Supplementary Information

Determining the feasibility of characterising cellular senescence in human skeletal muscle and exploring associations with muscle morphology and physical function at different ages: findings from the MASS_Lifecourse Study

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Appendix 1

Materials and Methods

Participant characteristics

Skeletal muscle health and function

Sarcopenia categories were based on the revised EWGSOP2 definition of sarcopenia (the European Working Group on Sarcopenia in Older People) [9]. Briefly, participants were classified as having probable sarcopenia if having low grip strength (<27 kg in men and <16 kg in women) and/or longer time for 5-chair stands (>15 s); confirmed sarcopenia if additionally having low appendicular lean mass index (ALMI; ALM in kg divided by height^2 in m (<7.0 kg/m^2 in men and <5.5 kg/m^2 in women)), and severe sarcopenia if having also slow gait speed (≤ 0.8 m/s).

Lifestyle

Daily moderate to vigorous physical activity (minutes): Participants were provided with a GENEActiv® wrist-worn accelerometer (calibrated to 100Hz) and were instructed to wear it continuously for 7 days on a dominant wrist. This device measures the acceleration of the wearing arm along three different axes of space in milligravity units (mGal) as the arm moves, thus denoting the amount/intensity of physical activity of the wearer. Participants were provided with a form to record any time intervals during which they removed the device. The accelerometers were collected at a clinical visit during which muscle biopsy and imaging were performed. From the GeneActiv

accelerometer recordings, the mean daily duration of moderate to vigorous physical activity (MVPA) was calculated using the GGIR package in R (https://cran.r-project.org/web/packages/GGIR/GGIR.pdf) [S1]. MVPA was defined as time periods for which measured acceleration was >0.1 g calculated using the Euclidean norm minus one algorithm, which were sustained for at least 80% of a minimum bout of 5 minutes as described in the MASS_Lifecourse Study protocol [11].

Appendix 2

Supplementary Results

Associations between muscle-related outcomes and age

Men

The following significant Spearman correlations were observed in men between measures of muscle strength and mass with age: grip strength (r = -0.53), and ALM (r = -0.48) showing a decline in muscle health with ageing. Associations with gait speed, 5-chair stands, BMI, ALMI, MVPA and 5-chair stands were weak (r = -0.18, r = 0.33, r = -0.24, r = -0.37, and r = -0.38, respectively).

Women

Similarly, a significant Spearman association was found between ALM (r = -0.66) and age in women, but not with grip strength (r = -0.49), gait speed (r = -0.08), BMI (r = -0.31), ALMI (r = -44), and MVPA (r = -0.36) compared with men.

Supplementary Tables

Supplementary Table 1. Description of cellular senescence markers and corresponding variables in the MASS_Lifecourse Study

