAGAGTTCCACA	89
TFAM	NM_001270782.1	GCTGAAGACTGTAAAGGAAAACTGG	CCTGTGCCTATCCATTGTGA	240
RUNX1	NM_001754.5	CCGTGGTCCTACGATCAGTC	GTCGGGTGCCGTTGAGAGT	147
CHRNA1	NM_000079.4	AGGAGTCTAACAATGCGGCG	GGTTCCAGGGCAGAGCTAAG	183
NRCAM	NM_001037132.4	GACGAAAGCCTCAGAACCAGA	AGGGCTTCCACGTAATCACC	89
RPLP0 (reference)	NM_001002.4	GGCAGCATCTACAACCCTGA	GCCTTGACCTTTTCAGCAAGT	215
TBP (reference)	NM_003194.5	AGCCAAGAGTGAAGAACAGTCC	AACTTCACATCACAGCTCCCC	129
RPS15 (reference)	NM_001018.5	AGAAGCCGGAAGTGGTGAAGAC	AGAGGGATGAAGCGGGAGGAG	220
B2M (reference)	NM_004048.4	TGCCGTGTGAACCATGTG	GCGGCATCTTCAAACCTC	92

[a]From 5′ to 3′, bp = fragment length.

protein loading was done using Revert 700 Total Protein Stain (LI-COR) and used for normalization of target protein after quantification using ImageJ (NIH, USA).

Data availability

The RNAseq data of this study have been deposited under accession number GSE159217.

Statistical analyses

Data were presented as mean ± SD. Statistical analyses, other than RNA sequencing analysis and differential gene expression analysis (see above), were performed using GraphPad Prism v.8 (GraphPad Software, CA, USA). Means between the two groups were compared using a Student's unpaired *t*-test or Welch's *t*-test when variances were unequal. Normality was tested using D'Agostino and Pearson normality test. Not normally distributed data were transformed or compared using a Mann–Whitney test. Correlations between variables were calculated using regression analysis. Significance was accepted at $P < 0.05$.

Results

Distinct transcriptional signature in older muscle despite similar physical activity levels

Total physical activity energy expenditure and physical activity level, as defined by accelerometry, was similar between the groups. Time spent in MVPA was similar between the groups, yet older males spent higher proportion of time in LPA compared with young males, possibly at expense of SPA (*Table* 1). PCA on transformed count RNAseq data using a variance stabilizing transformation showed that the samples from young and older subjects separate in distinct groups based on principal component 1, explaining 20% of the variation in the data (*Figure* 1A). This showed that in an early ageing population with similar physical activity, a clear distinction in the transcriptional signature was observed. When comparing the gene counts of the young and older group, 4327 DEGs were identified using an adjusted *P*-value cut-off of <0.05. Three thousand seven hundred ninety-seven of those genes were identified coding genes, of which 1891 were higher expressed and 1906 were lower expressed in the older individuals (*Figure* 1B). Gene ontology gene set enrichment analysis revealed an enrichment in gene sets related to oxidative metabolism (e.g. oxidative phosphorylation and cellular respiration), cell adhesion (e.g. cell adhesion and cell–cell adhesion), inflammation (e.g. defence response and inflammatory response) and innervation (e.g. synapse organisation and regulation of the synapse structure or activity). Most of these enriched gene sets were upregulated, except for the gene sets related to oxidative metabolism, which were downregulated in the older muscle (*Figure* 1C).

Cell adhesion and the matrisome belong to most significantly regulated processes with ageing

Cell adhesion was the most significantly regulated process in older compared with young muscle based on adjusted *P*-value. Furthermore, using the curated C2 canonical gene sets Molecular Signatures Database (Msigdb), a database of gene sets that are canonical representations of a biological process compiled by domain experts, the matrisome was the most significant enriched gene set in the older group (*Figure* S1). We therefore compared the expression of genes between the groups according to the classification of the Matrisome Project, a research effort devoted to characterize and classify genes in the ECM.[34] This classification divides the matrisome into the core matrisome, subdivided in collagens, proteoglycans and ECM glycoproteins, and in matrisome-associated proteins, subdivided in ECM-affiliated proteins, ECM regulators and secreted factors. Out of the 44 genes that encode the 28 different collagen proteins, 20 genes were differentially regulated between the groups. Collagens form a network with which glycoproteins and proteoglycans in the ECM associate. Out of 195 genes encoding ECM glycoproteins, 44 were differentially regulated, and out of the 35 genes that encode proteoglycans, eight were differentially regulated between the young and older muscle. Of the 171 genes that encode for ECM-affiliated proteins and the 238 genes that encode for ECM regulators, respectively, 28 and 48 genes were differentially expressed between the groups. Out of 344 genes that encode for secreted factors, 51 genes were differentially regulated between the young and older muscle. Secreted factors encompass all secreted proteins that can be secreted by the cells that reside in the ECM and play a role in tuning communication between cells, such as growth factors. Moreover, it encompasses interleukins that regulate inflammatory processes, for example, interleukin 15 (*IL15*; FC = 2.1, padj = 0.002), interleukin 25 (*IL25*; FC = 6.75, padj = 0.001) and its receptor interleukin 17 Receptor B (*IL17RB*; FC = 1.24 and padj = 0.037) (*Table* S1). Overall, a substantial number of matrisome components were differentially regulated with age (*Figure* 2A).

The ECM forms a framework for muscle fibres and other cells that reside in the ECM, playing an important role in development,[35] growth,[36] repair[37] and contractile force transmission.[38] Collagen is the most abundant ECM protein and makes up the intramuscular connective tissue (IMCT), organized in the endo-, peri- and epimysium. The sarcolemma associates with the ECM through a specialized IMCT

