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Adiposity is associated with widespread transcriptional changes and downregulation of longevity pathways in aged skeletal muscle

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Abstract

Background Amongst healthy older people, a number of correlates of impaired skeletal muscle mass and function have been defined. Although the prevalence of obesity is increasing markedly in this age group, information is sparse about the particular impacts of obesity on ageing skeletal muscle or the molecular mechanisms that underlie this and associated disease risk.

Methods Here, we examined genome-wide transcriptional changes using RNA sequencing in muscle biopsies from 40 older community-dwelling men from the Hertfordshire Sarcopenia Study with regard to obesity (body mass index [BMI] >30 kg/m^2, $n = 7$), overweight (BMI 25–30, $n = 19$), normal weight (BMI < 25, $n = 14$), and per cent and total fat mass. In addition, we used EPIC DNA methylation array data to investigate correlations between DNA methylation and gene expression in aged skeletal muscle tissue and investigated the relationship between genes within altered regulatory pathways and muscle histological parameters.

Results Individuals with obesity demonstrated a prominent modified transcriptional signature in muscle tissue, with a total of 542 differentially expressed genes associated with obesity (false discovery rate ≤0.05), of which 425 genes were upregulated when compared with normal weight. Upregulated genes were enriched in immune response ($P = 3.18 \times 10^{-41}$) and inflammation (leucocyte activation, $P = 1.47 \times 10^{-41}$; tumour necrosis factor, $P = 2.75 \times 10^{-15}$) signalling pathways and downregulated genes enriched in longevity ($P = 1.5 \times 10^{-3}$) and AMP-activated protein kinase (AMPK) ($P = 4.5 \times 10^{-3}$) signalling pathways. Furthermore, differentially expressed genes in both longevity and AMPK signalling pathways were associated with a change in DNA methylation, with a total of 256 and 360 significant cytosine–phosphate–guanine–gene correlations identified, respectively. Similar changes in the muscle transcriptome were observed with respect to per cent fat mass and total fat mass. Obesity was further associated with a significant increase in type II fast-fibre area ($P = 0.026$), of which key regulatory genes within both longevity and AMPK pathways were significantly associated.

Conclusions We provide for the first time a global transcriptomic profile of skeletal muscle in older people with and without obesity, demonstrating modulation of key genes and pathways implicated in the regulation of muscle function, changes in DNA methylation associated with such pathways and associations between genes within the modified pathways implicated in muscle regulation and changes in muscle fibre type.

Keywords adiposity; AMPK; DNA methylation; gene expression; longevity; skeletal muscle

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Introduction

The proportion of people aged over 65 years is projected to continue to increase, reaching one in six globally by 2050.[1] Ageing is accompanied by physiological changes, the most prominent being loss of muscle mass and function. Up to 50% of muscle mass is lost by age 70 years, resulting in multiple adverse outcomes including impaired mobility, falls, fractures, increased insulin resistance and associated co-morbidities and mortality.[2]

Age-related muscle decline is driven by lifestyle, systemic and cellular causes, including physical inactivity,[3] poor nutrition,[4] inflammation,[5] myosteatosis,[6] mitochondrial dysfunction[7] and a decline in satellite cell function.[8] Amongst older people, the prevalence of obesity is rising markedly; in the United Kingdom, 81% of those aged 65–74 are currently overweight or obese.[9] Obesity alone affects skeletal muscle metabolic homeostasis, muscle mass and muscle function, for example, decreasing muscle contractile function by repressing AMP-activated protein kinase activity and calcium signalling, increasing fat deposition and inducing a shift from slow to fast muscle fibre type.[10] In animal models, obesity is linked to impaired satellite cell function and reduced muscle regenerative capacity.[11] Furthermore, low-grade inflammation associated with obesity alters systemic levels of proinflammatory cytokines and markers of chronic inflammation including C-reactive protein and interleukin-6.[12] Such inflammatory consequences of excess adiposity and intramuscular fat deposition are thought to contribute to muscle dysregulation, loss of quality and function. However, when obesity occurs in older individuals as is now common, effects on muscle function have been less studied, and consequences may be accentuated compared to younger individuals living with obesity.

To date, skeletal muscle transcriptomic studies have identified changes in gene expression associated with ageing, insulin resistance and type 2 diabetes (T2DM); however, muscle transcriptomic data on the impact of obesity in older individuals compared to healthy aged controls are sparse. Comparison of old with young muscle has been associated with differential expression of genes involved in cellular senescence, protein catabolism and oxidative phosphorylation, whereas skeletal muscle[13] and myoblast[14] transcriptome analyses from individuals with T2DM identified downregulation of mitochondrial pathways and myogenesis and upregulation of apoptosis and inflammation pathways.[14] Changes in gene transcription associated with ageing and metabolic dysfunction have been suggested to be mediated through epigenetic processes such as DNA methylation, which induce stable changes in gene expression without a change in gene sequence; genome-wide changes in DNA methylation have been reported in both ageing muscle and myoblasts from individuals with T2DM.[14,15] Here, we aimed to identify changes in the muscle transcriptome with respect to obesity, per cent fat mass and total fat mass in older community-dwelling adults and characterize the transcriptional pathways modified. In addition, using methylation arrays, we investigated correlations between DNA methylation and gene expression as a putative mechanism driving obesity-related phenotypic perturbations and investigated the relationship between genes within altered pathways and muscle histological parameters.

Methods

Study participants

Participants were recruited from the UK Hertfordshire Sarcopenia Study (HSS), investigating life course influences on muscle function in community-dwelling older people.[16] Ethical approval was received from the Hertfordshire Research Ethics Committee (Number 07/Q0204/68) and was conducted in accordance with the 1964 Declaration of Helsinki and its later amendments. Of the 105 male HSS participants, 40 with sufficient remaining muscle biopsy tissue were selected for RNA sequencing (RNAseq) analysis. Participants were classed as obese (body mass index [BMI] >30 kg/m^2, n = 7), overweight (BMI 25–30, n = 19) or normal weight (BMI < 25, n = 14) (*Table 1*). Six participants were classified as sarcopenic[17]: three healthy weight (BMI < 25 kg/m^2) and three overweight (BMI 25–30).

Procedures

Body composition (appendicular lean mass index [ALMi, kg/m^2]) was assessed by dual-energy X-ray absorptiometry (DXA) (Hologic Discovery, Software Version 12.5). Fasting (overnight) percutaneous *vastus lateralis* muscle biopsies (Weil–Blakesley conchotome) were conducted (local anaesthetic), and muscle tissue was snap frozen in liquid nitrogen-cooled isopentane and stored (-80°C) until analysis.

