ONLINE SUPPLEMENTARY INFORMATION

Patient exclusion criteria and ethics

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. 25 healthy age-matched controls (11 female and 14 male) 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).

Physiological measurements

Lung volumes measured using plethysmography, carbon monoxide transfer factor assessed using the single breath technique (CompactLab, Jaeger, Germany) and post-bronchodilator spirometry according to ATS/ERS guidelines [1]. Blood gas tensions were measured in arterialised capillary earlobe blood. Fat-free mass index (FFMI) was calculated using bioelectrical impedance (Bodystat 1500, Bodystat, UK) as described previously [2] and corrected in the case of patients with a disease specific equation [3]. FFMI <15 kg/m^2 in females and 16kg/m^2 in males were used as cut offs for low fat free mass.

Quadriceps strength was determined by measuring supine isometric maximal voluntary contraction (MVC) of the leg ipsilateral to the dominant hand, using an adaptation of the technique of Edwards *et al* [4] and correcting for weight (the main independent predictor of MVC in patients and controls), and by measuring the unpotentiated twitch quadriceps force (TwQ) as described by Polkey *et al* [5]. Exercise performance was assessed 5 minutes post-bronchodilator with the 6 minute walking test (6MW), performed according to ATS 2002 guidelines [6].

Measurement of circulating plasma miRNA levels

The method followed was adapted from Kroch *et al* [7] . Briefly, 4ml of Qiazol Lysis reagent solution (Invitrogen) was added to 400µl of thawed plasma sample, vortexed and left to stand for 5 minutes. A stock of synthetic *C. elegans* miRNA*,* (concentration 5 fmol/µL) was prepared to use as a spiked-in exogenous control Sequences: Cel-miR-39, 5'-UCACCGGGUGUAAAUCAGCUUG-3'; Cel-miR-54, 5'-CGUAAUCUUCAUAAUCCGAG-3' (Invitrogen). 5µl of this was added to the denatured plasma sample and vortexed.

300µl of molecular grade chloroform was then added to the sample, vortexed for 30 seconds and centrifuged for 25 minutes at 2,500 x g at 4°C. The aqueous phase for each sample was then transferred to a new collecting tube and RNA was isolated using the miRNeasy Mini RNA isolation kit according to the manufacturer's protocol (Qiagen, Inc). RNA Samples were eluted from the columns in 50µl RNAse free water and stored at -80°C.

For microRNA cDNA synthesis and poly (A) tailing from RNA, an NCode ™ VILO™ miRNA cDNA Synthesis kit was used according to the manufacturer's guidelines. 11µl of RNA was pipetted into one well on a 96 well plate, in which the cDNA was later stored. RNA was heated to 65°C for 5 minutes, and then placed on ice for 2 minutes. cDNA master mix was prepared (each reaction contained 4µl of 5X reaction, 2µl of 10X SuperScript enzyme mix and 3µl of DPEC water). 9µl of this master mix was then added to the heated RNA. The reaction was then incubated at 37°C for one hour and terminated by heating to 95°C for 5 minutes. 80µl of sterile water was then added to give a total RT product volume of 100µl of cDNA. cDNA was stored at -20°C.

Polymerase chain Reaction (PCR)

50 µl PCR reactions were performed to optimise annealing temperatures and validate custom made miRNA primers prior to quantitative PCR. Each reaction contained 25µl Express Sybr® Green JumpStart™ Taq Ready Mix (Sigma) detection reagent, 1µl of 10µM universal qPCR Primer, 20µl sterile water, 1µl 10µM miRNA forward primer and 3µl cDNA.

PCR amplification programme: 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds, 60°C for 1 minute, 72°C for 1 minute, the reaction was terminated at 72°C for 10 minutes. Different annealing temperatures were tested.

Forward miRNA gene primer sequences (Invitrogen):

miRNA- specific forward primer	*Primer sequence 5' to 3'*
C. elegans- mir 39	TCA CCG GGT GTA AAT CAG CTT G
miR-1	CCG GTG GAA TGT AAA GAA GTA TGT AT
miR-16	TAG CAG CAC GTA AAT ATT GGC G
miR-122	TGG AGT GTG ACA ATG GTG TTT
miR-499	GGC TTA AGA CTTGCA GTG ATG TTT
miR- 133	UUU GGU CCC CUU CAA CCA GCU G
miR- 206	UGG AAU GUA AGG AAG UGU GUG G
miR-181	ATT CAA CGC TGT CGG TGA GT

PCR products were separated by electrophoresis through a 2% agarose gel and visualised with ethidium bromide to confirm the size of the correct base pair size. A negative control was tested per reaction.