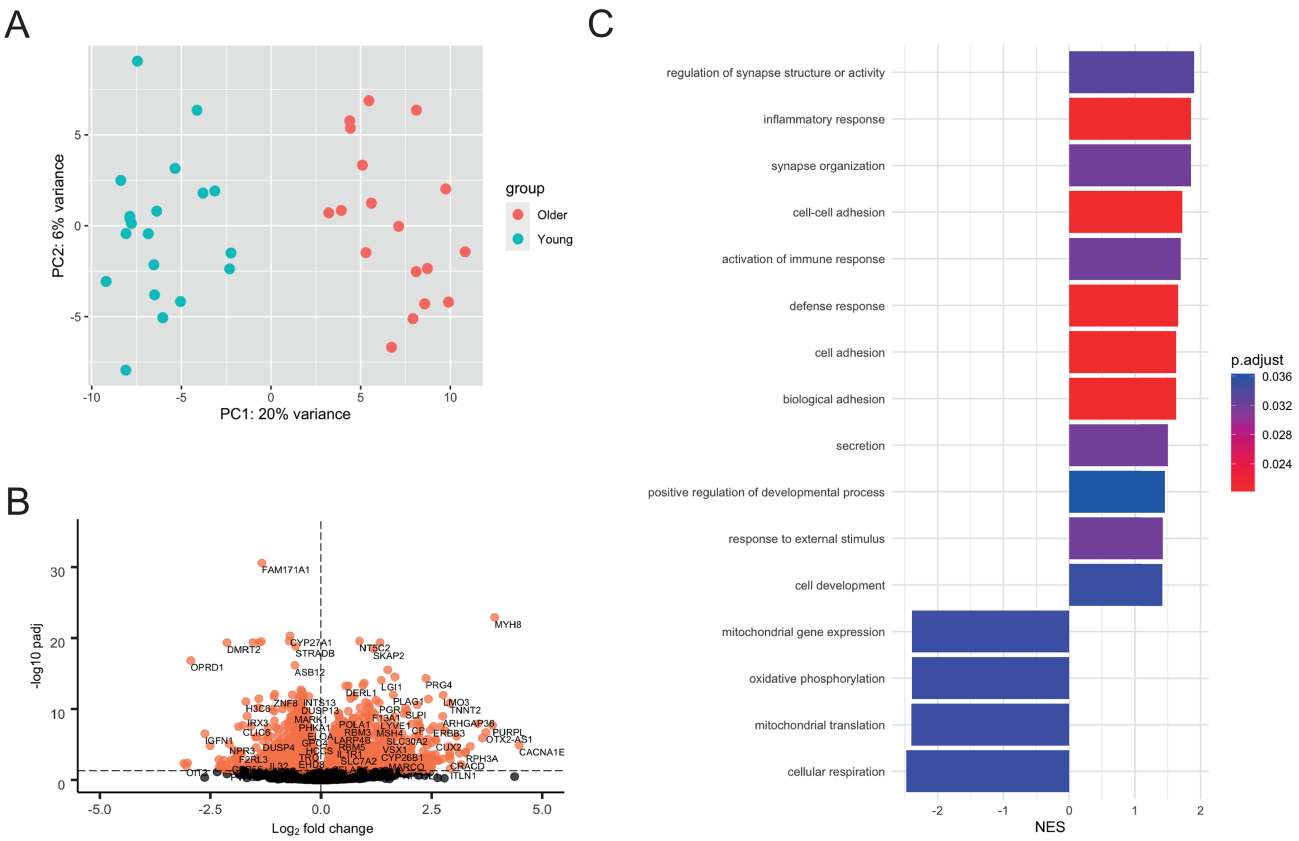

Figure 1 *(A)* PCA blot of Euclidean distance between samples on variance stabilising transformed data. Samples were coloured based on group. *(B)* Volcano plot displaying the DEGs. Three thousand seven hundred ninety-seven genes with entrez annotation were differentially regulated between the young and older *vastus lateralis* muscle using an adjusted *P*-value of 0.05 (adjusted according to Benjamini–Hochberg). *(C)* Gene set enrichment analysis of older versus young using GO biological process gene sets (C5 BP MSigdb) using an adjusted *P*-value of 0.05. GO gene sets were filtered based on 75% overlap in genes.

membrane that contains mostly collagen IV and the glycoprotein laminin. Here, laminin serves as a ligand for the transmembrane receptors dystrophin-associated glycoprotein complex (DGC) and integrins in the sarcolemma, which in their turn bind actin proteins in the sarcoplasm.[39] The interaction between the ECM and the sarcolemma establishes cell adhesion and is important for muscle contraction, as it is necessary for lateral transfer of contractile force.[38] Dysregulation in cell adhesion is hypothesized to contribute to the decrease in muscle strength observed in older rats.[40] Therefore, we specifically examined expression of genes involved in cell–ECM adhesion. Various genes that encode for the DGC complex and 11 out of 27 genes that encode for integrins, classified according to the HUGO Gene Nomenclature Committee,[41] were found to be differentially regulated in older muscle (*Figure* 2B). Alpha7 integrin (ITGA7), part of the alpha7beta1 integrin dimer, is important in mechanical adhesion and force transduction in the muscle.[42] Therefore, we examined expression of *ITGA7* by qPCR and verified its differential expression with age (FC = 0.86 *P* = 0.037) (*Figure* 2C).

Besides force transmission and mechanical adhesion, the ECM also plays an important role in muscle hypertrophy and regeneration, by interaction with muscle stem cells, or satellite cells, that reside in the ECM.[43] These cell–cell interactions are primarily regulated via cadherins, a group of transmembrane proteins that play a role in regulating myogenesis and differentiation via interaction with catenins, which, in their turn, link with the actin cytoskeleton.[44] Of the 125 cadherins and catenins, classified according to the HUGO Gene Nomenclature Committee,[41] 12 catenins and 30 cadherins were differentially regulated between young and older muscle, of which were 26 upregulated (*Figure* 2B). Using qPCR, we examined gene expression of muscle cadherin (CDH15 or M-cadherin), which is important for cell adhesion between muscle fibres and satellite cells.[44,45] Indeed, gene expression of *CDH15* was confirmed to be significantly higher in older compared with young muscle (FC = 1.22, *p* = 0.014) (*Figure* 2D). This suggested that not only cell–ECM adhesion was altered with ageing but also cell–cell adhesion within the ECM.