RNA extraction

RNA was extracted using the mirVana miRNA Isolation Kit (Thermo Fisher Scientific). RNA was eluted in nuclease-free

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Table 1 Cohort characteristics

	Normal weight	Overweight	Obese	
n	14	19	7	*P* value
Age (years)	71.71 ± 1.80	73.92 ± 2.48	72.56 ± 2.56	$P = 0.029$*
Height (m)	1.70 ± 0.05	1.71 ± 0.07	1.74 ± 0.04	$P = 0.452$
Weight (kg)	69.66 ± 3.53	80.88 ± 9.53	101.87 ± 10.53	$P = 1.83 \times 10^{-9}$***
BMI	23.98 ± 0.82	27.39 ± 1.29	33.6 ± 2.58	$P = 9.41 \times 10^{-16}$***
Total fat mass	15.99 ± 3.05	22.57 ± 4.54	33.03 ± 5.69	$P = 1.61 \times 10^{-9}$***
% fat mass	23.24 ± 4.33	28.21 ± 3.77	33.04 ± 4.29	$P = 2.28 \times 10^{-5}$***
Sarcopenic status	3	3	0	N/A

Abbreviation: BMI, body mass index.
*$P < 0.05$.
***$P < 0.001$.

water at >25 ng/μL (40–100 μL), and quantity/quality was measured by Qubit 2.0 Fluorometer (Thermo Fisher Scientific) and Bioanalyzer (Agilent Technologies). All RNA had an RNA integrity number (RIN) of >8.0 and stored at −80°C.

DNA extraction

Genomic DNA was extracted from muscle using the QIAamp DNA Mini Kit (Qiagen). Genomic DNA was quantified (NanoDrop ND1000, Thermo Scientific) and quality checked by agarose gel electrophoresis.

RNA sequencing

RNAseq was carried out as previously described.[7] Briefly, sequencing libraries were prepared from 250-ng total RNA (TruSeq Stranded Total RNA HT Kit with the Ribo-Zero Gold module, Illumina), followed by 13 cycles of PCR amplification (KAPA HiFi HotStart ReadyMix, Kapa Biosystems). Libraries were quantified with PicoGreen (Life Technologies), and size pattern was controlled with the DNA High Sensitivity Reagent Kit on a LabChip GX (PerkinElmer). Libraries were pooled at an equimolar ratio and clustered at 7 pM on paired-end sequencing flow cells (Illumina). Sequencing was performed for 2 × 101 cycles (HiSeq 2500, Illumina, with v3 chemistry). Generated data were demultiplexed using Casava. Reads were aligned to the human genome (hs_GRCh38.p2) using TopHat[18] and t mapped reads within genes quantified by HTSeq[19] (Version HTSeq-0.6.1p1, mode = union, strand = reverse, quality alignment >10). This resulted in a sequencing depth of 51–110 million reads per sample, of which 38–84 million were uniquely mapped. Data are publicly available from Gene Expression Omnibus (GEO) (Accession Number GSE111006).

RNA sequencing bioinformatic analysis

Differentially expressed genes with respect to obesity, total fat mass and per cent fat mass were determined using edgeR[20] in R (Version 3.4.2). Briefly, data were normalized using the weighted trimmed mean of M values implemented in edgeR. Lowly expressed genes across the dataset were removed using the filterByExpr function implemented in edgeR. *P* values were corrected for multiple testing using Benjamini–Hochberg method, with genes with a false discovery rate (FDR) <0.2 classed as differentially expressed for inclusion in exploratory pathway analyses. As a sensitivity analysis, the edgeR model was additionally adjusted for sarcopenia status.

Infinium Human MethylationEPIC BeadChip array

A total of 750 ng of genomic DNA was treated with sodium bisulfite (Zymo EZ DNA Methylation-Gold Kit, Zymo Research, Irvine, CA, USA) and hybridized to the Infinium Human MethylationEPIC BeadChip array (Illumina, Inc., CA, USA) at the Centre for Molecular Medicine and Therapeutics (CMMT, http://www.cmmt.ubc.ca). EPIC array data were processed using Bioconductor minfi 27 in R (Version 3.4.2). We applied beta-mixture quantile (BMIQ) normalization to remove array biases and correct for probe design. Cytosine–phosphate–guanine (CpG) sites known to cross-hybridize to other genomic locations (*n* = 14 759), coinciding with single-nucleotide polymorphisms (SNPs) (*n* = 77 261), probes with a detection *P* value >0.01 (*n* = 5604), beadcount <3 (*n* = 1051) and 2928 non-CpG probes were removed from analysis. After pre-processing and quality control (QC), 34 samples and 744 547 CpG probes remained in the dataset. Data are available from GEO (Accession Number GSE154980). Robust regression models using limma were run for total fat mass with or without adjustment for age. Details are in the supporting information.

Histological analysis

Previously collected and published immunohistochemistry data[21] were used to examine correlations between muscle morphological measures and gene expression. Briefly, muscle

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tissue was fixed overnight (−20°C) and embedded in glycol methacrylate resin, and 7-μm serial cross-sections were cut and stained for type II fast-twitch myofibres using monoclonal anti-myosin fast antibody (1:6000 clone MY-32; Sigma-Aldrich, Dorset, UK). Stained sections were examined under a photomicroscope (Zeiss Axioskop II, Carl Zeiss Ltd, Welwyn Garden City, UK), viewed at ×5 magnification and myofibre number (type I, slow fibre vs. type II, fast fibre) expressed as percentages of total fibre number and myofibre cross-sectional areas (μm^2). Full details are in the supporting information.

Pathway analysis

Protein–protein interaction (PPI) networks were carried out using the Search Tool for Retrieval of Interacting Genes/Proteins (STRING) and visualized in Cytoscape. Large networks were segmented using MCODE, and Gene Ontology (GO) enrichment was determined using BiNGO. Pathway analysis was performed using Ingenuity Pathway Analysis (IPA) (Qiagen, UK) Version 68752261. Full details are in the supporting information.

Statistical analysis

Statistical analyses were undertaken in R (Version 3.4.2). Demographic characteristics were compared across the three groups using one-way analysis of variance (ANOVA). Correlations between DNA methylation and gene expression were performed using Spearman's rank correlation, and muscle morphological variables were compared across adiposity groups (one-way ANOVA with Tukey's multiple comparison test). Analysis of fibre associations was performed using linear regression of normalized, log-transformed counts of AMP-activated protein kinase (AMPK) and longevity genes against different muscle fibre measures. All models were count ~ fibre measure + age.

Results

Participant characteristics

Table 1 shows anthropometric and physical function characteristics of the 40 male participants. Mean age was 72.91 years, BMI 27.39 kg/m^2 and per cent fat mass 27.42. Total and per cent fat mass were higher in the overweight (n = 19) and obese (n = 7) groups compared to controls; the overweight group (73.92 ± 2.48) was older than the normal-weight (71.71 ± 1.80) and obese (72.56 ± 2.56) groups. Three (normal weight) and three (overweight) participants were categorized as sarcopenic.

