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Gene polymorphisms associated with heterogeneity and senescence characteristics of sarcopenia in chronic obstructive pulmonary disease

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Abstract

Background Sarcopenia, or loss of skeletal muscle mass and decreased contractile strength, contributes to morbidity and mortality in patients with chronic obstructive pulmonary disease (COPD). The severity of sarcopenia in COPD is variable, and there are limited data to explain phenotype heterogeneity. Others have shown that COPD patients with sarcopenia have several hallmarks of cellular senescence, a potential mechanism of primary (age-related) sarcopenia. We tested if genetic contributors explain the variability in sarcopenic phenotype and accelerated senescence in COPD.

Methods To identify gene variants [single nucleotide polymorphisms (SNPs)] associated with sarcopenia in COPD, we performed a genome-wide association study (GWAS) of fat free mass index (FFMI) in 32 426 non-Hispanic White (NHW) UK Biobank participants with COPD. Several SNPs within the fat mass and obesity-associated (*FTO*) gene were associated with sarcopenia that were validated in an independent COPDGene cohort (n = 3656). Leucocyte telomere length quantified in the UK Biobank cohort was used as a marker of senescence. Experimental validation was done by genetic depletion of FTO in murine skeletal myotubes exposed to prolonged intermittent hypoxia or chronic hypoxia because hypoxia contributes to sarcopenia in COPD. Molecular biomarkers for senescence were also quantified with FTO depletion in murine myotubes.

Results Multiple SNPs located in the *FTO* gene were associated with sarcopenia in addition to novel SNPs both within and in proximity to the gene *AC090771.2,* which transcribes long non-coding RNA (lncRNA). To replicate our findings, we performed a GWAS of FFMI in NHW subjects from COPDGene. The SNP most significantly associated with FFMI was on chromosome (chr) 16, rs1558902A > T in the *FTO* gene ($\beta = 0.151$, SE = 0.021, $P = 1.40 \times 10^{-12}$ for UK Biobank $|\beta = 0.220$, SE = 0.041, $P = 9.99 \times 10^{-8}$ for COPDGene) and chr 18 SNP rs11664369C > T nearest to the *AC090771.2* gene ($\beta = 0.129$, SE = 0.024, $P = 4.64 \times 10^{-8}$ for UK Biobank $|\beta = 0.203$, SE = 0.045, $P = 6.38 \times 10^{-6}$ for COPDGene). Lower handgrip strength, a measure of muscle strength, but not FFMI was associated with reduced telomere length in the UK Biobank. Experimentally, in vitro knockdown of *FTO* lowered myotube diameter and induced a senescence-associated molecular phenotype, which was worsened by prolonged intermittent hypoxia and chronic hypoxia.

Conclusions Genetic polymorphisms of *FTO* and *AC090771.2* were associated with sarcopenia in COPD in independent cohorts. Knockdown of *FTO* in murine myotubes caused a molecular phenotype consistent with senescence that was exacerbated by hypoxia, a common condition in COPD. Genetic variation may interact with hypoxia and contribute to variable severity of sarcopenia and skeletal muscle molecular senescence phenotype in COPD.

Keywords Prolonged intermittent hypoxia; Genetic variability; Sarcopenia in COPD; Fat mass and obesity gene; Murine myotubes; Gene knockout

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Introduction

Sarcopenia is common in patients with COPD and is associated with poor quality of life and mortality.[1] The reported prevalence of sarcopenia in COPD ranges from 7.9 to 66.7% and is influenced by a number of risk factors, including severity of lung disease and clinical setting (i.e. more prevalent in nursing homes).[1] However, variability in prevalence and severity of sarcopenia in COPD may also be due to an interaction between environmental and endogenous (i.e. genetic) factors. Importantly, genetic variability can explain differential responses to interventions for sarcopenia in COPD, including nutritional supplementation and pulmonary rehabilitation,[2] emphasizing the need for personalized therapeutic interventions.

An estimated 55–80% of variation in body mass index (BMI) is believed to be related to genetic factors.[3] Within COPD, genetic association studies have been performed with BMI and fat free mass index (FFMI) as continuous traits.[4] However, such studies have limited generalizability to sarcopenia that includes both the loss of skeletal muscle mass and strength. As such, strategies for defining sarcopenia include limb lean mass normalized to height or appendicular skeletal muscle index (ASMI), basal metabolic rate (BMR) and handgrip strength (HGS), which measures skeletal muscle strength. Given that significant peripheral skeletal muscle loss occurs in COPD, measures that incorporate limb lean mass like ASMI have been identified as highly relevant to defining sarcopenia for this population.[5] BMR has been associated with exercise capacity,[6] a negative contributor to progression of sarcopenia. Although hypermetabolism with increased BMR is frequent in early stages of COPD,[7] reduction in BMR is associated with disease progression, weight loss and sarcopenia.[8] At the molecular level, COPD patients with sarcopenia have several hallmarks of cellular senescence, which include cell-cycle arrest that contributes to accelerated ageing.[9] Whether genetic susceptibility to sarcopenia is associated with an increased risk for cellular senescence in the skeletal muscle of COPD patients is currently unknown. In the present studies, we tested whether genetic variants were associated with sarcopenia using multiple measures (i.e. FFMI, ASMI, BMR, HGS) in COPD patients.

Previous smaller studies of BMI and FFMI in COPD have identified associations of genetic variants in the first intron of the *FTO* gene,[4] although this study did not incorporate skeletal muscle strength (HGS) or BMR, analysed a limited number of SNPs and did not account for hypoxia responses. *FTO* was one of the first genes identified as a locus for adult and childhood obesity in a genome-wide association study (GWAS) of type 2 diabetes mellitus and has been extensively studied in relation to obesity.[10] Others have noted that FTO is necessary for myogenic differentiation[11] and mitochondrial biogenesis in skeletal muscle cells.[12] The protein product of the *FTO* gene regulates epitranscriptomic modifications of RNA.[13] During hypoxia, a common condition in respiratory diseases including COPD, FTO protein levels are lower.[14] Moreover, lower functional FTO protein product has been linked to an early senescence phenotype in cultured fibroblasts.[15] Previous studies have also noted that the first intron of the *FTO* gene directly interacts with the promoter of *IRX3* (Iroquois Homeobox 3) and that certain *FTO* genetic risk variants modulate *IRX3* expression to impact body mass and composition.[16] In particular, IRX3-deficient mice demonstrate loss of fat mass and increased basal metabolic rate with browning of white adipose tissue.[16]

To further investigate the role of genetic variants of sarcopenia in COPD, we conducted a genome-wide association analysis of 32 426 COPD subjects utilizing data from the UK Biobank analysing FFMI both as a continuous variable and as a categorical variable defining sarcopenia as an FFMI $\leq$17.4 kg/m^2 for males, $\leq$15 kg/m^2 for females.[17] For FFMI-associated genetic variants, we replicated our findings in an independent cohort of 3656 subjects from COPDGene. We found multiple SNPs located in the *FTO* gene associated with sarcopenia and discovered a novel association nearest to or within the AC090771.2 gene [which transcribes long non-coding RNA (lncRNA)]. To determine if other anthropometric phenotypes were associated with FTO or AC090771.2 genes, a phenome wide association study (PheWAS) for *FTO* and *AC090771.2* in the UK Biobank showed a number of additional associations with body composition. We then depleted *FTO* in murine skeletal myotubes and noted lower myotube diameter and a senescence-like molecular phenotype. This was worsened by prolonged intermittent hypoxia (PIH) or chronic hypoxia (CH), which is consistent with the oxygen sensitivity of FTO. Leucocyte telomere length in COPD, which may be associated with skeletal muscle senescence, was associated with HGS-defined sarcopenia in the UK Biobank cohort. Our studies lay the foundation for genetic

contributors to sarcopenia in COPD that can explain heterogeneity in clinical presentations in these patients.