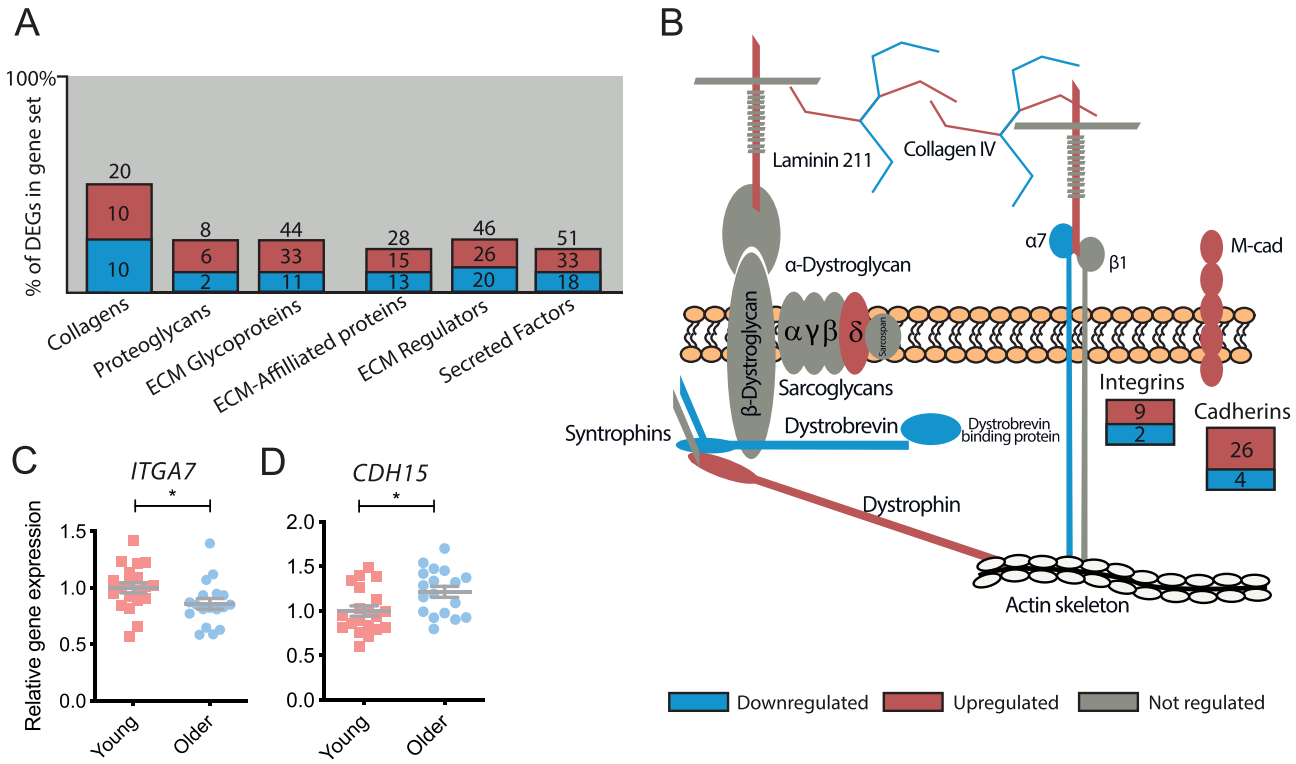

Figure 2 *(A)* Percentage of DEGs for each extra cellular matrix (ECM) component according to 'Matrisome' classification. *(B)* Schematic presentation of sarcolemma–ECM adhesion complexes. Downregulated (blue) and upregulated (red) are indicated. *(C)* qPCR analysis of integrin 7a (*ITGA7*) and *(D)* muscle cadherin (*CDH15*). Values are mean ± SD. *$P < 0.05$.

Higher expression of genes related to synaptogenesis at neuromuscular junctions

Another cadherin that was significantly upregulated in the older group was neural cadherin (*CDH2* or N-cadherin; FC = 2.5, padj < 0.001). *CDH2* is upregulated during muscle regeneration and induces neurite outgrowth.[44] Both *CDH2* and *CDH15* play an important role in synaptogenesis, the formation of synapses and synapse plasticity, the ability of the synapse to change.[46] These processes that were also identified among the most significant upregulated gene sets in the older muscle, namely, 'synapse organization' and 'regulation of the synapse structure or activity' (*Figure* 1C). Therefore, we examined the expression of genes related synaptogenesis. We used a selection of genes based on a selection of DEGs involved in synaptogenesis and genes that encode for proteins at the neuromuscular junction (NMJ), the synapse between a myofibre and a motor neuron, in older compared with young mice muscle.[47] Many of the genes related to synaptogenesis were upregulated in older muscle, such as genes that encode for neurotrophins, proteins that stimulate nerve and synapse growth (*Figure* 3A). We verified the expression of seven genes by qPCR. Significant differential gene expression between the groups was confirmed for: brain-derived neurotrophic factor (*BDNF*; FC = 2.9, $P < 0.001$), a growth factor for neurons and synapses in the central and peripheral nervous system; neurotrophic tyrosine kinase receptor type 2 (*NTRK2*; FC = 1.35, $P = 0.0058$), a gene that encodes for the *BDNF* receptor; neuronal acetylcholine receptor subunit alpha 1 (*CHRNA1*; FC = 1.79, $P = 0.0012$), a subunit of the acetylcholine receptor at the NMJ; neuronal cell adhesion molecule (*NRCAM*; FC = 2.18, $P = 0.0045$) that regulates axon guidance and growth and muscle-specific receptor kinase (*MUSK*; FC = 1.38, $P < 0.013$), a key player in the formation of NMJs, although a trend was observed for the expression of runt-related transcription factor 1 (*RUNX1*; FC = 1.41, $P = 0.094$) (*Figure* 3B). Moreover, we verified differential expression of one of the top most regulated DEG (*Table* 3), myosin heavy chain 8 (*MYH8*; FC = 10.8, $P < 0.001$) (*Figure* 3B), encoding a perinatal myosin that is only expressed during development and is associated with denervated muscle.[48,49] Additionally, MUSK protein levels in *vastus lateralis* biopsies of seven older and seven young individuals were measured using western blot (*Figure* 3C). In agreement with the gene expression data, a trend for higher MUSK protein levels in the older group was observed ($P = 0.08$; *Figure* 3D).