PCR products were successively diluted to generate dilution series per miR tested. qPCR reactions (as above) were performed on these dilutions to generate standard curves. A reference standard curve per miR tested was used to align the results across the plates tested.

Quantitative real-time PCR

Express Sybr® GreenER™ miRNA qRT-PCR kit was used according to the manufacturer's guidelines (Invitrogen). Each reaction contained 10 µl Express Sybr® GreenER™ qPCR SuperMix with Premixed ROX , 0.4µl forward miR primer 10µM, 0.4µM universal qPCR primer 10µM, 6.2µl sterile water and 3µl cDNA.

The qPCR reactions were run on the 7500 Fast Real-Time PCR System (Applied Biosystems, UK.), with the following cycle program: 50 °C for 2 minutes, 95 °C for 10 minutes, then 40 cycles of 95 °C for 15 seconds, 60°C for 60 seconds. A dissociation curve/ melt curve stage was run with the first reaction. Each sample was tested in duplicate on 96 well plates, equal number of controls were on each plate. We measured miR-1, miR-499, miR-206, miR-181 and miR-133. Two miRNAs known not to be muscle restricted (miR-16 and miR-122) served as controls.

Analysis was performed as previously described. Results were normalised to an exogenous spiked in control, *C. elegans* miR- 39. Samples where duplicates gave inconsistent results were excluded from the analysis. Average Ct value for the patients was 18.9 ± 2.1 and for the controls was 19.4 ± 2.3 (mean ± SD) these values were not significantly different.

Muscle analysis

MHC mRNA analysis

Real time quantitative PCR (RT-PCR): RNA was extracted from muscle biopsies using the Qiagen RNeasy® kit (Qiagen, UK), the concentration of RNA was quantified using a spectrophotometer (Nanodrop (ND1000, Wilmington USA) and first strand cDNA generated using Superscript® II Reverse Transcriptase (Invitrogen). The qPCR analysis was carried out in duplicate on each cDNA sample for every target gene and for the reference genes RPLPO using a 10 µl reaction of SYBR® Green Quantitative RT-PCR Kit (Sigma Aldrich, UK) and the primer pair (2pmol/µl) in 96 well plates (MicroAmp, Fast optical 96 well reaction plate (0.1 ml) (Applied Biosystems, UK.), covered by an optical plate cover (MicroAmp, Optical adhesive film (PCR compatible), Applied Biosystems, UK.). The qPCR reactions were run on the 7500 Fast Real-Time PCR System (Applied Biosystems, UK.), with the following cycle program: 95 ºC for 10 minutes, then 40 cycles of 95 ºC for 15 seconds, 64°C for 30 seconds, 72°C for 30 seconds. The annealing temperature was optimised for each individual pair of primers. The PCR products were run on a 2% agarose gel to confirm the size of the correct base pair size. Q-PCR data for each gene was normalised to the value for RPLPO from the same sample as previously described [8].

The Primer Sequences used are as follows:

RPLPO Forward TCTACAACCCTGAAGTGCTTGATATC,

RPLPO Reverse GCAGACAGACACT GGCAACATT,

MHC type I, IIa and IIX primers have previously been described [9 10].

Transcription factor Enzyme linked Immunsorbent Assays (TF ELISAS) to quantify NFκβ P65 and P50 subunits in quadriceps muscle biopsies

10ug protein per sample was analysed. Assays were performed by the US Panomics/Affymetrix testing Service in California, USA using the Panomics/Affymetric TF ELISA plates, solutions and

nuclear extraction kit (#EK1111, #EK1121 and EK1041). Samples were run in duplicate with appropriate positive controls (HeLa cell nuclear extracts treated with TNF-α) and negative controls. The average result for each sample was calculated for purposes of analysis.

Luminex assays for Plasma cytokines.

