

Increased expression of H19/miR-675 is associated with a low fat free mass index in patients with COPD

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ABSTRACT

Background: Loss of muscle mass and strength are significant co-morbidities in patients with COPD that limit their quality of life and have prognostic implications but do not affect everyone equally. To identify mechanisms that may contribute to the susceptibility to a low muscle mass, we investigated miRNA expression, methylation status, and regeneration in quadriceps muscle from COPD patients and the effect of miRNAs on myoblast proliferation *in vitro*. The relationships of miRNA expression with muscle mass and strength was also determined in a group of healthy older men.

Methods: We identified miRNAs associated with a low fat free mass (FFM) phenotype in a small group of patients with COPD using a PCR screen of 750 miRNAs. The expression of two differentially expressed miRNAs (miR-675 and miR-519a) was determined in an expanded group of COPD patients and their associations with FFM and strength identified. The association of these miRNAs with FFM and strength was also explored in a group of healthy community-dwelling older men. As the expression of the miRNAs associated with FFM could be regulated by methylation, the relative methylation of the H19 ICR was determined. Furthermore, the proportion of myofibres with centralized nuclei, as a marker of muscle regeneration, in the muscle of COPD patients was identified by immunofluorescence.

Results: Imprinted miRNAs (miR-675 and from a cluster, C19MC which includes miR-519a) were differentially expressed in the quadriceps of patients with a low fat-free mass index (FFMI) compared to those with a normal FFMI. In larger cohorts, miR-675 and its host gene (H19) were higher in patients with a low FFMI and strength. The association of miR-519a expression with FFMI was present in male patients with severe COPD. Similar associations of miR expression with lean mass and strength were not observed in healthy community dwelling older men participating in the Hertfordshire Sarcopenia Study. Relative methylation of the H19 ICR was reduced in COPD patients with muscle weakness but was not associated with FFM. *In vitro*, miR-675 inhibited myoblast proliferation and patients with a low FFMI had fewer centralised nuclei suggesting miR-675 represses regeneration.

Conclusion: The data suggest that increased expression of miR-675/H19 and altered methylation of the H19 imprinting control region are associated with a low FFMI in patients with COPD but not in healthy community dwelling older men suggesting that epigenetic control of this loci may contribute to a susceptibility to a low FFMI.

INTRODUCTION

Skeletal muscle is central to all aspects of physical function. Consequently the loss of muscle mass and strength that accompanies a range of chronic diseases impairs the quality of life of many individuals. Quadriceps muscle wasting[1] and weakness [2] are common complications of Chronic Obstructive Pulmonary Disease (COPD), and have been associated with reduced survival. Loss of muscle mass and function can occur even in mild COPD [3], yet some patients with severe COPD maintain muscle mass and function [4]. In patients with severe disease, measures of muscle mass and strength are better predictors of mortality than FEV₁ [1]. Pulmonary rehabilitation, an exercise-based regimen that principally augments muscle mass and function, is an effective treatment in COPD [5, 6] highlighting the impact of skeletal muscle on a patient's clinical condition.

In healthy adults muscle mass is maintained by balancing loss (through protein degradation, autophagy and apoptosis) with synthesis (through protein synthesis and satellite cell recruitment) [7]. In patients with chronic disease the relative rates of these processes are altered such that catabolic processes outweigh anabolic processes causing muscle loss. However, individuals are likely to have their own balance of anabolic and catabolic processes against which the insult of COPD is superimposed. If the balance of catabolism and anabolism is variable between individuals, it follows that individuals will differ in their response to an identical magnitude of atrophic insult. This suggestion is consistent with the observation that patients with the same degree of lung function impairment display a large variation in degree of muscle wasting. Susceptibility to muscle wasting may result from influences operating in early life as can be seen from the increased prevalence of sarcopenia in individuals who had a low birth weight [8, 9].

MicroRNAs (miRNAs) are important regulators of cell phenotype involved in both normal development and disease [10]. We have recently shown that there is a reduction in miR-1 in the *quadriceps* muscle of COPD patients, associated with an increase in its target, HDAC4 [11]. Others have shown that changes in miRNA are important in atrophy associated with congenital myopathies

[12], maintaining fibre type [13] and muscle adaptation to exercise [14]. MiRNAs exported into the circulation are also potential biomarkers for specific disease phenotypes and for following disease progression [15, 16]. We have previously shown levels of muscle-specific miRNA are elevated in the plasma of COPD patients relative to controls [17].

As many miRNAs are processed from RNA polymerase II dependent transcripts, miRNA levels will be affected by the same mechanisms that control mRNA production, including DNA methylation,. Consistent with this suggestion, hypermethylation of the loci for miR-9, -148 and -34c inactivates expression of these miRNA in cancer cells[18-20]. Other miRNAs are also expressed from imprinted regions of the genome so expression of one or other allele is suppressed often by methylation. Indeed two of the largest miRNA clusters in the human genome, one on chromosome 19 (C19MC) and one of chromosome 14 (C14MC) are imprinted with the C19MC expressed only from the paternal chromosome [21] and the C14MC expressed from the maternal chromosome [22]. Similarly, miR-675 is processed from the maternally expressed lincRNA, H19. This gene is widely expressed during development, becomes down regulated in most adult tissues but is maintained in adult muscle[23]. Consistent with a role in muscle development or maintenance, miR-675 has been shown to inhibit myoblast proliferation and promote differentiation [24].

Smoking, a primary cause of COPD, is known to modify DNA methylation through a number of mechanisms including the introduction of DNA breaks and nicotine dependent suppression of DNA methylase (DNMT1) expression (reviewed in [25]). Consequently changes in DNA methylation are likely to contribute to the changes in gene expression including that of miRNAs. The extent to which these effects occur in an individual will be dependent on factors including the relative activity of the DNA methylation cycle enzymes. Polymorphisms that lead to reduced activity of the DNA methylation cycle and hypomethylation of DNA are over-represented in elite athletes suggestive of a role in muscle biology[26].

The targeted studies we have performed previously have identified altered miRNA expression in the muscle and plasma of COPD patients [17, 11]. This led us to hypothesise that the miRNA pattern would differ between patients with a low FFMI and those with a normal FFMI and that these differences would provide mechanistic insight into the susceptibility of some individuals with COPD to lose significant muscle mass. We compared miRNA expression profiles in samples of quadriceps muscle from COPD patients with a low FFMI and those with a normal FFMI. This analysis identified miRNAs from two imprinted regions of the genome, miR-675 (from the maternally imprinted lincRNA H19) and C19MC miRNAs (from a paternally expressed cluster) as the most differentially expressed miRNAs. We validated the findings by analyzing the expression of miR-675 and miR-519a and the host gene for miR-675 (H19) in a larger group of COPD patients and determined their association with FFMI and strength. As these miRNAs were derived from imprinted loci we next analysed relative DNA methylation on the H19 imprinting control region (ICR) in DNA isolated from the quadriceps of COPD patients. If the differences identified were just a measure of size rather than susceptibility to a loss of muscle mass we would have expected the same associations to exist in a normal population. We therefore determined the expression of these miRNAs in the quadriceps of healthy community dwelling older men participating in the Hertfordshire Sarcopenia Study. Finally to understand the contribution of regeneration to the difference between low FFMI and normal FFMI we determined the proportion of fibres with centralized nuclei in samples from COPD patients with a low or normal FFMI.

MATERIALS AND METHODS

Subjects

COPD cohort: Patients with COPD according to the Global Initiative in Obstructive Lung Disease (GOLD) guidelines 2004 [27] were enrolled from clinics at the Royal Brompton Hospital. Patients with a diagnosis of heart, renal or liver failure, a systemic inflammatory or metabolic disorder or a moderate/severe exacerbation (i.e. requiring antibiotics, oral steroids, or hospitalisation) in the preceding 4 weeks were excluded. Healthy age-matched controls were recruited by advertisement. All subjects gave written informed consent and the protocol was approved by the Royal Brompton & Harefield NHS Trust Research Ethics Committee (Studies 06/Q0404/35 and 06/Q0410/54). 16 control subjects were recruited by local advertisement. A low fat free mass index (low FFMI) was defined as below 16.0 kg/m²[28] .

Physiological measurements: COPD subjects in this study form part of a larger well-phenotyped cohort described by Natanek et al [4]. Measurements of lung volume, using plethysmography, carbon monoxide transfer factor, using the single breath technique (CompactLab, Jaeger, Germany) and post-bronchodilator spirometry were performed according to ATS/ERS guidelines [29]. Blood gas tensions were measured in arterialised capillary earlobe blood. Fat-free mass index (FFMI) was calculated using bioelectrical impedance (Bodystat 1500, Bodystat, UK) measured in patients after resting supine for 10 min as described previously [30]. Due to the availability of samples, it was not possible to work on a single set of samples. The physiological characteristics of the groups analysed are given in Table 1 and Supplementary Tables 1, 2, 4 and 5.

Quadriceps strength was determined by measuring supine isometric maximal voluntary contraction (MVC) and unpotentiated Twitch force (TwQ) as described previously [30] and exercise capacity measured as 6 minute walk distance (6MW) 5 minutes after bronchodilator treatment (ATS 2002 guidelines[31]) as described previously. Muscle biopsy was performed by percutaneous needle

biopsy of the *vastus lateralis* in the mid-thigh of the leg that strength was tested was performed under local anaesthesia using the Bergstrom technique[32].

Hertfordshire Sarcopenia study cohort: Samples from the Herts Sarcopenia Study study [33] were used in this analysis as an additional control set to determine whether similar associations existed in a normal healthy population of older people as those identified in the COPD cohort and whether there were any associations with birth weight. Recruitment of this cohort has been described previously [33]. The protocol was approved by the Hertfordshire Research Ethics Committee (study 07/Q0204/68) and all participants gave written informed consent. The skeletal muscle characterisation carried out in this study included; body composition and lean mass by dual energy X ray absorptiometry scanning, hand-grip strength, determined using a Jamar hydraulic dynamometer and physical capability determined as 3m gait speed and '6m time to get up and go' test. These have been previously described [33] and the data for the cohort used in this study are shown in Supplementary Table 3. Muscle biopsy was performed by percutaneous needle biopsy of the *vastus lateralis* under local anaesthesia as described previously[34].

TaqMan® Array MicroRNA Cards: The amplified cDNAs were quantified using Human TaqMan® Array MicroRNA Cards (Cards A and B, V.3.0 Applied Biosystems) according to the manufacturer's instructions on an ABI 7900HT thermocycler. Array cards were run under the 384 well TaqMan® Low Density Array default thermal-cycling conditions defined by SDS 2.4 software. Once complete all cards were calibrated to a single control sample and one detection threshold set across all samples and assays using RQ manager software. The resultant Ct values were then exported and normalised to the geometric mean of U6, RNU44 and RNU48, using the $\Delta\Delta$ Ct method. As variance tended to increase with miRNA intensity the data were standardised by a log transformation.

Assessment of mRNA and miRNA levels: Messenger RNA was extracted and quantified by quantitative real time PCR (qRTPCR) as described previously [35] or using the allele specific primer kit for RS2075245 (Life Technologies). PCR values for H19 (obtained from 11 controls, 34 normal

FFMI patients and 22 low FFMI patients) were normalised to the geomean of RPLPO and HPRT. Primers were H19: For-TGCTGCACTTTACAACCACTG, Rev- TGGTGTCTTTGATGTTGGGC RPLPO: For-TCTACAACCCTGAAGTGCTTGATATC, Rev-GCAGACAGACACTGGCAACATT and HPRT: For-GCTATAAATTCTTTGCTGACCTGCTG, Rev- AATTACTTTTATGTCCCCTGTTGACTGG. MicroRNA expression was analysed in trizol extracted RNA. RNA isolated muscle was reverse transcribed using MultiScribe™ Reverse Transcriptase with, Megaplex™ RT Primers (human pools A and B, Version 3.0, Applied Biosystems) according to the manufacturer's instructions. The reactions were terminated by heat deactivation at 85°C for 5min and the cDNA stored at -80°C. The cDNAs were pre-amplified using Megaplex™ PreAmp Primers (Applied Biosystems) for 12 cycles of 95°C for 15 sec and 60°C for 4 min. The reaction was terminated by heating to 99.9°C for 10 min and pre-amplified cDNA diluted by addition of 75µl of 0.1× TE buffer pH 8.0 (Qiagen) and stored at -80°C.

Quantification of single microRNAs using TaqMan® probes: For the quantification of individual microRNAs, custom designed primers and probes were purchased for each test gene from Applied Biosystems and amplification was carried out on cDNA preamplified as described above according to the manufacturer's instructions. Each reaction was performed in duplicate and the average Ct value normalised to the corresponding geometric mean of U6, and RNU44 using the $\Delta\Delta$ Ct method. RNA isolated from cells was analysed using single RT reactions. MiRNAs were quantified in 16 controls, 24 low FFMI patients and 24 normal FFMI patients the COPD cohort and from 67 individuals in the Herts Sarcopenia Study cohort.