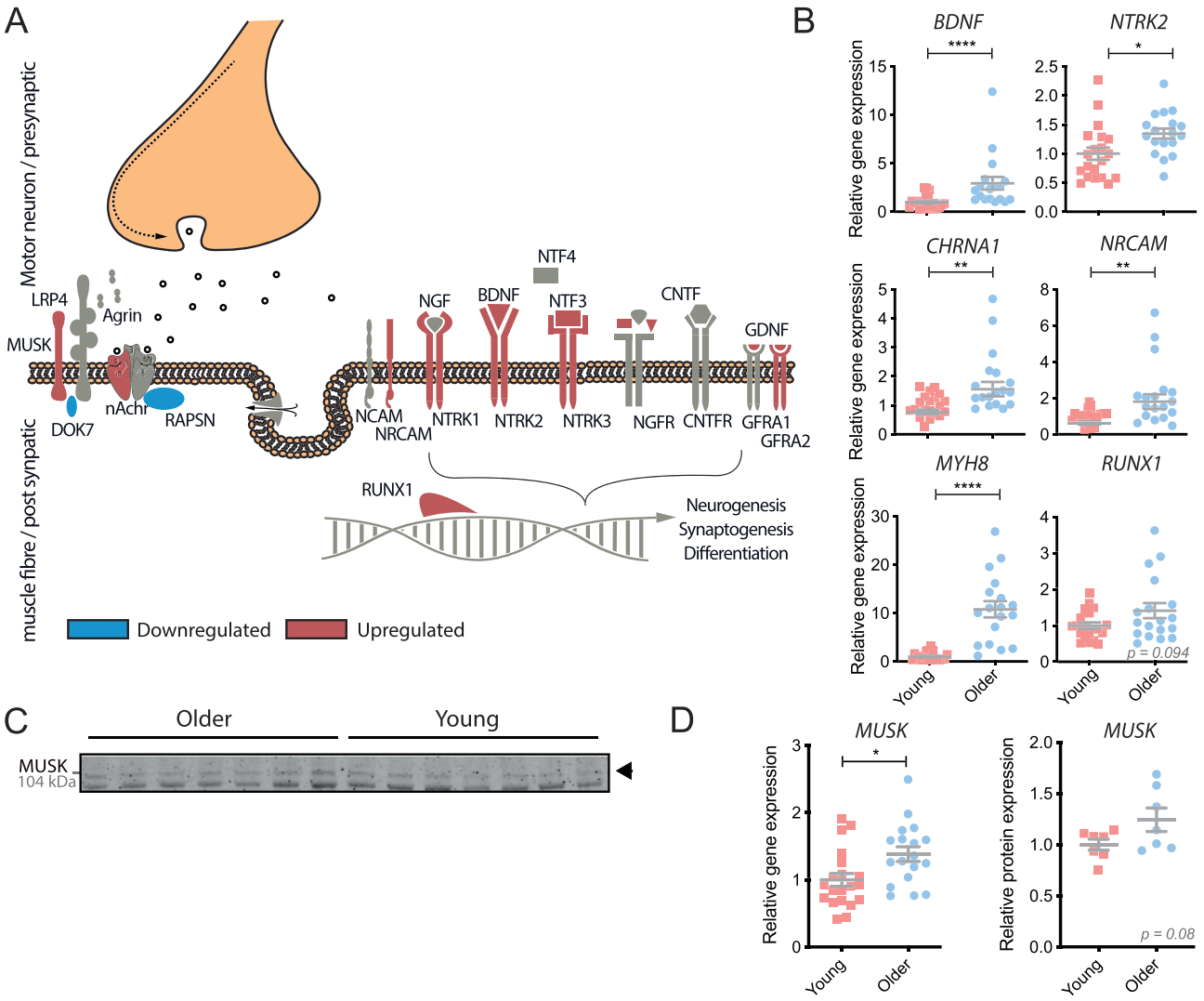

Figure 3 *(A)* Schematic presentation of neuromuscular junction and proteins involved in synaptogenesis. *(B)* qPCR analysis of brain-derived neurotrophic factor (*BDNF*), neurotrophic receptor tyrosine kinase 2 (*NTRK2*), neuronal acetylcholine receptor subunit alpha-1 (CHRNA1), neuronal cell adhesion molecule (NRCAM), muscle associated receptor tyrosine kinase (*MUSK*), runt-related transcription factor 1 (RUNX1) and myosin heavy chain (*MYH8*). *(C)* Western blot of protein expression of MUSK of seven older and seven young vastus lateralis muscle on nitrocellulose membrane. *(D)* mRNA expression by qPCR (left) and protein expression (right) corrected for total protein (averaged over two blots, after the average of the young subjects was set to 1). Values are mean ± SD. $^{*}P < 0.05$, $^{****}P < 0.0001$.

Oxidative respiration is the main downregulated process during ageing

Gene sets related to oxidative metabolism were significantly downregulated in the older group. Therefore, we further explored mitochondrial annotation of the DEGs using the MitoCarta2.0 gene set, a set of 1158 genes with strong support for mitochondrial localisation. Ten percent of all DEGs were identified as mitochondrial genes, and of the downregulated DEGs specifically, 17% of the genes were mitochondrial. Protein interaction network analysis of all downregulated mitochondrial genes showed that many genes were associated with mitochondrial respiratory complexes, mitochondrial ribosomal proteins and membrane-associated proteins (*Figure* 4A). In particular, many genes that encode subunits that make up mitochondrial respiratory Complex I, or NADH dehydrogenase, and Complex IV, or cytochrome C oxidase, were lower expressed in old muscle. Semi-quantitative protein levels were determined using western blot in the protein lysates of the *vastus lateralis* biopsies of seven older and seven young individuals. These individuals were selected because enough material was available and data on *in vivo* mitochondrial capacity in the *vastus lateralis* were previously collected.[21] The transcriptional differences translated into significant lower protein expression of Complex IV and Complex II, or succinate dehydrogenase ($P = 0.011$ and $P = 0.0009$, respectively) (*Figure* 4B), whereas a trend could be observed for Complex I, in older compared

Table 3 Top 20 significantly regulated genes between young and older males

Symbol	FC	Padj
FAM171A1	0.4	2.62E-31
MYH8	15.21	1.25E-23
CYP27A1	0.62	4.84E-21
CTNNBIP1	0.61	2.67E-20
NT5C2	1.83	2.75E-20
KAZALD1	0.39	3.10E-20
RADX	2.52	4.22E-20
DAAM2	0.38	4.22E-20
DMRT2	0.23	4.51E-20
STRADB	0.67	1.46E-19
SKAP2	2.27	3.24E-19
OPRD1	0.13	1.45E-17
ASB12	0.66	6.97E-17
PRG4	5.18	4.68E-15
LGI1	2.57	8.78E-15
ADAMTS5	1.96	2.30E-14
TENM3	1.92	4.35E-14
MALT1	1.53	5.21E-14
DERL1	1.48	5.74E-14
ZNF768	0.73	2.03E-13

Adj. *P*-value, adjusted *P*-values using Benjamini–Hochberg method; FC, fold change regulation or mean expression older versus young.

with young muscle ($P = 0.062$) (*Figure* 4C). The lower protein expression could explain, at least in part, the lower mitochondrial capacity observed in the older compared with the young group, as protein expression of Complexes I and IV was significantly correlated to mitochondrial capacity in the *vastus lateralis* as measured *in vivo* using NIRS ($P = 0.017$, $R^2 = 0.42$ and $P = 0.030$, $R^2 = 0.36$) (*Figure* 4D).

Mitochondrial mass is regulated by proliferator-activated receptor gamma coactivator 1-alpha (*PPARGC1A* or *PGC1α*), and gene expression of this transcriptional cofactor was lower in the older group (FC = 0.8, padj = 0.02). Furthermore, when identifying transcription factors that are significantly associated with the DEGs, four transcription factors gene sets were significantly regulated with an FDR q-value < 0.2, among which the gene set associated with *PGC1α* (*Figure* 5A). Mitochondrial transcription factor A (*TFAM*) is a major target for *PGC1α* and was also downregulated in the older group (FC = 0.85, padj = 0.03). We examined mRNA and protein levels of TFAM using qPCR and western blot. Although differential *TFAM* gene expression was not confirmed using qPCR (*Figure* 5D), TFAM protein levels were significantly lower in the older group ($P = 0.030$), which is in accordance with RNAseq data (*Figure* 5B and 5C). At the same time, mitophagy did not appear as a significantly regulated process, which was confirmed by close inspection of the reactome autophagy and mitophagy pathways, which showed upregulation as well as downregulation for all genes involved as well as for the significant genes (*Figure* S2A and S2B). The lower expression of *PGC1α* and TFAM could therefore be an explanation for the lower expression of mitochondrial complexes and the lower *in vivo* mitochondrial capacity in the older group.

In attempt to further explain the lower expression of *PGC1α* and its targets genes in the older muscle, we explored mechanisms upstream from *PGC1α*. Following muscle contraction, PGC1α is activated by the energy-sensing AMP-activated protein kinase (AMPK) protein complex.[50] During exercise, the α2/β2/γ3 heterotrimer of this complex is mostly activated, and activity of the AMPK complex was mainly associated with the phosphorylation of the skeletal muscle-specific subunit of this complex, namely, AMP-activated protein kinase non-catalytic subunit gamma 3 (*PRKAG3*).[51] The expression of *PRKAG3* was downregulated in older compared with young muscle (FC = 0.58, padj < 0.001), suggesting that the activation of PGC1α in response to exercise could be lower. When looking at other exercise-responsive genes, nuclear receptor subfamily 4 group A member 1 (*NR4A1*), nuclear receptor subfamily 4 group A member 3 (*NR4A3*) and the long non-coding RNA NR4A1 antisense RNA (*NR4A1AS*) were downregulated (FC = 0.45, padj < 0.001, FC = 0.42, padj = 0.04 and FC = 0.34, padj = 0.007, respectively). *NR4A1* and *NR4A3* are known as potent exercise response genes and are involved in the regulation of mitochondrial biogenesis and oxidative metabolism, possibly in part via AMPK.[52–54] *NR4A1AS* upregulates NR4A1 expression via stabilisation of its mRNA.[55] We verified the lower gene expression of *PGC1α* (FC = 0.77, $P = 0.0015$), *PRKAG3* (FC = 0.65, $P = 0.0005$), *NR4A1* (FC = 0.5, $P = 0.012$) and *NR4A3* (FC = 0.37, $P = 0.0017$) using qPCR older compared with young muscle (*Figure* 5E). This shows that on molecular level, there are differences in expression of important genes that regulate mitochondrial capacity, also associated with the response to exercise and physical activity.

