ORIGINAL ARTICLE

Downregulation of the serum response factor/miR-1 axis in the quadriceps of patients with COPD

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ABSTRACT

Rationale Muscle atrophy confers a poor prognosis in patients with chronic obstructive pulmonary disease (COPD), yet the molecular pathways responsible are poorly characterised. Muscle-specific microRNAs and serum response factor (SRF) are important regulators of muscle phenotype that contribute to a feedback system to regulate muscle gene expression. The role of these factors in the skeletal muscle dysfunction that accompanies COPD is unknown.

Methods 31 patients with COPD and 14 healthy age-matched controls underwent lung and quadriceps function assessments, measurement of daily activity and a percutaneous quadriceps muscle biopsy. The expression of muscle-specific microRNAs, myosin heavy chains and components of the serum response factor signalling pathway were determined by qPCR.

Results A reduction in expression of miR-1 (2.5-fold, p = 0.01) and the myocardin-related transcription factors (MRTFs) A and B was observed in patients compared with controls (MRTF-A mRNA: twofold, p = 0.028; MRTF-B mRNA: fourfold, p = 0.011). miR-1 expression was associated with smoking history, lung function, fat-free mass index, 6 min walk distance and percentage of type I fibres. miR-133 and miR-206 were negatively correlated with daily physical activity. Insulin-like growth factor 1 mRNA was increased in the patients and miR-1 was negatively correlated with phosphorylation of the kinase Akt. Furthermore, the protein levels of histone deacetylase 4, another miR-1 target, were increased in the patients.

Conclusions Downregulation of the activity of the MRTF-SRF axis and the expression of muscle-specific microRNAs, particularly miR-1, may contribute to COPD-associated skeletal muscle dysfunction.

INTRODUCTION

Skeletal muscle dysfunction in the locomotor muscles is an important systemic complication of chronic obstructive pulmonary disease (COPD). Quadriceps weakness and wasting predict mortality among patients with moderate to severe COPD independently of lung function and are common complications. Characteristically, the vastus lateralis of patients with COPD exhibits a shift towards a predominance of type IIA fibres and an increase in the proportion of type IIX fibres associated with a marked reduction in exercise capacity. In addition to these changes, fibre atrophy occurs and is particularly marked in type IIX fibres. However, the mechanisms that control these phenotypic changes have not been fully defined.

Recent data have implicated microRNAs (miRNAs, small non-coding RNAs that reduce mRNA half-life and translation) in the regulation of the skeletal muscle phenotype. Several miRNAs are highly expressed in skeletal muscle and regulate muscle phenotype. miR-1 and miR-206 promote myotube formation and miR-133 is associated with regeneration after nerve injury. Genetic knockout of miR-499 or miR-208-b reduces the proportion of type I fibres whereas overexpression of miR-499 increases type I fibres and endurance in mice. Conversely, miR-153 expression inhibits myotube formation and promotes proliferation.

Analysis of miRNA expression in primary skeletal myopathies identified changes in miRNAs that target conserved pathways, but the expression of individual miRNAs varied. Similarly, the profile of miRNAs is altered in the skeletal muscle of patients with insulin resistance. miRNA profiles are also markedly affected by activity, and studies in humans have shown altered miRNA profiles associated with endurance exercise training. As inactivity may be a major contributor to skeletal muscle dysfunction in COPD, changes in miRNA expression associated with inactivity may contribute to the phenotype. Studies of inactivity resulting from denervation, nerve entrapment or space flight all show initial reductions in
miR-1, but whether miR-1 levels remain suppressed varies. Interestingly, miR-1 did not change in response to change in MRTF/SRF activity is more important in activating expression of MHC expression. SRF activity is important in regulating MHC expression. SRF here have been reported in abstract form.

were matched for gender (p=0.74, Fisher exact test). Patients had a significantly reduced FFMI, quadriceps force and locomotion time compared with controls (table 1).

The patients had a marked difference in the size and proportion of muscle fibres compared with the controls (see figure S1 in online supplement). The proportion of type I fibres in the patients was reduced (from a median of 52 (IQR 39–62) to 25 (IQR 17–30)) and the proportion of type II fibres was increased (from mean±SD 47±14 to 69±15, table 1). The proportion of hybrid type I/II fibres was also increased in patients (from a median of 1.5 (IQR 0–3) to 3 (IQR 0–11), table 1). The fibres of the patients were smaller than those of the controls with a significant reduction in the size of the type I and type II fibres (table 1).