Methylation analysis

DNA from muscle biopsies (for 10 controls, 14 normal FFMI patients and 15 low FFMI patients) was extracted using the QIAamp DNA Mini kit (Life Technologies) according to manufacturer's instructions. The two resulting elutions were then pooled and precipitated. The resuspended DNA was diluted to 12.5ng/µl in AFA fiber pre-slit Snap-Cap microtubes (Covaris, Life Tech) and sheared using a Covaris S220 Focused-ultrasonicator to sonicate the DNA into 200bp fragments using a pre-

set protocol in the SONOLAB 7.2 software (Covaris, Life Tech). DNA (1µg) was applied to the MethylMiner Methylated DNA Enrichment kit (Life Tech) according to manufacturer's instructions. The resulting methylation enrichment DNA was eluted in 2000mM and 450mM NaCl, which were then concentrated and pooled. The DNA was then quantified using the Quant-iT PicoGreen dsDNA (Life Tech) according to manufacturer's instructions. The methylation enriched DNA was diluted to 0.1ng/µl whilst the original (non-enriched) DNA was diluted to 1ng/µl. 1.5µl of DNA was amplified in 20µl SYBR reactions as described for cDNA using primers UBE2B: For-CTCAGGGGTGGATTGTTGAC , Rev-TGTGGATTCAAAGACCACGA, H19 ICR: For-TTGGTGGAACACACTGTGATCA, Rev-GAGCCGCACCAGATCTTCAG.

Immunofluorescence: Frozen 10µm thick slices from quadriceps muscle biopsies of both low FFMI (n=5) and normal FFMI (n=10) patients (Supplementary Table 5) were fixed in 4% PFA for 10 min at room temperature. Following fixation sections were washed in 1× PBS-T (3× for 3min), prior to incubation in 5% BSA-PBS-T solution for 30 min at room temperature. The sections were then incubated with anti-laminin 2α antibody (1:300, AbCam) overnight at 4°C in a humidified chamber. The primary antibody solution was removed and the sections washed before incubation with anti-rabbit IgG conjugated to Alexafluor-658 (1:500, Invitrogen). The sections were incubated with the secondary antibody for 1h at room temperature, then washed and before be incubated with a DAPI solution (1:10,000) for 15 min.

Statistical analysis: All gene expression data were log transformed to stabilize variance and produce a normal distribution. To identify groupings within the data set principal components analysis (PCA) and hierarchical cluster analysis (HCA) were performed in Aabel (Gigawiz). The variables for HCA were scaled to unit variance and distances were calculated based on correlation coefficients and groups were defined based on centroid linkage. Correlation analysis was performed using Pearson correlations assuming that correlations would be linear (Aabel). Differences between groups were

calculated by Student's t-test for normally distributed data (Excel) and by Mann-Whitney U test for non-parametric data (Aabel, Gigawiz).

RESULTS

Patient characteristics

miRNA profiles were determined in RNA isolated from the quadriceps of, 7 patients with a low FFMI, 7 patients with a normal FFMI and 7 age-matched controls. All samples in this analysis were from males and their physiological characteristics are shown in Supplementary Table 1. Consistent with a diagnosis of COPD, both the low FFMI and normal FFMI patient groups had reduced FEV₁ and TL_{CO}% of predicted. *Quadriceps* strength, physical activity levels, and exercise performance were all reduced compared to controls. Both the low FFMI and normal FFMI COPD groups had a lower mean FFMI than the healthy controls. The low FFMI and normal FFMI groups did not differ in any characteristic measured including daily physical activity except, by design, FFMI and consequently weight and BMI (Supplementary Table 1). Furthermore, there was no difference in fibre type proportion between the patient groups although both the low FFMI groups had a lower proportion of type I fibres than the controls (Supplementary Table 1).

Low FFMI patients are a distinct sub-group of COPD patients

HCA and PCA, showed that the low FFMI patients formed a distinct group based on their miRNA profile. PCA but not HCA was also able to partially segregate normal FFMI patients and controls however, there was overlap in these two groups (Fig. 1A and B). This result was confirmed by linear discriminate analysis, which allowed us to correctly assign the entire low FFMI group, all controls and 5 of the 7 normal FFMI patients from their PCA scores.

Identification of FFMI associated miRNAs in muscle

To identify miRNAs associated with a low FFMI in COPD, the expression of individual miRNAs was compared by t-test between the groups in a pair wise manner. To increase stringency miRNA

associated with a low FFMI in COPD were defined as those that were statistically significantly different between low FFMI patients and controls/normal FFMI patients but not statistically different between controls and normal FFMI patients.

Forty-seven low FFMI-associated miRNAs were identified (Supplementary Table 5). Of these only two miRNAs were increased in low FFMI patients relative to normal FFMI patients of which miR-675 showed the greatest fold change of any miRNA (8.5 fold low FFMI vs normal FFMI $p=0.008$). The remaining 45 miRNAs were expressed at lower levels in low FFMI- compared to normal FFMI- patients. Median expression of all but 2 miRNAs was lower in low FFMI patients compared to controls. Comparison of miRNA expression in low FFMI and normal FFMI patients relative to controls showed that the median expression of 17 miRNAs changed in opposing directions in low FFMI vs. controls (statistically significant) compared to normal FFMI vs. controls (not statistically significant) (Fig 1C and Supplementary Table 6) including miR-675, miR-1 (consistent with our previous study [11]) and 9 miRNAs from a cluster located on chromosome 19q13.2 (C19MC).

Muscle Array validation:

To validate the screen, levels of miR-675 and miR-519a (derived from the C19MC) were quantified in an expanded cohort of muscle samples from patients including both males and females of all GOLD stages and healthy controls (controls $n=16$, normal FFMI $n=24$ and low FFMI $n=24$, freshly prepared cDNA samples from 5 normal FFMI, 1 low FFMI COPD patients and 4 controls from the original screen were used in this expanded study). The physiological characteristics of these groups are given in Table 1 and showed similar differences to those of the screen cohort. There was no difference in the expression of miR-675 between COPD patients (as a whole group) with controls. However, median miR-675 expression was higher in the low FFMI patients than either of the other two groups but was significantly different only compared to the controls (low FFMI vs cont; $p=0.013$, low FFMI vs normal FFMI; $p=0.027$, Bonferonni corrected p value for significance $=0.016$). There was no difference in the expression of miR-675 between normal FFMI patients and controls (Fig 2A). In

the patients, miR-675 was inversely correlated with FFMI ($r=-0.41$, $p=0.004$, Fig 2B). Furthermore, miR-675 was inversely correlated with quadriceps strength (vs. maximal voluntary contraction MVC, $r=-0.43$, $p=0.003$, vs. twitch force TwQ, $r=-0.47$, $p=0.001$, Fig 2C and D). MiR-675 was not associated with lung function judged by either FEV₁ or TL_{CO}% predicted. In the controls miR-675 was not associated with FFMI or quadriceps strength.

There was no difference in the expression of miR-519a between COPD patients as a whole group with controls. In the whole cohort (patients and controls together), consistent with the array, miR-519a was lower in individuals with a low FFMI than in those with a normal FFMI ($p=0.025$, Fig. 3A) and weakly correlated with FFMI ($r=0.24$, $p=0.044$, Fig. 3B). However, miR-519a was not different between patients with a low FFMI compared to patients with a normal FFMI. Restricting the analysis to samples with the original criteria used for patients in the screen (male GOLD III-IV, $n=27$) increased the association between miR-519a and FFMI ($r=0.48$, $p=0.017$, Fig. 3C). No association between miR-519a and FFMI was found in female patients with similar disease severity ($n=13$) raising the possibility of a sex-specific effect. Analysis of a second miRNA from the same locus (miR-518e) in GOLD III-IV males also showed a positive association of C19MC miRs with FFMI in these patients (Fig. 3D).

H19 linc RNA expression associates with muscle mass and strength in COPD

As miR-675 is processed from the H19 lincRNA, we determined the expression of H19. As the cDNAs for the miRNAs were generated separately from cDNAs for mRNA analysis and our samples were limited, it was only possible to obtain overlapping but distinct data sets for miR-675 and H19. This analysis used samples from 11 controls, 34 NFFMI patients and 22 low FFMI patients; 31 samples were included in both miRNA and mRNA analyses (see supplementary table 2 for the physiological characteristics). There was no difference in H19 expression between the patients and controls or between the low FFMI and normal FFMI patients (Fig 4). However, H19 expression was inversely associated with FFMI in the patients ($r=-0.39$, $p=0.001$) and with quadriceps strength (vs.

MVC $r=-0.44$, $p<0.001$ and vs. TwQ $r= -0.38$, $p=0.007$). H19 expression was also higher in those patients defined as weak based on an MVC less than 120% of their BMI ($p=0.01$). In those patients in which we analysed both miRNA and mRNA, expression of miR-675 and H19 showed a positive association ($r=0.49$, $p<0.018$, $n=22$ patients only and $r=0.47$, $p<0.007$ $n=31$ patients and controls, Supplementary Fig 1).

Methylation of the H19 locus is associated with muscle H19 expression and strength in COPD

To determine whether H19 was expressed from one or both alleles in adult muscle we used allele-specific PCR for the polymorphism RS2075245, which is in exon 1 of H19, close to miR-675. In this experiment homozygosity or mono-allelic expression was assumed when there was difference in Cycle threshold for each of the alleles of 4 cycles (i.e. when one allele contributed $>94\%$ of the detectable transcript). In this analysis, expression of a single SNP variant was detected in 60% of the samples whereas expression of both variants was detected in the remaining 40%. Within the European Caucasian population the A:T allele frequency is 48:52 so that the approximately 50% of individuals are heterozygous. Therefore, in the majority of individuals both alleles were active in the muscle. However in the subjects in whom both A and T alleles were detected, one allele contributed more than 75% of the transcript in all but 3 cases indicating that one allele was suppressed, probably by methylation. It was not possible to determine whether the same was true from homozygous individuals. As these primers are designed for genotyping and not mRNA quantification, to verify that the method quantified H19 RNA, the expression of each allele was normalised to RPLPO and the values summed. This value correlated strongly ($r=0.86$, $p<0.001$) to our measurements of H19 performed by non-allele specific PCR (Supplementary Fig 2) indicating that the approach was valid.

To determine whether methylation regulates H19 expression in muscle, we used methylated DNA immunoprecipitation (MeDIP) followed by qPCR using muscle samples from 10 healthy controls, 14 normal FFMI patients and 15 low FFMI patients. Methylation on the ICR of precipitated H19 DNA was quantified relative to a comparatively unmethylated gene UBE2B. There was no

significant difference between relative ICR methylation between patients and controls. Consistent with methylation suppressing H19 expression, the ICR:UBE enrichment ratio was inversely correlated with H19 expression in the COPD patients ($r=-0.498$, $p=0.008$, Fig. 5A). Furthermore, the ICR/UBE methylation ratio was higher in patients with a normal strength compared to weak patients ($p=0.026$, Fig 5B) and correlated with strength ($r=0.45$, $p=0.014$, Fig. 5C) but not with FFMI. In this cohort of patients H19 correlated with strength ($r=-0.45$, $p=0.017$) but not with FFMI and was different between patients with normal strength and weak patients ($p=0.009$, Fig 5D). In the controls the association of relative ICR methylation with strength did not reach statistical significance ($r=0.57$, $p=0.084$) unless normalized to FFMI ($r=0.64$, $p=0.045$, Supplementary Fig 3). Similarly strength normalized for FFMI was also correlated with ICR methylation in the patients alone ($r=0.48$, $p=0.009$ Supplementary Fig. 3) and in the cohort as a whole ($r=0.45$, $p=0.004$). Methylation of the C19MC was not determined because these miRNAs are not expressed in differentiated cells [36, 37]. As analysis of methylation in the biopsies would have profiled whole muscle, the majority of signal would have been from cells that did not express the miRNA rather than determining the effect of methylation on expression.

Are miR-675 and miR-519a markers of FFMI in the general population?

The association of miR-675 and miR-519a with FFFMI in COPD raises the possibility that they are markers of muscle mass in the general population rather than showing an adaptation to disease. We therefore determined the expression of both miRs in the quadriceps of a subset of individuals from the Hertfordshire Sarcopenia Study, which assessed muscle mass and function in healthy older adults. In this cohort neither miRNA was associated with FFMI or strength (measured as grip strength) or with physical capability marked by 3m walking time and 6m time to get up and go. Furthermore, there was no association of the expression of these miRNAs with birth weight. Comparison of the expression of miR-675 and miR-519a in people with a history of smoking and in non-smokers also showed no difference.