Because mitochondrial protein expression was lower in older compared with younger muscle, it is of interest to study whether a higher physical activity among individuals in the older group associates with a relative higher mitochondrial gene expression, providing a molecular mechanistic basis for a beneficial effect of physical activity on the ageing muscle. For this, the association between gene expression and the continuous variable 'time spent in MVPA' was calculated for the young older group separately. It must be noted that there is a substantial decrease in sample size due to the analysis in a single group. Six hundred sixty-two genes with an Entrez annotation were significantly associated with time spent in MVPA using an unadjusted *P*-value cut-off of 0.05 (*Figure* 5F). Ten percent of those genes were mitochondrial genes according to the MitoCarta gene set and included 11 out of the 13 genes encoded in the mitochondrial DNA, which all encode for subunits of mitochondrial respiratory complexes. GO enrichment analysis of the significantly associated genes with MVPA showed a clear mitochondrial signature. For the genes that were differentially expressed in the mitochondrial processes, such as 'ATP metabolic process' and 'oxidative phosphorylation', a higher MVPA was

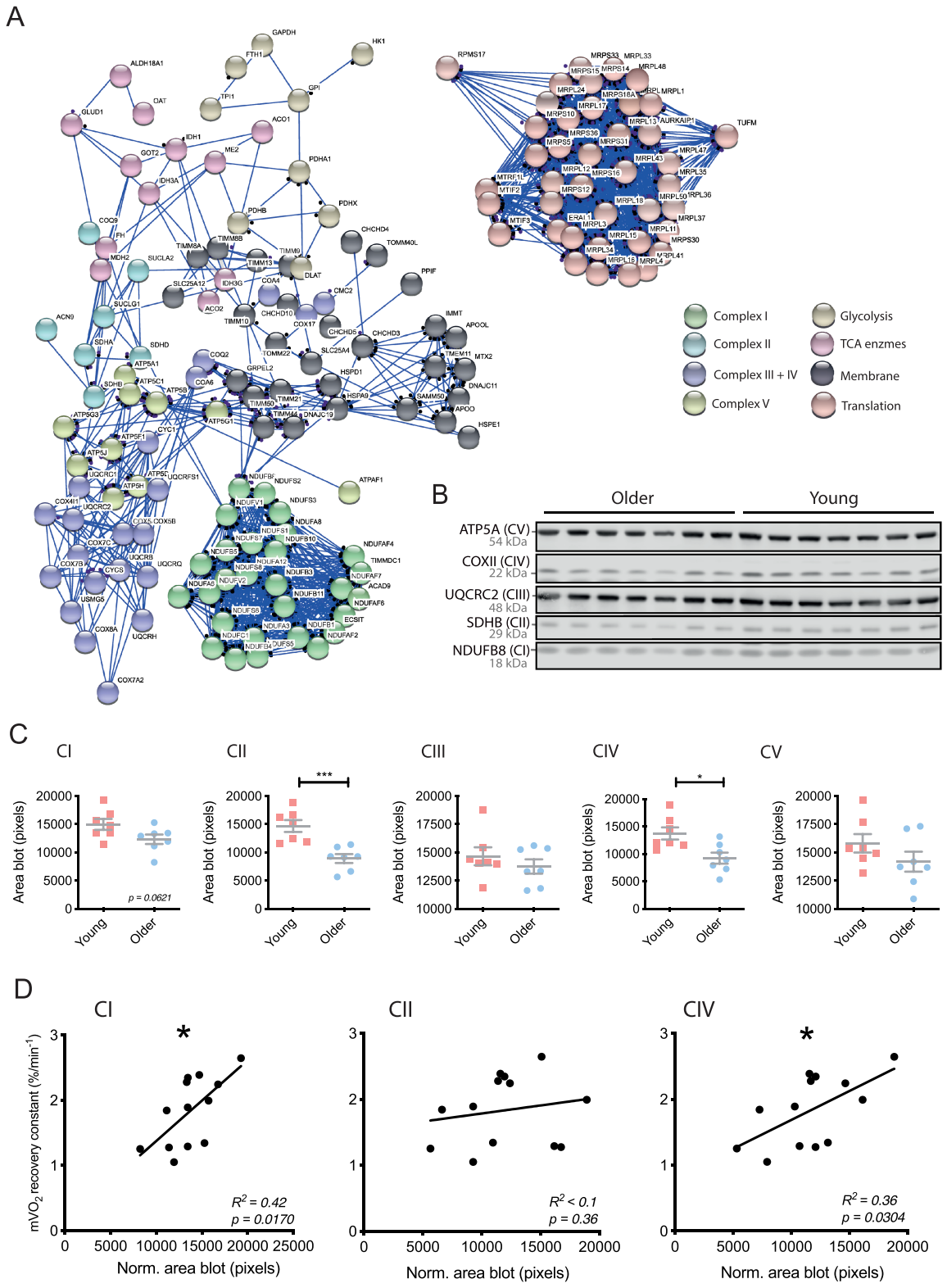

Figure 4 *(A)* Differentially expressed genes were compared with genes in MitoCarta genes set. Protein–protein interaction network for all mitochondrial downregulated genes. Nodes and edges with a molecular action are represented, and nodes without any connections are removed. Proteins were manually grouped based on function in mitochondria. *(B)* Western blot of protein expression of OXPHOS complexes of seven older and seven young *vastus lateralis* muscle on nitrocellulose membrane. *(C)* Quantification of protein expression corrected for total protein. *(D)* Correlation between protein expression and mitochondrial capacity in the *vastus lateralis* measured using NIRS.