Obesity-associated changes in the muscle transcriptome

Comparison of the muscle total RNAseq data for obese versus normal-weight participants identified 542 differentially expressed genes associated with obesity (FDR ≤ 0.05), with 425 genes upregulated and 117 downregulated. The top 2 upregulated genes were coagulation factor XIII-A chain (*F13A1*, FDR = 5.69×10^{-05}) and S100 calcium-binding protein A4 (*S100A4*, FDR = 8.64×10^{-05}); the top 2 downregulated genes were glial cell-derived neurotrophic factor (*GDNF*, FDR = 2.09×10^{-03}) and insulin receptor substrate 2 (*IRS2*, FDR = 2.61×10^{-03}) (*Figure 1* and *Tables 2*, *S1* and *S2*). As three participants (normal weight) were sarcopenic, which affects muscle structure and function, we carried out sensitivity analysis adjusting for sarcopenia status in the comparison of obesity to normal weight; there was considerable overlap between differentially expressed genes in unadjusted and adjusted analyses, with 8 of the top 10 genes identical, with the same direction of association and similar significance and effect size. The remaining two differentially expressed genes were also significantly associated with obesity in the adjusted analysis but fell outside the top 10 differentially expressed genes in the adjusted analysis (*Table S3*).

Comparison of overweight with normal-weight individuals identified 10 differentially expressed genes (FDR ≤ 0.05), 6 upregulated and 4 downregulated. The top 2 upregulated genes were spermatogenesis-associated 7 (*SPATA7*, FDR = 1.80×10^{-02}) and synaptotagmin-like 2 (*SYTL2*, FDR = 1.80×10^{-02}); the top 2 downregulated genes were RNA, 7SL, cytoplasmic 449, pseudogene (*RN7SL449P*, FDR = 1.80×10^{-02}) and *IRS2* (FDR = 1.98×10^{-02}) (*Table 3*). Four differentially expressed genes overlapped between the obese versus normal-weight and overweight versus normal-weight subjects: *IRS2*, unc-51-like autophagy-activating kinase 1 (*ULK1*), cholinergic receptor nicotinic alpha 5 subunit (*CHRNA5*) and *SPATA7*.

Inflammation and longevity were top pathways enriched amongst the obesity-associated genes

To understand the functional significance of the changes to the muscle transcriptome with respect to obesity, we inputted differentially expressed genes into Cytoscape to generate a PPI network. The PPI enrichment P values for the upregulated and downregulated gene networks were 1×10^{-16} and 2.33×10^{-6}, respectively, indicating that the proteins have more biological connections than expected by chance (*Figure S1A*,*B*). The top 3 pathways overrepresented amongst the upregulated gene set within GO or Kyoto Encyclopedia of Genes and Genomes (KEGG) were cell activation ($P = 1.47 \times 10^{-41}$), leucocyte activation ($P = 1.47 \times 10^{-41}$) and immune system process ($P = 3.18 \times 10^{-41}$), whereas

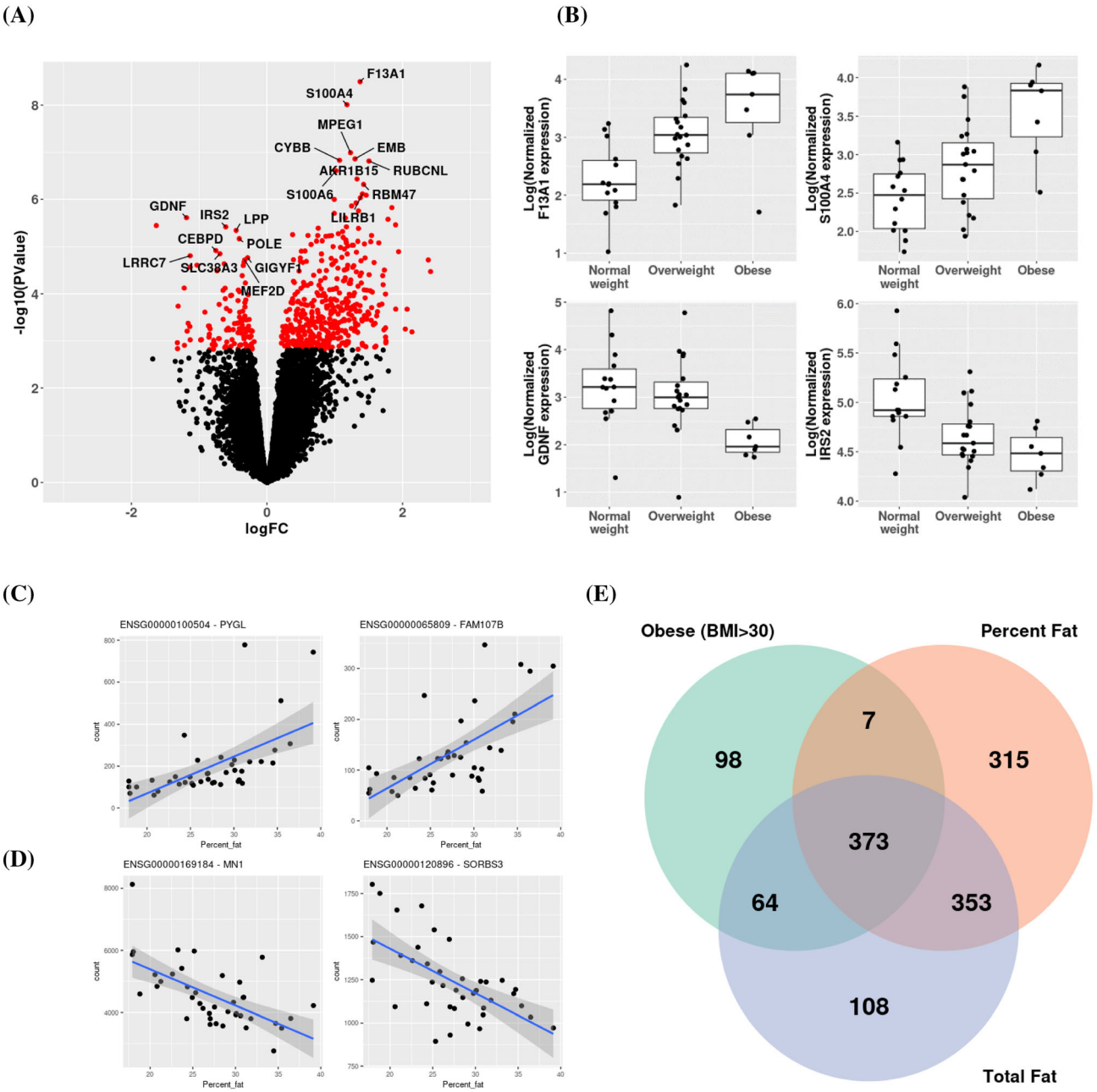

Figure 1 (A) Volcano plot showing the top 10 upregulated and downregulated genes (FDR < 0.05) in skeletal muscle with respect to obese versus normal weight. (B) Box plots of the top 2 upregulated and top 2 downregulated genes. Top 2 (C) positively and (D) negatively regulated transcripts with respect to per cent fat mass (*PYGL* and *MN1*) and obesity (*FAM107B* and *SORBS3*). (E) Differentially expressed gene overlap between obese, per cent fat mass and total fat mass analysis (FDR < 0.05).

amongst downregulated genes, the top pathways were longevity regulating pathway ($P = 1.5 \times 10^{-3}$), AMPK signalling pathway ($P = 4.5 \times 10^{-3}$) and double-stranded DNA binding ($P = 1.96 \times 10^{-2}$) (*Tables 4*, *S4* and *S5*).