Materials and methods

Details of the UK Biobank have been reported previously. In brief, the UK Biobank is a prospective cohort of 502 536 participants, age 37–73 years, across the United Kingdom. In our analysis, only non-Hispanic white participants (NHW) were included because other races represented ~2.4% (n = 782) of the UK biobank with COPD.

COPDGene is a large National Institutes of Health-funded multicentre study that enrolled ever-smokers with and without COPD, aged 45–80 years, with at least 10 pack-years of smoking history. Our replicative cohort consisted of NHW from COPDGene, which made up the majority of the population (~76.9%).

Access to both datasets was obtained, and analyses were performed after obtaining approval from the Institutional Review Board at the Cleveland Clinic (IRB #20-446). Details on phenotype and definitions of sarcopenia are included in *Data* S1.

Genomic analysis

GWAS were performed with binary traits using logistic regression for FFMI-defined sarcopenia and with continuous traits (FFMI, ASMI, HGS, BMR) using linear regression with sarcopenia as the response variables using PLINK V2.0 genetic analysis software. An additive model for each SNP was adjusted for age, sex, smoking status (never, past and current) and the first 10 principal components (PCs) from the UK Biobank cohort of participants with COPD. Linear regression analysis for CT-derived FFMI was adjusted for age, sex, smoking status and the first 10 PCs from COPDGene. The plots and the descriptive statistical analysis were performed using R version 4.2.0 (R Project for Statistical Computing, Vienna, Austria). Genome-wide significance was defined as previously described (P value $< 5 \times 10^{-8}$).[4] For our replication cohort, the P value for top SNPs for each locus using false discovery rate was P = 0.007. Effect size was compared between the same SNPs (rs1421085, rs1558902) in each cohort by calculating a z-score and multiplying by two times the normalized P value. Details of quality control, leucocyte telomere length, LocusZoom, PheWAS and eQTL are included in *Data S1*.

Experimental validation

Myotube cultures

All studies were performed in differentiated murine C2C12 myotubes as previously reported.[18] In brief, myoblasts (ATCC, Manassas, VA) were grown to near confluence in proliferation medium (Dulbecco's Modified Eagle Medium (DMEM), with 10% foetal calf serum) followed by differentiation medium (DMEM with 2% horse serum) for 48 h.

Genetic depletion of FTO gene

To determine if sarcopenia is a phenotype of decreased *FTO* gene expression, gene knockdown studies were performed in C2C12 myotubes as previously described.[19] C2C12 myoblasts were transfected with FTO shRNA or empty vector (shRandom) followed by differentiation to myotubes. Depletion efficiency was determined by immunoblots for expression of FTO.

Hypoxia responses

Hypoxia is a known contributor to sarcopenia in COPD. We therefore exposed C2C12 myotubes to either normoxia (21% oxygen), PIH (defined as 8 h of 1% oxygen followed by 16 h of 21% oxygen to re-create a model of nocturnal hypoxemia) or CH (1% oxygen) for a total of 72 h as reported earlier.[18] Details on immunoblots, in vitro telomere length and senescence associated molecular phenotype are included in *Data S1*.

Additional statistical analyses (other than genomic analyses)

Categorical variables were compared using chi square test and expressed as proportions or ratios. Quantitative variables were expressed as mean ± standard deviation (SD) and compared using analysis of variance, Student's t-test or appropriate non-parametric tests when normality assumptions were not met. Interaction terms were included between *FTO* genetic variants and smoking status as the dependent variables of sarcopenia. Unless stated otherwise, all statistical analyses were conducted with R, version 4.2.0 (R Project for Statistical Computing, Vienna, Austria).

Results

Baseline characteristics of COPD subjects from the UK Biobank are presented in *Table 1*. Of the total number of COPD subjects (n = 32 426), those with low FFMI-defined sarcopenia represented 9.8% (n = 3181) of the cohort. Subjects in the UK Biobank with COPD had an average FEV1% predicted of 80.0 ± 20.6% for those without sarcopenia and 76.4 ± 21.3% for those with sarcopenia. Patients with sarcopenia were more likely to be female (59.5% vs. 42.5%, $P < 0.001$) and to be current smokers (53.1% vs. 32.9%, $P < 0.001$), but the total pack-years of smoking was similar (34.1 ± 20.8 vs. 34.4 ± 21.3 pack-years, P = 0.524) between the two groups. The average FFMI in subjects without sarcopenia was 19.1 ± 2.5 kg/m^2 and for those with sarcopenia was 15.2 ± 1.2 kg/m^2. Severity of COPD defined by GOLD stages in the UK Biobank cohort showed that 92.7% of pa-

Table 1 Baseline characteristics of COPD subjects in the UK Biobank

	No sarcopenia, n = 29 245	Sarcopenia, n = 3181	P value
Age (mean (SD))	59.6 (7.2)	59.9 (7.0)	0.014
Number of female sex (%)	12 431 (42.5)	1894 (59.5)	<0.001
Number of self-reported medical conditions (non-cancer)	2.8 (2.4)	2.6 (2.2)	<0.001
Overall health rating (mean (SD))	2.4 (0.9)	2.5 (0.9)	<0.001
FEV1 per cent predicted (mean (SD))	80.0 (20.6)	76.4 (21.3)	<0.001
FVC per cent predicted (mean (SD))	94.9 (29.7)	94.81 (33.8)	0.843
FEV1/FVC ratio (mean (SD))	0.66 (0.08)	0.63 (0.09)	<0.001
Body mass index (BMI)	28.2 (4.74)	21.3 (2.3)	<0.001
Fat-free mass index (mean (SD))	19.1 (2.47)	15.2 (1.2)	<0.001
ASMI (mean (SD))	11.7 (1.8)	9.1 (0.8)	<0.001
Handgrip strength (mean (SD))	31.3 (11.2)	25.8 (9.2)	<0.001
Basal metabolic rate (mean (SD))	6855.7 (1345.8)	5358.3 (730.9)	<0.001
Summed MET minutes per week for all activity	2841.9 (3069.1)	2602.3 (2937.5)	<0.001
GOLD stage (%)			<0.001[a]
Stage 1 (FEV1 > 80%, FEV1/FVC < 0.7)	9871 (43.0)	1007 (40.2)	
Stage 2 (50% < = FEV1 < 80%, FEV1/FVC < 0.7)	11 404 (49.7)	1188 (47.4)	
Stage 3 (30% < = FEV1 < 50%, FEV1/FVC < 0.7)	1470 (6.4)	251 (10.0)	
Stage 4 (FEV1 < 30%, FEV1/FVC < 0.7)	197 (0.9)	59 (2.4)	
Smoking status (%)			<0.001[a]
Never	3391 (11.6)	324 (10.2)	
Previous	16 188 (55.4)	1163 (36.6)	
Current	9596 (32.8)	1686 (53.0)	
Pack-years of smoking (mean (SD))	34.4 (21.3)	34.1 (20.8)	0.524

ASMI, appendicular skeletal muscle index; MET, metabolic equivalent minutes.
Handgrip strength represents the average of the right and left hand in kilograms. FFMI, ASMI and BMI were in kg/m^2. Basal metabolic rate was measured in kJ. Overall health rating was a questionnaire ranging from 1 to 4 with 1 representing *excellent health*, 2 representing *good health*, 3 representing *fair health* and 4 representing *poor health*.
[a]*P* values that are starred represent ANOVA analysis; otherwise, *P* values represent *t*-tests for quantitative variables and chi square for categorical variables.