Altered regeneration in low FFMI patients

The ability of miR-675 to inhibit C2C12 cell proliferation (as previously described [38] and as shown in supplementary information) raises the possibility that there is a difference in muscle regenerative activity in low FFMI patients and normal FFMI patients. We therefore determined the expression of MyoD, a marker of satellite cell activation, and the number centralized nuclei in samples from male patients with GOLD 3/4 COPD, the selection criteria for our original screen. MyoD was suppressed in the low FFMI patients compared to the normal FFMI patients (low FFMI= 0.031 ± 0.009 AU, normal FFMI= 0.041 ± 0.017 AU, $p=0.037$, Fig. 6A). The low FFMI patients also had fewer fibres with centralised nuclei indicating recent regeneration compared to normal FFMI patients (normal FFMI 6.9 ± 1.1 centralised nuclei/100 fibres vs low FFMI 3.2 ± 0.6 centralised nuclei/100 fibres $p=0.036$, Fig. 6B-D). These findings are consistent with increased muscle regeneration occurring in normal FFMI patients compared to low FFMI patients. In patients in which centralized nuclei, miR-675 and miR-519a were all measured, miR-675 was higher (LFFMI median was 2.4 fold higher than the NFFMI median, $p=0.014$) and miR-519a was lower (LFFMI median was 0.71 times the NFFMI median, $p= 0.008$) in the LFFMI patients than in the NFFMI patients.

DISCUSSION

The data presented show that a low FFMI and weakness in COPD patients are associated with increased expression of miR-675 and H19 in the muscle and that muscle weakness correlates in these patients with relative methylation of the H19 ICR. Furthermore, the expression of miRNAs from the C19MC is suppressed in the muscle of male patients with a low FFMI and associated with FFMI. However, there was no association of miRNA expression with FFMI or strength in healthy individuals from the Herts Sarcopenia Study nor did smoking affect the expression of the miRNAs, suggesting that the associations of miRNA with FFMI and strength occur only on the background of the disease. Finally we confirm that there are fewer centralized nuclei in the muscle of low FFMI patients compared to patients with a normal FFMI raising the possibility that poor regeneration is a contributory to the low FFMI phenotype. Together, these data indicate that the level of imprinted miRNAs contributes to the susceptibility to muscle wasting in the context of COPD, where the disease provides an atrophic stimulus that can be resisted in individuals to varying extents dependent on the expression of these miRNAs. The potential mechanisms by which these miRNAs contribute to the low FFMI phenotype are discussed separately below.

miR-675-H19

The H19-IGF-2 imprinted gene cluster is one of the best-studied imprinted regions[39]. In this cluster H19 is expressed from the maternal chromosome whereas IGF-2 is expressed from the paternal chromosome with expression of H19 suppressed by methylation of an imprinting centre. H19 is a long intergenic non-coding RNA the function of which appears to be to act as a source of miR-675 [24, 38]. The fact that the observed associations of H19 with strength and FFMI were not as strong as those of miR-675 is consistent with this suggestion. Previous studies of miR-675 have shown that it inhibits cell proliferation and consistent with this observation we also found that miR-675 could inhibit C2C12 proliferation. This inhibition of proliferation has been shown to occur at

least in part through suppressing IGF1R[38], therefore increased miR-675 in myofibres is likely to suppress hypertrophy.

Our data show that H19 and miR-675 are expressed at low levels in proliferating cells but are markedly elevated in cells that have stopped proliferating and are differentiating (see supplementary information). Recently it has also been shown that miR-675 promotes myoblast differentiation [24] by suppressing expression of SMAD-1 and-5 leading to reduced BMP signaling as well as a suppression of cdc6 (a protein required for the G1-S phase transition in the cell cycle). This study suggests 3 likely consequences of an elevated background of miR-675 that would contribute to a low FFMI phenotype. Firstly, in differentiated myofibres BMP signaling is pro-hypertrophic [40] and a reduction in BMP signaling has been shown to be pro-atrophic [40]. Secondly, as the analysis was performed in whole muscle homogenates we are unable to differentiate between changes in miR-675 expression in myofibres and satellite cells. Consequently, if the increase in miR-675 is not restricted to myofibres but is also present in myoblasts and satellite cells it will inhibit the expansion of the myoblast pool (by inhibiting cdc6 and reducing cell proliferation) and thirdly, it will promote the early differentiation of these cells reducing the time available for myoblast proliferation and further restricting the pool of cells available for regeneration.

C19MC miRNAs

The C19 microRNA cluster is the largest cluster of miRNAs in the human genome but arose relatively late in evolution and is only found in primates[21]. The miRNAs from this cluster are expressed in pluripotent cells and in the placenta but are not expressed in fully differentiated tissues [36, 37]. It therefore seems likely that the transcripts that we detected arose from satellite cells rather than from the myofibres. Consistent with this suggestion the levels of C19MC miRNAs were low. Functionally the C19MC miRNAs have been shown to promote the pluripotent state in

embryonic stem cells by targeting RBL-2 and thereby relieving RBL-2 dependent suppression of the expression of a methyltransferase DNMT3B a key regulator of embryonic differentiation[41]. Knockdown of DNMT3B results in a reduction in embryonic stem cell survival and in the formation of embryonic stem cell-derived clones *in vitro*. These data suggest that C19MC miRNAs may contribute to the normal FFMI phenotype by promoting stem cell survival.[36, 37]

It is important to note that the association of C19MC expression with FFMI was only evident in males with severe COPD. The lack of association of C19MCs in the cohort as a whole and in other groups individually may be due to confounding factors and/or statistical power as some of the subgroups were relatively small. It is also possible that it is only apparent in the severe subgroup as, in the absence of significant physiological stress due to severe disease, the requirement for regeneration will be limited to normal muscle cell turnover.

Imprinting and DNA methylation

Both C19MC and H19 expression are regulated by imprinting[42]. The C19MC locus is expressed from the paternal chromosome, whereas H19 is expressed from the maternal chromosome. In general, paternally imprinted genes promote growth whereas maternally imprinted genes inhibit growth and our data are consistent with this observation. In muscle, imprinted genes are predominantly expressed in stem/progenitor cells in adult mice[43]. This pattern of expression is consistent with an important functional role in the regulation of satellite cells and represents one way in which imprinted genes may contribute to the control of post-natal skeletal muscle mass [43]. As imprinting involves DNA methylation the involvement of imprinting suggests that DNA methylation makes an important contribution to the low FFMI and normal FFMI phenotypes. Methylation of the H19-IGF-2 ICR inhibits the expression of H19 [44] and our data showing an inverse correlation of H19 expression with relative H19-IGF-2 ICR methylation is consistent with this observation. Furthermore, our data showing that relative methylation of the H19-IGF-2 ICR is higher in normal FFMI patients and is correlated with muscle strength suggests that differences in DNA

methylation of particular loci may contribute to the control of muscle function in adulthood in response to the stress of COPD.

Most studies of imprinted genes focus on development and the early post-natal period. Consequently the association of H19 and miR-675 with muscle mass and strength in adults has not previously been noted and we did not find an association of miR-675 with FFMI or strength in the Herts Sarcopenia Study participants. This observation suggests that the association is only present or detectable in patients with a disease that predisposes to muscle wasting. It is therefore possible that differences in the expression of this miRNA are only of functional significance when there is an increased atrophic drive and/or an increase in the demand for muscle synthesis.

Reduced regeneration in low FFMI patients

Consistent with the recent studies of Theriault et al[45] who observed reduced centralised nuclei in patients with a mid thigh muscle cross sectional area $<70\text{cm}^2$ compared to those with a mid thigh cross sectional area $>70\text{cm}^2$ we found that patients with a low FFMI had reduced proportion of myofibres with centralized nuclei. This observation implies that a low level of regeneration contributes to the low FFMI phenotype. As described above both a low level of expression of C19MC miRNAs and an increased miR-675 expression may contribute to this reduction in regeneration.

Critique of the Method

The study is cross-sectional and as such the data can only demonstrate associations rather than causation. Nevertheless, our analysis of the muscle has highlighted a number of miRNAs with potential mechanistic relevance to the development of skeletal muscle dysfunction in COPD that warrant further investigation. Although one interpretation of our data is that there is a reduction in the number of satellite cells, we did not quantify satellite cell number directly due to the number and size of the samples available. Our analysis of centralized nuclei is consistent with the data of

Theriault [45] and in their study they did not see a difference in satellite cell number between patients with a low or normal mid thigh cross sectional area suggesting that any differences are small. Consequently if there is a difference in satellite cell number it will require a large number of biopsies to identify it. It is also possible that the number of satellite cells does not change either because these cells have become senescent as suggested by Theriault et al or because the effects of miR-675 are on the proliferation of committed myoblasts rather than the number of satellite cells. Whether any such senescence results from high miR-675 and/or low C19MC expression remains to be established.

Clinical Implications

There is significant variation in the amount or rate of loss of muscle mass in response to a range of diseases. In COPD, skeletal atrophy appears to be a significant factor in about 25% of patients that can start early in the disease[3]. In a second example, we have recently shown that following major cardiac surgery approximately 50% of patients will lose more than 10% of their muscle mass in the following 7 days whereas the others will not[46]. Furthermore, there is marked diversity in the response to treatment in patients with muscle wasting with only a subset responding well to pulmonary rehabilitation. This diversity hampers the development of methodologies to improve muscle mass. Understanding the basis for this variation in individual response to disease and treatment will help to identify those at greatest risk of wasting, to identify those likely to respond to therapies and to develop novel targeted therapies. Our data here suggest that expression levels of H19, miR-675 and the C19MC miRNAs may contribute to this diversity of response and that DNA methylation is a potential contributor to this differential expression. It is possible therefore that measurement of these factors may help to identify sub-groups of individuals likely to respond differently to anabolic therapies.

Conclusion

Our data show that increased expression of miR-675 and H19 are associated with a low FFMI in patients with COPD. Furthermore, increased expression of H19 is inversely correlated with relative methylation of the H19 ICR. In male patients with severe COPD, expression of the paternally expressed C19MC miRNAs were suppressed in patients with a low FFMI. We also confirmed that patients with a low FFMI had lower levels of muscle regeneration than patients with a normal FFMI. Together these data suggest that expression of imprinted miRNAs may contribute to the susceptibility to a low FFMI phenotype in COPD.

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Ethical Statements:

The authors of this manuscript certify that they comply with the ethical guidelines for authorship and publishing in the Journal of Cachexia, Sarcopenia, and Muscle 2010;1:7–8 (von Haehling S, Morley JE, Coats AJ and Anker SD)

Human studies: All subjects gave written informed consent; the COPD study protocol was approved by the Royal Brompton & Harefield NHS Trust Research Ethics Committee (Study numbers 06/Q0404/35 and 06/Q0410/54); the Herts Sarcopenia Study study protocol was approved by the Hertfordshire Research Ethics Committee (study 07/Q0204/68)

Author Contributions:

PK and AL proposed the hypothesis for the study. AL, JL, AD and SV performed the miRNA, mRNA DNA and cell culture analysis supervised by PK. SAN collected the human COPD muscle biopsies supervised by MP. The Herts Sarcopenia Study study samples were collected by HPP under the supervision of AAS. PK wrote the first draft of the paper and all authors contributed to the revision of the draft and the critical analysis of the project.

Conflict of Interest Statement:

Amy Lewis, Jen Lee, Anna Donaldson, Srividya Vaidyanathan, Holly Syddall, Avan Sayer and Cyrus Cooper do not have any conflicts of interest. Paul Kemp has received research grant funding from GSK and Astra Zeneca, Samantha Natanek has received grant funding from GSK, Michael Polkey has received research grant and./or consultancy funding from GSK, Lilly, Novartis, Biomezin Astra Zeneca and Pfizer (paid to his institution).