Patients had reduced MHCI mRNA compared with controls (table 1 and figure S2 in online supplement). MHCI mRNA was directly correlated with forced expiratory volume in 1 s (FEV1) percentage predicted and inversely correlated with smoking history across both patients and controls (see figure S2 in online supplement). MHCI mRNA was positively correlated with exercise performance (6MW, figure S3 in online supplement) but was not associated with strength. MHCI mRNA was inversely correlated with 6MW (figure S3 in online supplement) across all data as well as within the patient group alone, but also showed no association with strength. There was no significant difference in α-actin expression between the groups (data not shown).

Table 1  Clinical characteristics of study subjects

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=14)</th>
<th>COPD (n=31)</th>
<th>Significance (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (F:M)</td>
<td>6:8</td>
<td>11:21</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>68±8</td>
<td>65±7</td>
<td>0.192 (T)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>171±8</td>
<td>169±9</td>
<td>&gt;0.5 (T)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>79.8±18.4</td>
<td>70.9±16.5</td>
<td>0.114 (T)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.9±4.5</td>
<td>24.5±4.7</td>
<td>0.109 (T)</td>
</tr>
<tr>
<td>FFMI (kg/m³)</td>
<td>17.8±2.3</td>
<td>15.7±2.2</td>
<td>0.006 (T)</td>
</tr>
<tr>
<td>Pack-years*</td>
<td>4.1 (0–10)</td>
<td>45 (34–69)</td>
<td>&lt;0.001 (MW)</td>
</tr>
<tr>
<td>FEV1 (% pred)*</td>
<td>110 (103.6–112.6)</td>
<td>32 (25.1–47.1)</td>
<td>&lt;0.001 (MW)</td>
</tr>
<tr>
<td>RV/TLC (%)</td>
<td>37.1±4.7</td>
<td>56.6±9.9</td>
<td>&lt;0.001 (T)</td>
</tr>
<tr>
<td>TCO (% pred)*</td>
<td>86 (82.5–91.8)</td>
<td>33 (26.2–52.2)</td>
<td>&lt;0.001 (MW)</td>
</tr>
<tr>
<td>PaO₂ (kPa)*</td>
<td>5.33 (4.80–5.42)</td>
<td>5.18 (4.96–5.74)</td>
<td>&gt;0.5 (MW)</td>
</tr>
<tr>
<td>PaCO₂ (kPa)*</td>
<td>10.37–1.55</td>
<td>9.25±1.34</td>
<td>0.017 (T)</td>
</tr>
<tr>
<td>6MW (m)</td>
<td>623±89</td>
<td>378±134</td>
<td>&lt;0.001 (T)</td>
</tr>
<tr>
<td>6MW (% pred)</td>
<td>125±15</td>
<td>72±24</td>
<td>&lt;0.001 (T)</td>
</tr>
<tr>
<td>Locomotion time (min/12 h)*</td>
<td>86 (61–122)</td>
<td>45.5 (23–81)</td>
<td>0.006 (MW)</td>
</tr>
<tr>
<td>Mt (%M₄)</td>
<td>20.6±7.2</td>
<td>15.7±6.2</td>
<td>0.03 (T)</td>
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<tr>
<td>MI (%M₄)*</td>
<td>2.1 (1.7–2.4)</td>
<td>1.6 (1.4–2.0)</td>
<td>0.017 (MW)</td>
</tr>
<tr>
<td>SGRO*</td>
<td>5 (1–8)</td>
<td>52 (41–61)</td>
<td>&lt;0.001 (MW)</td>
</tr>
<tr>
<td>Best MVC</td>
<td>36.84±7.78</td>
<td>28.21±9.11</td>
<td>0.009 (T)</td>
</tr>
<tr>
<td>Best TwQ</td>
<td>9.58±3.04</td>
<td>7.89±2.41</td>
<td>0.077 (T)</td>
</tr>
<tr>
<td>MVC/BMI</td>
<td>1.4±0.3</td>
<td>1.2±0.3</td>
<td>0.075 (T)</td>
</tr>
<tr>
<td>Quadriceps endurance T80*</td>
<td>110 (70–160)</td>
<td>80 (60–105)</td>
<td>0.121 (MW)</td>
</tr>
<tr>
<td>MHCI mRNA (AU)*</td>
<td>28.0 (19.2–39.9)</td>
<td>7.0 (4.8–14.0)</td>
<td>&lt;0.001 (MW)</td>
</tr>
<tr>
<td>MHCIIA mRNA (AU)*</td>
<td>1.5 (0.8–2.2)</td>
<td>2.6 (1.3–3.6)</td>
<td>0.105 (MW)</td>
</tr>
<tr>
<td>Type I CSA (µm²)</td>
<td>5786±1371</td>
<td>4809±1327</td>
<td>0.048 (T)</td>
</tr>
<tr>
<td>Type II CSA (µm²)*</td>
<td>4533.5 (2946–6105)</td>
<td>3784 (2964–4615)</td>
<td>0.141 (MW)</td>
</tr>
<tr>
<td>Type IIX CSA (µm²)</td>
<td>6187±1868</td>
<td>3231±1403</td>
<td>&lt;0.001 (T)</td>
</tr>
<tr>
<td>% Type I fibres*</td>
<td>52 (39–62)</td>
<td>25 (17–30)</td>
<td>&lt;0.001 (MW)</td>
</tr>
<tr>
<td>% Type I/II fibres*</td>
<td>1.5 (0–3)</td>
<td>3 (0–11)</td>
<td>0.088 (MW)</td>
</tr>
<tr>
<td>% Type II fibres</td>
<td>47±14</td>
<td>69±15</td>
<td>&lt;0.001 (T)</td>
</tr>
<tr>
<td>% Type IIA fibres</td>
<td>44±13</td>
<td>60±15</td>
<td>0.002 (T)</td>
</tr>
<tr>
<td>% Type IIX fibres*</td>
<td>2.6 (0–4.0)</td>
<td>8.9 (3.0–13.0)</td>
<td>0.006 (MW)</td>
</tr>
</tbody>
</table>