Table 2 Top loci from genome-wide association results for sarcopenia defined by fat-free mass index from the UK Biobank

Rank	CHR	BP	ID	REF	ALT	OBS_CT	BETA	SE	TSTAT	P	Closest gene	Type
1	16	53806453	rs56094641	A	G	31338	0.849	0.028	−5.817	**5.99×10^{-9}**	FTO	Intronic
2	11	36137453	rs12276510	G	A	30921	1.336	0.056	5.148	2.63×10^{-7}	LDLRAD3	Intronic
3	18	57758855	rs35386941	G	A	31334	0.874	0.029	−4.617	3.89×10^{-6}	AC090771.2	Intergenic

ALT, alternative allele; BETA, effect size of alternative allele; BP, variant position; CHR, chromosome number; ID, variant identifier of allele; OBS_CT, number of individuals with non-missing data; *P, P* value of association between alternative allele and sarcopenia defined by low fat-free mass index (<17.4 kg/m^2 for males and <15 kg/m^2 for females); REF, reference genome sequence allele; SE, standard error of alternative allele; T_STAT, t-statistic.
All models adjusted for sex, smoking status, genotype measurement batch and principal components analysis that were statistically significant (1–10). SNPs that were genome-wide significant are bolded and are defined as a *P* value $< 5 \times 10^{-8}$.

tients with sarcopenia and 87.6% of those without sarcopenia were either stage 1 (mild) or stage 2 (moderate) disease.

On anthropometric evaluation, the sarcopenic UK biobank cohort had lower muscle mass, strength and metabolic activity compared with those without sarcopenia: ASMI (9.1 ± 0.8 vs. 11.7 ± 1.8 kg/m^2, $P < 0.001$); HGS (25.8 ± 9.2 vs. 31.3 ± 11.2 kg, $P < 0.001$); lower BMR (5358.3 ± 730.9 vs. 6855.7 ± 1345.8 kJ, $P < 0.001$); and lower total metabolic minutes per week of activity (2602.3 ± 2937.5 vs. 2841.9 ± 3069.1 min/week, $P < 0.001$). Patients with sarcopenia by various definitions (*Table S1*) had higher mortality: FFMI (8.7% vs. 4.7%, $P < 0.001$); ASMI (7.4% vs. 4.1%, $P < 0.001$); HGS (8.2% vs. 4.5%, $P < 0.001$); and BMI (11.3% vs. 4.9%, $P < 0.001$).

Genome-wide association analysis of the UK Biobank COPD cohort was performed using logistic regression with sarcopenia defined by low FFMI (≤17.4 kg/m^2 for males and ≤15 kg/m^2 for females).[17] The most significant associations (*Table 2*; **Manhattan plot:** *Figure 1A*) were with SNP (rs56094641A > G) located in the fat mass and obesity-associated (*FTO*) gene on chromosome 16 (OR 2.34 [95% CI 2.21–2.47], SE 0.028, $P = 5.99 \times 10^{-9}$). We then performed a genome-wide association analysis of the UK Biobank using FFMI as the continuous dependent variable. The most significant associations are shown in *Table 3*, and Manhattan plot is shown in *Figure 1B*. Several of the associations reached genome-wide significance with FFMI in COPD, including SNP (rs7188250T > C) located in the *FTO* gene

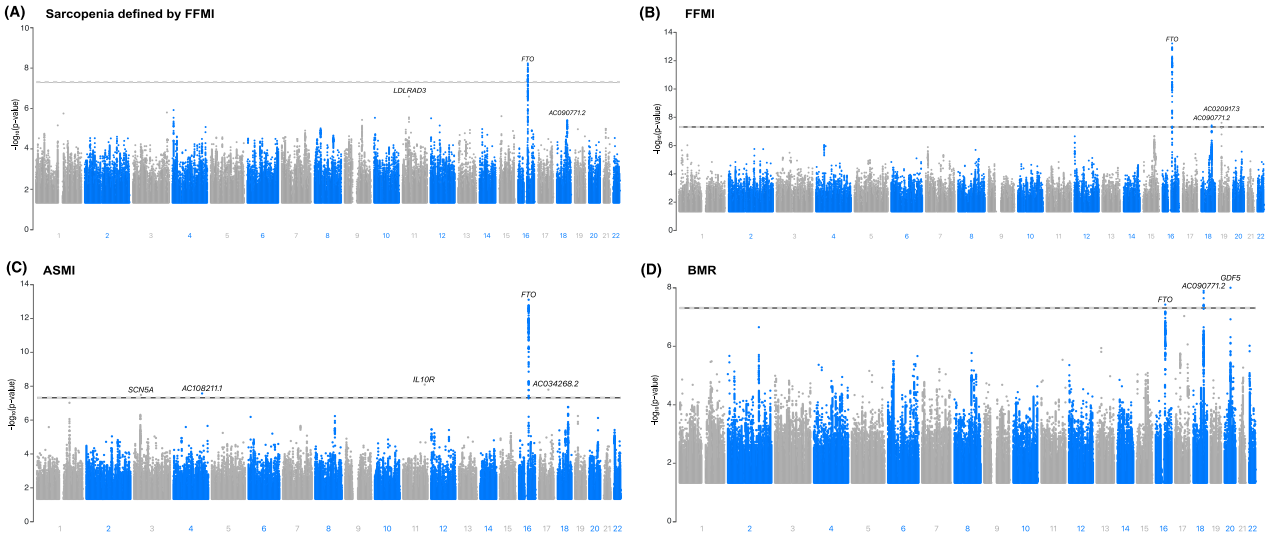


Figure 1 Manhattan plots of genes associated with sarcopenia in the UK Biobank. (A) Manhattan plot of genes associated with sarcopenia (defined by FFMI) in the UK Biobank cohort of COPD subjects. Manhattan plot showing *P* values for SNPs analysed in the UK Biobank cohort of COPD subjects and fat-free mass index (FFMI). Gene names are identified. The grey dashed line indicates the threshold for genome-wide significance (*P* value $< 5 \times 10^{-8}$). (B) Manhattan plot of genes associated with FFMI in the UK Biobank cohort of COPD subjects. Manhattan plot showing *P* values for SNPs analysed in the UK Biobank cohort of COPD subjects and fat-free mass index (FFMI). Linear regression was performed. Gene names are identified. The grey dashed line indicates the threshold for genome-wide significance (*P* value $< 5 \times 10^{-8}$). (C) Manhattan plot of genes associated with ASMI in the UK Biobank cohort of COPD subjects. Manhattan plot showing *P* values for SNPs analysed in the UK Biobank cohort of COPD subjects and appendicular skeletal muscle index (ASMI). Linear regression was performed. Gene names are identified. The grey dashed line indicates the threshold for genome-wide significance (*P* value $< 5 \times 10^{-8}$). (D) Manhattan plot of genes associated with basal metabolic rate in the UK Biobank cohort of COPD subjects. Manhattan plot showing *P* values for SNPs analysed in the UK Biobank cohort of COPD subjects and basal metabolic rate. Linear regression was performed. Gene names are identified. The grey dashed line indicates the threshold for genome-wide significance (*P* value $< 5 \times 10^{-8}$).

Table 3 Top loci from genome-wide association results for fat-free mass index from the UK Biobank

Rank	CHR	BP	ID	REF	ALT	OBS_CT	BETA	SE	TSTAT	P	Closest gene	Type
1	16	53834607	rs7188250	T	C	31302	0.159	0.021	7.506	**6.24×10^{-14}**	FTO	Intronic
2	19	16455773	rs17642401	A	G	31359	0.152	0.027	5.572	**2.53×10^{-8}**	AC020917.3	Intergenic
3	18	57739284	rs72323282	AATTT	A	31151	0.130	0.024	5.499	**3.86×10^{-8}**	AC090771.2	Intergenic
4	15	78857986	rs55781567	C	G	31359	−0.113	0.022	−5.186	2.16×10^{-7}	CHRNA5	5′ UTR
5	12	3201029	rs7980247	T	A	31236	0.108	0.021	5.175	2.30×10^{-7}	TSPAN9	Intronic
6	4	45186139	rs10938398	G	A	31284	0.104	0.021	4.902	9.52×10^{-7}	AC108467.1	Intergenic
7	1	45277021	rs11582453	G	T	30999	0.181	0.037	4.897	9.78×10^{-7}	BTBD19	Intronic

5′ UTR, 5′ untranslated region or the region of an mRNA that is directly upstream from the initiation codon; ALT, alternative allele; BETA, effect size of alternative allele; SE, standard error of alternative allele; BP, variant position; ID, variant identifier of allele; CHR, chromosome number; OBS_CT, number of individuals with non-missing data; *P*, *P* value of association between alternative allele and fat free mass index as the continuous dependent variable; REF, reference genome sequence allele; T_STAT, *t*-statistic.
All models adjusted for sex, smoking status, genotype measurement batch and principal components analysis that were statistically significant (1–10). SNPs that were genome-wide significant are bolded and are defined as a *P* value $< 5 \times 10^{-8}$.