Table 1 Patient characteristics of the validation cohort

	Control (n=16)	normal FFMI (n=24)	low FFMI (n=24)
Sex (M, F)	7, 9	15, 9	12, 12
Age (years)	66± 8	67±7	64 ±9
Smoking History ^a (pack-year)	0 (0, 10)	47 (35, 66)***	39 (24, 48)***
Weight ^a (kg)	67.1 (61, 74.1)	75.1 (65.3, 83.0)	61.4 (53.5, 65.8)*, +++
BMI ^a (kg/m ²)	24.8 (23.5, 26.2)	26.7 (24.0, 29.7)	21.8 (19.8, 22.9)**, +++
FFMI ^a (kg/m ²)	16.1 (15.3, 17.2)	17.1(16.2, 17.4)	14.5 (13.4, 14.6)***, +++
FEV ₁ ^a (% pred)	107.6 (100.6, 112)	42.8 (25.7, 49.6)***	36.6 (27.6, 45.2)***
RVTLC	35± 6	58±8***	60±8***
TLCO ^a (% pred)	92.3 (83.0, 98.1)	45.3 (34.8, 54.4)***	40.6 (26.7, 50.6)***
6 min walk (m)	621± 84	368±119***	400±130***
6min walk % pred	126±12	77±24***	77±24***
pVO ₂ ^a (% pred)	99 (88, 111)	51 (42, 65)***	45 (36, 51)***
SGRQ ^a	2 (0, 8)	53 (43, 60)***	56 (51, 63)***
Quadriceps MVC (kg)	34.7±10.6	27.7±9.8	25.7±7.2**
Quadriceps MVC (% pred)	78±19	66±17*	61±11**
Locomotion time ^a (min/12 hr)	96 (84, 127)	37 (23, 52)***	44 (26, 64)***
Movement time (as % of 12hrs)	23±6	12±6***	13±4***
Type I fibre %	54.4 ± 18.1	28.7 ± 12.1***	27.4 ± 13.2 ***
Type IIA fibre %	41.0 ± 14.0	58.5 ± 10.6***	63.6 ± 14.0***
Type IIX fibre %	2.5 ± 3.5	6.7 ± 8.2	4.8 ± 4.5

Definitions of abbreviations: ^a= Not normally distributed, BMI = Body Mass Index, FFMI= Fat-Free Mass Index, FEV₁ =Forced Expiratory Volume in 1 sec, RV =Residual volume, TLC = total lung capacity, TLCO= transfer coefficient of the lung for CO, Pa_{O2} = arterial oxygen partial pressure, Pa_{CO2} = arterial carbon dioxide partial pressure, pred = predicted, MVC = Maximal Voluntary Contraction, SGRQ, St George's respiratory questionnaire. Values are means ± SD for normally distributed variables and as median (interquartile range) for variables that were not normally distributed. Significance was calculated by t-Test for normally distributed variables and by Mann Whitney U test for variables that were not normally distributed. *(p < 0.05) ** (p = <0.01) ***(p= <0.001) low FFMI or normal FFMI vs control . ++ (p=<0.01), +++ (P=<0.001) low FFMI vs normal FFMI

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Figure legends

Figure 1. miRNA expression in patients with COPD

Hierarchical cluster analysis (A) and principal component analysis (B) of miRNA expression in the quadriceps of low FFMI, normal FFMI patients and controls show that the low FFMI group have a distinct pattern of miRNAs compared to normal FFMI patients and controls. Separation between the normal FFMI and control groups was weaker and had some degree of overlap. (C) Comparison of the median expression of miRNAs between low FFMI patients and controls and between normal FFMI patients and controls to identify 'low FFMI-associated miRNAs'. Median expression of miRNAs in the bottom right hand quadrant is lower in low FFMI patients than controls (statistically significant) but higher in the normal FFMI patients than controls (not statistically significant) whereas median expression of the miRNA in the top left hand quarter is higher in low FFMI patients than controls (statistically significant) but lower in normal FFMI patients than controls (not statistically significant).

Figure 2. MiR-675 is increased in low FFMI COPD patients compared to normal FFMI COPD patients and is associated with muscle mass and strength.

The expression of miR-675 was determined by qPCR in samples of quadriceps from the validation cohort of COPD patients and controls as described in Methods. MiR-675 was elevated in patients with a low FFMI compared to patients with a normal FFMI ($p=0.019$, A). In both patients alone and the whole cohort, miR-675 was correlated with FFMI ($r=-0.41$, $p=0.004$ patients alone $r=-0.35$, $p=0.04$ patients and controls, B). In the patients alone miR-675 was correlated with strength both voluntary (MVC, $r=-0.43$, $p=0.003$, C) and involuntary (TwQ= -0.47 , $p=0.001$, D) measurements of strength. Patients are shown as grey circles controls as black circles.

Figure 3. MiR-519a is suppressed in low FFMI individuals compared to normal FFMI individuals.

The miRNA expression was determined by qPCR in samples of quadriceps from the validation cohort of COPD patients and controls as described in Methods. In the whole cohort, miR-519a levels were reduced in individuals with a low FFMI compared to individuals with a normal FFMI (A) and weakly correlated with FFMI ($r=0.29$ $p=0.044$, B). In GOLD3/4 male patients quadriceps expression of miR-519a (C) and miR-518e (D) were correlated with FFMI ($r=0.48$, $p=0.01$ and $r=0.39$, $p=0.025$ respectively). Patients are shown as grey circles controls as black circles.

Figure 4. H19 is increased in low FFMI COPD patients compared to normal FFMI COPD patients and is associated with muscle mass and strength.

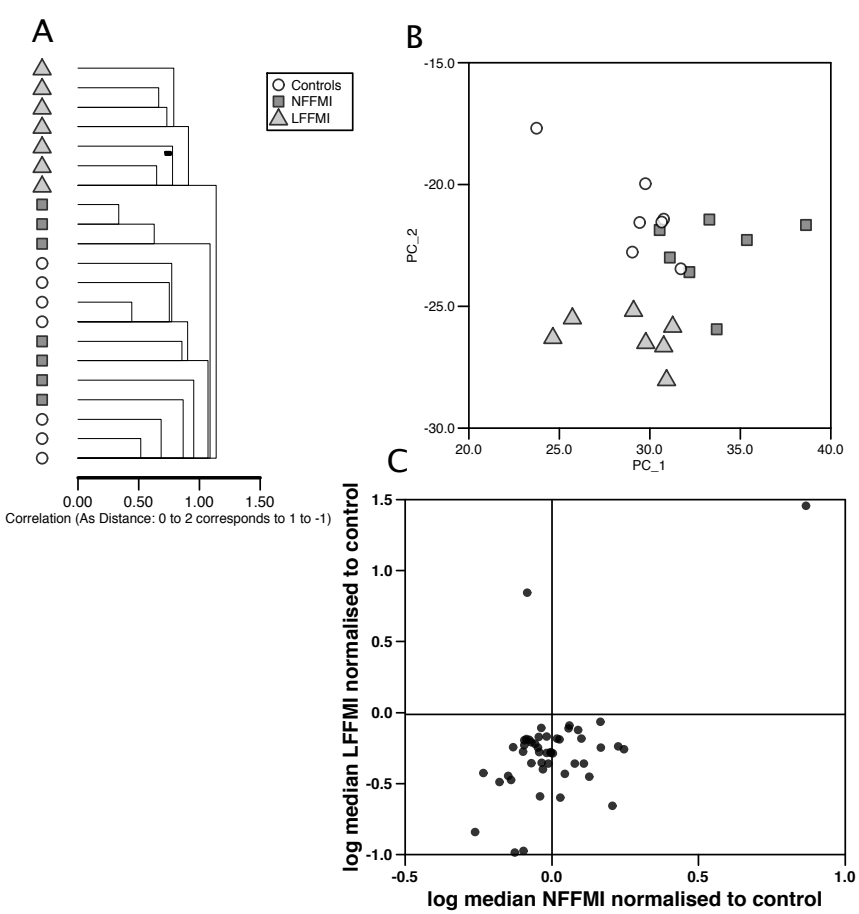
The expression of H19 was determined by qPCR in samples of quadriceps from the validation cohort of COPD patients and controls as described in Methods. H19 not different between patient groups or controls (A). In patients H19 was correlated with FFMI ($r=-0.385$, $p=0.001$, B) and with was correlated with quadriceps strength both voluntary (MVC, $r=-0.44$, $p<0.001$, C) and involuntary (TwQ= -0.38 $p=0.007$, D) measurements of strength. Patients are shown as grey circles controls as black circles.

Figure 5. Methylation of the H19 locus is associated with H19 gene expression and strength

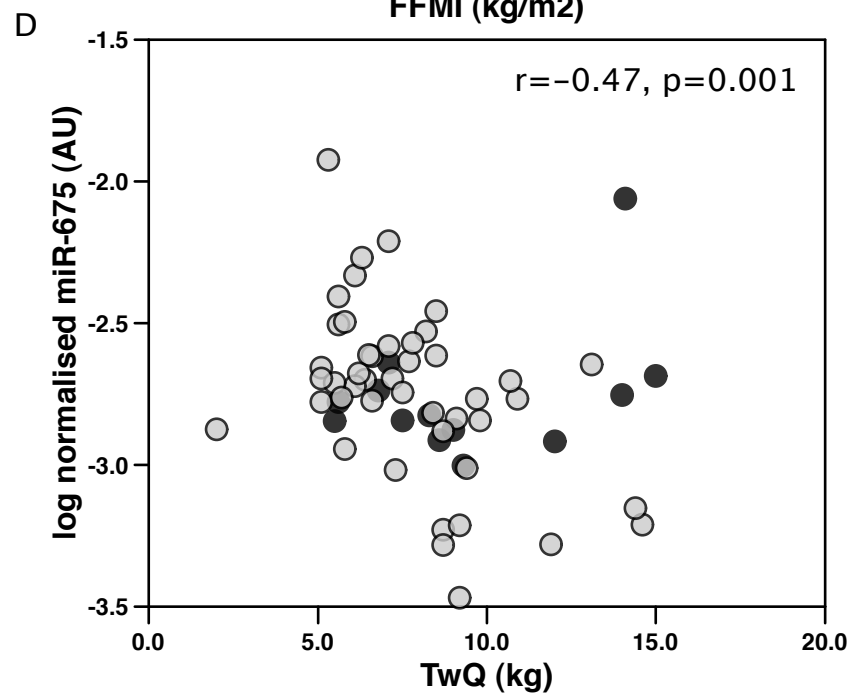
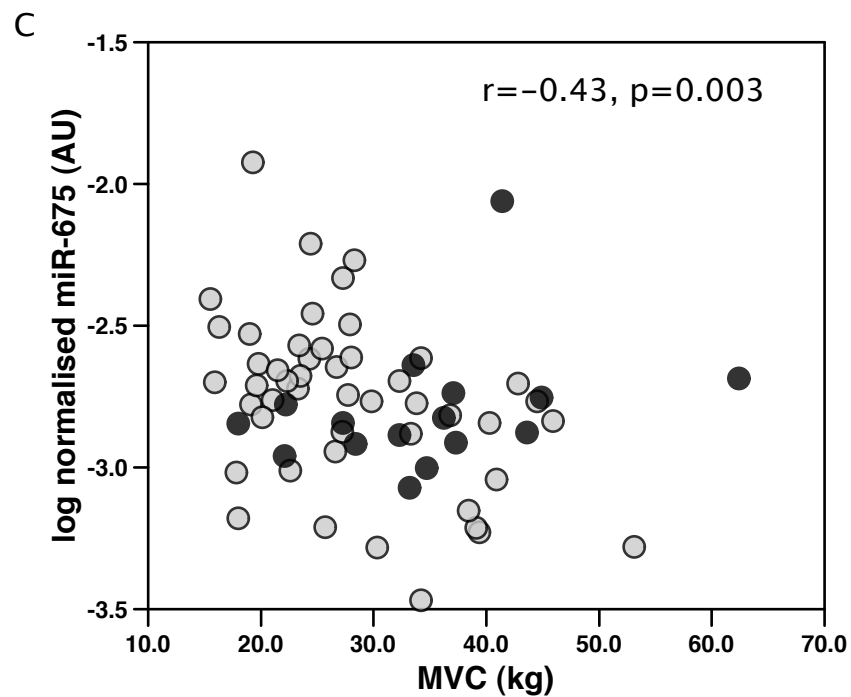
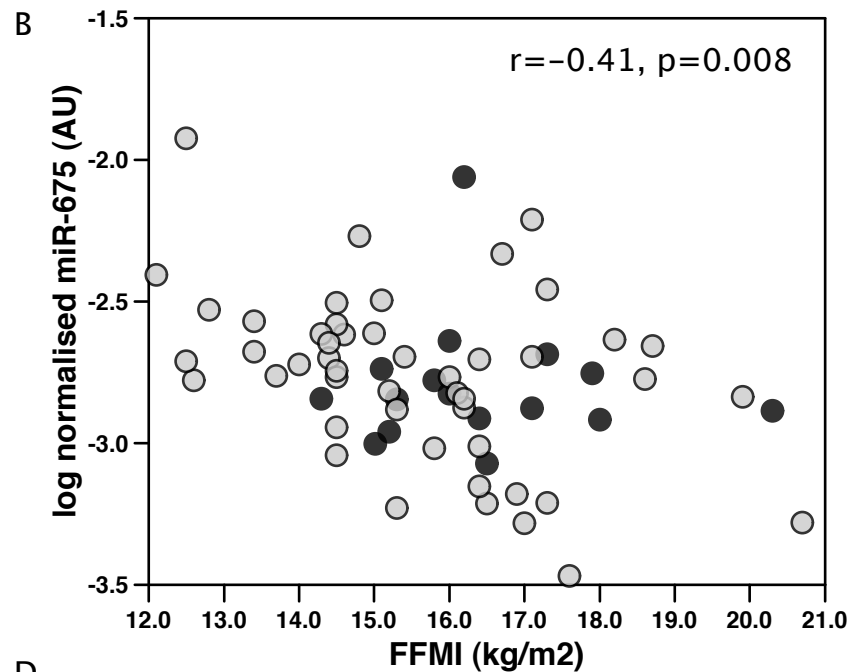
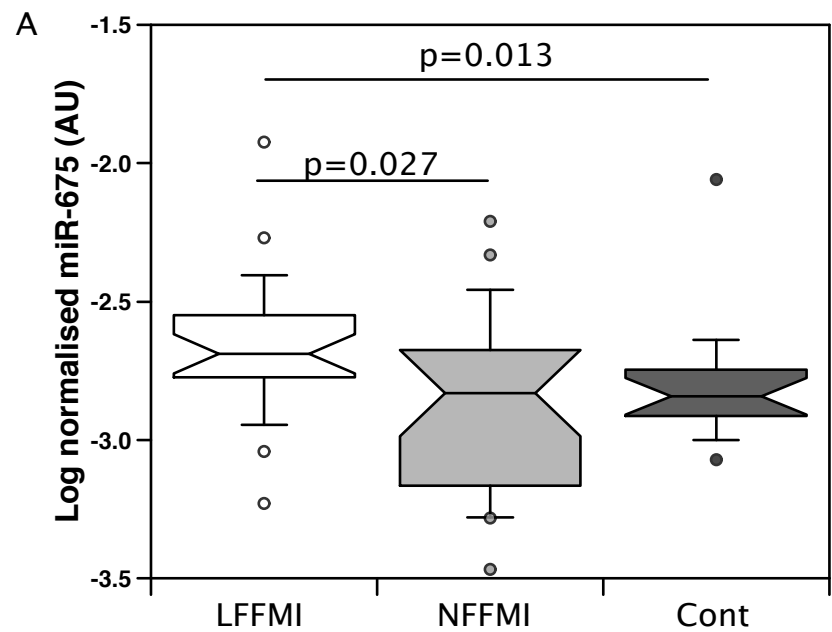
Methylated DNA was precipitated using the MeDiP kit as described in the Methods and input and precipitated DNA was quantified for the H19 ICR and for UBE2. ICR methylation is presented relative to the methylation of UBE2 (a sparsely methylated DNA region). H19 ICR methylation was inversely proportional to H19 gene expression (A), higher in weak patients compared to patients with normal strength (B) and correlated with quadriceps strength measured as % predicted (C). H19 expression was reduced in weak patients compared patients with normal strength (D)

