**Quadriceps miR-542-3p and 5p are elevated in COPD and reduce function by inhibiting ribosomal and protein synthesis**

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**New and Noteworthy:** miR-542-3p and -5p are elevated in the quadriceps muscle of patients with chronic obstructive pulmonary disease (COPD) in proportion to the severity of their lung disease. These miRNAs inhibit mitochondrial and cytoplasmic protein synthesis suggesting that they contribute to impaired exercise performance in COPD.

**Running title:** miR-542 in the control of mitochondrial and cytoplasmic protein synthesis

**Key words:** mitochondrial and cytoplasmic protein synthesis, chronic obstructive pulmonary disease, microRNA,

**Abstract**

Reduced physical performance reduces quality of life in patients with COPD. Impaired physical performance is, in part, a consequence of reduced muscle mass and function, which is accompanied by mitochondrial dysfunction. We recently showed that miR-542-3p and miR-542-5p were elevated in a small cohort of COPD patients and more markedly in critical care patients. In mice these miRNAs promoted mitochondrial dysfunction suggesting that they would affect physical performance in patients with COPD but we did not explore the association of these miRNAs with disease severity or physical performance further. We therefore quantified miR-542-3p/5p and mitochondrial rRNA expression in RNA extracted from quadriceps muscle of patients with COPD and determined their association with physical performance. As miR-542-3p inhibits ribosomal protein synthesis its ability to inhibit protein synthesis was also determined *in vitro*.Both miR-542-3p and -5p expression were elevated in patients with COPD (5-fold p<0.001) and the degree of elevation associated with impaired lung function (TLCO% and FEV1 %) and physical performance (6-minute walk distance %). In COPD patients, the ratio of 12S rRNA to 16S rRNA was suppressed suggesting mitochondrial ribosomal stress and mitochondrial dysfunction and miR-542-3p/5p expression was inversely associated with mitochondrial gene expression and positively associated with p53 activity. miR-542-3p suppressed RPS23 expression and maximal protein synthesis *in vitro*. Our data show that miR-542-3p and -5p expression is elevated in COPD patients and may suppress physical performance at least in part by inhibiting mitochondrial and cytoplasmic ribosome synthesis and suppressing protein synthesis.

**Introduction**

A reduction in muscle mass and strength occurs both in response to chronic disease and as part of normal human ageing (9), and loss of muscle function reduces quality of life by limiting ability to perform normal daily tasks and is associated with increased mortality (7, 36). One chronic disease, for which skeletal muscle wasting a common co-morbidity is chronic obstructive pulmonary disease (COPD) (19) and, similarly, in this condition, wasting and reduced muscle function is associated with poorer quality of life and increased mortality (8, 35).

Muscle function is affected both by the amount of muscle, which predominantly affects strength (12) and its oxidative capacity, which predominantly affects endurance. Muscle oxidative capacity is dependent on the number and function of mitochondria in the tissue (31). In COPD patients there is a reduction in both muscle mass (33) and mitochondrial oxidative capacity (13, 20). The loss of muscle mass arises from an imbalance in protein turnover with a relative increase in protein breakdown compared to protein synthesis and diverse studies have reported changes in components of both degradative and synthetic pathways, but, taken together, these data do not convey a consistent conclusion suggesting that there are multiple factors that regulate mass(15).

There are also a number of studies examining changes in mitochondria in the muscle of COPD patients. Together they show both reduced mitochondrial density and mitochondrial dysfunction. The factors contributing to reduced mitochondrial density include reduced mitochondriogenesis with lower levels of PGC1α and TFAM. The molecular mechanisms leading to mitochondrial dysfunction are less clear. Reduced activity of electron transfer complexes that contain mitochondrially encoded proteins has been reported (37) as have changes in the respiratory control ratio (29). Consistent with these observations a recent transcriptomic analysis of COPD muscle showed marked reduction in the expression of mitochondrial genes with weighted gene correlation network analysis identifying a mitochondrially enriched gene module as a major associated with pulmonary function and exercise capacity (41). Changes also seen in mitochondrial dysfunction associated with ageing and in patients with intensive care unit acquired weakness (3, 14, 37).