(β = 0.159, SE = 0.021, $p = 6.24\text{x}10^{-14}$). Other associations with FFMI in COPD included an intergenic SNP (rs1764240A > T; β = 0.152, SE = 0.027, $P = 2.53 \times 10^{-8}$) on chromosome 19 near *AC020917.3*, followed by an intergenic SNP (rs72323282AATTT > A; β = 0.130, SE = 0.024, $P = 3.86 \times 10^{-8}$) on chromosome 18 near *AC090771.2*. Other SNPs that were associated with FFMI but did not achieve genome-wide significance are presented in *Table 3*.

As ASMI is considered to be better than FFMI as a measure of muscle mass,[20] we performed a genome-wide association analysis with ASMI as the continuous dependent variable. The most significant associations are shown in *Table S2* and *Figure 1C*. Several associations reached genome-wide significance, including an SNP located in the *FTO* gene (rs56094641A > G; β = 0.092, SE = 0.012, $P = 8.05 \times 10^{-14}$) and several intergenic SNPs (rs139954366A > AATTTATTT near *IL10R [interleukin 10 receptor]* on CHR 2, β = −8.723, SE = 1.514, $P = 8.31 \times 10^{-9}$; rs12942047A > G near *AC034268*.2 on CHR 17, β = −8.528, SE = 1.510, $P = 1.65 \times 10^{-8}$; and rs4129759C > A near *AC108211.1* on CHR 4, β = −8.393, SE = 1.510, $P = 2.76 \times 10^{-8}$).

Because BMR correlates with exercise capacity,[6] we performed a genome-wide association of BMR as the continuous dependent variable (*Table S3* and *Figure 1D*). The most significant association was located on the *GDF5 (growth differentiation factor 5)* gene (rs143384G > A; β = 66.413, SE = 11.590, $P = 1.01 \times 10^{-8}$), followed by an intergenic SNP closest to *AC090771.2* (rs7231987G > T; β = 72.881, SE = 12.814, $P = 1.30 \times 10^{-8}$) and an intronic SNP located in the *FTO* gene (rs7188250T > C; β = 63.507, SE = 11.546, $P = 3.82 \times 10^{-8}$). We also performed GWAS of HGS as the continuous dependent variable and sarcopenia defined by combined low HGS and low FFMI. Even though none of the SNPs were significant at the genome-wide level, several were close to the $P = 5 \times 10^{-8}$ significance threshold for HGS (including intronic variants within genes *IFT88*, *FCSK*, *EFHB* and *DGKD*) and combined HGS/FFMI-defined sarcopenia (including an intron variant for *EPB41L4A* and intergenic variants closest to *RN7SL705P*, *HRH2*) (*Table S4* and *S5* and *Figure S1*). Locus zoom of the *FTO* gene was then performed for the UK Biobank cohort (FFMI), and associated SNPs showed a num-

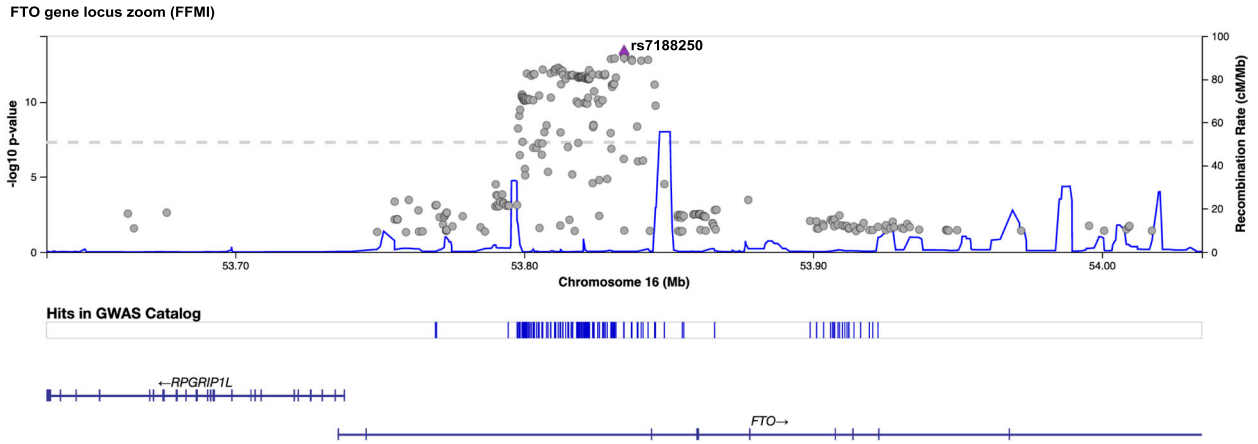


Figure 2 LocusZoom plot of the FTO gene from the UK Biobank. *P* values of SNPs associated with the FTO gene (log10 scale) for continuous variable FFMI from the UK Biobank. SNP rs7188250 is indicated with a purple arrow. Plot generated with LocusZoom (http://csg.sph.umich.edu/locuszoom/).

Table 4 Baseline characteristics of COPD subjects in COPDGene

	No sarcopenia, $n = 3070$	Sarcopenia, $n = 586$	*P* value
Age (mean (SD))	64.5 (8.0)	64.3 (8.5)	0.657
Number of female sex (%)	1224 (39.9)	359 (61.3)	<0.001
Health status (%)			0.160
Poor	231 (7.7)	53 (9.1)	
Fair	815 (26.5)	176 (30.1)	
Good	1302 (42.4)	228 (39.0)	
Very good	629 (20.5)	107 (18.3)	
Excellent	93 (3.0)	20 (3.4)	
FEV1 per cent predicted (mean (SD))	50.7 (17.3)	42.7 (18.2)	<0.001
FVC per cent predicted (mean (SD))	76.2 (16.7)	74.4 (17.9)	0.020
FEV1/FVC ratio (mean (SD))	0.50 (0.13)	0.42 (0.1)	<0.001
GOLD stage (%)			<0.001[a]
Stage 2 (50% < = FEV1 < 80%, FEV1/FVC < 0.7)	1708 (55.6)	218 (37.2)	
Stage 3 (30% < = FEV1 < 50%, FEV1/FVC < 0.7)	976 (31.8)	216 (36.9)	
Stage 4 (FEV1 < 30%, FEV1/FVC < 0.7)	386 (12.6)	152 (25.9)	
Waist circumference (mean (SD))	105.4 (14.3)	82.6 (11.5)	<0.001
Arm span circumference (mean (SD))	169.4 (10.8)	166.6 (17.1)	0.006
Pectoralis muscle CSA cm^2 (mean (SD))	37.2 (11.6)	27.4 (8.1)	<0.001
Fat free mass index (mean (SD))	19.2 (2.3)	15.1 (1.4)	<0.001
Not utilizing O_2 therapy (%)	977 (29.1)	226 (36.2)	<0.001
Pack-years of smoking (mean (SD))	56.1 (27.4)	53.5 (25.0)	0.031
Smoking status (%)			<0.001[a]
Current	930 (30.3)	229 (39.1)	
Former	2140 (69.7)	357 (60.9)	

[a]*P* values that are starred represent ANOVA analysis; otherwise, *P* values represent *t*-tests for quantitative variables and chi square for categorical variables. FFMI defined according to CT-based pectoralis muscle definition. Age represents the age that their visit to the study centre was performed. Waist circumference and arm span circumference were measured in centimetres.

ber of associations that were genome-wide significant from 53.80 to 53.85 Mb on chromosome 16 (*Figure 2*).