Plasma cytokines were determined using luminex assays run by European Invitrogen Testing Service. An ultrasensitive panel was run to determine TNF-α, IL-2 and IL-5 with a sensitivity of <1pg/ml for each analyte (#LHC6004) and standard panels used to quantitate CRP (#LHP0031) and TNF-α receptors I and II (TNFRI and TNFRII, #LHC0006).

Statistical analysis:

PCR data were log transformed to stabilise the variance in the dataset as variance increases with Ct value and to give equal weighting to samples with low levels. Pearson's correlation coefficient was calculated to analyse correlations assuming linearity. Differences between patients and controls were calculated by Student's t-test for normally distributed data and by Mann-Whitney U test for data that did not fit a normal distribution. Comparison of single variables across the GOLD stages was performed by ANOVA and the analysis corrected for multiple testing using a Bonferroni-Dunn Correction.

ROC analysis: samples were defined as exhibiting type I fibre shift if the proportion of fibres in the biopsy was below the 2.5th percentile for the control group (less that 31% type I fibres). Physiological values and log normalised miRNA values were used alone or combined by multiplication to give a score as the variable for ROC analysis. All values were multiplied by -1 to classify patients according to the presence of type I fibre-shift. ROC analysis was performed in Aabel (Gigawiz). Statistical significance was taken at $p<0.05$ for all analyses. Analyses were performed using Prism 5 (Graphpad Software inc., San Diego, CA, USA) or Aabel (Gigawiz).

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	Control	GOLD I	GOLD II	GOLD III	GOLD IV
FEV1%	110 (101, 113)	88 (87,89)**	61 (57, 68)**	41 (36, 44)**	25 (20, 27)**
FFMI (kg/m^2)	16.4 (15.2, 19.9)	16.9 (16, 17.9)	17.3 (15.5, 18.5)	15.3 (14.3, 16.5)**††	14.9 (14.1, 16.7)**††
MVC/Wt (kg/kg)	1.4 (1.2, 1.6)	1.4 (1.1, 1.6)	1.0 (0.74, 1.4)	1.2 (1.0, 1.5)	1.2 (0.9, 1.5)
Type I %	52, (46, 59.5)	37 (35, 40)	31 (23, 38)**	30 (23, 37)**	28 (11, 35)**
Type IIa %	40 (35, 46)	53 (47, 60)**	58 (52, 67)**	62 (56, 68)**	62 (55, 70)**
Type IIx %	1.0 (0, 5.5)	2.5 (1.5, 6.3)	4.5 (1.0, 7.5)	4.0 (1.0, 7.0)**	6.0 (1.5, 11.5)
Type I CSA (um2)	5320 (4821, 6104)	4941 (4408, 5367)	5095 (4267, 6521)	4924 (4052, 5634)	5233 (3850, 5840)
Type IIa CSA (um2)	4329 (3406, 5837)	3935 (3616, 4358)	3877 (2658, 4549)	3920 (3001, 4758)	4908 (3599, 4914)
Type IIx CSA (um2)	4819 (4186, 6811)	3698 (3124, 4475)	2746 (1754, 3979)	2944 (1853, 3406)**	3024 (1962, 4001)
plasma miR-1 (AU)	1.36 (0.87, 1.60)	1.42 (1.08, 1.74)	1.40 (1.15, 1.70)	1.70 (1.42, 1.99)	1.68 (1.40, 1.89) **
plasma miR-499 (AU)	3.70 (3.09, 3.96)	4.23 (3.99, 4.43)	4.06 (3.79, 4.44) **	4.05 (3.82, 4.33) **	3.82 (3.59, 4.05)
plasma miR-133 (AU)	2.25 (2.05, 2.46)	2.68 (2.52, 2.88)	2.58 (2.28, 2.77)	2.55 (2.40, 2.65)	2.40 (2.15, 2.60)
plasma miR-206 (AU)	2.11 (1.93, 2.35)	2.59 (2.43, 2.76)	2.69 (2.42, 2.96) **	2.43 (2.24, 2.74)	2.36 (2.20, 2.62)
plasma miR-181 (AU)	3.40 (2.75, 3.72)	3.53 (3.46, 3.58)	3.47 (3.32, 3,63)	3.56 (3.39, 3.74)	3.95 (3.37, 3.81) **
plasma miR-16 (AU)	4.03 (3.82, 4.22)	4.15 (4.06, 4.21)	3.95 (3.69, 4.28)	4.14 (3.88, 4.35)	4.13 (3.92, 4.31)
plasma miR-122 (AU)	3.37 (3.15, 3.42)	3.61 (3.61, 3.62)	3.60 (3.30, 3.91)	3.40 (3.27, 3.51)	3.30 (3.22, 3.54)
NF-kB p50 (AU)	987 (510, 1424)	970 (614,1296)	1072 (549, 1469)	1038(550.1586)	1096 (492, 1570)
NF-kB p65 (AU)	283 (155, 418)	271 (224, 303)	274 (184, 395)	209 (129, 318)	285 (214, 433)

