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

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**Abstract**

**Background**: Among healthy older people a number of correlates of impaired skeletal muscle mass and function have been defined. While 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 RNAseq in muscle biopsies from 40 older community dwelling men from the Hertfordshire Sarcopenia Study (HSS) with regards

to obesity (BMI>30 kg/m2, n=7), overweight (BMI 25-30, n=19), normal weight (BMI<25, n=14), and percentage 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 (FDR)≤0.05), of which 425 genes were upregulated when compared with normal weight. Upregulated genes were enriched in immune response (p=3.18x10-41) and inflammation (Leukocyte Activation p=1.47x10-41,Tumour Necrosis Factor (TNF), p=2.75 x10-15) signalling pathways, and downregulated genes enriched in Longevity (p=1.5x10-3) and AMPK (p=4.5x10-3) 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 (CpG)-gene correlations identified, respectively. Similar changes in the muscle transcriptome were observed with respect to percent 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.

**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]. Among older people the prevalence of obesity is rising markedly; in the UK, 81% of those aged 65-74 are currently overweight or obese [9]. Obesity alone affects skeletal muscle metabolic homeostasis, muscle mass and 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 is sparse. Comparison of old versus young muscle has been associated with differential expression of genes involved in cellular senescence, protein catabolism and oxidative phosphorylation, while 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, percent and total fat mass in older community dwelling adults, and characterise 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 RNAseq analysis. Participants were classed as obese (BMI >30 kg/m2, n=7), overweight (BMI 25-30, n=19) or normal weight (BMI<25, n=14) (Table 1). Six participants were classified as sarcopenic[17]; 3 healthy weight (BMI < 25 kg/m2), three overweight (BMI 25-30).

**Procedures**

Body composition (appendicular lean mass index (ALMi, kg/m2) 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 anesthetic) and muscle tissue snap frozen in liquid nitrogen cooled isopentane and stored (-80°C) until analysis.

**RNA extraction**

RNA was extracted using the mirVana miRNA Isolation Kit (ThermoFisher Scientific). RNA was eluted in nuclease free water at >25 ng/µl (40-100 µl) and quantity/quality measured by Qubit 2.0 Fluorometer (ThermoFisher Scientific) and Bioanalyzer (Agilent Technologies). All RNA had a RIN of >8.0 and stored at -80°C.

**DNA extraction**

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

**RNAseq**

RNAseq was carried out as previously described [7]. Briefly, sequencing libraries were prepared from 250ng 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 controlled with the DNA High Sensitivity Reagent kit on a LabChip GX (Perkin Elmer). Libraries were pooled at an equimolar ratio and clustered at 7 pM on paired-end sequencing flow cells (Illumina). Sequencing was performed for 2x101 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 is publicly available from GEO (accession number GSE111006).

**RNAseq Bioinformatic analysis**

Differentially expressed genes with respect to obesity, total fat mass and percent fat mass were determined using edgeR [20] in R (v3.4.2). 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 an 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**

750ng of genomic DNA was treated with sodium bisulfite (Zymo EZ DNA Methylation-Gold kit, ZymoResearch, Irvine, California, USA) and hybridised 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 was 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-hybridise to other genomic locations (n=14,759), coinciding with SNPs (n=77,261), probes with a detection p-value >0.01 (n=5,604), beadcount <3 (n=1,051) and 2,928 non-CpG probes were removed from analysis. After pre-processing and QC, 34 samples and 744,547 CpG probes remained in the dataset. Data is available from GEO (accession number GSE154980). Robust regression models using limma were run for total fat mass with or without adjustment for age. Details in Supplementary materials.

**Histological Analysis**

Prevoiusly collected and published immunohistochemistry data [21] was used to examine correlations between muscle morphological measures and gene expression. Briefly, muscle tissue was fixed overnight (−20°C) embedded in glycol methacrylate resin and 7μm serial cross-sections 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 Ziess Ltd, Welwyn Garden City, UK) viewed at ×5 magnification and digitized obtaining tissue area, myofibre number (type I, slow fibre vs. type II, fast fibre, expressed as percentages of total fibres), and myofibre cross-sectional areas (μm2). Full details in Supplementary materials.