Values are mean±SEM for normally distributed data or median (IQR) for non-normally distributed data. p values were calculated by t test (normally distributed data), indicated by (T), or the Mann–Whitney test (non-normally distributed data), indicated by (MW), and are shown in bold when p<0.05.

MHCI mRNA was determined by qPCR and normalised to the expression of RPLPO in the same samples as described in the online supplement.

*p Not normally distributed.

MHC IIa was inversely correlated with exercise performance (6MW, figure S3 in online supplement) but was not associated with strength. MHCI mRNA was inversely correlated with 6MW (figure S3 in online supplement) across all data as well as within the patient group alone, but also showed no association with strength. There was no significant difference in α-actin expression between the groups (data not shown).

miRNA expression in quadriceps of patients with COPD

Multivariate statistical analyses (eg, hierarchical clustering and PCA) offer a powerful unsupervised approach for the identification of structure and relationships between samples, allowing for the visualisation of natural groupings in data. Hierarchical clustering and PCA of miRNA expression in quadriceps of patients with COPD compared with matched controls. The expression of miRNAs was determined by qPCR and normalised to the expression of SS RNA in the same sample, as described in the Methods section. The heat map shows the expression of miRNAs in each sample organised by hierarchical clustering in which the expression of each miRNA was given equal weighting. This analysis shows clear separation of the majority of patients (closed circles) from the control group (open circles).
Chronic obstructive pulmonary disease

The association of the miRNAs with different physiological characteristics showed that miR-1 was negatively correlated with FEV1 (% predicted, r = -0.34, p = 0.023 and locomotion time; miR-133: r = -0.33, p = 0.029) and negatively with smoking history (r = -0.33, p = 0.026) and MVC/BMI (r = -0.29, p = 0.049). Like miR-1, miR-499 was positively associated with FFMI (r = 0.46, p = 0.006, not shown). Again these associations were not present in individual groups. To tie this reduction in MRTF expression to miR-1, we showed that MRTFs could activate the miR-1 promoters (figure 6D and supplementary results).

Comparison of miRNA expression with the muscle characteristics showed that miR-1 was positively correlated with the percentage of type I fibres (r = 0.33, p = 0.029) and miR-499 showed a trend towards association with percentage of type I fibres (r = 0.28, p = 0.063). None of the other miRNAs was associated with muscle fibre characteristics (figure 5B).