To validate the association of SNPs in the *FTO* gene and SNPs closest to *AC090771*.2 with FFMI, we performed analyses in an independent cohort of subjects from COPDGene. Baseline characteristics of patients from COPDGene are shown in *Table 4*. There were 3656 subjects with COPD, of which 586 (16.0%) had sarcopenia as defined by CT-derived fat-free mass index.[21] Similar to the UK Biobank, COPD participants with sarcopenia were more likely to be female (61.3% vs. 39.9%, $P < 0.001$) and to have a lower FEV1% (42.7 ± 18.2 vs. 50.7 ± 17.3, $P < 0.001$). Current smokers were also more likely to have sarcopenia in COPDGene (39.1% vs. 30.3%, $P < 0.001$). Pack-years of smoking were lower in subjects with sarcopenia (53.5 ± 25.0 vs. 56.1 ± 27.4, $P = 0.031$) in COPDGene but not in the UK Biobank cohort. Even though measures of sarcopenia in the two cohorts studied are different, we used currently accepted definitions to allow for validation of the observations from the UK Biobank cohort in the COPDGene cohort.

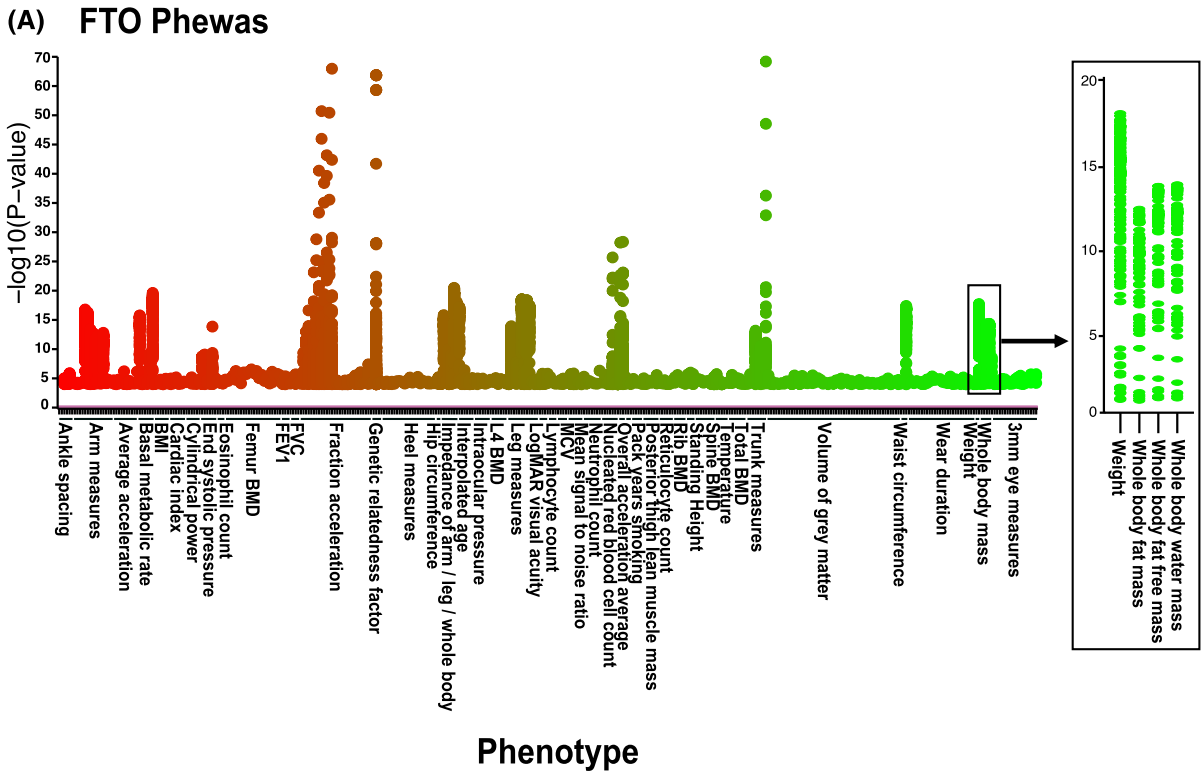
Multiple SNPs from the *FTO* gene that were associated with FFMI as a continuous variable in the UK Biobank cohort were replicated in COPDGene cohort (*Table S6*). SNPs most significant in the COPDGene cohort included rs1421085T > C ($\beta = 0.208$, SE = 0.041, $P = 1.26 \times 10^{-12}$ in UK Biobank |$\beta = 0.151$, SE = 0.021, $P = 3.68 \times 10^{-7}$ in COPDGene) and rs1558902T > A ($\beta = 0.220$, SE = 0.041, $P = 1.40 \times 10^{-12}$ in UK Biobank |$\beta = 0.151$, SE = 0.021, $P = 9.99 \times 10^{-8}$ in COPDGene). There was no significant difference in effect size between the UK Biobank cohort and the COPDGene cohort for SNPs rs1421085T > C ($P = 0.990$) or rs1558902T > A ($P = 0.134$). Given that SNP rs1558902T > A was most significantly associated with FFMI in the UK Biobank and COPDGene cohorts, clinical features and anthropometric measures from the UK Biobank for the SNP were evaluated and showed that the TT genotype of rs1558902 was associated with lower FFMI, ASMI and BMR (*Table S7*). Similar findings were observed with the TT genotype in SNP rs1421085 (*Table S8*). SNPs from chromosome 18 in the *AC090771*.2 gene that were associated with FFMI as a continuous variable in the UK Biobank cohort were also associated with FFMI in COPDGene (*Table S9*). The SNP rs11664369C > T from the UK Biobank and COPDGene cohorts was most associated with FFMI ($\beta = 0.129$, SE = 0.024, $P = 4.64 \times 10^{-8}$ in UK Biobank|$\beta = 0.203$, SE = 0.045, $P = 6.38 \times 10^{-6}$ in COPDGene).

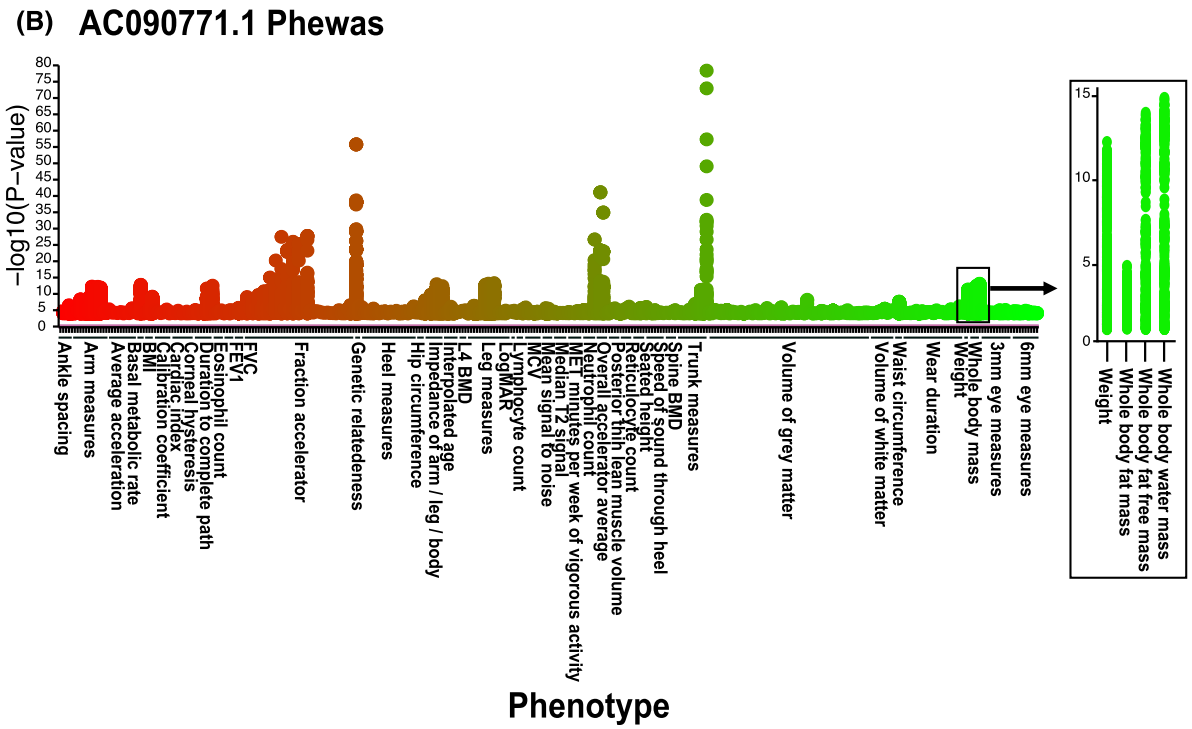
Because current smokers and those with FTO polymorphisms were more likely to have sarcopenia in the UK Biobank, we performed interaction analyses to determine whether the effect of one covariate was dependent on the other covariate. Our analysis of top SNPs from the FTO gene (*Table S6*) did not demonstrate significant interactions between current smokers and FTO polymorphisms (*Table S10*), although smoking status and the FTO polymorphisms were individually associated with sarcopenia.