One mechanism by which this change in the activity of mitochondrial complexes that contain mitochondrially encoded proteins may occur is through the inhibition of mitochondrial translation. We recently showed that a microRNA (miRNA, miR-542-3p) inhibited the expression of mitochondrial ribosomal proteins, reduced mitochondrial rRNA in particular the 12S rRNA leading to a reduction in the 12S:16S mitochondrial rRNA ratio and reduced mitochondrial membrane potential (11). This miRNA also targets cytoplasmic ribosomal proteins suggesting that it would reduce maximal protein synthesis so may contribute to a loss of muscle mass. Elevation of this miRNA was observed in a small cohort of patients with severe COPD but the association of this miRNA with muscle function in COPD patients was not analysed further in our prior report (11). Nor did we quantify the expression of mitochondrial RNAs to determine whether there was evidence for mitochondrial ribosomal stress in COPD patients.

The current study therefore was designed to investigate the expression of miR-542-3p/5p in the quadriceps of a second, larger cohort of COPD patients, compare it to physical performance and quantify the expression of mitochondrial rRNAs. The expression of these miRNAs was determined in the muscle of healthy older individuals to identify any association with muscle dysfunction in the absence of overt disease. We also determined the effect of miR-542-3p on cytoplasmic protein synthesis.

**Methods**

***Subjects*:** All subjects gave informed consent prior to inclusion in the relevant study which had been approved by the appropriate ethical review bodies as detailed for each cohort. All procedures were carried out in accordance with the Helsinki Declaration.

***COPD cohort:*** COPD subjects (n=52) and controls (n=16) were part of a larger study described by Natanek et al (23). The patients had COPD according to the Global Initiative in Obstructive Lung Disease (GOLD) guidelines 2004 (30) and 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 (16 in total) were recruited by advertisement. All subjectsgave 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). Measurements of lung volume, using plethysmography, carbon monoxide transfer factor, using the single breath technique (CompactLab, Jaeger, Germany) and post-bronchodilator spirometry were performed accordingto ATS/ERS guidelines (40). Blood gas tensions were measured in arterialised capillary earlobe blood. Bioelectrical impedance was used to determine fat-free mass index (FFMI) (Bodystat 1500, Bodystat, UK)as described previously (34).

Quadriceps strength was determined by measuring supine isometric maximal voluntary contraction (MVC) as described previously (35) and physical performance measured as 6 minute walk distance (6MW), 5 minutes after bronchodilator treatment (ATS 2002 guidelines (1)). V*astus lateralis* muscle biopsies were obtained under local anaesthesia by percutaneous needle biopsy of the in the mid-thigh using the Bergstrom technique (2).

***Hertfordshire Sarcopenia Study (HSS) cohort:*** The study protocol was approved by the Hertfordshire Research Ethics Committee (study 07/Q0204/68) and all participants gave written informed consent. Muscle mass and strength together with gait speed and a timed-up-and go (TUG) test were ascertained; HSS methods have been previously described (25). Muscle biopsy was performed by percutaneous conchotome biopsy of the *vastus lateralis* under local anaesthesia (24).

***Quantification of rRNA,*** RNA was extracted using Trizol, and transcripts were reverse transcribed into cDNA using random primers using the Quantitect RT kit (Qiagen) and quantified by quantitative real time PCR (qRTPCR) as described previously using the primers described in Table 1 (10). rRNA expression was normalised to HPRT expression (human samples) or to the geometric mean of HPRT and β2 microglobin (cell culture samples) in the same sample using the CT method.

***Quantification of microRNAs:*** miRNAs were reverse transcribed using MultiScribe™ Reverse Transcriptase and Megaplex™ RT Primers (human pool A, Version 3.0, Applied Biosystems) according to the manufacturer’s instructions. The samples were heated to 85°C for 5min to terminate the reactoins then 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 and the reactions were terminated by heating to 99.9°C for 10 min. The resulting cDNA was diluted to 100μl by addition of 0.1× TE buffer pH 8.0 (Qiagen) and stored at -80°C. Primers and probes for miR-542-3p and miR-542-5p were purchased for each test gene from Applied Biosystems and amplification was carried out 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 RNU48 using the ΔΔ Ct method. RNA isolated from cells was analysed using single RT reactions.

***Cell culture:*** HLCN-M2 cells were maintained in skeletal muscle growth medium (PromoCell) supplemented with 20% FCS, as previously described (42). All experiments were performed on cells as myoblasts. RNA was extracted using Trizol; mRNA and miRNAs were quantified as described above.

**MicroRNA transfection:** 6250 cells were seeded into each well of a 96-well plate. After 24 hours, cells were transfected with mirVana miRNA mimics with each well treated with a mixture containing 0.5µL of 20µM mirVana miRNA mimic(s) and 0.5µL Lipofectamine 2000 (ThermoFisher) prepared according to the manufacturer’s instructions.