As genes within the AMPK signalling and longevity pathways play critical roles in muscle function, we further examined muscle gene expression in these pathways in individuals who were overweight compared to normal weight (*Tables S6* and *S7*). Amongst overweight individuals, AMPK and longevity pathway gene changes were similar to those in obese individuals; genes such as *IRS2* and *ULK1* (FDR ≤ 0.05), regulatory-associated protein of mTOR complex 1 (*RPTOR*), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PPARGC1A*), insulin receptor (*INSR*), insulin-like growth factor 1 receptor (*IGF1R*), *FOXO1* and *FOXO3* (nominal $P \leq 0.05$) were also downregulated in overweight individ-

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Table 2 Top 15 upregulated and downregulated differentially expressed transcripts (FDR < 0.05) in skeletal muscle from obese versus normal-weight participants

ID	Hgnc_Sym	logFC	*P* value	FDR	Description
Upregulated					
1	*F13A1*	1.372932	3.22E − 09	0.0000569	Coagulation factor XIII-A chain
2	*S100A4*	1.179204	9.78E − 09	0.0000864	S100 calcium-binding protein A4
6	*RUBCNL*	1.503885	1.54E − 07	0.000453	Rubicon-like autophagy enhancer
4	*EMB*	1.298071	1.38E − 07	0.000453	Embigin
3	*MPEG1*	1.231345	1.02E − 07	0.000453	Macrophage-expressed gene 1
5	*CYBB*	1.06915	1.48E − 07	0.000453	Cytochrome *b*-245 beta chain
7	*S100A6*	1.018021	2.48E − 07	0.000626	S100 calcium-binding protein A6
8	*AKR1B15*	1.327127	3.70E − 07	0.000817	Aldo-keto reductase family 1 member B15
9	*RBM47*	1.424659	4.83E − 07	0.000948	RNA-binding motif protein 47
11	*AIF1*	1.46209	8.18E − 07	0.001314	Allograft inflammatory factor 1
10	*LILRB1*	1.407698	7.68E − 07	0.001314	Leucocyte immunoglobulin-like receptor B1
12	*TLR6*	1.376415	9.27E − 07	0.001364	Toll-like receptor 6
13	*CD44*	0.991163	1.00E − 06	0.001364	CD44 molecule (Indian blood group)
14	*KYNU*	1.314581	1.19E − 06	0.001504	Kynureninase
15	*BTK*	1.248046	1.38E − 06	0.00163	Bruton tyrosine kinase
Downregulated					
1	*GDNF*	−1.18851	2.46E − 06	0.002091	Glial cell-derived neurotrophic factor
2	*IRS2*	−0.60895	3.85E − 06	0.002614	Insulin receptor substrate 2
3	*Unknown*	−1.63356	3.61E − 06	0.002614	Novel transcript
4	*LPP*	−0.45601	4.58E − 06	0.002789	LIM domain-containing preferred translocation partner in lipoma
5	*POLE*	−0.40932	6.83E − 06	0.003016	DNA polymerase epsilon, catalytic subunit
6	*CEBPD*	−0.75442	1.21E − 05	0.0038	CCAAT enhancer-binding protein delta
7	*SLC38A3*	−0.69563	1.41E − 05	0.004001	Solute carrier family 38 member 3
8	*LRRC7*	−1.13415	1.56E − 05	0.004304	Leucine-rich repeat-containing 7
9	*GIGYF1*	−0.28589	1.72E − 05	0.004508	GRB10-interacting GYF protein 1
10	*MEF2D*	−0.32935	1.92E − 05	0.00471	Myocyte enhancer factor 2D
11	*KMT2B*	−0.33862	2.15E − 05	0.004789	Lysine methyltransferase 2B
12	*LGR5*	−0.63125	2.31E − 05	0.004985	Leucine-rich repeat-containing G protein-coupled receptor 5
13	*ERICD*	−1.03278	2.47E − 05	0.005142	E2F1-regulated inhibitor of cell death
14	*DIP2C*	−0.35095	2.52E − 05	0.005159	Disco-interacting protein 2 homologue C
15	*SLC22A3*	−1.13538	2.72E − 05	0.005442	Solute carrier family 22 member 3

Abbreviations: FDR, false discovery rate; Hgnc, HUGO Gene Nomenclature Committee; logFC, log fold change.

Table 3 Upregulated and downregulated differentially expressed transcripts (FDR < 0.05) in skeletal muscle from overweight versus normal-weight participants

ID	Hgnc_Sym	logFC	*P* value	FDR	Description
Upregulated					
1	*SPATA7*	0.499988	4.08E − 06	0.018047	Spermatogenesis-associated 7
2	*SYTL2*	0.695393	4.01E − 06	0.018047	Synaptotagmin-like 2
3	*ZNF300P1*	1.114316	2.38E − 06	0.018047	Zinc finger protein 300 pseudogene 1
4	*Novel Transcript*	0.592078	7.88E − 06	0.019882	Novel transcript
5	*CHRNA5*	1.487575	6.24E − 06	0.019882	Cholinergic receptor nicotinic alpha 5 subunit
6	*SLCO2B1*	0.564605	1.65E − 05	0.036197	Solute carrier organic anion transporter family member 2B1
Downregulated					
1	*RN7SL449P*	−0.90611	2.73E − 06	0.018047	RNA, 7SL, cytoplasmic 449, pseudogene
2	*IRS2*	−0.49369	6.81E − 06	0.019882	Insulin receptor substrate 2
3	*AMPD3*	−0.83289	1.84E − 05	0.036197	Adenosine monophosphate deaminase 3
4	*ULK1*	−0.30328	2.33E − 05	0.041199	Unc-51-like autophagy-activating kinase 1

Abbreviations: FDR, false discovery rate; Hgnc, HUGO Gene Nomenclature Committee; logFC, log fold change.

uals in the longevity pathway, with *RPTOR*, solute carrier family 2 member 4 (*SLC2A4*), *PPARGC1A/PGC1α*, member RAS oncogene family (*RAB11B*), *INSR*, *FOXO1* and *FOXO3* (nominal $P \leq 0.05$) also downregulated in overweight compared to normal-weight individuals in the AMPK pathway (*Figure 2*).