**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 determined using BiNGO . Pathway analysis was performed using Ingenuity Pathway Analysis (IPA) (Qiagen, UK) version 68752261. Full details in Supplementary materials.

**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 compared across adiposity groups (one-way ANOVA with Tukey’s multiple comparison test). Analysis of fibre associations were performed using linear regression of normalised, log transformed counts of 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/m2 and percent fat mass 27.42. Total and percent 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 participants (normal weight) and three (overweight) were categorised 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 XIIIA Chain (*F13A1*, FDR=5.69 x10-05) and S100 Calcium Binding Protein A4 (*S100A4*, FDR=8.64 x10-05); the top 2 downregulated genes were Glial-Derived Neurotrophic Factor (*GDNF*, FDR=2.09 x10-03) and Insulin Receptor Substrate 2 (*IRS2*, FDR=2.61 x10-03) (Figure 1, Table 2, Tables S1, S2). As 3 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 2 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=1.80x10-02) and Synaptotagmin Like 2 (*SYTL2*, FDR=1.80x10-02); the top 2 downregulated genes were RNA 7SL Cytoplasmic 449 Pseudogene (*RN7SL449*, FDR=1.80x10-02) and *IRS2* (FDR=1.98 x10-02) (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 Spermatogenesis Associated 7 (*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 protein-protein interaction (PPI) network. The PPI enrichment p-values for the upregulated and downregulated gene networks were 1x10-16 and 2.33x10-6, 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 Gene Ontology (GO) or Kyoto Encyclopaedia of Genes and Genomes (KEGG) were Cell Activation (p=1.47x10-41), Leukocyte Activation (p=1.47x10-41), Immune System Process (3.18x10-41) whilst among downregulated genes the top pathways were Longevity Regulating Pathway (p=1.5x10-3), AMPK Signalling Pathway (p=4.5x10-3), Double-Stranded DNA Binding (p=1.96x10-2) (Table 4, Tables S4, 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, S7). Among 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*, *FOXO3* (nominal p≤0.05) were also downregulated in overweight individuals in the longevity pathway, with *RPTOR*, Solute Carrier Family 2 Member 4 (*SLC2A4*), *PPARGC1a/PGC1α*, Member RAS Oncogene Family(*RAB11B*), *INSR*, *FOX01*, *FOX03* (nominal p≤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 Ingenuity Pathway Analysis (IPA) to identify potential causal networks and upstream regulators that may mediate such changes. Within the upregulated gene set, top causal networks were Translocation-Ets-Leukemia Virus-Runt-Related Transcription Factor 1 (*ETV6-RUNX1*, p=1.88x10-18), Membrane Attack Complex (*MAC*, p=4.04x10-17) and Plasminogen Activator Urokinase Receptor (*PLAUR*, p=1.38 x10-15). Top upstream regulators were Translocation-Ets-Leukemia Virus-Runt-Related Transcription Factor 1 (p=4.15x10-17), Granulin Precursor (*GRN*, p=3.28 x10-12) and CCAAT Enhancer Binding Protein Alpha (*C/EBPA*, p=4.05 x10-10) (Table S8).

Within the downregulated gene set, the top causal networks were **Serine/Threonine-Protein Kinase D1 (***PRKD1*, p=1.39x10-05), *SMARCB1* (p=1.63x10-05) and AKT Serine/Threonine Kinase 1 (*AKT1*, p=1.63x10-05). Top upstream regulators were Dihydrolipoamide S-Succinyl transferase (*DLST*, p=6.47x10-05), **SMAD**Family Member 4 (*SMAD4*, p=2.22x10-03) and Methyl-CpG Binding Domain Protein 3 (*MBD3*, p=2.81x10-03) (Table S9).