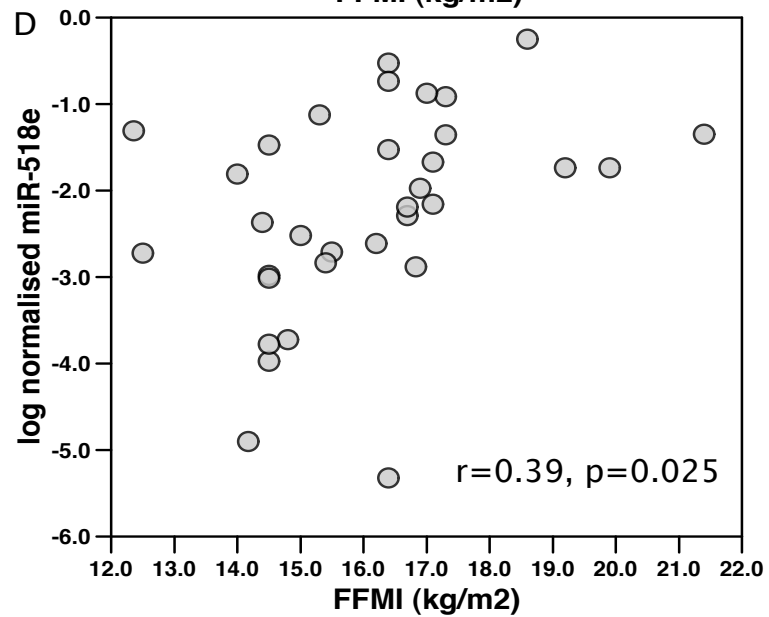
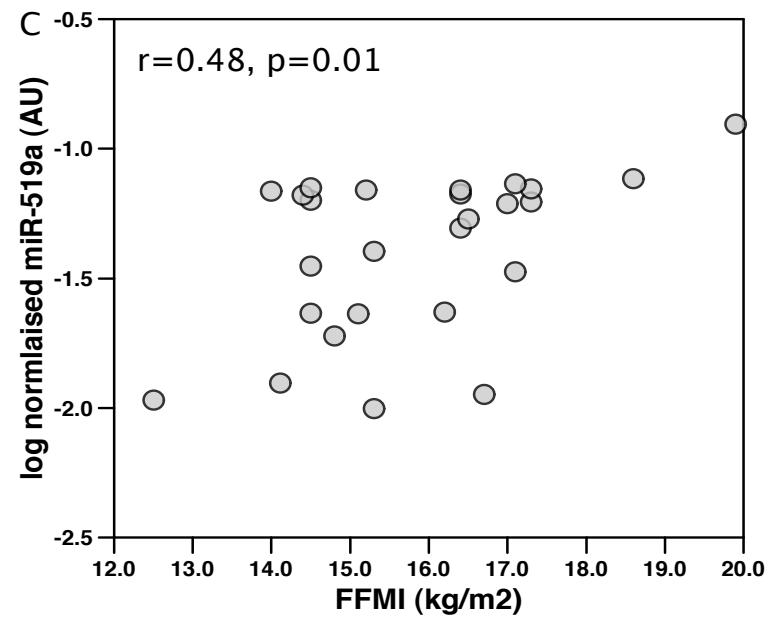
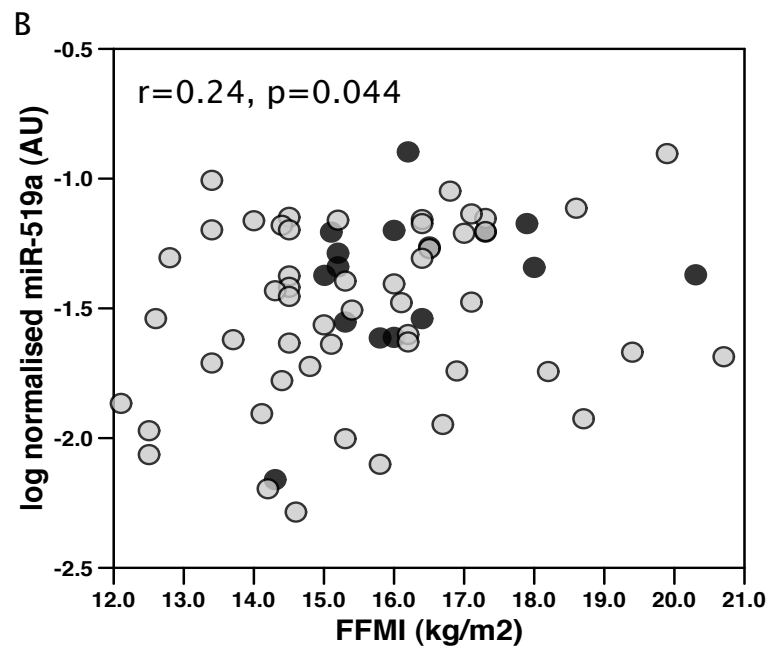
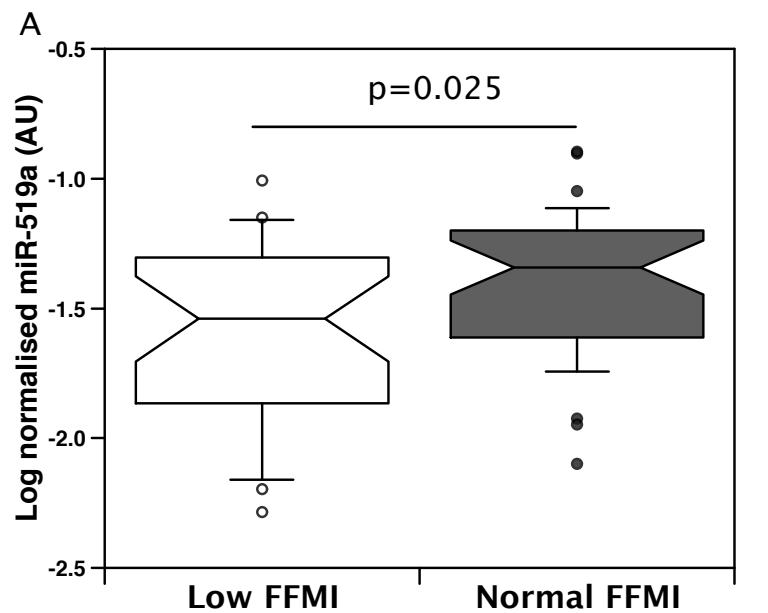
Figure 6. Reduced regeneration in the muscle of low FFMI patients

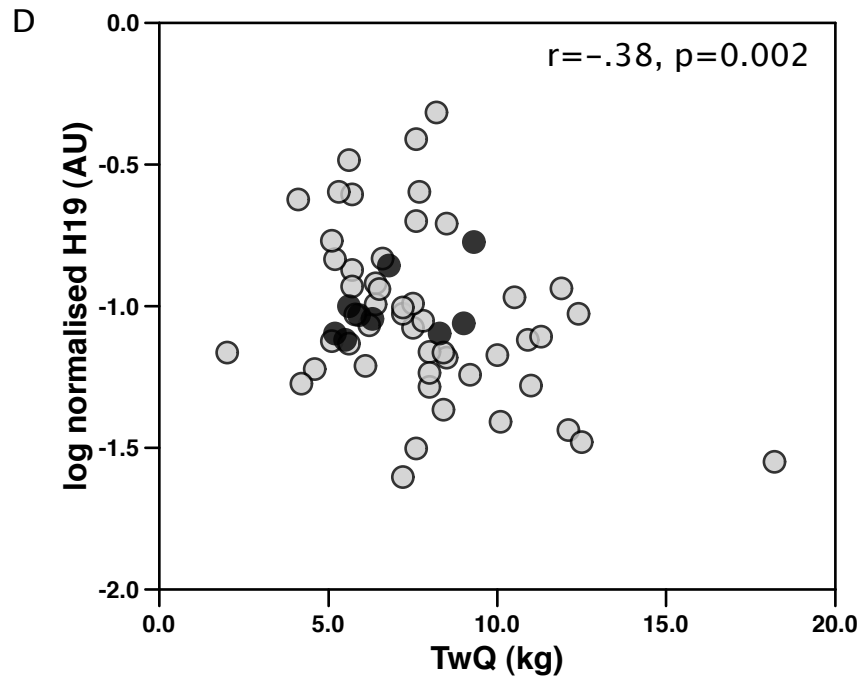
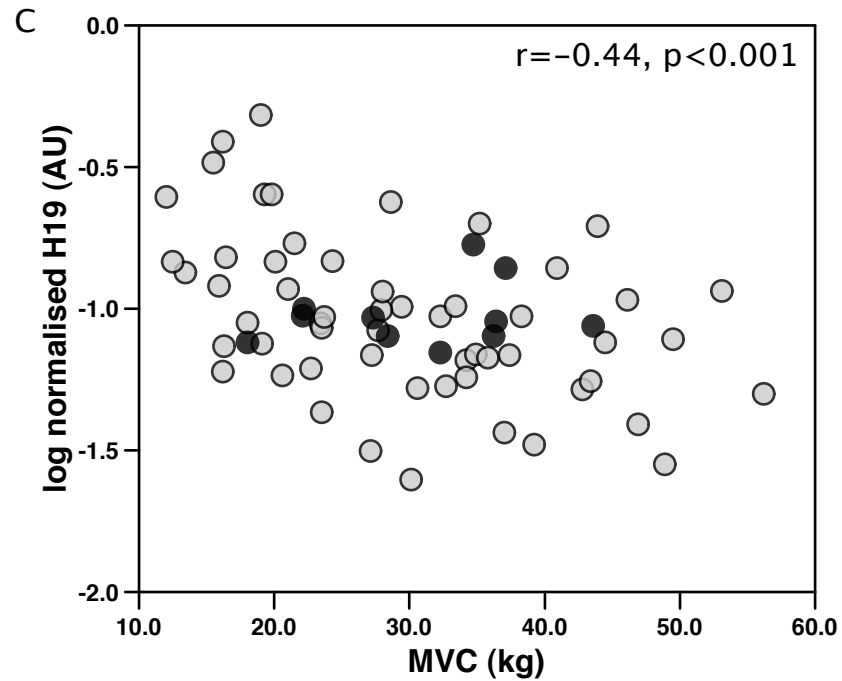
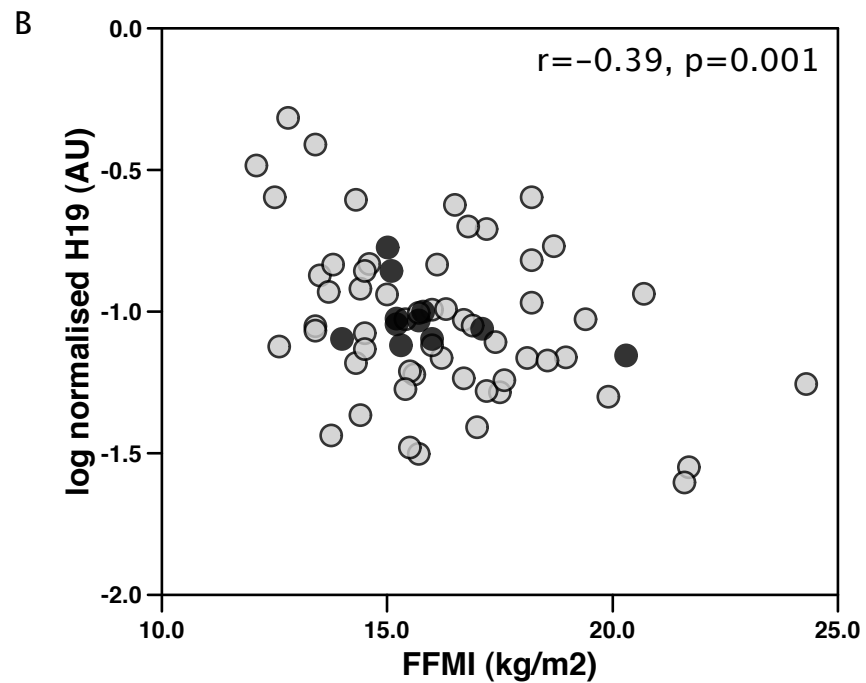
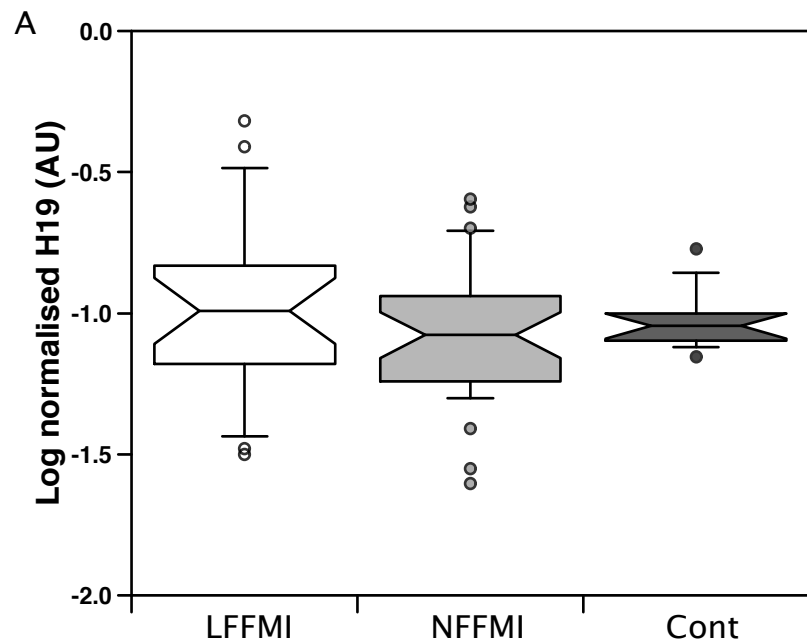
(A) sections of muscle were stained for laminin and DAPI as described in Methods. The number of centralised nuclei was determined by counting the number of transverse cut fibres with an intact laminin outline and the number of centralised nuclei in the whole section by an individual blinded to the group assignment of each image. Centralised nuclei are presented as number per 100 fibres. The number of centralised nuclei was higher in normal FFMI patients than in low FFMI patients. (C and D) Segments of representative images used in counting the number of centralised nuclei from a normal FFMI patient (C) and a low FFMI patient (D). White arrows indicate centralised nuclei. (B) MyoD mRNA expression was determined in the quadriceps of patients with COPD as described in Methods. Normal FFMI patients expressed significantly more MyoD than low FFMI patients ($p < 0.05$).