**RNA extraction:** 48h after microRNA transfection, RNA was extracted from LHCN-M2 cells using the CellAmp Direct RNA Prep Kit (TaKaRa) according to the manufacturer’s instructions. miRNA, and rRNA were analysed as described above.

 **Western Blotting:** Western blotting for RPS23 was carried out as previously described (18) but using Rabbit anti-RPS23 (Novus) at 1:100.

**Protein synthesis:** 48 hours after miRNA transfection LHCN-M2 cells were serum and leucine-starved by incubation in leucine free DMEM. 2 hrs later the medium was replaced with leucine-containing DMEM supplemented with 130nM of IGF-1 (Cambridge Bioscience) and the cells were incubated for 45 minutes, before addition of 100ng/mL puromycin (Sigma) and harvested 30 minutes later and protein extracted. 300 ng of the extracted protein was diluted in 200 µL of 50 mM sodium bicarbonate and incubated in a 96 well plate well at 37°C for 2 hours. The wells were washed (1 X PBS) then blocked with 200 µL PBS containing 5% BSA (w/v) (PBS-BSA) for 30 minutes at RT. The solution was replaced with 100 µL of 100 ng/mL anti-puromycin (Millipore) diluted in PBS-BSA and the samples were incubated for 1 hour at RT. After 2 X PBS washes 100 µL of sheep anti-mouse (GE Healthcare) was added at a 1/10,000 dilution in PBS-BSA. The samples were washed 4 X in PBS before 100 µL of TMB substrate (Sigma) was added and the samples incubated for 15 min. The reaction was stopped with 100 µL of stopping solution (Sigma) and absorbance determined at 450 nm.

***Statistical analysis:*** Gene expression data from human studies were log transformed to stabilize variance and produce a normal distribution. Gene expression data from *in vitro* studies were normalized to the mean value for the appropriate control dataset. Statistical analysis was performed in Aabel (Gigawiz). Pearson correlations were used to identify linear correlations after visual inspection of scatter plots. Differences between groups were calculated by Student’s t-test for normally distributed data and by Mann-Whitney U test for non-parametric data. Differences between multiple groups were identified by ANOVA with post-hoc testing using a Bonferroni-Dunn correction.

**Bioinformatic analysis:**

The quadriceps gene expression profiles of the majority of the patients included in this study have been published previously(41). Correlation analysis was performed within the WGCNA R package using robust biweight mid-correlation to determine the correlation coefficients. Gene Set Enrichment Analysis (GSEA) was then performed to identify groups of genes associated with the miRNAs comparing the correlation coefficients for genes that were significant at p<0.02. To determine the association of the miRNAs with mRNAs for mitochondrial ribosomal proteins the geometric mean of the target proteins individually and for all detected mitochondrial small ribosomal proteins was calculated and compared with miR-542 expression in the same sample.

**Results**

**Patient Demographics**

By design and consistent with a diagnosis of COPD, the patients had poorer lung function than controls by all measures. The patient cohort included 11 patients with GOLD 1-2, 25 patients with GOLD 3 and 16 patients with GOLD 4 COPD. There was no difference in fat free mass index (FFMI) between patients and controls but patients had poorer exercise performance measured both as 6-minute walk distance and maximal quadriceps voluntary contraction (MVC). The patients also had lower daily activity levels than the controls. The demographic data are presented in Table 2.

**Expression of miR-542-3p/5p in the quadriceps of patients with COPD**

The expression of miR-542-3p/5p were determined in a cohort of COPD patients (both sexes and all GOLD grades) and controls. Both miRNAs were elevated in the muscle of COPD patients compared to controls (542-3p, 5.5-fold, p<0.001 and 542-5p, 5.9-fold p<0.001, Fig. 1A, B) and highly correlated with each other (r=0.95, p<0.001). Quadriceps expression of these miRNAs was inversely associated with lung function measured either as TLCO% predicted (-3p r=-0.60, p<0.001 and -5p r=-0.62, p<0.001 for the whole cohort and -3p r=-0.44, p=0.001, -5p r=-0.42, p=0.002, for the patients alone, Fig. 1 C, D) or as FEV1% predicted (-3p r=-0.54 p=<0.001 and -5p r=-0.59, p<0.001 for the whole cohort and -3p r=-0.38, p=0.006, -5p r=-0.29, p=0.035 for the patients only, Fig. 1 E, F). Furthermore, the miRNAs were inversely proportional to exercise performance measured as 6MW expressed, as % predicted, in both the whole cohort (-3p r=-0.58, p<0.001, -5p r=-0.59, p<0.001 Fig. 2 A, B) and in the patients alone (-3p, r=-.037, p=0.006 and -5p, r=-0.35, p=0.012). Although both miRNAs were weakly inversely associated with strength in the patients and controls considered together (measured as MVC% predicted, -3p r=-0.37, p=0.002 and -5p r=-0.43, p<0.001, Fig. 2 C, D) and FFMI (-3p r=-0.27, p=0.028, -5p r=-0.26, p=0.034, Fig. 2 E, F), neither miRNA was associated with strength in the patients alone (-3p r=-.22, p=0.109, -5p r=-0.27, p=0.054) or FFMI (-3p r=-0.177, p=0.210, -5p r=-0.172, p=0.224). The associations did not differ significantly between males and females (data not shown).