Ingenuity Pathway Analysis

The obesity-associated upregulated and downregulated gene sets (FDR < 0.05) were analysed using IPA to identify potential causal networks and upstream regulators that may

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Table 4 Top 5 upregulated and downregulated GO or KEGG pathways with respect to obese versus normal skeletal muscle (Cytoscape)

Description	No. of background genes	No. of genes	FDR	Genes (upregulated top 10, downregulated all)
Upregulated				
Cell activation	1075	111	1.47×10^{-41}	*CD4*|*LCP2*|*STK10*|*CMTM6*|*LGALS1*|*PYGL*|*TIMP1*|*CORO1A*|*RELB*|*SPI1*
Leucocyte activation	929	104	1.47×10^{-41}	*CD4*|*LCP2*|*STK10*|*CMTM6*|*LGALS1*|*PYGL*|*CORO1A*|*RELB*|*SPI1*|*SELPLG*
Immune system process	2481	163	3.18×10^{-41}	*CD4*|*LCP2*|*STK10*|*CMTM6*|*LGALS1*|*PYGL*|*CORO1A*|*RELB*|*TGFB1*|*SPI1*
Immune response	1588	129	1.22×10^{-39}	*CD4*|*LCP2*|*STK10*|*CMTM6*|*LGALS1*|*PYGL*|*CORO1A*|*RELB*|*SELL*|*LILRB3*
Immune effector process	969	93	5.03×10^{-32}	*STK10*|*CMTM6*|*LGALS1*|*PYGL*|*CORO1A*|*RELB*|*SELL*|*LILRB3*|*PYCARD*|*DOCK2*
Downregulated				
Longevity regulating	87	6	0.0015	*INSR*|*ULK1*|*EIF4EBP1*|*IRS2*|*FOXO3*|*PIK3R1*
AMPK signalling	120	6	0.0044	*INSR*|*ULK1*|*EIF4EBP1*|*IRS2*|*FOXO3*|*PIK3R1*
Double-stranded DNA binding	1156	18	0.0196	*MCM4*|*PATZ1*|*MEF2D*|*KLF15*|*PKNOX2*|*PRKDC*|*GCFC2*|*PLAG1*|*H1FX*|*ZBTB16*|*MAPT*|*TEAD3*|*SOX13*|*BCL6*|*FOXO3*|*CEBPD*|*CTCFL*|*PER3*
Longevity regulating pathway—Multiple species	61	4	0.0263	*INSR*|*IRS2*|*FOXO3*|*PIK3R1*
FOXO signalling pathway	127	5	0.0311	*INSR*|*IRS2*|*BCL6*|*FOXO3*|*PIK3R1*

Abbreviations: AMPK, AMP-activated protein kinase; FDR, false discovery rate; FOXO, forkhead box O; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

mediate such changes. Within the upregulated gene set, top causal networks were translocation–Ets–leukaemia virus–runt-related transcription factor 1 (*ETV6–RUNX1*, $P = 1.88 \times 10^{-18}$), membrane attack complex (*MAC*, $P = 4.04 \times 10^{-17}$) and plasminogen activator urokinase receptor (*PLAUR*, $P = 1.38 \times 10^{-15}$). Top upstream regulators were translocation–Ets–leukaemia virus–runt-related transcription factor 1 ($P = 4.15 \times 10^{-17}$), granulin precursor (*GRN*, $P = 3.28 \times 10^{-12}$) and CCAAT enhancer-binding protein alpha (*CEBPA*, $P = 4.05 \times 10^{-10}$) (*Table S8*).

Within the downregulated gene set, the top causal networks were serine/threonine-protein kinase D1 (*PRKD1*, $P = 1.39 \times 10^{-05}$), *SMARCB1* ($P = 1.63 \times 10^{-05}$) and Akt serine/threonine kinase 1 (*AKT1*, $P = 1.63 \times 10^{-05}$). Top upstream regulators were dihydrolipoamide *S*-succinyltransferase (*DLST*, $P = 6.47 \times 10^{-05}$), SMAD family member 4 (*SMAD4*, $P = 2.22 \times 10^{-03}$) and methyl-CpG-binding domain protein 3 (*MBD3*, $P = 2.81 \times 10^{-03}$) (*Table S9*).

Similar changes in the muscle transcriptome were observed with respect to per cent fat mass and total fat mass

BMI reflects both fat and fat-free mass, which may have very different influences on health outcomes; we therefore examined changes in the muscle transcriptome with respect to per cent fat mass and total fat mass; 1048 genes were differentially expressed (FDR ≤ 0.05) with respect to per cent fat mass, of which 891 were upregulated (*Figure 1* and *Tables 5* and *S10*); 898 genes were differentially expressed with respect to total fat mass, of which 746 were upregulated (*Table S11*). There was considerable overlap between differentially expressed genes in muscle tissue associated with BMI, per cent fat mass and total fat mass, with 373 genes associated with all 3 measures, 726 common genes between per cent fat mass and total fat mass, 437 between obesity and total fat and 380 between obesity and per cent fat mass (*Figure 1E*).

PPI analysis of the differentially expressed upregulated genes associated with per cent fat mass produced a PPI network with an enrichment *P* value of 1×10^{-16}; the top 5 enriched pathways in GO and KEGG were immune system process ($P = 1.43 \times 10^{-66}$), cell activation ($P = 1.41 \times 10^{-64}$) and leucocyte activation ($P = 1.93 \times 10^{-61}$). The downregulated genes produced a PPI network enrichment *P* value of 3.6×10^{-3}, with the top-enriched pathways in GO and KEGG being AMPK signalling pathway ($P = 4.8 \times 10^{-03}$), longevity regulating pathway ($P = 4.8 \times 10^{-03}$) and forkhead box O (FOXO) signalling pathway ($P = 2.02 \times 10^{-02}$) (*Tables S12* and *S13*). A number of the same genes were also associated with total fat mass, with genes such as adiponectin, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta (*PIK3CD*), RAC-beta serine/threonine-protein kinase (*AKT2*), *IRS2*, *INSR*, *IGF1R* and eukaryotic translation initiation factor 4E-binding protein 1 (*EIF4EBP1*) downregulated amongst the longevity pathway and eukaryotic translation elongation factor 2 (*EEF2*), tuberous sclerosis 1 (*TSC1*), *IRS2*, *INSR*, *PRKAA2*, carnitine palmitoyltransferase 1B (*CPT1B*),