We then examined additional phenotypes associated with the *FTO* and *AC090771.2* genes using complementary PheWAS analysis (*Figure 3A and Figure 3B*) in the UK Biobank. Anthropometric measures that were found to be associated with these genes included whole-body FFM and arm/leg FFM after adjustment for multiple testing using the false discovery rate method. Additional phenotypes of body composition and muscle function that were significantly associated with the identified SNPs included hip circumference and activity measures (see Methods section).

Loss of FTO functional protein in mice is associated with reductions in lean body mass.[22] We analysed publicly available GTEx data, which showed that the genetic variant for FTO (rs1421085) that was most associated with sarcopenia in the UK Biobank and COPDGene cohorts alters expression of FTO in skeletal muscle (*Figure S2A*). Expression of FTO varied based on the genotype and was lowest in the TT genotype (*Figure S2B*). We then determined whether depletion of *FTO* results in a sarcopenic phenotype in murine skeletal muscle myotubes. The protein product of *FTO* is sensitive to cellular oxygen concentrations, and previous studies have demonstrated that hypoxia lowers *FTO* expression.[23] To determine a causal link between FTO expression and myotube responses, we evaluated the impact of PIH and CH[18] on myotube diameter in controls and myotubes with genetic depletion of FTO. Our studies showed that myotubes exposed to PIH and CH had lower myotube diameter, which was worsened by FTO depletion (*Figure 4*). FTO knockdown also causes a senescence-like phenotype,[15] which has been linked to sarcopenia of ageing. We therefore probed for previously reported markers of post-mitotic senescence in myotubes.[24] We noted increased expression of known molecular markers of senescence including P16, P21 and phospho-P53 in FTO knockdown cells as compared with controls. We also observed increased senescence associated β-galactosidase activity in FTO knockdown cells consistent with a senescence-like phenotype in myotubes (*Figure 4*). The first intron of the *FTO* gene interacts with the *IRX3* gene, which influences body mass and composition.[16] We therefore probed for IRX3 expression in myotubes with FTO knockdown. We found no significant change in IRX3 expression during normoxia but a significant decline of IRX3 with hypoxia (both PIH and CH) in myotubes with FTO knockdown (*Figure 4*).

Sarcopenia in chronic diseases mimics a senescence-related phenotype, and replicative senescence has been associated with reductions in telomere length.[25] We performed complementary analyses of C2C12 cells exposed to PIH and CH (*Figure S3*). Our findings demonstrated no significant difference in telomere length for myotubes exposed to PIH when compared with normoxia, whereas CH demonstrated increased telomere length. We then analysed


(A) FTO Phewas
−log10(P−value)
Phenotype
Ankle spacing
Arm measures
Average acceleration
Basal metabolic rate
BMI
Cardiac index
Cylindrical power
End systolic pressure
Eosinophil count
Femur BMD
FEV1
FVC
Fraction acceleration
Genetic relatedness factor
Heel measures
Hip circumference
Impedance of arm / leg / whole body
Interpolated age
Intraocular pressure
L4 BMD
Leg measures
LogMAR visual acuity
Lymphocyte count
MCV
Mean signal to noise ratio
Neutrophil count
Nucleated red blood cell count
Overall acceleration average
Pack years smoking
Posterior thigh lean muscle mass
Reticulocyte count
Rib BMD
Standing Height
Spine BMD
Temperature
Total BMD
Trunk measures
Volume of grey matter
Waist circumference
Wear duration
Weight
Whole body mass
3mm eye measures
Weight
Whole body fat mass
Whole body fat free mass
Whole body water mass


(B) AC090771.1 Phewas
−log10(P−value)
Phenotype
Ankle spacing
Arm measures
Average acceleration
Basal metabolic rate
BMI
Calibration coefficient
Cardiac index
Corneal hysteresis
Duration to complete path
Eosinophil count
FEV1
FVC
Fraction accelerator
Genetic relatedeness
Heel measures
Hip circumference
Impedance of arm / leg / body
Interpolated age
L4 BMD
Leg measures
LogMAR
Lymphocyte count
MCV
Mean signal to noise
Median T2 signal
MET minutes per week of vigorous activity
Neutrophil count
Overall accelerator average
Posterior thin lean muscle volume
Reticulocyte count
Seated height
Speed of sound through heel
Spine BMD
Trunk measures
Volume of grey matter
Volume of white matter
Waist circumference
Wear duration
Weight
Whole body mass
3mm eye measures
6mm eye measures
Weight
Whole body fat mass
Whole body fat free mass
Whole body water mass

leucocyte telomere length from COPD subjects in the UK Biobank. Sarcopenia defined by HGS was associated with reductions in telomere length ($P < 0.001$), whereas sarcopenia as defined by FFMI and BMI were associated with increased telomere length ($P = 0.020$), and ASMI defined sarcopenia was not significantly different based on t-tests (*Table S11*). Linear regression analysis to determine whether COPD participants with sarcopenia had a greater reduction in telomere length in unadjusted and adjusted models. COPD participants with HGS-defined sarcopenia had shorter telomeres on both univariate and multivariate models (adjusted for age, sex and smoking status). ASMI-defined sarcopenia was not significant in univariate or multivariate models. FFMI and BMI-defined sarcopenia were associated with longer telomeres in univariate models, which was not significant after multivariate adjustment (*Figure S3* and *Table S12*).

Discussion

Our analysis of a large cohort of COPD patients demonstrated that genetic variants in or near the *FTO* and *AC090771.2* genes are associated with anthropometric measures of sarcopenia including FFMI, ASMI and BMR from the UK Biobank. For FFMI-associated variants from the UK Biobank, we replicated these findings in CT-derived FFMI from COPDGene. On PheWAS analysis, we showed that these genes are associated with other anthropometric measures related to body composition and physical activity. In vitro, we found that *FTO* depletion causes a sarcopenic phenotype with a molecular phenotype of senescence exacerbated by hypoxia. We also noted that leucocyte telomere length was associated with HGS-defined sarcopenia. In addition to discovering novel SNPs near or within *AC090771.2* associated with sarcopenia in COPD, our findings suggest that both genetic variants and hypoxia responses contribute to decreased *FTO* gene expression, which may cause skeletal muscle loss in COPD.

