**Effects of respiratory muscle training on endothelium and oxidative stress biomarkers in hemodialysis patients: A randomized clinical trial**

Nataly Gurgel Campos\(^a\), Débora Fortes Marizeiro\(^a\), Ana Carolina Lins Florêncio\(^a\), Ítalo Caldas Silva\(^a\), Gdayllon Cavalcante Meneses\(^b\), Gabriela Freire Bezerra\(^b\), Alice Maria Costa Martins\(^b\), Alexandre Braga Libório\(^a,c\), Nataly Gurgel Campos\(^a\), Débora Fortes Marizeiro\(^a\), Ana Carolina Lins Florêncio\(^a\), Ítalo Caldas Silva\(^a\), Gdayllon Cavalcante Meneses\(^b\), Gabriela Freire Bezerra\(^b\), Alice Maria Costa Martins\(^b\), Alexandre Braga Libório\(^a,c\).

\(^a\) Medical Sciences Post-graduate Program, Department of Clinical Medicine, Universidade Federal do Ceará, Brazil

\(^b\) Department of Clinical and Toxicological Analysis, Faculty of Pharmacy, Federal University of Ceará, Fortaleza, Ceará, Brazil

\(^c\) Medical Sciences Post-graduate Program, Centro de Ciências da Saúde, Universidade de Fortaleza – UNIFOR, Brazil

**ABSTRACT**

**Introduction:** Hemodialysis (HD) patients have altered pulmonary function and this is associated with impaired endothelial function and cardiovascular events. Respiratory muscle training (RMT) has the potential to improve cardiovascular outcomes in patients undergoing maintenance HD. Here, we evaluated the effects of RMT on endothelium/glycocalyx, oxidative stress biomarkers and pulmonary function test in HD patients.

**Methods:** This is a randomized controlled clinical trial including 41 patients undergoing thrice-weekly maintenance HD. Patients were randomly assigned at a 2:1 ratio to receive or not RMT during HD sessions for 8 weeks. Main outcomes were changes in levels of the biomarkers related to endothelium activation (vascular cell adhesion molecule 1, VCAM-1, and intercellular adhesion molecule 1, ICAM-1), glycocalyx derangement (syndecan-1), aberrant angiogenesis (angiopoietin-2) and oxidative stress (malondialdehyde) compared to baseline. Also, maximal inspiratory/expiratory pressure (MIP, MEP), Forced vital capacity (FVC) and forced expiratory volume in the first second (FEV1) were evaluated. Other outcomes included changes in functional capacity and pulmonary function test. We also performed a post-hoc analysis of plasma endothelin-1 levels.

**Results:** Of 56 randomly assigned patients, 41 were included in the primary final analyses. RMT increased all pulmonary function parameters evaluated and significantly reduced plasma syndecan-1 levels at 8 weeks compared to placebo (between-group difference: −84.5; 95% CI, −148.1 to −20.9). Also, there was a reduction in plasma levels of angiopoietin-2 (between-group difference: −0.48; 95% CI, −1.03 to −0.097). Moreover, there was a significant reduction in mean blood pressure at rest (between-group difference: −12.2; 95%CI, −17.8 to −6.6) associated with a reduction in endothelin-1 levels (between-group difference: −0.164; 95% CI, −0.293 to −0.034). There was no difference regarding biomarkers of endothelial activation or oxidative stress.

**Conclusion:** A short-term RMT program ameliorate FVC, FEV1 and reduces syndecan-1 and angiopoietin-2 biomarker levels. Finally, better blood pressure control was attained during training and it was associated with a reduction in endothelin-1 levels.