To further determine whether the expression of these miRNAs was also associated with muscle function in healthy seniors we also measured the expression of the miRNAs in samples from the Hertfordshire Sarcopenia Study (HSS, demographics in Table 3). In normal healthy older men from the HSS both miRNAs were associated with poorer physical performance as indicated by 3m walk time and time to up and go 6m (TUG) test (Fig. 3A-D). Although neither miRNA was associated with FFMI in the HSS participants, both were elevated in those defined as having sarcopenia (Fig. 3E, F).

**Do COPD patients show evidence of mitochondrial ribosomal stress?**

In patients with established ICUAW the marked elevation of miR-542-3p was associated with a significant reduction in 12S rRNA levels compared to 16S rRNA levels indicative of mitochondrial ribosomal stress (11). To determine if increased miR-542-3p in the quadriceps of COPD patients was associated with evidence of mitochondrial ribosomal stress in COPD, we quantified the expression of 12S and 16S rRNA in the patients with COPD (n=40) and controls (n=14) for which we had appropriate cDNAs. There was a reduction in both the 12S and 16S rRNAs that was larger in the 12S rRNA resulting in a significant reduction in the 12S:16S ratio (Fig. 4).

**MiR-542 expression is associated with mitochondrial gene expression and P53 activity in patients.**

We have previously shown that over-expression of miR-542 leads to a reduction in the 12S:16S ratio in cells in culture and in the muscle of mice. This observation suggests that increased miR-542 in the muscle of patients with COPD may contribute to the loss of mitochondrial function in COPD muscle. To determine whether miR-542 was associated with mitochondrial gene expression by exploring the recently published array data from this study (41). Biweight mid-correlation coefficients were calculated for all detected genes using the R package WGCNA and correlations that reached a statistical significance of 0.02 were used for gene set enrichment analysis (GSEA). The gene set most negatively associated with miR-542 was mitochondrial genes and this observation was true whether the analysis was performed using the patients only or the whole cohort. The gene sets positively correlated with miR-542 included sets associated with the inflammatory response raising the possibility that some aspect of inflammatory signalling contributes to the expression of miR-542. However, there was no simple association of miR-542 expression with inflammatory cytokines (IL2, IL6, IL8 or TNFa) or with global DNA bound NFkB p50 or p65 indicating that these cytokines alone are not sufficient to explain the association of miR-542 with disease severity (data not shown).The positively correlated gene sets also included the gene set for P53 activity. As mitochondrial ribosomal stress has been shown to promote P53 activation this observation is consistent with miR-542 driven mitochondrial ribosomal stress activating P53.

**Does miR-542-3p inhibit protein synthesis?**

miR-542-3p inhibits the expression of a number of proteins that make up the cytoplasmic small ribosomal subunit promoting cytoplasmic ribosomal stress and reducing 18S rRNA expression (39). Consequently, this miRNA should suppress maximal protein synthesis. We therefore confirmed the reduction in RPS23 and 18S rRNA in muscle cells (Fig. 5A, B) and determined the effect of miR-524-3p on leucine and IGF-1 stimulated proteins synthesis by quantifying puromycin incorporation. Transfection of LHCN-M2 muscle myoblasts with miR-542-3p decreased puromycin incorporation (Fig. 5C) suggesting that miR-542-3p inhibited the formation of the small ribosomal subunit and inhibited protein synthesis.