Cellular senescence marker	Abbreviation	Description	Method of detection
p16		The cyclin dependent kinase inhibitor (CDKI) *p16* is a primary mediator of cell-cycle arrest and a promoter of cellular senescence [S2, S3, 21].	RNA *in situ* hybridisation (RNA-ISH)
Percentage of nuclear foci positive for ≥2 *p16* (%)	%2+p16Nuclei	Percentage (%) of myonuclei expressing ≥2 *p16* mRNA transcript foci	"
Percentage of fibre foci positive for ≥2 *p16* (%)	%2+p16Fibre	Percentage (%) of myofibre expressing ≥2 *p16* mRNA transcript foci	"
Percentage of any foci positive for ≥2 *p16* (%)	%2+p16Any	Percentage (%) of both myonuclei and myofibre expressing ≥2 *p16* mRNA transcript foci	"
Percentage of nuclear foci positive for *p16* (%)	%p16+Nuclei	Percentage (%) of myonuclei expressing any number of *p16* mRNA transcript foci (≥1)	"
Percentage of fibre foci positive for *p16* (%)	%p16+Fibre	Percentage (%) of myofibres expressing any number of *p16* mRNA transcript foci (≥1)	"
Percentage of any foci positive for *p16* (%)	%p16+Any	Percentage (%) of both myonuclei and myofibre expressing any number of *p16* mRNA transcript foci (≥1)	"
TAF and γH2A.X		Telomeres are specialised structures the ends of chromosomes comprised of tandem 5'-TTAGGG-3' repeats that play a vital role in maintaining genomic stability [S4]. Telomere dysfunction results in the activation of a persistent DNA-damage response (DDR) which has been shown to trigger and maintain senescence in several tissues during ageing [S5]. γH2A.X, a phosphorylated histone variant H2A.X at serine 139 (Ser139), is the DDR protein that activates DNA-damage mediators [S6]. The colocalisation between telomeres and the DDR protein γH2A.X is otherwise known as TAF (telomere-associated DNA damage foci).	Immunofluorescence *in situ* hybridisation (immuno-FISH)
Percentage positive for ≥2 TAF positive (%)	%TAF2+	Percentage (%) of myonuclei expressing ≥2 TAF signals	"
Percentage positive for ≥3 TAF positive (%)	%TAF3+	Percentage (%) of myonuclei expressing ≥3 TAF signals	"
Percentage positive for γH2A.X (%)	% γH2A.X	Percentage (%) of myonuclei expressing % γH2A.X signal	Immunofluorescence
γH2A.X positive as a proportion of TAF positive (%)	TAF% γH2A.X	Percentage of myonuclei expressing % γH2A.X signal as a proportion of TAF positive nuclei	Immuno-FISH

HMGB1		HMGB1 (High Mobility Group Box 1) is a DNA binding non-histone protein that acts as an alarmin, signaling cellular damage when released extracellularly and creating an inflammatory response associated with the senescence-associated secretory phenotype (SASP). The loss of HMGB1 expression with age have been identified as a marker of senescence both *in vivo* and *in vitro* [S7].	Immunofluorescence (IF)
Percentage of nuclei positive for HMGB1 (%)	%HMGB1+	Percentage (%) of myonuclei expressing HMGB1 signal	"
Lamin B1		Lamin B1 is a nuclear envelope protein involved in nuclear stability. The loss of Lamin B1 with age have been identified as a marker of cellular senescence [S8].	Immunofluorescence
Percentage of nuclei positive for Lamin B1 (%)	%LaminB1+	Percentage (%) of myonuclei positive for Labin B1 signal	"

Supplementary Table 2. Reagents and Assays

Item	Supplier	Catalogue Number
4% Paraformaldehyde in PBS	Santa-Cruz Biotechnology	sc-281692
Avidin/Biotin Blocking Kit	Vector Laboratories	SP-2001
Bovine Serum Albumin (BSA)	Sigma-Aldrich	A3059
Cy-3-labelled telomere specific (CCCTAA) peptide nuclei acid (CCCTAA) peptide nuclei acid probe (594 nm)	Panagene	F1002-5
Deionised Formamide	Amresco	606
EcoMount	Biocare Medical	EM897L
EDTA	Sigma-Aldrich	E9884
Eosin Y	ScyTek Laboratories	EY0500
Ethanol, Absolute	Fisher Scientific	64-17-5
Fluorescein Avidin DCS	Vector Laboratories	A2011
Formamide	Sigma-Aldrich	F7508
Haematoxylin	Sigma-Aldrich	MHS16
Histo-Clear	National Diagnostics	HS-200
Hydrogen Chloride	Sigma-Aldrich	HX0603
Magnesium Chloride	BDH UK	101494V
Malic Acid	Sigma-Aldrich	M0375
Methanol, Absolute	Fisher Scientific	67-56-1
Normal Goat Serum	Vector Laboratories	S-1000
Phosphate Buffered Saline, 10X	Sigma-Aldrich	D1408
Picro Sirius Red Stain Kit	Abcam	ab150681
ProLong Gold Mounting Media with DAPI	Thermo Fisher	15260719
RNAscope 2.5 HD Detection Reagent - RED	Advanced Cell Diagnostics	322360
RNAscope H_2O_2 & Protease Plus Reagents	Advanced Cell Diagnostics	322330
RNAscope Probe Hs-CDKN2A	Advanced Cell Diagnostics	310181
RNAscope Target Retrieval Reagents	Advanced Cell Diagnostics	322000
RNAscope Wash Buffer Reagents	Advanced Cell Diagnostics	310091
Roche Blocking reagent	Roche	11096176001
Sodium Chloride	Sigma-Aldrich	S7653
Sodium Citrate	Sigma-Aldrich	W302600
Tris	Sigma-Aldrich	T1503
Wheat Germ Agglutinin (647nm)	Thermo Fisher Scientific	W11261