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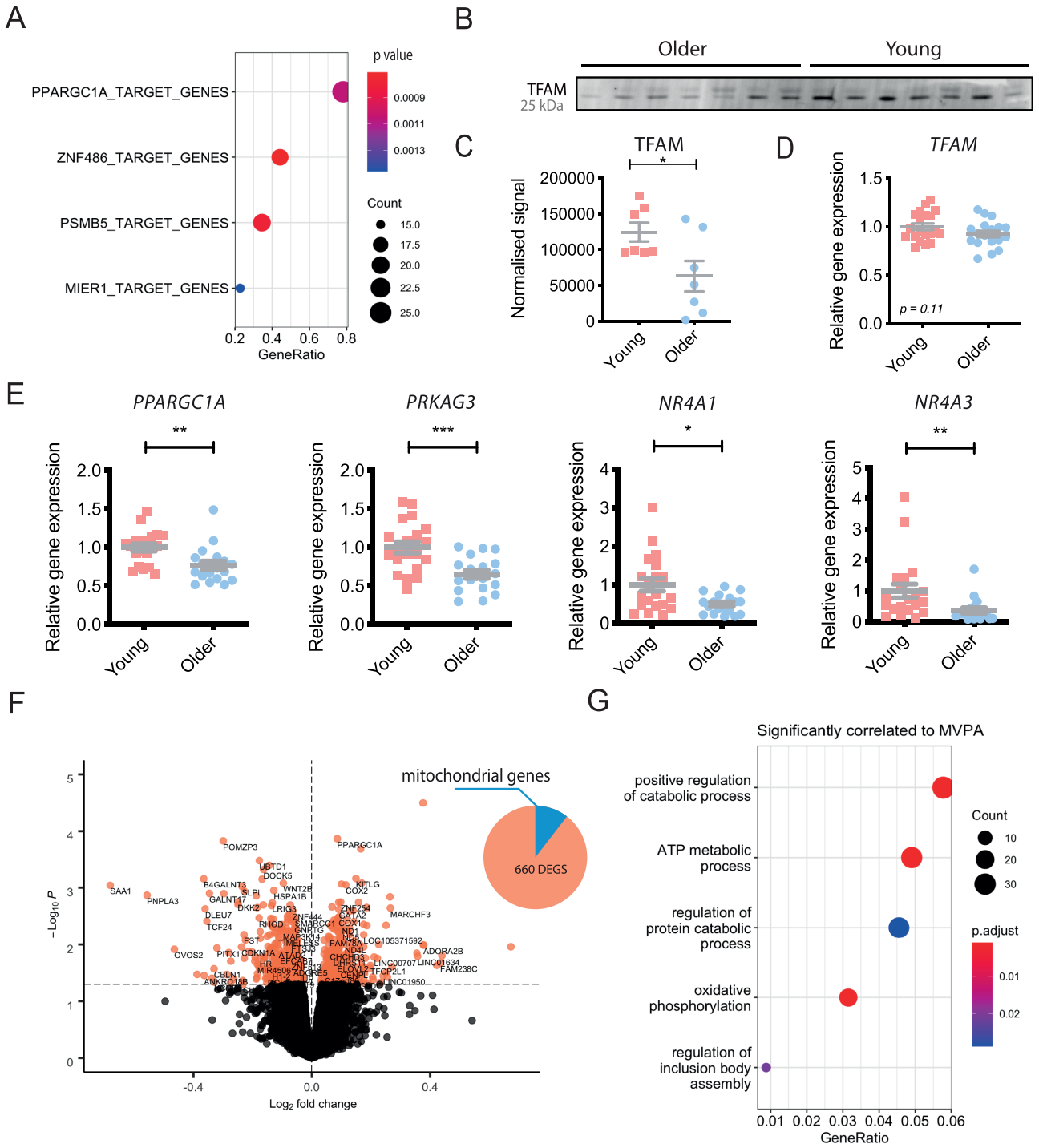

Figure 5 *(A)* Transcription factor enrichment analysis for all differentially expressed genes using C3 'TFT:GTRD' from MSigDB was used at FDR < 0.2. *(B)* Western blot analysis of mitochondrial transcription factor A (TFAM) on PVDF membrane. *(C)* Quantification of TFAM protein expression normalized for total protein. *(D)* qPCR analysis of *TFAM* expression. *(E)* qPCR analysis of peroxisome proliferator-activated receptor gamma coactivator 1 alpha (*PPARGC1A*), protein kinase AMP-activated non-catalytic subunit gamma 3 (*PRKAG3*), nuclear receptor subfamily 4 group A member 1 (*NR4A1*) and 3 (*NR4A3*). *(F)* Volcano plot displaying the 662 DEGs with entrez annotation that were significantly correlated with time spent in moderate-to-vigorous physical activity (MVPA) in older *vastus lateralis* muscle using an *P*-value of 0.05 and no FDR correction. Ten percent of those genes were mitochondrial genes. *(G)* GO enrichment analysis of genes significantly correlated to MVPA. The *P*-value was adjusted using the Benjamini–Hochberg procedure, and GO terms were filtered based on 50% overlap in genes. GeneRatio represents the ratio of enriched genes to total number of genes in the specific GO term.

correlated to a higher expression of those genes. But for the genes involved in catabolic processes, such as 'positive regulation of catabolic process' and 'regulation of protein catabolic process', the opposite relationship was observed (*Figure* 5G). This showed that despite a negative effect of age on mitochondrial capacity, still a higher level of physical activity attenuated this effect. A similar analysis in the younger group showed 1058 genes with an Entrez annotation that were significantly associated with time spent in MVPA using an unadjusted *P*-value cut-off of 0.05 (*Figure* S3A). Of these genes, <1% were mitochondrial genes according to the MitoCarta gene set. For the genes that were differentially expressed in the most significantly regulated gene sets, such as 'negative regulation of nervous system development' and 'negative regulation of neuron differentiation', a higher MVPA was correlated to a lower expression of genes in those gene sets (*Figure* S3B).

Discussion

The aim of this study was to better understand the effects of early ageing on muscle function by disentangling factors that affect the ageing process, such as sex and physical activity, and advancing age itself. We showed clear differences in the transcriptome signatures of the *vastus lateralis* muscle of healthy older and young males with similar physical activity. Because we corrected for physical activity, we were able to identify relatively early ageing processes that occur independent of age-related decline in physical activity. The processes identified to be largely upregulated in older males were cell adhesion, the matrisome, innervation and inflammation. Oxidative metabolism, on the other hand, was downregulated. Key genes in these processes were validated using qPCR. Our study encompasses a relatively homogeneous age group of older, but not yet old, individuals and suggests that a decline in mitochondrial function is not a late phenomenon in muscle ageing. Moreover, the decrease in mitochondrial function is robust, as a large number (±10% of all DEGs) of mitochondrial genes and processes are affected, including expression of the primary and essential process of mitochondrial ATP generation by the mitochondrial OXPHOS complexes. We further validated this by showing that the protein expression of these complexes is lower in older compared with young males, and we found that this is correlated with *in vivo* mitochondrial capacity in the older group. Because we previously measured lower *in vivo* mitochondrial capacity in the *vastus lateralis* muscle in older compared with young males using near-infrared spectroscopy,[21] we now strengthen our previous functional observations and show that the observed lower *in vivo* mitochondrial capacity could be caused by a lower expression of mitochondrial proteins.

Our data show for the first time that the observed decrease in mitochondrial capacity with age cannot be entirely prevented by maintaining physical activity levels, at least not with the amount and types of physical activity measured in the current study. Our finding is in contrast with previous studies that have reported similar mitochondrial ATP production rates in older compared with young individuals with similar physical activity.[14,15] Nevertheless, a clear trend can be observed in the aforementioned studies for a lower mitochondrial ATP production in older individuals. The absence of a significant effect might originate from a more heterogenic study populations due to inclusion of individuals of both sexes in a wider age range. Sex is known to be an important effect modifier in age-related mitochondrial decline,[56] and possibly, the ageing trajectory is sex dependent.[18] Furthermore, in accordance with the current study, a lower mitochondrial complex abundance, *TFAM* expression and *PGC1α* expression were observed, even when comparing similarly active, trained older and young individuals,[15] likewise suggesting that physical activity alone is not able to completely halt the age-related decline in mitochondrial capacity. Nonetheless, we do show that spending a higher amount of time in was associated with a higher expression of mitochondrial genes and a lower expression of genes involved in catabolic processes. Therefore, our findings support the current belief that increasing or maintaining physical activity is associated with higher mitochondrial capacity and that it is a useful strategy to attenuate the age-driven decline in mitochondrial capacity.[15,57,58]