**Discussion**

The data presented here show that increased expression of miR-542-3p and miR-542-5p are associated with muscle dysfunction in COPD patients. Quadriceps expression of these miRNAs is associated with lung function measured as either TLCO% predicted or FEV1% predicted. Consistent with promotion of mitochondrial ribosome stress by miR-542-3p, expression of the 12S rRNA was reduced. Furthermore, the expression of miR-542-3p/5p was inversely associated with the expression of mitochondrial genes and positively associated with the genes associated with activation of the p53 pathway, consistent with an association with mitochondrial dysfunction and ribosomal stress. We also show that miR-542-3p can reduce RPS23 and 18S rRNA expression and inhibit protein synthesis in muscle cells. Taken together the data suggest that disease situated in the lung promotes the muscle expression of miR-542-3p/5p where it promotes mitochondrial dysfunction and inhibits protein synthesis. The association of miR-542-3p/5p with inflammatory signalling raises the possibility that in this context, inflammatory mediators released as part of, or in response to, lung disease promote miR-542-3p/5p expression but the lack of correlation of miRNA expression with the classical mediators of inflammatory signalling mean that the factors underlying this association remain to be identified. The promoter that controls the expression of miR-542 has yet to be confirmed but the close proximity of this miRNA to the miR-424-503 host gene suggests that these genes are co-expressed from the same promoter. This suggestion is supported by relatively tight correlation of the expression of miR-542 expression with miR-424 in all the studies we have performed. This region of DNA contains multiple binding sites for STAT, NF-κB and glucocorticoid receptors suggesting that it functions as part of a stress response system. An alternative possibility for the elevation of these miRNAs in muscle is delivery from another tissue via exosomes. However, this route would require sufficient production of these miRNAs and exosomes in an organ to increase the levels in muscle 6-fold and where skeletal muscle accounts for about 40% of the body mass.

*Significance of the findings*

A significant proportion of COPD patients report leg fatigue as a more important locus of exercise limitation than breathlessness (16, 28). A well-recognized cause of this early fatigue is a reduction in mitochondrial function and oxidative capacity. One intriguing component of this reduction in oxidative capacity is a greater reduction in the activity of mitochondrial complex I and IV, which require translation to occur both in the cytoplasm and mitochondria, compared to complex II which only requires translation in the cytoplasm (37). Consequently, a relative reduction in translation in mitochondria would lead to a greater reduction in the activity of complexes I and IV. This reduction in activity of electron transfer components requiring mitochondrial translation is also seen in sarcopenia where an accumulation of mutations in the mitochondrial genome has been suggested to contribute (4, 38). Our previous study showing that miR-542-3p suppressed the expression of mitochondrial ribosomal proteins and the 12S rRNA provided an additional mechanism by which mitochondrial protein synthesis could be suppressed and mitochondrial dysfunction could occur (11). Furthermore, this reduction in the ability to form functional mitochondrial ribosomes also reduced the levels of the small ribosomal RNA (12S) compared to the large ribosomal subunit RNA (16S). Our observation of a reduced 12S:16S rRNA ratio is consistent with such a mechanism contributing to the mitochondrial dysfunction in COPD patients. This effect of miR-542-3p on mitochondrial function is sufficient to explain the association of miR-542-3p with physical performance that we see both in the COPD patients and in the healthy older individuals from the HSS.

This mechanism does not appear to be restricted to COPD patients as we have also demonstrated similar effects in patients with ICUAW (11), in whom there is also preferential loss of complex I and complex IV activity. Taken together these data suggest that changes mediated by miR-542-3p are not disease specific but a more general pathophysiologic process. In its most extreme form the effects of inhibition of mitochondrial translation can be seen via the occasional gene mutations in humans causing an inability to synthesise the small subunit of the mitochondrial ribosome (21, 32). These mutations have a similar, but more marked, effect on the electron transfer chain and on 12S rRNA levels to that observed here and ICUAW.

The observation that miR-542-3p mediated muscle dysfunction can complicate ICUAW, COPD and indeed ‘normal’ ageing suggests a currently unknown mechanism by which diverse aetiologies can trigger a single underlying molecular mechanism. Immobility (or more specifically contractile history) would be one candidate since it is present to some extent in all 3 conditions. However, we did not see any association of miR-542-3p expression with daily physical activity. Other possible candidates might include inflammation which is present in ICU and at least episodically in COPD (at time of acute exacerbation) and as demonstrated in this study there is an association of miR-542 expression with markers of the activation of inflammatory signalling in the muscle but no association with classic inflammatory cytokines. Identification of the mechanisms leading to increased expression of miR-542-3p would be insightful and could offer a novel therapeutic approach to this condition