Supplementary Table 3. Stock solutions

Solution	Components
2x Saline Sodium Citrate (SSC) Buffer (pH 7.0)	17.532g sodium chloride (0.3M) 8.823g sodium citrate (0.03M) 1L distilled water
4% Paraformaldehyde	40g paraformaldehyde 1L PBS
Citric Acid Buffer (0.1M, pH 6.0)	29.41g of trisodium citrate 1L distilled water
Tris-EDTA Buffer (1M, pH 7.0)	15.759g Tris (0.1M, pH 8.0) 2.92g EDTA (0.01M, pH 8.0) 1L distilled water
FISH Wash Buffer	70ml formamide 30ml 2x SSC
ImmunoFISH Hybridisation Buffer	2.5µl Tris (1M, pH 7.2) 21.4µl magnesium chloride buffer 175µl deionised formamide 1µl Cy-3-labelled telomere specific (CCCTAA) peptide nuclei acid probe 12.5µl blocking reagent (2µl 10x blocking reagent (Roche) in 18µl malic acid (pH 7.5)) 33.6µl distilled water
Magnesium Chloride Buffer (pH 7.0)	0.119g magnesium chloride 86.445mg citric acid 0.582g hydrogen phosphate 50 ml distilled water Maleic acid buffer 2.901g maleic acid 250ml deionised water
Malic Acid Buffer (pH 7.5)	100mM malic acid 150mM NaCl in distilled water
Roche Blocking Reagent	150mM NaCl in distilled water

Supplementary Table 4. Antibodies[a] and dilutions

Primary Antibodies					**Secondary Antibodies**		**Tertiary Antibodies/Development Kit**	
Antigen	Supplier		Species	Dilution	Antibody	Dilution	Antibody	Dilution
γH2A.X (S139)	Cell Signalling	9718S	Rabbit	1:250	Goat anti-rabbit, biotinylated (BA-1000)	1:200	DCS Fluorescein (Vector Labs)	1:500
HMGB1	Abcam	ab18526	Rabbit	1:500	Goat anti-rabbit, Alexa Fluor 594 (ab150080)	1:500		
Lamin B1	Abcam	ab16048	Rabbit	1:500	Goat anti-rabbit, Alexa Fluor 488 (ab150077)	1:500		

[a]All antibodies polyclonal unless otherwise stated.