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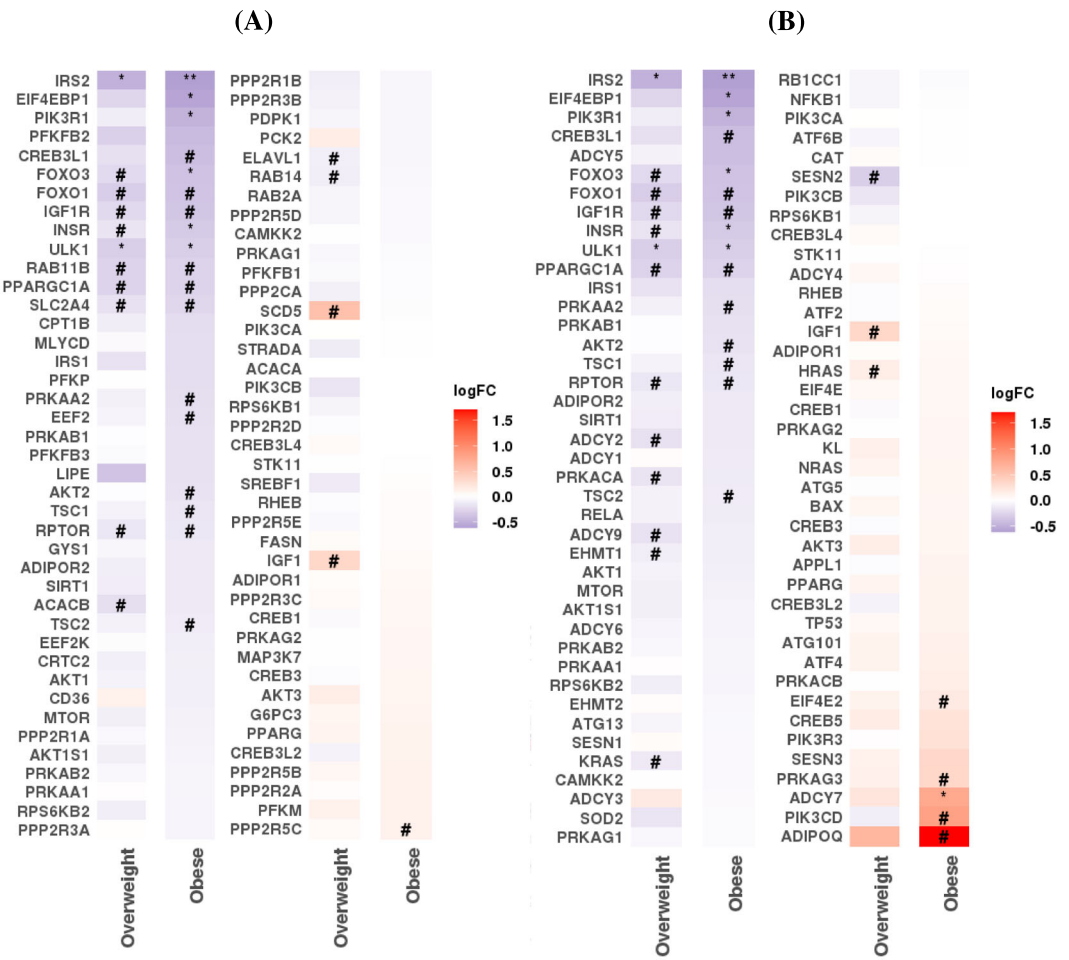

Figure 2 Comparison of the expression of genes in (A) the longevity regulating pathway and (B) the AMPK signalling pathway in overweight versus normal-weight and obese versus normal-weight participants. *FDR $<$ 0.05. **FDR $<$ 0.01. #Nominal *P* value $<$0.05.

IGF1R, *EIFEBP1*, *CREB3L1* and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 (*PFKFB2*) downregulated in the AMPK pathway.

Correlation between DNA methylation and gene expression in skeletal muscle tissue

A total of 34 HSS samples with RNAseq data also had EPIC DNA methylation data. To explore the correlation between gene expression and DNA methylation, we examined DNA methylation at CpGs annotated to longevity and AMPK signalling pathway genes; 62 of the differentially expressed genes in the longevity pathway were associated with a change in DNA methylation ($P < 0.05$), with 256 significant CpG–gene correlations identified. Genes associated with DNA methylation were found to be differentially expressed (FDR < 0.05) in obese versus normal subjects including three CpGs within *FOXO3*, four CpGs within *INSR*, five CpGs within phosphatidylinositol 3-kinase (*PIK3R1*) and five CpGs within *ULK1* (*Tables 6* and *S14* and *Figure S2*). In the AMPK pathway, 82 of the differentially expressed genes were associated with DNA methylation, with 360 CpG–gene correlations identified; genes associated with DNA methylation that were differentially expressed (FDR < 0.05) in obese versus normal subjects included three CpGs within *FOXO3*, four CpGs within *INSR*, five CpGs within *PIK3R1* and five CpGs within *ULK1* (*Tables 6* and *S15*). Although there was a narrow age range between individuals, we investigated whether the epigenetic changes could be attributed to age-related effects as opposed to obesity itself. DNA methylation analysis with respect to total fat mass was performed with and without age as a covariate; there was considerable overlap of the fat mass associated with differentially methylated CpGs (dmCpGs) between age-adjusted and age-unadjusted analyses, with the top 10 dmCpGs in the unadjusted analyses showing the same direction of association and similar significance and effect size in the adjusted analysis (*Tables S16* and *S17*).

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Table 5 Top 10 significant (FDR ≤ 0.05) positively associated and negatively associated differentially expressed genes from per cent fat mass analysis

Gene	logFC	FDR	Description
Positively associated genes			
PYGL	0.100787	1.23×10^{-09}	Glycogen phosphorylase L
FAM107B	0.098733	7.37×10^{-09}	Family with sequence similarity 107 member B
F13A1	0.098389	9.84×10^{-09}	Coagulation factor XIII-A chain
CMTM3	0.069715	2.72×10^{-07}	CKLF-like MARVEL transmembrane domain-containing 3
DOCK11	0.091248	2.72×10^{-07}	Dedicator of cytokinesis 11
PDE3B	0.135029	2.72×10^{-07}	Phosphodiesterase 3B
APBB1IP	0.145752	4.96×10^{-07}	Amyloid beta precursor protein-binding family B member 1-interacting protein
MVP	0.036987	5.23×10^{-07}	Major vault protein
IL10RA	0.085479	5.23×10^{-07}	Interleukin-10 receptor subunit alpha
S100A4	0.075423	8.84×10^{-07}	S100 calcium-binding protein A4
Negatively associated genes			
MN1	−0.03109	8.44×10^{-05}	MN1 proto-oncogene, transcriptional regulator
SORBS3	−0.02761	0.000194	Sorbin and SH3 domain-containing 3
DIP2C	−0.02488	0.000263	Disco-interacting protein 2 homologue C
RTN4RL2	−0.07329	0.000305	Reticulon 4 receptor-like 2
PKD1P2	−0.14019	0.000362	Polycystin 1, transient receptor potential channel-interacting pseudogene 2
POLE	−0.02728	0.00054	DNA polymerase epsilon, catalytic subunit
GDNF	−0.07286	0.001044	Glial cell-derived neurotrophic factor
CYP4F35P	−0.06165	0.001374	Cytochrome P450 family 4 subfamily F member 35, pseudogene
TMEM201	−0.02364	0.001446	Transmembrane protein 201
TNIK	−0.02504	0.002154	TRAF2- and NCK-interacting kinase

Abbreviations: FDR, false discovery rate; logFC, log fold change.