The *FTO* gene, one of the first genes identified as a locus for adult and childhood obesity in a genome-wide association study of type 2 diabetes mellitus, has been extensively studied in relation to obesity.[10] Previous studies have noted that the first intron of the *FTO* gene directly interacts with the promoter of *IRX3* to influence *IRX3* expression, which impacts body mass and composition.[16] Our data show that knockdown of the *FTO* gene resulted in significant reduction in IRX3 expression during PIH and CH but not normoxia. This suggests that the protective effect of *FTO* on skeletal muscle may be due to IRX3 expression during hypoxic stress, which is particularly relevant for patients with respiratory diseases. Emerging data have demonstrated a strong association between *FTO* and skeletal muscle mass,[4,26] and our studies provide a potential mechanistic basis of these observations. The association between variants in *FTO* and FFMI in COPD has been previously reported in a combined analysis of the ECLIPSE, COPDGene, NETT trial and the Norway–Bergen cohort (total $n = 3707$).[4] By utilizing the UK Biobank and COPDGene datasets, we were able to validate our findings in multiple SNPs on the *FTO* gene and also discovered novel variants near or within gene *AC090771*.2.

The protein product of the *FTO* gene regulates epitranscriptomic modifications of RNA in an oxygen-dependent manner, which is decreased during hypoxia.[11] *FTO* is important for myogenic differentiation in C2C12 cells, and in a mouse model, down-regulation of *FTO* suppressed mitochondrial biogenesis in skeletal muscle.[12] In mouse myocardial cells, *FTO* overexpression inhibited apoptosis in response to hypoxia and reoxygenation by regulat-

Figure 3 PheWAS plots of phenotypes from the UK Biobank. (A) PheWAS plot of phenotypes associated with the FTO gene from the UK Biobank cohort of COPD subjects. PheWAS plot showing *P* values for phenotypes analysed in the UK Biobank cohort of COPD subjects for the FTO gene. Linear regression was performed. Phenotype names are identified on the x-axis. Ankle spacing represents ankle width. Arm measures include arm fat mass, fat-free mass and total mass. Average acceleration represents the physical activity measured by an accelerometer. Cylindrical power represents an eye measurement (autorefraction). FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity. Fraction acceleration represents the acceleration intensity distribution also measured by an accelerometer. BMD, bone mineral density. Heel measures include bone mineral density and heel quantitative ultrasound index. Leg measures include leg fat mass, fat-free mass and total mass. LogMAR is a visual acuity measure. MCV, mean corpuscular volume. Mean single-to-noise ratio is a hearing test. Overall acceleration average represents the average physical activity measured by an accelerometer. Trunk measures represent bone mineral density, fat mass, fat-free mass and total mass. Three-millimetre eye measures represent visual acuity measures. Whole-body measures were zoomed in on to demonstrate significant associations with whole body fat-free mass, which was used to calculate FFMI in our analysis. (B) PheWAS plot of phenotypes associated with AC090771.1 from the UK Biobank cohort of COPD subjects. PheWAS plot showing *P* values for phenotypes analysed in the UK Biobank cohort of COPD subjects for the AC090771.1 gene. Linear regression was performed. Phenotype names are identified on the x-axis. Arm measures include arm fat mass, fat-free mass and total mass. Average acceleration represents the physical activity measured by an accelerometer. Cylindrical power represents an eye measurement (autorefraction). FEV1 = forced expiratory volume in 1 second. FVC = forced vital capacity. Fraction acceleration represents the acceleration intensity distribution also measured by an accelerometer. BMD, bone mineral density. Heel measures include bone mineral density and heel quantitative ultrasound index. Leg measures include leg fat mass, fat-free mass and total mass. LogMAR is a visual acuity measure. MCV, mean corpuscular volume. Mean single-to-noise ratio is a hearing test. Overall acceleration average represents the average physical activity measured by an accelerometer. Trunk measures represent bone mineral density, fat mass, fat-free mass and total mass. Three-millimetre and 6-mm eye measures represent visual acuity measures. Whole-body measures were zoomed in on to demonstrate significant associations with whole body fat-free mass, which was used to calculate FFMI in our analysis.

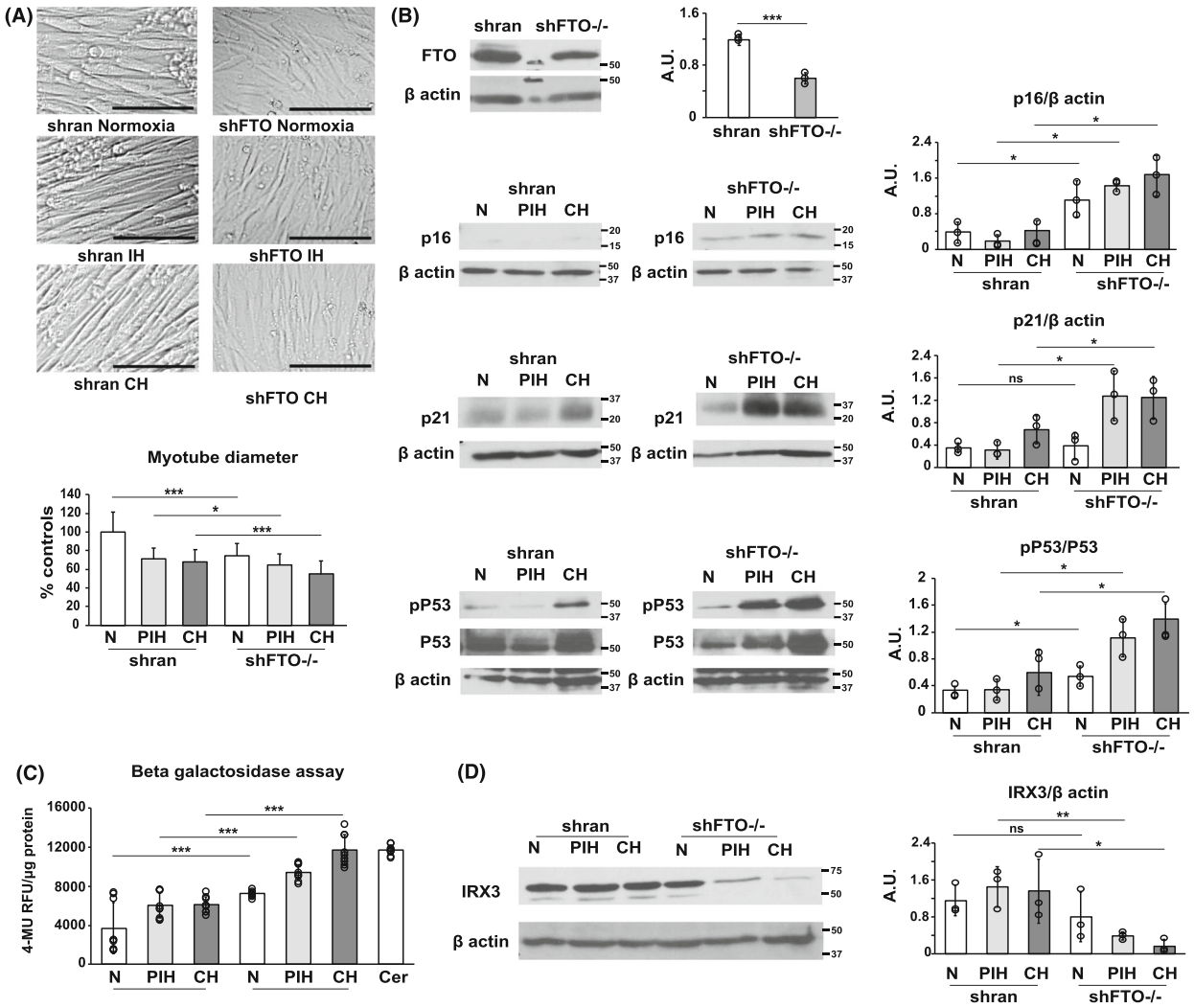


Figure 4 FTO knockdown in an in vitro model of skeletal muscle results in a sarcopenic phenotype. (A) Representative photomicrographs of differentiated myotubes (shrandom and shFTO) exposed to normoxia (N), prolonged intermittent hypoxia (PIH: 8 h hypoxia/16 h normoxia) and chronic hypoxia (CH) for 72 h. Scale bar is 100 µm. Myotube diameter of differentiated myotubes (shrandom and shFTO) for groups N, PIH and CH. All data mean ± SD from 80 myotubes in four fields for each biologic replicate (n = 3). (B) Representative immunoblots and densitometry of shFTO knockdown. Representative immunoblots and densitometry of biomarkers for senescence: p16 normalized to β-actin, p21 normalized to β-actin and phospho-p53 normalized to total p53. (C) Senescence-associated β-galactosidase activity was quantified and expressed as 4-methylumbelliferone (4-MU) fluorescence normalized to protein content shran cells exposed to N, PIH and CH, and FTO knockdown cells exposed to N, PIH and CH. Myotubes treated with 100 mM of ceramide serves as a positive control. (D) Representative immunoblots and densitometry of IRX3 normalized to β-actin. *$P < 0.01$, **Pp < 0.01, ***$P < 0.001$ as t-tests comparing the same groups in shrandom versus shFTO.