Table S1: Physiological, muscle and plasma miR levels according to disease status

Parameters were measured as described in Materials and Methods and are shown as median (inter-quartile range). ** $p<0.005$ as determined by ANOVA, adjusted $\alpha=0.005$. These data describe the values from the subjects included in this study.

FIGURE LEGENDS

Figure S1. Association of physiological characteristics with disease status

FFMI, 6 MW (% predicted) and strength measured as MVC/wt as described in methods were compared across the GOLD stages. FFMI was suppressed in GOLD III and GOLD IV patients compared to controls and to GOLD II patients (there were too few GOLD I patients for proper analysis). GOLD II, GOLD II and GOLD IV patients had lower endurance marked by 6MW% predicted than controls with GOLD IV patients having the lowest endurance. There were no significant differences in strength between the groups. (** $p<0.005$ vs control, ††$p<0.005$ vs GOLD II, †††$p<0.005$ GOLD III vs GOLD IV, ANOVA adjusted $\alpha=0.005$).

Figure S2. Plasma levels of non muscle derived miRNAs are not different between patients and controls

Plasma miR levels were measured as described in Materials and Methods and normalised to a spiked *C.Elegans* control. Data are presented as log normalised levels with the notched boxes showing median and IQR, error bars at 10^{th} and 90^{th} percentile, outliers are also shown. COPD GOLD stage I and II patients are represented by filled grey circles, GOLD stage III and IV patients are represented by filled black circles and controls are shown as open grey circles. Statistical significance was calculated by t test or Mann-Whitney as the non-parametric alternative.

Figure S3. Plasma levels of myomiRs according to disease status

Plasma miRNAs were determined as described in Materials and Methods and compared across GOLD stages. MiR-1 was significantly elevated in GOLD IV patients compared to controls. MiR-499 was elevated in both GOLD II and GOLD III patients compared to controls but was not elevated in GOLD IV patients. MiR-206 was elevated in GOLD II patients compared to controls but not in GOLD III or

GOLD IV patients. Distribution of the data points are shown as are the confidence intervals 10th and 90th percentile and the outliers. (** p<0.005 vs control, ANOVA adjusted α=0.005).

Figure S4. Plasma levels of miRs according to disease status

Plasma miRNAs were determined as described in Materials and Methods and compared across GOLD stages. GOLD stage did not affect plasma levels of miR-16 or miR-122 but miR-181 levels were elevated in GOLD III and GOLD IV patients. Distribution of the data points are shown as are the confidence intervals 10th and 90th percentile and the outliers. (** p<0.005 vs control, ANOVA adjusted α=0.005)

Figure S5. Association of plasma miRNAs with FFMI or strength in ex-smoking COPD patients

Physiological parameters and plasma miRNA levels were determined as described in Materials and Methods. (A) Plasma miR-1 levels negatively correlated with FFMI, in ex-smoking COPD patients (r=0.30, p=0.005). Mir-206 (B), -499 (C) and -133 (D) weakly correlated with quadriceps MVC corrected for weight in the ex-smoking patients. In ex-smoking patients the associations were stronger than in the whole cohort (r=0.28, p=0.009, r=0.27, p=0.013, r=0.29, p=0.008 respectively).

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Figure S6 ROC analyses of plasma miRNA levels to discriminate patients according to presence of type I fibre-shift

GOLD III and IV patients were defined as having type I fibre-shift or not as described in Results. ROC analysis was then performed as described in Materials and Methods using plasma levels of; miR-1 (A), miR-206 (B), miR-181 (C), miR- 499 (D). Areas under the curves are shown for each miR.