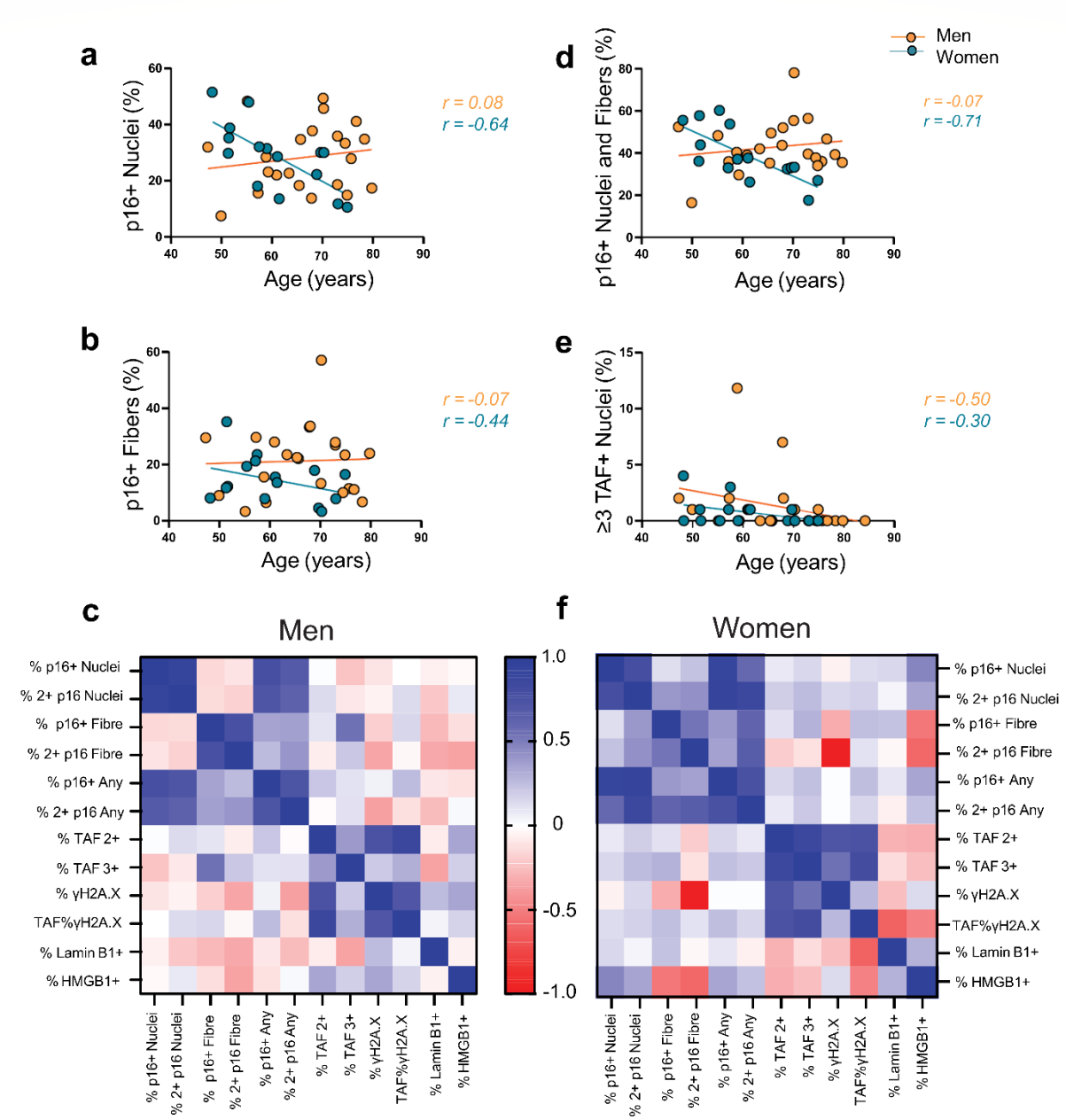
Supplementary Table 5. Quantification of additional cellular senescence markers in skeletal muscle in men and women in the MASS_Lifecourse Study

Cellular senescence marker[a]	**Abbreviation**	**Men**	**Women**	***P* value**[b]
Percentage of nuclear foci positive for p16 (%)	%p16+Nuclei	27.6 (17.8, 35.4)	30 (23.8, 34.4)	0.61
Percentage of fibre foci positive for p16 (%)	%p16+Fibre	22.5 (11.3, 28.0)	12.9 (7.9, 19.0)	0.09
Percentage of any foci positive for p16 (%)	%p16+Any	39.5 (35.7, 48.9)	36.6 (33.0, 51.3)	0.49
Percentage positive for γH2A.X (%)	% γH2A.X	54.0 (47.0, 65.5)	52.0 (46.0, 58.5)	0.39

[a]Values shown are median (interquartile range, IQR) of within participant value.
[b]Differences between men and women analysed using Wilcoxon rank-sum tests.