ing m6A modification of Mhrt (myosin heavy chain-associated RNA transcript).[27] In cultured fibroblasts homozygous for a rare non-synonymous *FTO* mutation, an early senescence phenotype[15] was demonstrated, which was similar to our findings in skeletal muscle C2C12 cells with *FTO* knockdown. Future studies are needed to dissect the mechanism by which *FTO* knockdown causes reductions in myotube diameter and promotes a senescence-like phenotype in skeletal muscle.

Interestingly, a higher proportion of active smokers met criteria for sarcopenia in the UK Biobank and COPDGene. Although smoking increases the risk for sarcopenia,[28] previous studies have noted that associations of SNPs on the *FTO* gene were more strongly associated with BMI in current smokers than former.[29] However, though current smoking was associated with sarcopenia, we did not find an interaction between current smoking and *FTO* polymorphisms in our analyses.

Although our study demonstrated a number of phenotypic associations with polymorphisms in the *FTO* gene, the novel association of SNPs near or within the *AC090771*.2 gene with sarcopenia has not been previously reported in patients with COPD. A number of risk loci for traits and diseases that are intergenic or intronic variants have been identified by GWAS, but have not been matched to biologic roles.[30] These variants

may influence conformational changes of DNA structure, disrupt protein–DNA or RNA–DNA interactions, alter the binding of proteins to promoters or are responsible for epigenetic markers.[30] The *AC090771*.2 gene has been previously designated as lncRNA, which may have diverse activities including remodelling of chromatin and genomic architecture, or stabilization of RNA and transcription regulation.[31] Our data therefore lay the foundation for future studies in the skeletal muscle multiome of COPD subjects.

Other genetic associations of ASMI included rs139954366, an SNP known to be a super enhancer[32] of transcriptional activity in close proximity to the *IL-10* receptor gene. IL-10 is an anti-inflammatory cytokine associated with the pathophysiology of sarcopenia in older adults.[33] We also found rs143384, an SNP in the *GDF5* gene, which has previously been associated with muscle weakness in the elderly.[34] *GDF5* encodes a protein in the transforming growth factor beta (TGF-β) family important for the maintenance of muscle mass.[35] These observations suggest future areas of research on the genetic susceptibility to sarcopenia in COPD.

Previous studies have shown that FTO protein dysfunction causes a senescence-like molecular phenotype even without additional hypoxic stress.[15] However, because hypoxia causes reductions in FTO protein expression,[14] we hypothesized that hypoxia (both PIH and CH) would exacerbate senescence with greater expression of p16, p21 and pP53. Consistently, we show increased expression of p16 and pP53 in all $FTO^{-/-}$ models (N/PIH/CH), whereas expression of p21 was higher in PIH and CH models of $FTO^{-/-}$ compared with normoxia (N). The function of FTO remains an ongoing area of research, and our findings suggest that $FTO^{-/-}$ promotes both the initiation of senescence and the maintenance of senescence. Interestingly, others have reported that pP53 and p21 initiate senescence and that p16 is responsible for the maintenance of senescence.[36] These findings are consistent with our hypothesis that FTO dysfunction induces a senescence-like molecular phenotype in the skeletal muscle of COPD patients and is exacerbated by hypoxia.

As *FTO* knockdown resulted in increased expression of senescence markers, we also quantified telomere length in C2C12 myotubes in response to PIH and CH. Interestingly, there was no difference between normoxia and PIH, but significant increases in telomere length were observed with CH. Although others have shown that transient intermittent hypoxia models of obstructive sleep apnoea cause reductions in telomere length,[37] our model is unique in that it recreates a phenotype of nocturnal hypoxemia in COPD patients.[18] Greater telomere length in myotubes with chronic hypoxia is consistent with previous reports that chronic hypoxia increases the enzyme activity of telomerase to promote telomere length as an adaptive mechanism.[38] In the UK Biobank cohort, using different definitions for sarcopenia, we analysed leucocyte telomere length and found that only HGS-defined sarcopenia was significantly associated with shorter telomeres. Our findings show that genetic variants related to both skeletal muscle mass (i.e. FFMI and associations with *FTO*) and skeletal muscle strength (i.e. HGS and reduced telomere length) may play complementary roles in promoting senescence in the skeletal muscle of COPD patients.

Whereas our exploratory analysis of the UK Biobank in subjects with COPD yielded multiple genome-wide associations with sarcopenia that were validated in COPDGene, we recognize limitations to our study. Even though our study used different methods to identify FFMI (bioelectric impedance in the UK Biobank and CT-derived pectoralis cross-sectional area in COPDGene), recent data suggest a high correlation between these methods,[39] supporting our interpretation and validation across different datasets. The UK Biobank participants are predominantly NHW, and therefore, in our validation studies, we compared this population to the NHW COPDGene cohort. Therefore, our findings need to be validated in other populations. A significant proportion of subjects in the UK Biobank population had either stage 1 (mild) or stage 2 (moderate) COPD, whereas the majority of subjects in COPDGene were stage 2 (moderate) and stage 3 (severe). However, observational studies have demonstrated that sarcopenia is present at all stages of COPD, with an average FEV1 in the moderate range for those with sarcopenia.[40] Given that the same SNPs associated with sarcopenia were demonstrated in severe-stage COPD (from COPDGene) and early-stage COPD (from the UK Biobank), these associations are likely not dependent on disease severity and may represent true genetic associations. We also utilized HGS for our analyses that is also, however, influenced by the skeletal dimensions of the hand rather than only muscle mass and/or function.[41] Strength measurements of other large muscle groups may be more closely related to functional impairment such as walking or stair climbing[42] and need to be evaluated in future. Our study does not establish the role of FTO expression in vivo in the skeletal muscle of COPD subjects. eQTL analysis demonstrated sarcopenia-associated *FTO* variants influence expression of FTO protein in skeletal muscle. SNPs in the first intron of *FTO* can act as enhancers for distal genes and are known to regulate the expression of *IRX3* and other genes within a 2-Mb topologically associated domain.[16] Moreover, *IRX3* has been known to affect body weight and composition and impact adipose tissue browning.[16] Our studies on genetic depletion of *FTO* resulted in lower expression of IRX3 during hypoxia (both PIH and CH) but not normoxia, which suggests a skeletal muscle protective role for FTO during hypoxic states. Future studies are needed to confirm the causal genes for the genetic associations that we identified for sarcopenia within COPD cohorts. Our study shows

a strong link between genetic variants in the *FTO* and *AC090771.2* genes and loss of muscle mass in two large cohorts of COPD patients, but whether this association is unique to COPD is currently not known. Identifying the contribution of smoking that is dependent on and independent of lung dysfunction to sarcopenia in COPD also needs evaluation because active smokers with normal lung function can have sarcopenia.[43]

In conclusion, we show that genetic variation contributes to heterogeneity in severity of sarcopenia in large cohorts of patients with COPD and identify consistent associations of gene variants with body composition. We also found that knockdown of *FTO* decreased myotube diameter and caused post-mitotic senescence that was worse with hypoxia. These data lay the foundation for an improved understanding of the mechanisms of sarcopenia in COPD.

Conflict of interest

No other conflicts of interest.

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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.

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