**1. Introduction**

Chronic kidney disease (CKD) patients, mainly those undergoing maintenance renal replacement therapy (peritoneal dialysis or hemodialysis - HD), have a complex syndrome with various effects on the cardiovascular, nervous, respiratory, musculoskeletal, immune, endocrine, and metabolic systems. In these patients, altered pulmonary function [1] may be the direct/indirect result of circulating uremic toxins, volume overload, muscle atrophy, malnutrition, anemia, inflammation, oxidative stress, extrasosseous calcification and others [2]. Several studies have described an association between reduced lung function, irrespective of previous smoking status, and cardiovascular events/mortality [3–5]. It has also been demonstrated that patients with reduced pulmonary function also have impaired endothelial function [6–8]. Because the endothelium and its glycocalyx have a central role in all phases of the atherosclerotic process [9], a causal sequence of events, although controversial [10], has been suggested: reduced lung function leads to endothelial alterations and, consequently, to cardiovascular disease [11].

Cardiovascular diseases are, collectively, the main cause of death in...
patients undergoing hemodialysis [12]. Besides hypertension, these patients have endothelial glycocalyx derangement [13], altered endothelial function [14], dysregulated angiogenesis [15] and endothelial cell activation [16]. Moreover, they have increased oxidative stress [17]. All these processes are associated, directly or indirectly, to the atherosclerotic process.

In this study, we hypothesized that respiratory muscle training (RMT) would lead to improvements in Ref. [1] endothelial activation [2], glycocalyx derangement [3]; aberrant angiogenesis and [4] oxidative stress biomarkers in patients undergoing maintenance HD. We also assessed effects of RMT on respiratory function, respiratory muscle strength, functional capacity, blood pressure and endothelin-1 levels, a potent vasoconstrictor.

2. Methods

2.1. Study design and participants

This was a randomized clinical trial comparing patients undergoing an 8-week RMT program using a variable pressure device (Threshold PEP®) with a control group. It was performed at an outpatient dialysis facility in the city of Fortaleza, Brazil. This center was chosen because it has infra-structural facilities for research and previous experience with clinical studies. All patients undergoing HD (n = 118) in that service were screened for eligibility (December 2016 to January 2017) by study coordinators. Data collection and study intervention were initiated in all patients by February 2017. Criteria for study participation included the following: patients receiving thrice-weekly maintenance HD for at least 3 months, age older than 18 and younger than 70 years, adequate cognitive and physical capacity to perform the study protocol procedures and to provide informed consent. Exclusion criteria included any chronic respiratory disease (patients with established diagnosis of chronic obstructive pulmonary disease – COPD, interstitial lung disease, asthma in treatment, chronic pleural effusion, pleuroscopy, bronchiectasis and others) and patients with acute coronary syndrome, decompensated heart failure, major infectious processes, or hospitalization within the last 3 months. The study is registered at www.clinicaltrials.gov under number NCT 03041155.

A total of 84 patients were assessed for eligibility, and 56 gave their consent and were randomly allocated using a computer-generated random numbers, at a 2:1 ratio, to the RMT or the control group. Prior to starting the study interventions, 8 participants voluntarily chose not to participate in the study, 3 participants were hospitalized because cardiac disease and another had an infectious complication – Fig. 1. During the study period, one patient in the RMT group voluntarily gave up participating in the study; additionally, one patient assigned to the control group was admitted to renal transplantation and another was removed from the study because he missed more than one dialysis session during study period. Thus, 41 patients completed the study (RMT group, 29; control group, 12) and were included in the analysis.

2.2. Study procedures

Participants were screened for eligibility criteria and clinical data were collected from the dialysis facility records. Before the midweek dialysis session, blood was collected for biomarker analysis, centrifuged, and the plasma aliquoted for storage at −80 °C until sent for analysis. Participants underwent pulmonary function tests: forced expiratory volume in 1 s (FEV1); forced vital capacity (FVC); maximal inspiratory pressure (MIP); and maximal expiratory pressure (MEP). All pulmonary function tests were obtained before the midweek dialysis session and performed according to American Thoracic Society recommendations [18]. In addition, patients performed the 6-min walk test (6MWT) in the corridor of the dialysis facility, which is 30 m long. After resting on a chair for at least 10 min, we encouraged the patients to walk back and forth along the corridor for 6 min. We measured the total distance walked in 6 min. Blood pressure, pulse oxygen saturation (SpO2, using a Nellcor PM10N Handheld Pulse Oximeter), heart and respiratory rates were measured before and immediately after the 6MWT. Dyspnea was assessed according to the Borg modified scale from 0 (no dyspnea) to 10 (maximal dyspnea) [19]. Oxygen desaturation was considered in the presence of a decrease greater than 4% from the baseline saturation [20]. All procedures described were performed before and after the study intervention (described below).