Appendix 3

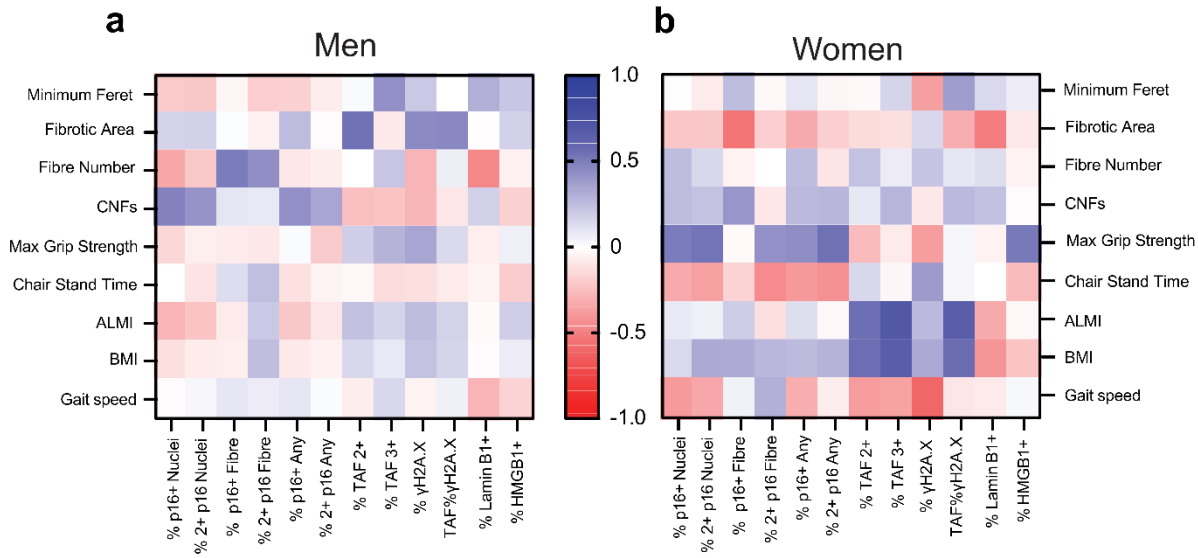
Supplementary Figures and Figure Legends



Supplementary Figure 1. Changes in cellular senescence marker expression in skeletal muscle with age and associations between senescence markers in men and women in the MASS_Lifecourse Study.