Expression of SRF, MRTF-A, MRTF-B

To investigate the SRF system we analysed the expression of SRF and two SRF co-activators (MRTF-A and MRTF-B) by qPCR. Mean SRF mRNA was not significantly different between the groups (COPD 3.42 (IQR 2.49–3.63) AU, n = 19; controls 3.51 (IQR 3.22–3.89) AU, n = 16, p = 0.185 (MW), figure 6A). MRTF-A and MRTF-B mRNA levels were significantly lower in patients (3.73 (IQR 3.48–3.87) AU and 3.94±0.394 AU respectively, n = 25) than in controls (3.85 (IQR 3.72–4.22) AU, p = 0.028 (MW), figure 6B and 4.42±0.634 AU, p = 0.011 (T) respectively, n = 15, figure 6C). MRTF-A expression was correlated with FEV1 (% predicted, r = 0.46, p = 0.027) and MRTF-B was correlated with smoking history (r = -0.576, p = 0.006, not shown). Again these associations were not present in individual groups. To tie this reduction in MRTF expression to miR-1, we showed that MRTFs could activate the miR-1 promoters (figure 6D and supplementary results).

Figure 3 Principal component analysis of the miRNA expression pattern in patients with chronic obstructive disease (COPD) and controls. (A) Scatter plot comparing PC1 and PC2 for each sample. (B) Principal component analysis of miRNA expression in patients and controls shows a significant difference in the pattern of miRNA expression between the two groups as a difference in PC2 (p<0.0001). (C) Loading plots for PC1 showing equal contribution of the miRNAs to this component. (D) Loading plots for principal component analysis identifies miR-1, miR-208 and miR-499 as the major contributors to the separation of the two groups. Patients are shown as closed circles and controls are shown as open circles.

Comparison of the expression of the miRNAs with the physiological data showed that miR-1 was negatively correlated with smoking history (r = -0.39, p = 0.007) but positively associated with FEV1 (r = 0.34, p = 0.022), FFMI (r = 0.35, p = 0.025), 6MW (r = -0.33, p = 0.026) and MVC/BMI (r = -0.29, p = 0.049). Like miR-1, miR-499 was positively associated with FFMI (r = 0.37, p = 0.012) and negatively with smoking history (r = -0.33, p = 0.027). miR-133 and miR-206 were negatively associated with physical activity (movement intensity; miR-133: r = -0.50, p = 0.057; miR-206: r = -0.35, p = 0.023) and locomotion time; miR-133: r = -0.55, p = 0.027; miR-206: r = -0.34, p = 0.029). The association of the miRNAs with different physiological characteristics is shown in figure 5A. Associations with p<0.05 were only present if both the control and patient groups were combined; this feature may result from a lack of power but also raises the possibility that disease is an important component of the difference.
SRF activity is also regulated by the localisation of the protein. We therefore determined the localisation of SRF in muscle sections from a subset of patients with COPD and controls. In the controls, SRF was readily detectable in the nuclei of all of the samples analysed. However, in the patients there was a reduction in nuclear staining and the appearance of nuclei with perinuclear SRF staining (figure 7).

Reduced expression of targets of miR-1 in patients with COPD
miR-1 targets include IGF-1 and the IGF-1 receptor, indicating that it should modify the activity of the IGF-1 pathway, and we have found that phosphorylation of the kinase Akt (also known as protein kinase B) is inversely proportional to activity in our patient group. We therefore analysed the expression of IGF-1 and the activity of the IGF-1 pathway by examining the phosphorylation of Akt in patients and controls. IGF-1 mRNA was increased in patients (figure 8A) and miR-1 expression was inversely proportional to the phospho-Akt/total Akt ratio (figure 8B, r=-0.43, p=0.022). A trend towards this association was present in the patients alone (r=-0.46, p=0.053). There were no other associations of the phospho-Akt/total Akt ratio with any of the other miRNAs.

We also determined the expression of histone deacetylase 4 (HDAC4) mRNA and protein in an additional set of patients with COPD and controls. This analysis showed that, while there was no difference in the expression of HDAC4 mRNA between patients and controls (figure 8C), HDAC4 protein was higher in patients with COPD than in controls (figure 8D).