Why mitochondrial protein and gene expression is not comparable in different age-groups despite similar physical activity levels is currently unknown. However, we did find a lower of expression of important genes that regulate mitochondrial biogenesis in the older muscle, such as *TFAM* and *PGC1α*, and genes involved in energy regulation, such as *PRKAG3*. The γ3 subunit of the AMPK complex, which is encoded by the *PRKAG3* gene, has been shown to be most important in the activation of AMPK following physical activity.[51] AMPK activation is essential for the activation of PGC1α, which in turn regulates mitochondrial biogenesis via TFAM.[50,59] In addition, we show a lower expression of *NR4A1* and *NR4A3*, which are potent exercise response genes that enhance oxidative capacity[53] and are regulated in response to local contractile stimuli, possibly in part via AMPK activation.[54] Together, this suggests that the lower mitochondrial gene and protein expression that was observed in the older males might be explained by a decreased sensitivity to exercise. Indeed, an age-related decrease in AMPK activation and mitochondrial biogenesis was previously observed in old compared with young mice.[60] Moreover, the response of *PGC1α* to an exercise intervention after a period of bed rest was lower in old compared with young individuals and failed to restore expression of some mitochondrial complexes in the older individuals.[61] Likewise, although some of the

beneficial effects of exercise interventions are evident and age independent,[62] there is evidence that a similar physical intervention increases mitochondrial capacity more in young than older individuals.[57] However, although *PRKAG3* is important for AMPK activity following exercise,[51] paradoxically, its expression is downregulated following an exercise intervention.[63]

An alternative explanation for the lower expression of exercise response genes despite similar physical activity could be differences in patterns of muscle use between the old and young group. For example, the *vastus lateralis* is thought to be less activated during locomotion with advancing age,[64–66] which could elicit a detraining-like molecular response, whereas physical activity levels remain similar. Hence, the *vastus lateralis* muscle might not be representative for ageing in all skeletal muscles. Nonetheless, muscle biopsies are almost exclusively sampled from the *vastus lateralis* muscle, potentially giving a biased view of skeletal muscle ageing. Therefore, it is of interest to study the transcriptomic signature in muscles that do not show a lower mitochondrial capacity with age, such as the *tibialis anterior*.[21,67,68]

Apart from oxidative metabolism, we showed the differential expression of genes that regulate innervation between young and older muscle. The observed gene expression pattern is largely in agreement with the observations in the *vastus lateralis* muscle of older mice, where denervation and a smaller fibre size were observed,[47,69] suggesting increased denervation in the older group. We also observed increased expression of the neonatal myosin *MYH8*. *MYH8* is associated with the developing muscle, and expression has been reported to be low to undetectable in healthy adult muscle.[49,70] Nevertheless, our results show a higher expression in the older group, confirming previous observations where older and young males and females in a similar age range were used as those in the current study.[71–73] Increased expression of MYH8 has been associated with denervation,[74,75] and indeed, we found that the expression of *MYH8* was significantly, positively correlated to genes that are associated with denervation, such as *CHRNA1, RUNX1* and NRCAM (data not shown). Although NRCAM itself is not well studied in relation to NMJs, it is structurally similar to NCAM, a gene that is often associated with denervation.[76] *NCAM* expression was not different between the old and young group, although various other genes associated with denervation were significant and showed similar patterns as previously observed in elderly muscle, such as increased *MUSK* and *CHRNA1*.[73] These findings reinforce a possible association of MYH8 with denervation. Of note is that *MYH8* protein expression was found to be unaffected by fibre type.[77] Therefore, a change in fibre type is unlikely to explain the difference in *MYH8* expression (*Figure* S3C).

Denervation and muscle remodelling events are a normal neuromuscular processes. Orphan muscle fibres send signals that stimulate sprouting of neighbouring motor neurons to become reinnervated.[78] However, when denervation wins from innervation, some fibres will lose their innervation, and this will affect muscle strength,[79] likely due to atrophy of denervated fibres.[69] Denervation events are thought to be caused by degradation at the NMJ, but the underlying mechanisms are still unclear.[80,81] Distortion of NMJs due to accumulative muscle injury[82] and age-related changes in mitochondria close to the NMJ or in the motor neurons themselves have been suggested as mechanisms that could play a role in age-related denervation.[83,84] However, what is cause and what is effect are currently unknown. For example, denervation can also directly affect, and by some is thought to precede, mitochondrial dysfunction.[85,86] Nevertheless, we show that expression of genes encoding essential parts of the NMJ and synaptogenesis are upregulated in older individuals. We therefore think that a better mechanistic understanding of NMJ degradation is critical to understand denervation and muscle ageing, which may play an important role even when physical activity is maintained.

Although many anti-ageing interventions are aimed at improving muscle fibre functionality, it is often overlooked that the fibres are embedded in an ECM to establish contractile force transmission[38] and which regulates development,[35] growth [36] and repair.[37] We showed that cell adhesion and the matrisome were the most significantly regulated processes in the ageing muscle, and we therefore argue that dysregulation of the ECM is an important mechanism in early muscle ageing. Although this process has not been identified as a signature ageing process in a large reannotation of 3000 muscle samples,[87] it was previously observed in a large population of muscle samples from humans of varying ages.[88] In this study, we showed that a large percentage of genes that encode for proteins involved in the ECM are differentially regulated between young and older individuals. By interrogation of the current dataset with the matrisome gene set,[34] we showed that this is especially true for genes that encode collagens, because approximately 50% of all the genes encoding for collagens are upregulated in older compared with young muscle. Increased collagen disposition, at least in rats,[89] and higher collagen crosslinking[90] have been observed with age. The higher collagen deposition and collagen crosslinking increase the rigidity of the ECM, which has been put forward as a cause for the increased muscle stiffness with age.[91] Increased muscle stiffness might negatively affect muscle regeneration, as it was shown that satellite cells cultured on a rigid membrane displayed decreased survival and increased differentiation.[92,93]

Apart from increased collagen deposition and crosslinking, the increased rigidity could also be caused by a lower ECM protein turnover, which is regulated by ECM remodelling factors such as matrix metalloproteases (MMPs). Lower

MMPs were observed in the circulation of old compared with young mice.[94] Nevertheless, our data suggest that remodelling of the ECM by metallopeptidases might not play a major role in early muscle ageing, because only matrix metallopeptidase 15 was downregulated in older muscle (FC = 0.8, padj = 0.002). However, some disintegrin and metallopeptidase domain (ADAM) and disintegrin-like metallopeptidases with thrombospondin type 1 motif (ADAMTSs) were differentially upregulated in older compared with young muscle. These ECM remodelling genes are implicated in muscle development[95,96] and could therefore play a role in muscle ageing.