*Critique of the Method*

Even though we showed that miR-542-3p can suppress maximal protein synthesis *in vitro*, expression of this miRNA was not strongly associated with FFMI. There are a number of likely reasons for this lack of association. Firstly, FFM is not a good tool for detecting quadriceps weakness in COPD (22), presumably because the muscle wasting observed in COPD is regional in nature. Secondly there are multiple inputs into the regulation of protein synthesis and the increase of miR-542-3p would have to be one of the predominant regulators for a significant association to be observed. For example, we have already shown that an miRNA from the same locus as miR-542 (miR-424-5p) suppresses the expression of rRNAs by targeting PolR1A the RNA polymerase required for rRNA synthesis and UBTF a key transcription factor that activates RNA polymerase I (5) as well as targeting IGF-1 (6). This miRNA whilst closely correlated with miR-542-3p expression in COPD and ICUAW is associated with FFMI in the COPD patients suggesting that suppression of rRNA synthesis is more important in terms of protein synthesis than the effects of miR-542 on small ribosomal protein expression. Thirdly, altering the number or activity of ribosomes by one miRNA is also unlikely to be the only mechanism contributing to protein synthesis. For example, increasing or reducing the number of nuclei will affect the capacity to transcribe all genes implicating the rate of regeneration in muscle retention. Again, regeneration is controlled by miRNAs and we have shown that the expression of the imprinted miRNAs from a cluster on chromosome 19 (C19MC, expressed from the paternal chromosome) and miR-675 (expressed from the maternal chromosome) associate with FFMI in COPD patients but not controls (17). Finally, the loss of muscle mass over-time is not only due to reduced protein synthesis and protein breakdown will also contribute. The rate of protein breakdown will be dependent on the strength of any signal promoting atrophy and the sensitivity of an individual to that signal. Previous studies have shown that levels of GDF-15, a protein that promotes muscle breakdown and reduces appetite, are associated with muscle mass and function in COPD (26) suggesting that this is one pro-atrophic signal contributing to muscle breakdown. Variation in the sensitivity to atrophic signals also comes from miRNA dependent regulation of the expression of components of signaling pathways with miR-422a reducing the activity of myostatin/TGF-β signaling by suppressing SMAD4 (27).

The work presented here is a cross sectional study into the expression of a microRNA and mitochondrial rRNAs in patients with COPD. We therefore cannot prove that the associations or relationships are causal. We are also unable to confirm that there is reduced mitochondrial function in the individual samples that we examined as there is insufficient sample available. However, our *in vitro* and animalstudies presented here and in our previous study (11) are consistent with such a role for the miRNAs and there are a number of studies showing reduced mitochondrial function in patients with COPD (reviewed in (20)). Furthermore, we identify an altered 12S:16S ratio in the COPD patients consistent with mitochondrial dysfunction. The data are also consistent with the miRNA suppressing maximal protein synthetic capacity. This altered miRNA and rRNA expression pattern is also seen in the ICUAW patients we have also studied.

In conclusion our data identify the targeting of both cytoplasmic and mitochondrial ribosomal proteins by miR-542-3p as contributing to the loss of physical performance in patients with COPD. This reduction in ribosomal proteins reduces the availability of small ribosomal subunits inhibiting protein synthesis in each compartment. The expression of these miRNAs is elevated in response to components of the disease process that are known to cause tissue hypoxia, and *in vitro* data reported here confirm this as a plausible mechanism.

**Disclosures**

The authors declare no competing financial interests

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**Author contributions**

The study was conceived by PRK with input from MIP and SAN. The muscle biopsies were collected by SAN and HP. The biochemical analysis was performed by RF-G and JYL with assistance from MC. The first draft of the paper was written by PRK with input from all authors.

**Figure Legends**

**Figure 1.** **Expression of miR-542-3p and miR542-5p are increased in the quadriceps of COPD patients and associated with disease severity.**

miR-542-3p and miR-542-5p were quantified in muscle biopsies from patients with COPD (n=52) and controls (n=16). (A) miR-542-3p was elevated 5.5 fold (p<0.001) and (B) miR-542-5p was elevated 5.9 fold (p<0.001). Comparison of the expression of both miRNAs with measurements of lung function (TLCO % predicted, C, D and FEV1% predicted) showed that expression of these miRNAs in the quadriceps was associated with disease severity. miRNA expression was normalized to the expression of U6 and RNU48 in the same samples. Data were analysed by T-test and Pearson correlations shown are for the entire cohort. Patients are shown as closed circles and controls as empty circles.