DISCUSSION
This study shows that the profile of miRNAs in the quadriceps of patients with COPD differs from that in controls. The data also suggest that MRTF/SRF activity is downregulated in patients. Together with previous animal data showing a role for SRF and the miRNAs in the control of muscle phenotype, these data support the hypothesis that altered miRNA expression and reduced SRF activity contribute to COPD-associated muscle dysfunction.

Significance of the findings
The most striking finding is the reduction in the expression of miR-1 in patients compared with controls. miR-1 expression is increased by SRF, and we show that it is increased in an SRF-dependent manner by the MRTFs. The reduced expression of MRTFs, as well as altered SRF localization, suggest that reduced activity of the MRTF/SRF axis contributes to the reduction in MHCI and miR-1 expression. Our data also indicate functional consequences of reduced miR-1 with increased expression of IGF-1, an inverse correlation of miR-1 with Akt phosphorylation and increased HDAC4 protein in the patients. Whether the observed increase in IGF-1 mRNA is a direct response to reduced miR-1 is not clear, as miRNAs are suggested to inhibit translation in muscle rather than increase RNA degradation. Consistent with this suggestion, we found that patients had normal HDAC4 mRNA levels but increased HDAC4 protein. The interaction of miR-1 with IGF-1 signalling and HDAC4 have been documented previously. These observations raise two points regarding the role of the SRF/miR-1 axis in muscle, which are discussed separately.

miR-1 in muscle biology
miR-1 has an accepted role in skeletal muscle differentiation. One likely effect of the reduced miR-1 observed is the increase in HDAC4 protein. HDAC4 inhibits the activity of MEF-2 and SRF, both of which are important regulators of MHCI expression, providing a mechanism by which the reduction in miR-1 may

Figure 4 Expression of microRNAs in patients with chronic obstructive pulmonary disease (COPD) and controls. Expression of miRNAs was determined by qPCR and normalised to the expression of the 5S RNA in the same sample as described in the Methods section. The expression of miR-1 (A) was lower in patients than controls but there was no significant difference in the expression of miR-499 (B) or the other miRNAs (C) analysed. Data are presented as log normalised expression with the box showing median and IQR, error bars to maximum and minimum points. Patients are shown as closed circles and controls are shown as open circles. Statistical significance was calculated by t test.
**Figure 5** Pearson correlation matrices for miRNAs with physiological characteristics and muscle fibre parameters in the cohort. MicroRNA expression was correlated with non-muscle physiological characteristics for all the samples (A) or with the muscle-specific physiological and histochemical parameters (B). The direction and intensity of the correlation is colour-coded according to the bar. The miRNAs are organised in the order of their contribution to PC2 as determined by principal components analysis. Correlations reaching a statistical significance where \( p < 0.05 \) are indicated by * and the \( p \) values are given in table S1 in the online supplement. Characteristics were ordered by hierarchical clustering (figure S4 in online supplement). BMI, body mass index; CSA, cross-sectional area; FFMI, fat-free mass index; FEV1, forced expiratory volume in 1 s; Lo, locomotion; MVC, maximal voluntary contraction; MI, movement intensity; Mt, movement time; 6MW, 6 min walk test; SGRQ, St George Respiratory Questionnaire; TwQ, twitch force in the quadriceps; Ty, type.

**Figure 6** Effect of myocardin-related transcription factors (MRTFs) on miR-1 promoter activity and the expression of MRTF and serum response factor (SRF) in the quadriceps muscle of patients with chronic obstructive pulmonary disease (COPD). Expression of MRTF-A (A), MRTF-B (B) and SRF (C) mRNA in the quadriceps of patients and healthy age-matched controls was quantified by real-time PCR and normalised to the expression of RPLPO. Data are presented as log normalised expression with the box showing median and IQR, error bars to maximum and minimum points. The expression of MRTF-A and MRTF-B was suppressed in the patients compared with controls but SRF expression did not differ significantly between groups (AU, arbitrary units). (D) C2C12 cells were transfected with miR-1-1, miR-1-2 promoter reporter vectors as described in the online supplement in the presence or absence of expression vectors for MRTF-A and MRTF-B. Luciferase activity was determined 24 h later. MRTF-A and MRTF-B increased the activity of the miR-1-1 and miR-1-2 promoters (\( p < 0.001 \)) but did not increase the activity of the delta enhancer promoter. Data are presented as mean ± SEM. Patients are shown as closed circles and controls are shown as open circles. Statistical significance for the mRNA expression was calculated by the Mann–Whitney U test.
miR-1 also inhibits the expression of IGF-1 and, consistent with previous reports, we found an inverse correlation between miR-1 and IGF-1 signalling. A similar increase in IGF-1 mRNA has previously been observed by mRNA profiling in COPD. The increase in IGF-1 mRNA in patients likely to show muscle wasting seems paradoxical. However, other studies have also shown increased activity of the hypertrophy signalling pathway in patients with COPD, which they hypothesised to be part of a compensation pathway but could also result from synthetic resistance.