Tissue remodelling is associated with increased inflammation in the muscle and is, to some degree, necessary to attract cells that are involved in removing cell debris and regulating remodelling.[97] Indeed, when comparing old versus young subjects, a significant differential expression was observed in several inflammatory pathways, such as 'activation of immune response' and 'defence response'. In those gene sets, the most significantly upregulated inflammatory gene was *IL15*. IL15 is important for T-cell homeostasis and cell survival *in vitro*[98] and can be classified as a myokine, as it is expressed by skeletal muscle.[99] We found that the most upregulated interleukin was *IL25*, which is a cytokine that is produced by immune cells and plays a role in differentiation of T-helper cells by binding to its receptor IL17RB, which was also upregulated in the older group. IL25 activates natural killer cells and T-helper cells that produce pro-inflammatory cytokines such as interleukins 4, 5 and 13.[100] The suggestion that a pro-inflammatory response is induced is enforced by the upregulation of interleukin 17D (*IL-17D*; FC = 1.5 and Padj $<$ 0.001) in the older group, which is an inducer of, among others, pro-inflammatory interleukin 6. The regulation of these genes suggests an infiltration of immune cells in the tissue that secrete pro-inflammatory cytokines and growth factors that could be involved in tissue remodelling.

In addition to the components and regulators of the ECM, we also showed that genes that code for adhesion between cells and the ECM were significantly regulated with age. We hypothesized that dysregulation of cell–ECM adhesion could contribute to the loss of force transmission, which was previously observed in rats.[40] Indeed, many genes encoding for proteins in adhesion complexes were differentially regulated. We verified the expression of two key genes by qPCR, namely, *ITGA7* and *CHD15*. *ITGA7* and *CDH15* encode for major adhesion proteins expressed in skeletal muscle fibres and satellite cells.[101,102] For example, *ITGA7* encodes for part of the $\alpha 7\beta 1$ integrin that is thought to be a major contributor to force transmission,[42] supporting our hypothesis that a dysregulation of force transmission via adhesion complexes could be an important mechanism in early muscle ageing. Moreover, lower expression of *ITGA7* has been previously observed in satellite cells of old mice.[45] Therefore, our finding shows that these adhesion molecules now also provide an interesting target in human muscle ageing.

Nonetheless, though the ECM is clearly implicated in muscle ageing, the gene expression changes included both up- and downregulations, which makes the outcome difficult to interpret without dedicated studies. For example, loss of dystrophin protein expression, the main component of the DGC cellular adhesion complex, was previously observed in the *tibialis anterior* muscle in old compared with adult rats, whereas gene expression was upregulated twofold. Interestingly, other components of the DGC were upregulated, possibly in an attempt to compensate for loss of adhesion.[40] Likewise, in a mouse model for Duchene's muscular dystrophy, a muscular dystrophy disease that is caused by a dysfunctional dystrophin protein, cell adhesion proteins such as integrins are upregulated,[102] suggesting a similar compensatory mechanism. Moreover, mRNAs encoding for ECM proteins must to be translated into proteins, excreted and assembled in complex networks in the ECM, and can be subjected to post-translational modifications and degradation.[103] The reservation that mRNA expression might not closely correlate to changes in protein expression extends to all observed differences in gene expression. Although mRNA expression seems to be a good indicator for expression of ECM proteins, changes in mRNA might be less intimately linked to the expression of proteins involved in muscle contraction and the mitochondrion.[104] Hence, this limits straightforward mechanistic interpretation of transcriptomics data, despite verification by protein expression for key genes, and requires further mechanistic studies on the identified targets and pathways presented here. For example, verification using histological analysis would be a logical continuation, but no tissue was preserved for this purpose.

Another limitation of the current design is that although we controlled for time spent in exercise and exercise intensity with accelerometry measurements, the type of exercise was not standardized between the groups. Therefore, it could be that different types of exercise, such as resistance training, could have affected gene expression between the groups. For example, higher expression of adhesion genes, in particular protocadherin gamma genes, was previously observed in a population of older and frail older individuals compared with a young control group.[105] Although the latter study did not control for physical activity between the groups, it showed that expression of this gene cluster decreased after training and was correlated with muscle strength. Thus, differences in resistance-type exercise between the young and older group could have affect the results because we likewise observe an upregulation of protocadherin gamma genes in the older group. Moreover, this may not be limited to adhesion genes, because ECM remodelling factors such as metallopeptidases were also found to be regulated in an age-dependent manner after resistance training.[39] Therefore,

future cross-sectional research should aim to not only control for exercise time and intensity but additionally standardize for exercise type between the age groups. Besides, we assessed physical activity using accelerometry for a 5-day period and used the Baecke questionnaire to assess physical activity in the year prior to the study. However, physical activity throughout one's lifetime is likely to be a more important driver for muscle ageing. Therefore, because longitudinal studies over an entire lifetime present obvious challenges, research tools to assess physical activity retrospectively, such as data from wearable physical activity monitors, should be further developed.

Conclusions

We showed a clear ageing signature in the gene expression profile of the *vastus lateralis* muscle in older compared with young males and identified cell adhesion, the matrisome, innervation and inflammation as the main upregulated processes associated with muscle ageing despite similar physical activity levels. Additionally, a strong downregulation of mitochondrial proteins was observed, suggesting an impaired ability for energy generation. These data underpin the previously observed lower *in vivo* mitochondrial capacity at a molecular level. Yet, whether these results can be extended to other skeletal muscles needs to be examined, because we previously reported differences in the effect of age on mitochondrial capacity between muscles.[21] Why similar physical activity was not associated with similar mitochondrial respiratory complex expression needs to be further investigated. Possibly, the downregulation in upstream activators of mitochondrial biogenesis hints at a lower molecular sensitivity to cues for mitochondrial biogenesis, which needs to be confirmed. Nevertheless, our study highlights that mitochondrial function is lower in the older muscle, and this strongly associates with impaired sensitivity to physical activity, possibly due to structural changes involving cell adhesion, the matrisome and innervation. Because the older males in our study consisted of older, but not old individuals, the identified processes can be considered as a relatively early signature. In the future, an improved understanding of the hypotheses generated here will be key to design targeted anti-ageing therapies to ultimately sustain muscle health with advancing age.

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Author contributions

BL: data acquisition, principal data analysis, drafting the manuscript; AB: qPCR and data interpretation; BL, AGN, VCJB, JK: conception and design of research; data analysis and interpretation, drafting of manuscript. All authors edited, revised and approved final version of manuscript.

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Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 Differently regulated matrisome genes between young and older males

Figure S1. (A) Gene set enrichment analysis of older vs young using curated C2 canonical gene sets Msigdb. Gene sets were considered enriched with a Benjamini-Hochberg adjusted p-value < 0.05

Figure S2 Transcriptional fold change profiles (heat map) of older vs young males of genes sets: A) reactome_autophagy, B) reactome_mitophagy and C) all myosin genes

Figure S3. (A) Volcano plot displaying the 1058 DEGs with entrez annotation that were significantly correlated with time spent in moderate-to-vigorous physical activity (MVPA) in young *vastus lateralis* muscle using an p-value of 0.05 and no FDR correction. $<$1% of those genes were mitochondrial genes. (B) GO enrichment analysis of genes significantly correlated to MVPA, showing 8 most significantly regulated gene sets. The p-value was adjusted using the Benjamini-Hochberg procedure and GO terms were filtered based on 50% overlap in genes

Conflict of interest

No conflicts of interest, financial or otherwise, are declared by the authors. FrieslandCampina and Danone Research B. V. are sponsors of the TIFN programme and partly financed the project. They had no role in data collection and analysis, decision to publish or preparation of the manuscript, but commented on the study design.

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