**Figure 2.** **miR-542-3p and 542-5p are associated with physical performance in COPD**

miR-542-3p and miR-542-5p were quantified in muscle biopsies from patients with COPD (n=52) and controls (n=16). Comparison of the expression quadriceps of both miRNAs with 6 minute walk distance (6MW) % predicted showed an inverse association both in the whole cohort and in the patients as a subgroup [miR-542-3p, (A) and miR-542-5p (B)]. Comparison of the expression of both miRNAs with strength (maximal voluntary contraction (MVC) % predicted showed a weak inverse association in the whole cohort but not in the patients as a subgroup [miR-542-3p, (C) and miR-542-5p (D)]. Comparison of the expression quadriceps of both miRNAs with FFMI showed a weak inverse association in the whole cohort but not in the patients as a subgroup [miR-542-3p, (E) and miR-542-5p (F)]. miRNA expression was normalized to the expression of U6 and RNU48 in the same samples. Pearson correlation coefficients shown are for the entire cohort. Patients are shown as closed circles and controls as empty circles.

**Figure 3. miR-542-3p and -5p are associated with physical performance in older people**

miR-542-3p and miR-542-5p were quantified in quadriceps biopsies from older individuals enrolled in the Hertfordshire Sarcopenia Study (HSS) (n=64) and correlated with physical performance measured using the timed up and go test (A and B) and 3m gait speed (C and D). Both miR-542-3p (A and C) and miR-542-5p (B and D) were associated with poor physical performance on both tests. Furthermore, in the patients identified as sarcopenic according to EWGSOP guidelines (n=5) both miRNAs (E, miR-542-3p and F, miR-542-5p) were elevated compared to those who were not sarcopenic (n=59). The data shown for miR-542-3p are taken from all 64 samples, however, four samples did not amplify for miR-542-5p (3 non sarcopenic and 1 sarcopenic) so these data come from 56 control and 4 sarcopenic individuals. miRNA expression was normalized to the expression of U6 and RNU48 in the same samples. Pearson correlation coefficients shown and statistical difference was calculated by Student’s t-test.

**Figure 4. Mitochondrial RNA expression is reduced in COPD patients**

Quadriceps expression of 12S and 16S rRNAs were quantified in samples from COPD (n=40) and controls (n=14). Expression of both the 12S rRNA (A) and 16S rRNA (B) were reduced in the patients compared to controls. The reduction in the 12S rRNA was larger than that in the 16S rRNA leading to a reduction in the 12S:16S ratio (C).

**Figure 5. miR-542-3p suppresses the expression of RPS23 and inhibits protein synthesis in muscle cells.**

LHCN-M2 cells were transfected with miR-542-3p or scrambled control as described in Methods and incubated for 2 days. (A) transfected cells were lysed and the expression of RPS23 was determined by Western Blotting. The experiment was repeated 3 times and expression of RPS23 was normalized to PonceauS stain. RPS23 expression was suppressed by transfection with the miRNA. (B) 18S rRNA was quantified and normalized to the geometric mean of β2-microglobiulin and HPRT in transfections performed in hextuplicate and the experiment repeated 3 times. miR-542-3p suppressed 18S rRNA expression. (C) protein synthesis was analysed by quantifying puromycin incorporation as described in Supplement with the transfections performed in quadruplicate and the experiment repeated 3 times. Puromycin incorporation was lower in miR-542-3p transfected cells than in control transfected cells indicating a suppression of protein synthesis. Data are presented as mean ± SEM p values were calculated by Student’s t-test. Abbreviations: 3p; miR-542-3p, 3p + Ant; miR-542-3p + antagomiR, cont; control, RPS23; ribosomal protein S23, untrans; untransfected.

**Tables**

**Table 1: Primers used in this study**

|  |  |  |
| --- | --- | --- |
|  | Forward | Reverse |
|  12S | CCCAAACTGGGATTAGATACCC  | GTTTGCTGAAGATGGCGGTA |
|  16S | GCCTGTTTACCAAAAACATCAC  | CTCCATAGGGTCTTCTCGTCTT |
|  HPRT  | GCTATAAATTCTTTGCTGACCTGCTG | AATTACTTTTATGTCCCCTGTTGACTGG |
|  beta 2 microglobulin | TGCTGTCTCCATGTTTGATGTATCT | TCTCTGCTCCCCACCTCTAAGT |