miRNA in muscle disease

In addition to the reduction in miR-1 expression, the pattern of miRNA expression was also different between patients and controls, suggesting a more general change in miRNA expression. The expression of miR-133 and miR-206 were inversely associated with daily physical activity. This change is consistent with the observations of Nielsen et al. and Keller et al. who showed that endurance training suppresses the expression of these and other myo-miRs. The fact that we observed no association of miR-1 and miR-499 with physical activity suggests that factors other than daily physical activity are more important in determining their levels in patients with COPD.

Limitations of the study

The conclusions drawn from this study are limited by the cross-sectional design that only enables us to detect associations...
Figure 8  Altered insulin-like growth factor 1 (IGF-1) and histone deacetylase 4 (HDAC4) in patients with chronic obstructive pulmonary disease (COPD). IGF-1 (A) and HDAC4 (C) mRNA were quantified by real-time PCR as described in the online supplement. IGF-1 mRNA was increased whereas HDAC4 mRNA was unchanged in patients relative to controls. Data are presented as log normalised expression with the box showing median and IQR, error bars to maximum and minimum points. Akt levels and phospho-Akt levels were determined by luminex assay in muscle homogenates prepared as described in the online supplement. miRNA expression was quantified by qPCR and normalised to the expression of 5S RNA. Pearson correlation coefficients showed that miR-1 (B) was correlated with the ratio of phospho-Akt to total Akt. Patients are shown as open circles and controls are shown as closed circles. (D) HDAC4 protein levels were detected by western blotting (inset) quantified by densitometry and normalised to total protein loaded onto the blot determined by Ponceau Red staining. HDAC4 protein levels were increased in patients compared with controls. Data are presented as normalised protein levels with the box showing median and IQR, error bars to maximum and minimum points. Statistical significance for mRNA and protein levels was determined by the Mann—Whitney U test. Due to limitations on specimen size, we were unable to measure HDAC4 protein and miRNA in the same sample set.

between the gene expression and physiological characteristics. Furthermore, the data are correlative and, although they highlight potential mechanisms, they do not prove causality. It should be noted, however, that the highlighted associations have been demonstrated mechanistically through in vitro and in vivo studies.

We have also proposed that patients have reduced SRF activity but could not make direct measurements of SRF activity in our muscle samples. However, from the reduced MRTF and increased MHCI and miR-1 expression, together with altered SRF localization, it seems reasonable to conclude that SRF activity is reduced.

CONCLUSIONS
In this study we show that the expression of miRNAs is different in the quadriceps of patients with COPD than in control subjects. In particular, the expression of miR-1 is reduced and is associated with changes in fibre proportion, probably leading to the observed association with muscle mass and exercise performance. Furthermore, the expression of the MRTFs is also suppressed in the patients and again the expression of one of these genes is associated with disease severity. Together with data from studies identifying the MRTFs and SRF as critical regulators of skeletal muscle phenotype, these findings suggest that reduced activity of the SRF pathway contributes to COPD skeletal muscle dysfunction, in part by downregulating miR-1 expression.

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Competing interests None.

Patient consent Obtained.

Ethics approval Ethics approval was provided by Royal Brompton and Harefield NHS Trust research ethics committee.

Contributors AL, JR-C, SAN, PRK, AD, MIP: experimental design, data collection and analysis. AL, SAN, PRK, MIP: analysis and interpretation of data. All authors made an important intellectual contribution to the manuscript drafting and editing.

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REFERENCES