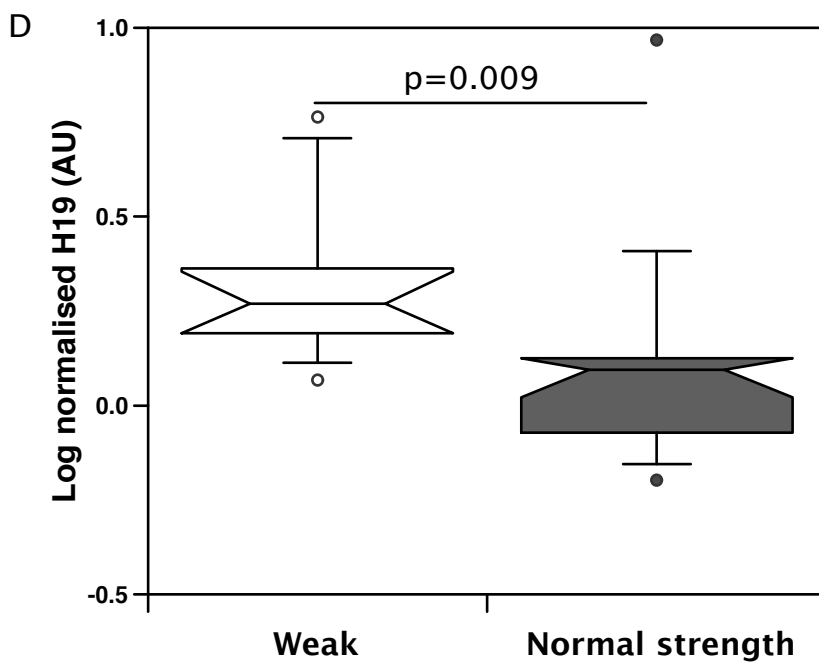
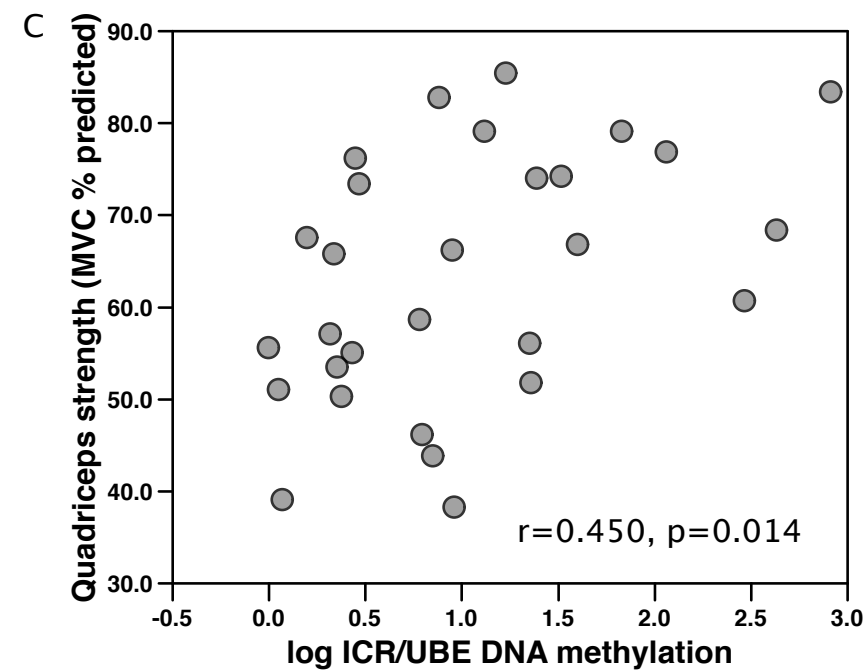
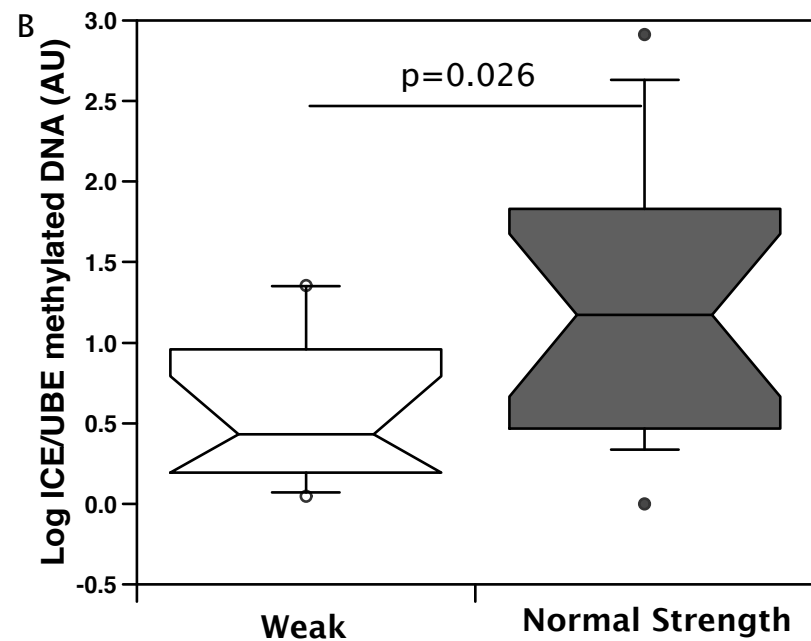
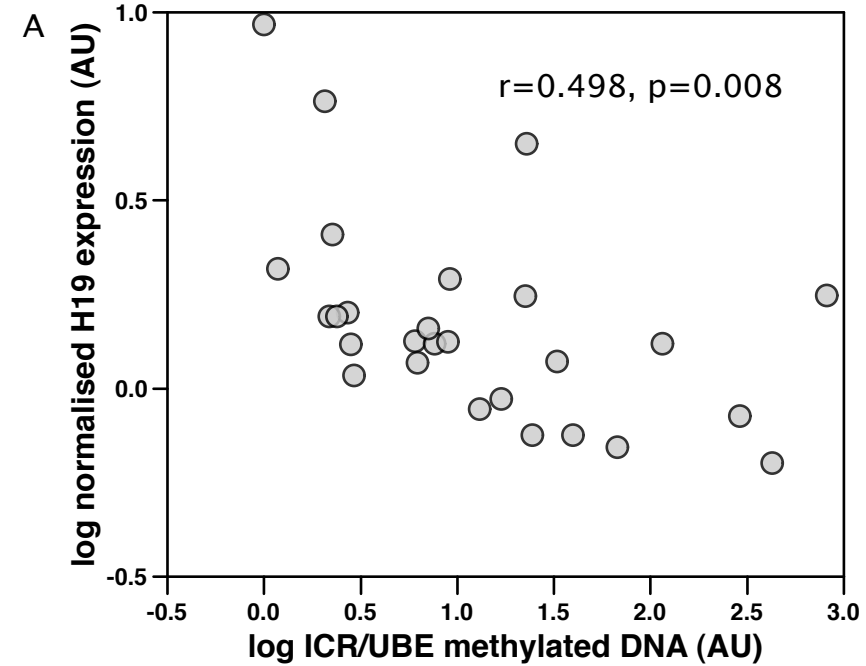