**Table 2: Physiological characteristics of the COPD cohort**

|  |  |  |
| --- | --- | --- |
|  | Control (n=16) | COPD (n=52) |
| Sex (M, F) | 7, 9 | 30, 22 |
| Age (years) | 66 ± 8 | 66 ± 8 |
| Smoking Historya (pack-year) | 0 (0, 10) | 44 (30,60)\*\*\* |
| Weight a (kg) | 67 (61, 74) | 66 (59, 77) |
| BMIa (kg/m2) | 24.8 (23.5, 26.2)  | 23.3 (21.4, 26.2) |
| FFMI a (kg/m2) | 16.1 (15.3, 17.2) | 15.3 (14.5, 16.8) |
| FEV1a(% pred) | 107.6 (100.6, 112) | 40.7 (27.2, 48.4)\*\*\* |
| RVTLC  | 38± 6 | 59 ± 8\*\*\* |
| TLCO a (% pred) | 92.3 (83.0, 98.1) | 45.0 (32.1, 52.4)\*\*\* |
| 6 min walk (m) | 621± 84 | 387± 125\*\*\* |
| 6min walk % pred | 126±12 | 76±24\*\*\* |
| pVO2 a(% pred) | 99 (88, 111) | 46 (38, 58)\*\*\* |
| SGRQ a | 2 (0, 8) | 54 (45, 61)\*\*\* |
| Quadriceps MVC (kg) | 34.7±10.6 | 28.2 ± 8.9\* |
| Quadriceps MVC (% pred) | 78±19 | 63 ± 14\*\* |
| Locomotion timea (min/12 hr) | 96 (84, 127) | 41 (26, 56)\*\*\* |
| Movement time (as % of 12hrs) | 23±6 | 13 ± 5\*\*\* |

BMI (Body mass index), FFMI (fat free mass index), FEV1 (Forced expiratory volume in 1 sec), RVTLC (ratio of the reserve volume to total lung capacity), TLCO (transfer capacity of the lung for CO), SGRQ (St George’s respiratory questionnaire) MVC (maximal voluntary contraction.

**Table 3: Physiological characteristics of HSS cohort**

|  |  |  |  |
| --- | --- | --- | --- |
|  | Whole cohort (n=67) | Non-sarcopenic (n=59) | Sarcopenic (n=5) |
| Weight a (kg) | 83 (73, 92) | 83 (72, 92) | 69(66, 71)\* |
| BMIa (kg/m2) | 27 (25, 29)  | 27 (25, 29) | 26 (25, 27) |
| FFMI a (kg/m2) | 18.4 (17.4, 19.3) | 18.5 (17.5, 19.3) | 16.2 (15.9, 17.1)\*\* |
| FEV1a(% pred) | 105 (98, 115) | 106 (98, 117) | 108 (103, 111) |
| TUG time (s)  | 10.5 (9.2,12) | 10.4 ± 1.7 | 13.7 ± 4.7\*\* |
| 3m walk time a(s) | 2.7 (2.5, 3.1) | 2.7 ± .4 | 3.4 ± 1.0\*\* |

BMI (Body mass index), FFMI (fat free mass index), FEV1 (Forced expiratory volume in 1 sec), TUG time (time to ‘get up and go’)

**Table 4: Gene set enrichment for genes associated with miR-542**

|  |  |  |  |
| --- | --- | --- | --- |
| **HALLMARK** | **NES** | **NOM p-val** | **FDR q-val** |
| **Positive** |  |  |  |
| EPITHELIAL MESENCHYMAL TRANSITION | 4.212 | 0.000 | 0.000 |
| INTERFERON GAMMA RESPONSE | 2.879 | 0.000 | 0.000 |
| IL2 STAT5 SIGNALING | 2.716 | 0.000 | 0.000 |
| INFLAMMATORY RESPONSE | 2.621 | 0.000 | 0.000 |
| APOPTOSIS | 2.616 | 0.000 | 0.001 |
| COAGULATION | 2.605 | 0.000 | 0.001 |
| COMPLEMENT | 2.421 | 0.000 | 0.002 |
| TNFA SIGNALING VIA NFKB | 2.087 | 0.006 | 0.013 |
| UV RESPONSE DN | 2.054 | 0.002 | 0.014 |
| HYPOXIA | 1.913 | 0.008 | 0.022 |
| APICAL JUNCTION | 1.775 | 0.020 | 0.042 |
| P53 PATHWAY | 1.767 | 0.028 | 0.040 |
| KRAS SIGNALING UP | 1.762 | 0.015 | 0.038 |
|  |  |  |  |
| **Negative** |  |  |  |
| OXIDATIVE PHOSPHORYLATION | -5.046 | 0.000 | 0.000 |
| FATTY ACID METABOLISM | -3.058 | 0.000 | 0.000 |
| ADIPOGENESIS | -2.231 | 0.000 | 0.002 |

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