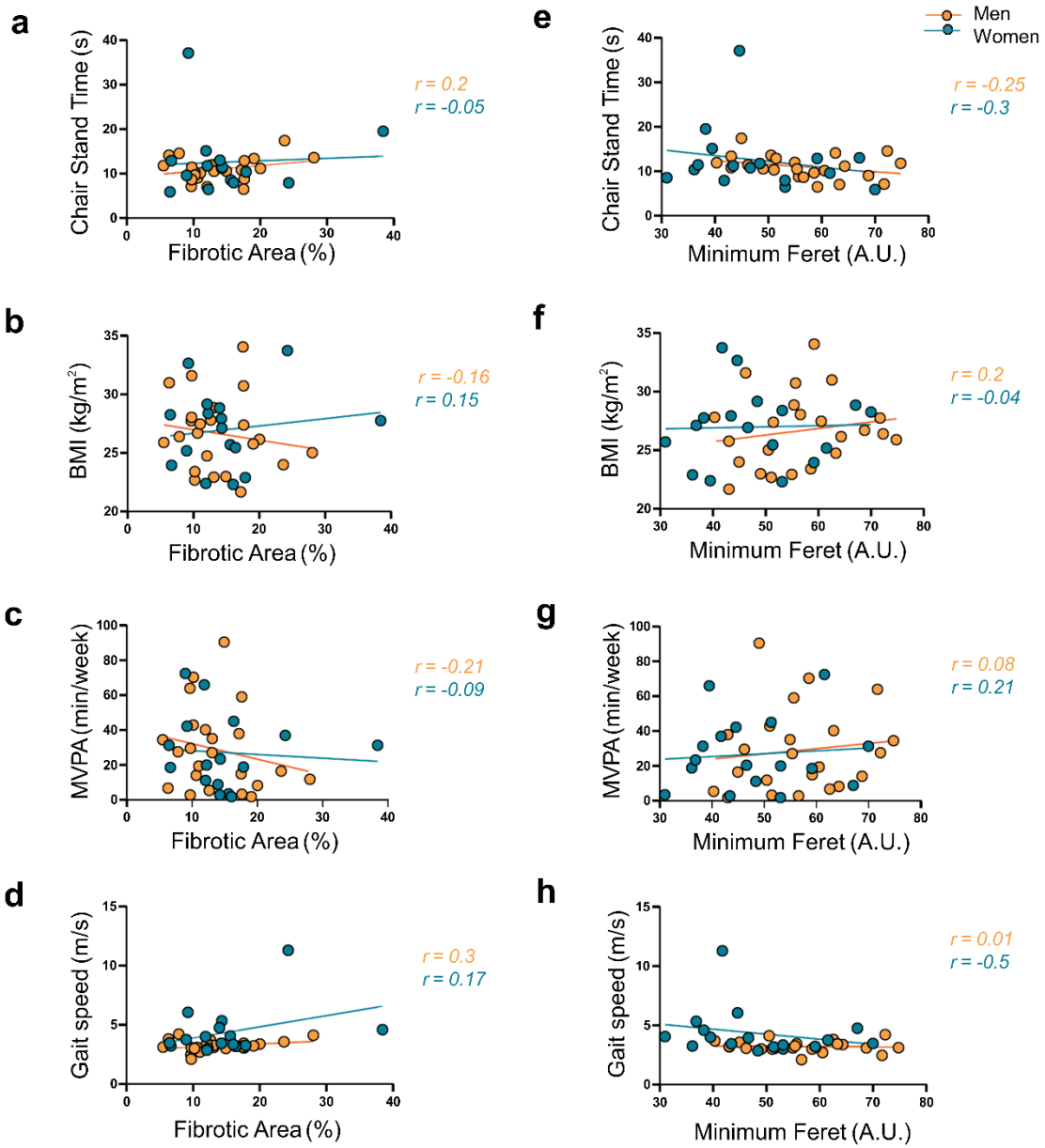
Graphs portraying changes in (**a**) percentage *p16* positive nuclei, (**b**) percentage *p16* positive fibers, (**d**) percentage *p16* positive fibers and nuclei, and (**e**) percentage nuclei positive for at least 3 TAF. Heatmaps portraying correlation coefficients between senescence markers in middle-aged and old (**c**) men and (**f**) women in the

MASS_Lifecourse Study. Correlations were examined using Spearman's correlation test. Regression lines are presented in orange for men, and in teal for women. Graphs and heatmaps were generated in Prism 9.0.



Supplementary Figure 2. Associations between markers of cellular senescence, morphological characteristic, and physical function with age in men and women in the MASS_Lifecourse Study

Heatmaps portraying correlation coefficients between senescence markers, muscle function, and indicators of muscle ageing in middle-aged and old (**a**) men and (**b**) women in the MASS_Lifecourse Study. Correlations were determined using Spearman's correlation test. Heatmaps were generated in Prism 9.0.



Supplementary Figure 3. Associations between morphological characteristics and physical function with age in men and women in the MASS_Lifecourse Study.

Graphs portraying correlations between percentage fibrotic area and (**a**) 5-chair stand time, (**b**) body mass index (BMI), (**c**) moderate to vigorous physical activity (MVPA; daily minutes), and (**d**) gait speed (meters per second). Graphs portraying correlations between minimum feret (fibre size) and (**e**) 5-chair stand time, (**f**) body mass index (BMI), (**g**) moderate to vigorous physical activity (MVPA; daily minutes), and (**h**) gait speed (meters per second). Correlations were determined using Spearman's correlation test. Graphs were generated in Prism 9.0.

Supplementary References

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