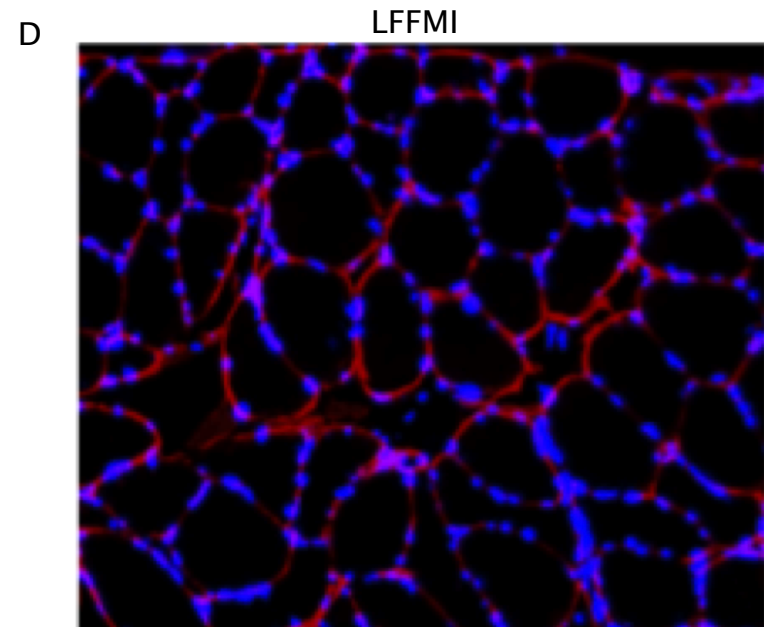
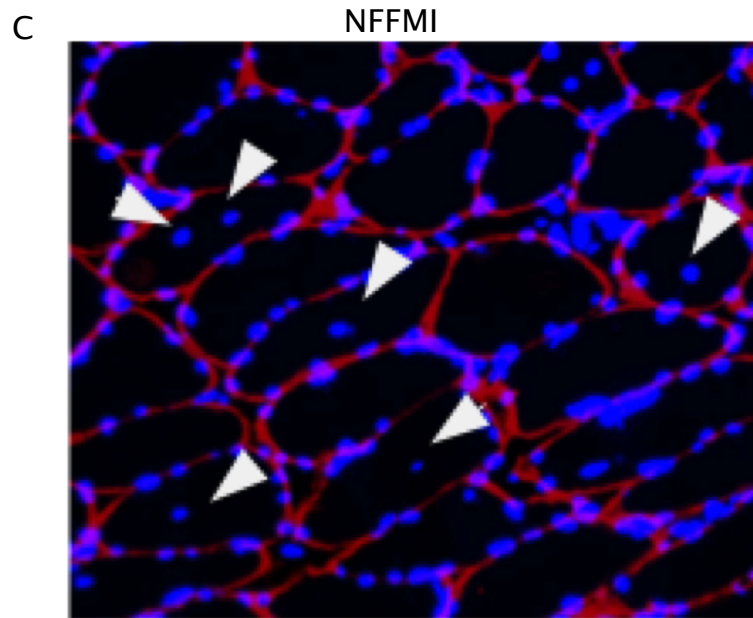
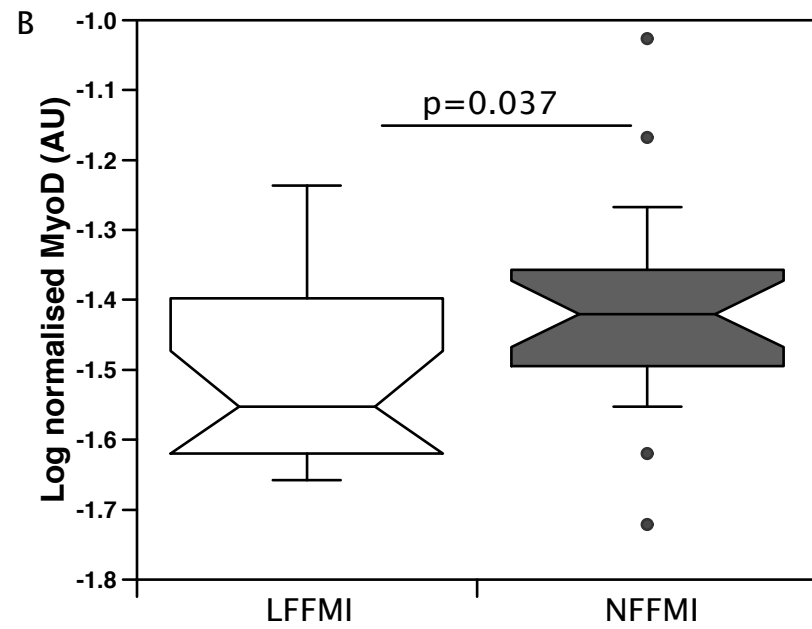
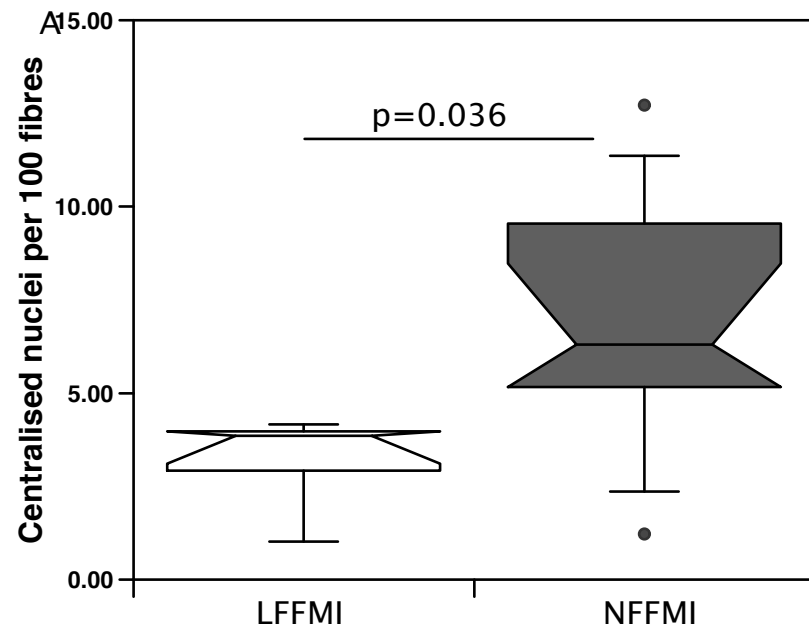
Lewis et al fig 1











Supplementary Methods

Cell culture: C2C12 cells were maintained in culture as described[33]. RNA was extracted using Trizol and mRNA and miRNAs were determined as described above. miR mimic transfection: cells were seeded into 96 well plates at 500 cells/well in octuplet. Each well was transfected the following day with 0.5 µl of 20 µM mimic with 0.5 µl lipofectamine 2000 in 50 µl Opti-MEM and 100 µl DMEM for 4h before the medium was replaced with DMEM supplemented with 10%FCS. Cells were quantified using CyQuant NF cell proliferation kit (Invitrogen) according to the manufacturer's instructions.

Supplementary Results

Mir-675 increases as myoblasts withdraw from the cell cycle and inhibits their proliferation *in vitro*

miR-675 has been associated with cell cycle inhibition [34]. To determine whether it inhibited myoblast proliferation, we determined the expression profile of miR-675 and H19 in C2C12 cells during differentiation. The expression of cell cycle markers (cyclinE1, Chk1 and cdc25) was reduced, while those of differentiation (MHCs and myogenic bHLH proteins) were increased over the time course, consistent with previous studies of C2C12 differentiation [35, 36](Supplementary Fig. 4). Expression of H19, miR-675-3p, and -5p was also higher in myotubes compared to proliferating myoblasts (Supplementary Fig. 4).

To determine whether these miRNAs could directly inhibit myoblast proliferation, C2C12 cells were transfected with these miRNAs (675-5p and 3p) and proliferation was analysed. Transfection with miR-290 (a close murine equivalent to C19MC miRNAs that has the same AAGUGC seed sequence as miR-519a) or a scrambled miRNA served as negative controls and transfection with miR-1 as a positive control. miR-675 inhibited C2C12 cell

proliferation to the same extent as miR-1. Neither the scrambled miRNA nor miR-290 suppressed cell proliferation (Supplementary Fig. 4) compared to untransfected cells.

Supplementary table 1 Screen cohort

	Control (n=7)	Normal FFMI (n=7)	Low FFMI (n=7)
Age (years)	67± 11	64±4	68 ±6
Smoking History ^a (pack-year)	6.25 (0, 24)	49 (41, 60) *	51 (32, 71) *
Weight ^a (kg)	75.5 (74.1, 86.8)	83.3 (72.1, 94.0)	60.8 (54.5, 69.4)*
BMI ^a (kg/m ²)	25.7 (24.8, 26.9)	24.5 (24.4, 27.1)	20.8 (19.9, 22.7) **, †
FFMI ^a (kg/m ²)	18.5 (17.5, 20.0)	17.6 (16.4, 17.9)	14.3 (13.9, 14.8)***, ††
FEV ₁ ^a (% pred)	107.7 (104.0, 123.2)	25.1 (22.0, 38.0)**	31.5 (27.4, 41.5)**
RVTLC	35± 4	61±9***	60±7***
TLCO ^a (% pred)	86.7 (84.5, 90.8)	31.9 (28.2, 50.7)**	29.0 (22.7, 43.3)**
6 min walk (m)	657± 102	367±159***	342±161***
6min walk % pred	116±12	67±31***	63±25***
pVO ₂ ^a (% pred)	90 (84, 99)	38 (33, 48)**	44 (31, 46)**
SGRQ ^a	1 (0, 8)	53 (51, 62)**	58 (51, 66)**
Quadriceps MVC (kg)	39.9±6.6	32.5±7.9	27.0±9**
Quadriceps MVC (% pred)	77 ± 15	62 ± 15	57±11*
Locomotion time ^a (min/12 hr)	90 (67, 115)	30 (18, 59)	61 (30, 77)
Movement time (as % of 12hrs)	19.2±6.4	13.7±9.2	15.7±8.2
Type I fibre %	45.9±13.7	27.0±17.7*	24.9±5.7**
Type IIA fibre %	48.0±12.3	59.0±12.5	61.8±10.4
Type IIX fibre %	3.4±3.4	11.7±10.6**	7.4±5.9**

Definitions of abbreviations: ^a= Not normally distributed, BMI = Body Mass Index, FFMI= Fat-Free Mass Index, FEV₁ =Forced Expiratory Volume in 1 sec, RV =Residual volume, TLC = total lung capacity, TL_{CO}= transfer coefficient of the lung for CO, Pa_{O2} = arterial oxygen partial pressure, Pa_{CO2}= arterial carbon dioxide partial pressure, pred = predicted, MVC = Maximal Voluntary Contraction, SGRQ, St George's respiratory questionnaire. Values are means ± SD for normally distributed variables and as median (interquartile range) for variables that were not normally distributed. Significance was calculated by t-Test for normally distributed variables and by Mann Whitney U test for variables that were not normally distributed. *(p < 0.05) ** (p = <0.01) *** (p= <0.001) low FFMI or normal FFMI vs control . †† (p=<0.01), ††† (P=<0.001) low FFMI vs normal FFMI

Supplementary table 2: H19 cohort

	Control (n=11)	Normal FFMI (n=34)	Low FFMI (n=22)
Sex (M, F)	2, 9	19, 15	5, 17
Age (years)	68± 10	68±9	63 ±9
Smoking History ^a (pack-year)	0 (0, 6)	45 (34, 655) ***	40 (30, 47) ***
Weight ^a (kg)	64.7(61.0, 65.4.8)	78.3 (73.9, 85.3)**	59.7 (51.0, 65.1)+++
BMI ^a (kg/m ²)	24.5 (23.6, 25.5)	28.1 (25.9, 30.8)**	21.7 (19.9, 23.5) *, +++
FFMI ^a (kg/m ²)	15.3 (15.2, 15.9)	17.7 (16.2, 18.5)**	14.0 (13.4, 14.5)***, +++
FEV ₁ ^a (% pred)	110.5 (99.1, 113.0)	58.5 (44.1, 71.9)***	43.8 (27.4, 59.0)***, ++
RVTLC	39± 5	51±9***	57±8***, ++
TLCO ^a (% pred)	82.5 (79.6, 95.1)	52.5 (40.0, 61.8)**	41.2 (26.1, 52.9)**
6 min walk (m)	603± 69	426±102***	374±136***
6min walk % pred	127±12	90±19***	72±26***, ++
pVO ₂ ^a (% pred)	97 (80, 110)	60 (50, 70)**	45 (34, 54)**
SGRQ ^a	2 (1, 7)	50 (37, 59)***	54 (47, 62)***
Quadriceps MVC (kg)	30.7±7.8	33.9±10.7	20.2±8.6*,+++
Quadriceps MVC (% pred)	77 ± 17	72 ± 19	56±13***,+++
Locomotion time ^a (min/12 hr)	96 (59, 141)	46 (36, 80)**	40 (21, 55)***
Movement time (as % of 12hrs)	23.8±6.5	14.4±5.7**	13.1±5.3***
Type I fibre %	53.8±12.5	32.6±10.6***	27.2±13.9***
Type IIA fibre %	42.0±11.6	56.4±11.0***	63.0±13.7***
Type IIX fibre %	1.9±3.1	5.1±5.7	5.3±5.3

Definitions of abbreviations: ^a= Not normally distributed, BMI = Body Mass Index, FFMI= Fat-Free Mass Index, FEV₁ =Forced Expiratory Volume in 1 sec, RV =Residual volume, TLC = total lung capacity, TL_{CO}= transfer coefficient of the lung for CO, Pa_{O2} = arterial oxygen partial pressure, Pa_{CO2}= arterial carbon dioxide partial pressure, pred = predicted, MVC = Maximal Voluntary Contraction, SGRQ, St George's respiratory questionnaire. Values are means ± SD for normally distributed variables and as median (interquartile range) for variables that were not normally distributed. Significance was calculated by t-Test for normally distributed variables and by Mann Whitney U test for variables that were not normally distributed. *(p < 0.05) ** (p = <0.01) *** (p= <0.001) low FFMI or normal FFMI vs control . ++ (p=<0.01), +++ (P=<0.001) low FFMI vs normal FFMI

Supplementary table 3: Herts Sarcopenia Study cohort

	Whole cohort (n=67)	Non-smokers (n=32)	Current and ex smokers (n=35)
Weight ^a (kg)	83.2 (72.8, 91.8)	81.2 (70.9, 86.4)	84.1 (80.0, 93.3)
BMI ^a (kg/m ²)	27.1 (24.8, 29.1)	25.8 (24.0, 28.7)	27.4 (26.2, 29.3)
FFMI ^a (kg/m ²)	18.4 (17.4, 19.3)	18.4 (17.1, 19.3)	18.4 (17.8, 19.3)
FEV ₁ ^a (% pred)	105.2 (98.2, 114.8)	106.1 (103.1, 117.2)	104.5 (92.3, 112.5)
TUG time (s)	10.5 (9.2, 12)	10.1 (9.2, 10.9)	10.9 (9.6, 12.25)
3m walk time ^a (s)	2.7 (2.5, 3.1)	2.7 (2.5, 2.9)	2.8 (2.5, 3.3)
Handgrip strength (kg)	38.6±8.9	38.6±7.8	38.5±10.0
Birth weight ^a (kg)	3.2 (3.0, 3.9)	3.2 (3.0, 4.0)	3.2 (3.0, 3.9)
Log miR-675 (AU)	-2.8 ± 19	-2.81 ± 0.58	2.83±0.54
Log miR-519a (AU)	-4.22 ± 0.36	-4.17 ± 0.28	-4.26 ± 0.40

Definitions of abbreviations: ^a= Not normally distributed, BMI = Body Mass Index, FFMI= Fat-Free Mass Index, FEV₁ =Forced Expiratory Volume in 1 sec, TUG time = time to up and go.