Obesity is associated with increased type II fibre area

To determine functional implications of increased adiposity, we examined the per cent of slow and fast fibres, slow/fast-fibre area, capillaries per fibre and satellite cells per fibre in muscle biopsies taken from the obese, overweight and normal-weight subjects. Obesity was associated with a significant increase in fast-fibre area ($P = 0.022$—one-way ANOVA; $P = 0.026$—Tukey's multiple comparison test), with no significant differences observed in the other parameters (*Table S18*). We further investigated associations of genes within the longevity and AMPK pathways with the above muscle fibre parameters. Nine genes significantly associated ($P < 0.05$) with fast-fibre area including *PPARGC1A* ($P = 1.8 \times 10^{-3}$), Klotho (*KL*) ($P = 1.9 \times 10^{-2}$), calcium-binding protein 39-like (*CAB39L*) ($P = 2.0 \times 10^{-2}$), *PRKAA2* ($P = 2.3 \times 10^{-2}$), TBC1 domain family member 1 (*TBC1D1*) ($P = 2.3 \times 10^{-2}$), cAMP-responsive element-binding protein 3 (*CREB3*) ($P = 2.6 \times 10^{-2}$), *INSR* ($P = 2.6 \times 10^{-2}$), protein phosphatase 2 regulatory subunit B delta (*PPP2R2D*) ($P = 4.1 \times 10^{-2}$) and *RAB11B* ($P = 4.7 \times 10^{-2}$) of which *CAB39L*, *INSR*, *PPARGC1A*, *PRKAA2*, *TBC1D1* and *RAB11B* were differentially expressed in muscle from individuals with obesity (*Table S19*).

Discussion

Here, we report differential expression of genes in aged skeletal muscle tissue with respect to adiposity, identifying considerable overlap between genes associated with obesity, per cent fat mass and total fat mass. Upregulated genes were enriched in pathways associated with inflammation, whereas downregulated genes were enriched for longevity and AMPK pathways. Expression of obesity-associated genes within longevity and AMPK pathways strongly correlated with DNA methylation, suggesting that DNA methylation may mediate or consolidate the changes in gene expression. Obesity was further associated with a significant increase in type II fast-fibre area, of which key regulatory genes within both longevity and AMPK pathways were significantly associated. These findings highlight that transcriptional regulation of key components of longevity and AMPK pathways are altered in muscle of older individuals with obesity, providing putative targets for epigenetic manipulation to improve muscle health in old age.

RNAseq analysis identified that expression of 542 genes in muscle was altered in obese compared to normal-weight individuals, with the majority (78.4%) upregulated. Far fewer genes were differentially expressed when comparing overweight to normal weight, with *IRS2*, *ULK1*, *CHRNA5* and *SPATA7* altered in both obesity and overweight phenotypes. Differential gene expression was also observed in relation to total fat mass and per cent fat mass; although there was considerable overlap between differentially expressed genes and pathways enriched between these groups, there were differences. Such differences most likely reflect total fat mass quantifying fat mass irrespective of body size, whereas per cent fat mass measures per cent fat relative to body size. There was also substantial overlap of obesity-related genes and pathways with those associated with per cent fat mass and total fat mass, suggesting that BMI is capturing the transcriptomic signature of increased adiposity in these individuals.

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Table 6 Top 15 significant ($P \leq 0.05$) DNA methylation CpG–gene correlations in the longevity or AMPK pathways

CpG	Gene	Rho	*P* value	CpG location relative to gene
Longevity pathway				
cg26556014	*EIF4E2*	−0.651	4.60×10^{-05}	Body/3′UTR
cg10364945	*EIF4E2*	−0.624	1.14×10^{-04}	3′UTR
cg20315150	*CREB5*	−0.574	4.86×10^{-04}	Body
cg16779321	*CREB5*	−0.557	7.56×10^{-04}	5′UTR/body
cg00718401	*CREB3L2*	−0.548	9.54×10^{-04}	Body
cg19092708	*AKT2*	−0.542	1.11×10^{-03}	5′UTR
cg13882251	*AKT2*	−0.541	1.13×10^{-03}	5′UTR
cg14844401	*ADCY5*	−0.535	1.33×10^{-03}	Body
cg06055845	*AKT2*	−0.532	1.42×10^{-03}	TSS1500/5′UTR
cg05335604	*RPTOR*	−0.525	1.68×10^{-03}	Body
cg25974308	*EIF4E2*	−0.519	1.91×10^{-03}	TSS1500
cg00098799	*IGF1R*	0.513	2.18×10^{-03}	Body
cg03317412	*CREB3L1*	−0.508	2.45×10^{-03}	Body
cg24070942	*ADCY4*	0.506	2.56×10^{-03}	Body
cg25076452	*EIF4E2*	−0.506	2.57×10^{-03}	Body
AMPK pathway				
cg04602990	*PFKFB4*	−0.621	1.23×10^{-04}	Body
cg02233614	*PFKFB2*	−0.620	1.28×10^{-04}	5′UTR
cg13805711	*PPP2R2C*	0.604	2.10×10^{-04}	Body
cg01733324	*TBC1D1*	−0.599	2.41×10^{-04}	Body
cg18953861	*PPP2R5E*	−0.588	3.33×10^{-04}	Body
cg09038531	*PFKFB2*	−0.587	3.41×10^{-04}	ExonBnd/body
cg02232863	*PFKFB2*	−0.583	3.78×10^{-04}	Body
cg21650716	*PPP2R2C*	0.576	4.62×10^{-04}	Body/5′UTR
cg20315150	*CREB5*	−0.574	4.86×10^{-04}	Body
cg12026039	*PPP2R5C*	−0.570	5.41×10^{-04}	Body
cg19081843	*CPT1A*	−0.569	5.59×10^{-04}	5′UTR
cg24913613	*RAB10*	0.568	5.72×10^{-04}	Body
cg15247669	*PFKL*	0.567	5.86×10^{-04}	5′UTR/body
cg19417588	*CPT1A*	0.565	6.20×10^{-04}	Body
cg17664711	*PPP2R2C*	0.561	6.83×10^{-04}	Body

Abbreviations: AMPK, AMP-activated protein kinase; CpG, cytosine–phosphate–guanine.

The gene most strongly upregulated in individuals with obesity was *F13A1*, encoding factor XIII-A blood-clotting factor, a transglutaminase enzyme identified in genome-wide association studies associated with BMI and insulin resistance in polycystic ovary syndrome.[22] In vitro studies suggest roles for factor XIII-A in preadipocyte differentiation and modulation of insulin signalling via promoting plasma fibronectin assembly into the extracellular matrix (ECM).[23] Upregulated genes associated with obesity showed enrichment for pathways involved in inflammation and immune response, suggesting inflammatory/immune cell infiltration in skeletal muscle tissue in individuals with obesity. This is consistent with reported higher levels of multiple proinflammatory lipids in muscle tissue from individuals with obesity,[24] although amongst middle-aged twins discordant for BMI, the main transcriptional pathway upregulated in muscle from the twin with a higher BMI was ECM remodelling.[25] The strong inflammatory signal that we observed in skeletal muscle may reflect synergistic effects of obesity and ageing on the transcriptome, uncovering a previously uncharacterized effect of combining such phenotypes.