**Similar changes in the muscle transcriptome were observed with respect to percent 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 percent fat mass and total fat mass. 1048 genes were differentially expressed (FDR≤0.05) with respect to percent fat mass, of which 891 were upregulated. (Figure 1, Table 5, Table 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, percent fat mass and total fat mass, with 373 genes associated with all 3 measures, 726 common genes between percent fat mass and total fat mass, 437 between obesity and total fat, and 380 between obesity and percent fat mass (Figure 1E).

PPI analysis of the differentially expressed upregulated genes associated with percent fat mass, produced a PPI network with an enrichment p-value of 1 x10-16; the top 5 enriched pathways in GO and KEGG were Immune System Process (p=1.43x10-66), Cell Activation (p=1.41x10-64) and Leukocyte Activation (p=1.93x10-61). The downregulated genes produced a PPI network enrichment p-value of 3.6 x10-3, with the top enriched pathways in GO and KEGG being AMPK Signalling Pathway (p=4.8x10-03), Longevity Regulating Pathway (p=4.8x10-03) and FOXO Signalling Pathway (p=2.02x10-02) (Tables S12, 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,*  Insulin-like Growth Factor 1(*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*)*, 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**

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 found to be differentially expressed (FDR<0.05) in obese vs normal subjects including 3 CpGs within *FOXO3*, 4 CpGs within Insulin Receptor (*INSR*), 5 CpGs within Phosphatidylinositol 3-Kinase (*PIK3R1*) and 5 CpGs within *ULK1* (Table 6, Figure S2, Table S14). 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 vs normal subjects included 3 CpGs within *FOXO3*, 4 CpGs within *INSR,* 5 CpGs within *PIK3R1* and 5 CpGs within *ULK1*(Table 6, Table 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 performedwith and without age as a covariate; there was considerable overlap of the fat mass associated differentially methylated CpGs (dmCpGs) between age adjusted and 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 (Table S16, S17).

**Obesity is associated with increased type II fibre area.**

To determine functional implications of increased adiposity we examined the percent 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. 9 genes significantly associated (p<0.05) with fast fibre area including *PPARGC1A* (p=1.8x10-3), Klotho (*KL)* (p=1.9x10-2), Calcium-binding protein 39-like *CAB39L* (p=2.0x10-2), *PRKAA2* (p=2.3x10-2), TBC1 Domain Family Member 1 (*TBC1D1*)(p=2.3x10-2), cAMP Responsive Element Binding Protein 3 (*CREB3*)(p=2.6x10-2), *INSR* (p=2.6x10-2), Protein Phosphatase 2 Regulatory Subunit B delta(*PPP2R2D*)(p=4.1x10-2) and *RAB11B* (p=4.7x10-2) 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, percent fat mass and total fat mass. Upregulated genes were enriched in pathways associated with inflammation, whilst 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 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 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 expression of 542 genes in muscle were 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 percent fat mass; while 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, while percent fat mass measures percent fat relative to body size. There was also substantial overlap of obesity related genes and pathways with those associated with percent fat mass and total fat mass, suggesting BMI is capturing the transcriptomic signature of increased adiposity in these individuals.

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 [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 among 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 uncharacterised 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 IL-13, and 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, while 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 (PI 3-kinase)-Akt -mTOR/FOXO signalling cascade [31]. *IRS-2* knockout mice show insulin resistance of muscle, fat and liver [32], while 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 PI-3 kinase-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 stimulates FOXO activity, increasing MuRF-1 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 *FOXO3* and *IRS2* transcripts levels are reduced in muscle from elderly individuals with obesity, suggesting 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 were highly correlated with changes in DNA methylation, suggesting 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*., (2020) has 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, 9 genes within Longevity and AMPK pathways were associated with type-II fast-fibre area, including Calcium-binding protein 39-like (*CAB39Ll*), 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.

While the activity of many of the pathways identified in this study have 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 our knowledge this is the first 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 percent 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 is 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 percent and total fat mass. Furthermore, 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**

K.M. Godfrey and H.P. Patel have received reimbursement for speaking at conferences sponsored by companies selling nutritional products. C. Cooper 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. Burton, E.S. Garratt, E. Antoun, K.M. Godfrey, and K.A. Lillycrop 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.

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