Supplementary Table 4 Physiological parameters for the Methylation cohort

	Control (n=10)	Normal FFMI (n=14)	Low FFMI (n=15)
Age (years)	66± 11	67±7.	67 ±7
Smoking History ^a (pack-year)	7 (0, 9)	55 (40,72)***	37 (29, 50) **
Weight ^a (kg)	77.5 (74.0, 96.9)	70.6 (63.2, 81.6)	60.8 (54.7, 68.8)***, †
BMI ^a (kg/m ²)	26.3 (24.7, 29.1)	24.2 (22.8, 25.1)	21.7 (19.6, 22.1)** , ††
FFMI ^a (kg/m ²)	19.0 (17.5, 20.5)	16.9 (16.4, 17.1)*	14.5 (14.5, 15.1)***, †††
FEV ₁ ^a (% pred)	107.7 (101.4, 111.1)	28.5 (24.1, 35.9)***	31.6 (27.0, 39.5)***
RVTLC	34± 4	63±7***	60±9***
TLCO ^a (% pred)	90.5 (86.7, 97.3)	40.3 (28.3, 46.2)***	39.3 (27.1, 54.6)***
6 min walk (m)	630± 101	332±119***	360±151***
6min walk % pred	120±14	72±23***	80±27***
pVO ₂ ^a (% pred)	96 (89, 104)	42 (34, 46)***	44 (31, 49)***
SGRQ ^a	3 (0, 8)	50 (42, 61)***	58 (49, 66)***
Quadriceps MVC (kg)	42.5±9.3	32.6±8.3**	28.1±7.3***
Quadriceps MVC (% pred)	82 ± 20	67 ± 15*	59±12**
Locomotion time ^a (min/12 hr)	85 (61, 97)	37 (20, 41)**	47 (20, 71)*
Movement time (as % of 12hrs)	17.0±4.9	11.0±5.3	12.7±6.4
Type I fibre %	53.1±18.1	29.2±14.3***	25.9±13.6 ***
Type IIA fibre %	39.8±17.4	62.7±8.6***	64.1±15.7***
Type IIX fibre %	3.6±3.6	5.6±8.9	5.7±5.1

Definitions of abbreviations: ^a= Not normally distributed, BMI = Body Mass Index, FFMI= Fat-Free Mass Index, FEV₁ =Forced Expiratory Volume in 1 sec, RV =Residual volume, TLC = total lung capacity, TL_{CO}= transfer coefficient of the lung for CO, Pa_{O2} = arterial oxygen partial pressure, Pa_{CO2}= arterial carbon dioxide partial pressure, pred = predicted, MVC = Maximal Voluntary Contraction, SGRQ, St George's respiratory questionnaire. Values are means ± SD for normally distributed variables and as median (interquartile range) for variables that were not normally distributed. Significance was calculated by t-Test for normally distributed variables and by Mann Whitney U test for variables that were not normally distributed. *(p < 0.05) ** (p = <0.01) *** (p= <0.001) low FFMI or normal FFMI vs control . †† (p=<0.01), ††† (P=<0.001) low FFMI vs normal FFMI

Supplementary table 5 Physiological Parameter centralized nuclei cohort

	Normal FFMI (n=10)	Low FFMI (n=5)
Sex (M, F)	10,0	5,0
Age (years)	63±5	68 ±6
Smoking History ^a (pack-year)	55 (38, 68)	75 (68, 80)
Weight ^a (kg)	79.8 (72.6, 90.8)	64.2 (60.8.5, 71.7),
BMI ^a (kg/m ²)	25.2 (24.3, 26.7)	22.0 (20.3, 23.4)
FFMI ^a (kg/m ²)	16.8 (16.4, 17.9)	14.5 (14.5, 14.6) ††
FEV ₁ ^a (% pred)	33.7 (25.7, 49.6)	27.6 (27.6, 45.2)
RVTLC	59±9	62±4
TLCO ^a (% pred)	44.3 (29.2, 52.4)	26.2 (19.1, 29.9)
6 min walk (m)	387±162	357±118
6min walk % pred	70±29	65±18
pVO ₂ ^a (% pred)	41 (32, 49)	34 (31, 45)
SGRQ ^a	55 (50, 67)	45(41, 60)
Quadriceps MVC (kg)	34.2.7±9.8	27.4±7.2
Quadriceps MVC (% pred)	66±15	56±7
Locomotion time ^a (min/12 hr)	37 (23, 66)	61 (52, 82)
Movement time (as % of 12hrs)	14±8	16±6
Type I fibre %	28.6 ± 18.1	23.4 ± 8.4
Type IIA fibre %	59.0 ± 15.1	60.6 ± 11.6
Type IIX fibre %	9.5 ± 9.4	10.6 ± 8.6

Definitions of abbreviations: ^a= Not normally distributed, BMI = Body Mass Index, FFMI= Fat-Free Mass Index, FEV₁ =Forced Expiratory Volume in 1 sec, RV =Residual volume, TLC = total lung capacity, TLCO= transfer coefficient of the lung for CO, Pa_{O2} = arterial oxygen partial pressure, Pa_{CO2}= arterial carbon dioxide partial pressure, pred = predicted, MVC = Maximal Voluntary Contraction, SGRQ, St George's respiratory questionnaire. Values are means ± SD for normally distributed variables and as median (interquartile range) for variables that were not normally distributed. Significance was calculated by t-Test for normally distributed variables and by Mann Whitney U test for variables that were not normally distributed. *(p < 0.05) ** (p = <0.01) *** (p= <0.001) low FFMI or normal FFMI vs control . †† (p=<0.01), ††† (P=<0.001) low FFMI vs normal FFMI

Supplementary Table 6. Low FFMI associated miRNAs in the quadriceps muscle of COPD patients

miRNA	Median fold change			P value		
	LFFMI vs NFFMI	LFFMI vs cont	NFFMI vs cont	LFFMI vs NFFMI	LFFMI vs cont	NFFMI vs cont
miR-519c-3p	0.137	0.220	1.611	0.000	0.008	0.393
miR-518a-3p	0.265	0.354	1.338	0.000	0.038	0.171
miR-517a	0.366	0.438	1.197	0.000	0.000	0.079
miR-517c	0.386	0.568	1.471	0.000	0.004	0.068
miR-519a	0.451	0.438	0.971	0.000	0.002	0.300
miR-512-3p	0.523	0.519	0.991	0.000	0.005	0.325
miR-525-3p	0.341	0.438	1.286	0.001	0.003	0.524
miR-518b	0.428	0.398	0.931	0.001	0.013	0.385
miR-519d	0.511	0.515	1.009	0.001	0.004	0.422
miR-517b	0.137	0.103	0.748	0.002	0.004	0.398
miR-523	0.314	0.553	1.760	0.002	0.036	0.139
miR-101	0.531	0.527	0.993	0.002	0.001	0.862
miR-515-5p	0.334	0.370	1.106	0.003	0.017	0.342
miR-518e	0.541	0.518	0.959	0.003	0.011	0.562
miR-520g	0.132	0.106	0.801	0.004	0.003	0.751
miR-522	0.280	0.256	0.912	0.004	0.006	0.544
miR-32	0.505	0.359	0.711	0.004	0.000	0.118
miR-15a	0.343	0.577	1.680	0.005	0.001	0.071
miR-26b	0.579	0.525	0.906	0.005	0.001	0.253
miR-342-3p	0.632	0.658	1.041	0.007	0.000	0.788
miR-342-5p	0.263	0.144	0.547	0.008	0.010	0.651
miR-126	0.734	0.591	0.805	0.008	0.001	0.255
miR-340	0.771	0.570	0.739	0.010	0.001	0.082
miR-489	0.521	0.656	1.259	0.011	0.018	0.268
miR-140-5p	0.687	0.602	0.876	0.011	0.001	0.492
miR-186	0.745	0.672	0.903	0.013	0.010	0.599
miR-518d-5p	0.235	0.251	1.070	0.015	0.006	0.675
miR-148b	0.635	0.568	0.896	0.015	0.014	0.623
miR-139-5p	0.724	0.617	0.851	0.017	0.004	0.216
miR-190	0.489	0.325	0.664	0.018	0.001	0.151
miR-146a	0.615	0.754	1.226	0.018	0.026	0.784
miR-195	0.770	0.645	0.837	0.018	0.004	0.102
miR-374a	0.516	0.440	0.851	0.019	0.004	0.166
miR-148a	0.665	0.531	0.799	0.024	0.011	0.437
let-7f	0.798	0.653	0.818	0.024	0.015	0.703
miR-193a-3p	0.462	0.336	0.727	0.025	0.006	0.388
miR-1	0.679	0.775	1.141	0.029	0.041	0.662
miR-208b	0.642	0.375	0.584	0.030	0.002	0.567
miR-628-5p	0.589	0.862	1.464	0.031	0.029	0.327
miR-454	0.481	0.444	0.923	0.034	0.008	0.475
miR-125a-5p	0.703	0.676	0.961	0.037	0.036	0.633
miR-30b	0.848	0.780	0.920	0.037	0.008	0.621
miR-98	0.610	0.649	1.063	0.038	0.017	0.513
miR-590-5p	0.794	0.639	0.805	0.039	0.003	0.080
miR-361-5p	0.706	0.811	1.148	0.041	0.029	0.329
miR-424#	3.893	28.569	7.340	0.009	0.000	0.220
miR-675	8.506	7.002	0.823	0.008	0.030	0.182

miRNAs that were suppressed in LFFMI patients compared to NFFMI patients are shown above the line in order of statistical significance, those increased in LFFMI patients compared to LFFMI patients are shown below the line in order of statistical significance.

Supplementary Figure legends

Supplementary Figure 1: Expression of miR-675 is correlated with H19 in the muscle of COPD patients and controls.

The expression of miR-675 and H19 were determined by qPCR as described and compared in the individuals where both determinations were possible. The expression of miR-675 was positively correlated with H19 expression in these samples. Patients are shown as grey circles and controls are shown as black circles

Supplementary Figure 2: Comparison of two methods of determining H19 expression.

H19 expression was determined using Sybr Green and by combining the results of two independent measures using the SNP assay. There was a tight correlation of the values obtained by these two methods. Patients are shown as grey circles and controls are shown as black circles

Supplementary Figure 3. Relative DNA methylation is associated with MVC normalized for FFMI in COPD patients and controls

Methylated DNA was precipitated using the MeDiP kit as described in the Methods and input and precipitated DNA was quantified for the H19 ICR and for UBE2. (A) Relative ICR methylation was not different between patients and controls. Relative ICR methylation was associated with QMVC normalized for FFMI in patient (B) and in controls (C).

Supplementary Figure 4. miR- 675 and H19 are increased in differentiating myoblasts *in vitro*

RNA was extracted from C2C12 and differentiating C2C12 cells 120h prior to (proliferating cells) and 48h, 96h, and 144h after the induction of differentiation by placing the cells into medium supplemented with 2% horse serum as described in Methods. QPCR was used to determine the expression of myosin heavy chains (MHCI, MHCIIA, MHCIIIX and MHCIIIB, A), myogenic transcription factors (myoD, myogenin and myf5, B) and cell cycle genes (Chk1, CDC25A and cyclin E1, C). The expression of H19, miR-675 is shown in D. Data are shown as mean +/- SEM from quadruplicates. (E) C2C12 cells were transfected with miR mimics for miR-675-3p, miR-675-5p, miR-290, a scrambled control or no oligonucleotide (lipo) as described in Methods. The cells were counted after 24, 48 or 96 h in growth medium. MiR-675-3p and miR-675-5p inhibited cell proliferation compared to scrambled or no transfected controls whereas miR-290 did not affect cell number. The data were from 2 separate experiments each performed in 8 biological repeats at each time point.