Consistent with upregulation of inflammatory genes, top upstream regulators and casual pathways, included GRN, implicated in inflammation and immune pathways, of which progranulin (the precursor protein for granulin) is an adipokine involved in diet-induced obesity and insulin resistance,[26] CEBPA involved in transcriptional activation of obesity genes, required for adipogenesis and normal adipocyte function,[27] as well as interleukin-13 (IL-13), and tumour necrosis factor (TNF). Modulation of such pathways in individuals with obesity suggests inflammatory response dysregulation[28] and altered adipogenic potential associated with decreased muscle function in obesity.[29]

The top downregulated gene in skeletal muscle of obese versus normal-weight individuals was *GDNF*, a potent survival factor for motor neurons important in maintaining hyperinnervation of skeletal muscle in adulthood[30]; its downregulation may reflect denervation of skeletal muscle of obese aged individuals. AKT was identified as one of the top causal networks amongst the downregulated genes, whereas pathway analysis of downregulated genes showed enrichment amongst longevity, AMPK and FOXO signalling pathways. Differentially expressed genes associated with these causal networks and pathways showed considerable overlap, with decreased expression of *IRS2*, *INSR*, *FOXO3* and *PIK3R1* contributing to enrichment of these networks and pathways. IRS2 alongside IRS1 mediates the many effects of insulin on cellular metabolism through activation of phosphatidylinositol 3-kinase (PI3k)–Akt–mTOR/FOXO signalling cascade.[31] *IRS2* knockout mice show insulin resistance of

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the muscle, fat and liver,[32] whereas in humans, *IRS2* mRNA expression has been reported to decline in insulin resistance.[33] Thus, reduced expression of *IRS2* in overweight and obese individuals observed in this study may reflect increased insulin resistance in elderly individuals with obesity. The insulin–PI3k–Akt–mTOR/FOXO signalling cascade has been linked to lifespan in many species from yeast to humans.[31,34] The link between this signalling cascade and longevity is likely to reflect the central role insulin plays in many cellular processes including glucose metabolism, lipid homeostasis, autophagy, cell cycle arrest, DNA damage repair, apoptosis and oxidative stress resistance.[31]

In muscle, insulin signalling via activation of PI3k–Akt–mTOR/FOXO cascade has also been shown to play a key role in preserving skeletal muscle mass by increasing amino acid uptake and promoting protein synthesis.[35] Under anabolic conditions, Akt phosphorylates and inhibits TSC2, which results in activation of mammalian target of rapamycin complex 1 (mTORC1), promoting protein synthesis by activating ribosomal protein S6 and by releasing the translation initiation factor eIF-4E. Akt also phosphorylates FOXO3, which inhibits its transcriptional activity, leading to the downregulation of atrogin-1 (FBXO32) and muscle RING finger 1 (MuRF1),[36] which promote muscle protein degradation. Moreover, Akt through direct phosphorylation of AMPK also attenuates AMPK activity.[37] In contrast, catabolic stimuli, via AMPK activation, stimulate FOXO activity, increasing MuRF1 and atrogin-1 expression in muscle leading to muscle atrophy.[36] *FOXO3* expression has not been found to be altered in old versus young muscle, and no change in FOXO3 protein expression has been reported in sarcopenic mice[38]; however, here, we show that *FOXO3* and *IRS2* transcript levels are reduced in muscle from elderly individuals with obesity, suggesting that the combination of obesity and age results in a downregulation of these important mediators of muscle mass. Expression of many of the genes in longevity and AMPK pathways was highly correlated with changes in DNA methylation, suggesting that DNA methylation may mediate or consolidate such changes. Age has been shown to be a strong driver of DNA methylation changes, and epigenetic clocks predictive of chronological age have been developed based on age-related changes in DNA methylation. The original pan-tissue epigenetic clock did not include skeletal muscle samples, and its accuracy to predict chronological age in muscle tissue is limited. However, more recently, Voisin et al. have developed a muscle-specific epigenetic clock (Muscle Epigenetic Age Test [MEAT]) based on methylation status of 200 CpGs, which can accurately predict chronological age,[15] suggesting muscle-specific changes in DNA methylation during ageing. However, the analysis of DNA methylation with respect to obesity, with or without adjustment for age in this study, suggested that the narrow participant age range is not a strong driver of the methylation changes we observed; rather, the changes in DNA methylation reflect differences in adiposity.

Muscle fibre analyses found that obesity was associated with an increase in type II fast-fibre area. Furthermore, nine genes within longevity and AMPK pathways were associated with type II fast-fibre area, including *CAB39L*, whose expression is altered in muscle tissue from individuals with obesity. Obesity-induced attenuation of calcium signalling has also been shown to modulate excitation–contraction coupling and excitation–transcription coupling in the myocyte, potentially affecting contractile function and muscle performance.[10] Downregulation of *CAB39L*, associated with fast-fibre area, may therefore be associated with fibre shift and modified contractile function within obese muscle.

Although the activity of many of the pathways identified in this study has previously been implicated in ageing, obesity or insulin resistance, here, we show that the transcriptional regulation of genes within these pathways is altered; whether this is a long-term response to the ageing or obese state is not known. However, we show that obesity in older individuals is associated with substantial changes in the muscle transcriptome of genes involved in metabolism, muscle atrophy and protein synthesis. Strengths of this study are that to the best of our knowledge this is the first study to examine the effect of obesity in older individuals on the global muscle transcriptome and to investigate the correlation between DNA methylation and gene expression. Furthermore, changes in gene expression observed in relation to obesity measured by BMI were also observed in relation to per cent fat mass and total fat mass. Limitations are that we compared the effect of obesity on the muscle transcriptome of older individuals with no younger comparator group. However, there are limited studies on obesity in older individuals where rates of obesity are rapidly increasing, making this an important population to investigate. Secondly, muscle tissue RNA and DNA were only available from male participants and there are well-reported sexual dimorphisms in muscle fibre-type composition, stem cell regeneration, endurance and recovery, fat deposition and insulin resistance.[39] As pathways linked to these functions and phenotypes were altered with respect to obesity in aged male individuals, differences in the transcriptional response to obesity are likely to be evident between sexes and further studies are required to determine transcriptional changes in muscle with respect to obesity in older females. Thirdly, due to limited muscle material from the biopsies, we were not able to determine if the change in gene expression was accompanied by a change in protein expression.

Conclusions

These findings show widespread changes in the muscle transcriptome associated with obesity with similar changes observed with respect to per cent and total fat mass. Further-

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more, we show correlation between downregulation of longevity and AMPK signalling pathways in obesity and associated changes in DNA methylation in skeletal muscle tissue. These findings support the premise that epigenetic processes play central roles within these pathways. Such findings demonstrate that the transcriptional regulation of components of the insulin–AKT–mTOR/FOXO signalling cascade is altered in muscle of older individuals with obesity, increasing our understanding of aged/obese muscle phenotype and providing novel targets for gene/epigenetic manipulation of such pathways in the treatment of muscle dysregulation in obesity.

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Conflict of interest statement

K.M.G. and H.P.P. have received reimbursement for speaking at conferences sponsored by companies selling nutritional products. C.C. has received consultancy fees and honoraria from Amgen, Danone, Eli Lilly, GlaxoSmithKline, Medtronic, Merck, Nestlé, Novartis, Pfizer, Roche, Servier, Shire, Takeda and UCB. M.A.B., E.S.G., E.A., K.M.G. and K.A.L. are part of academic research programmes that have received research funding from BenevolentAI Bio Ltd., Nestec and Danone. The other authors declare that they have no conflicts of interest.

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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