2.3. Study intervention

RMT was undertaken thrice weekly during 8 weeks using the threshold load training method supervised by a qualified trainer. Training was provided in the dialysis facility during hemodialysis sessions. Participants were required to breathe in (inspiratory training) or breathe out (expiratory training) against the resistance using a commercially available device (Threshold PEP; Respicor, Parsippany, NJ). Training consisted firstly of 12 sessions lasting 30 min each and resistance of 15 cmH2O; the following 12 sessions lasted 40 min each and resistance was set at 20 cmH2O. In the first half of each session, the resistance was against breathing in and in the last half, against breathing out.

2.4. Outcomes

The primary outcome was change in levels of the plasma markers of glycocalyx derangement (syndecan-1). Secondary outcomes included plasma markers of endothelium activation (VCAM and ICAM), aberrant angiogenesis (angiopoietin-2) and oxidative stress (malondialdehyde) compared to baseline. Other variables included changes in blood pressure and heart rate before and after the 6MWT; changes in functional capacity and changes in pulmonary function test and pulmonary muscle strength. We also decided to perform a post-hoc analysis of plasma endothelin-1 to investigate its role in blood pressure reduction.

2.5. Biomarker measurements

Syndecan-1 (Abcam, Cambridge, MA, USA), ICAM-1 and VCAM-1 (Life Technologies Brasil, São Paulo, Brazil) and angiopoietin-2 (Abcam, Cambridge, MA, USA) were measured using commercially available enzyme-linked immunosorbent assay kits. Malondialdehyde was measured using a TBARS Assay Kit (Cayman Chemical, Ann Arbor, MI). To measure ET-1, an ELISA kit from Biomedica Medizinprodukte GmbH & Co KG Divischgasse 4 1210 Vienna, Austria (cat. No. BI-20052) was used.

2.6. Statistical analysis

The study was designed to include 48 (2:1) patients to provide a 90% power to detect a difference of 40% in baseline-adjusted values between the intervention and control groups in the primary outcome. The power calculation assumed a common SD of 0.40 and two-sided α = 0.05. All analyses were performed on an intention-to-treat basis.

All variables were tested for normal distribution. Results were expressed as mean ± standard deviation for normally-distributed continuous variables, median and range for skewed variables, and frequency and percentage for categorical variables. Comparisons of baseline characteristics of the intervention versus control groups were conducted using t-test or χ² tests, depending on data type. Changes from baseline to week 8 for each study outcome were assessed using linear regression - ANCOVA [21]. All models included baseline values, age, gender, race, dialysis vintage, body mass index, presence of diabetes mellitus, cause of end-stage renal disease, current or previous (last 6 months) use of corticosteroids, hemoglobin, serum parathormone, serum calcium and serum phosphorus as covariates. Mean between-group difference was used for comparison of within-group changes
between intervention and control groups (intervention – control) after adjusting for baseline covariates using ANCOVA. We also performed paired t-test to compare variables before and after the intervention. All analyses were performed using SPSS 19.0 for Windows (Chicago, IL, USA). The nominal significance level was defined as \( P < 0.05 \).

3. Results

3.1. Participants’ characteristics

Overall, patients had a mean age of 50.0 ± 13.4 years; 24 (58.5%) were males and 10 (24.4%) had diabetes. All except one of the participants were dialyzed through an arteriovenous fistula. Two patients (one from each group) had a FEV1/FVC ratio lower than 65–70%. Because they had predicted FEV1 greater than 80%, no clinical diagnosis of COPD, no smoking past habit and as the post-bronchodilator pulmonary function test could not be performed, we preferred not to exclude these patients. There was no significant difference in patients assigned to control or RMT groups in relation to cardiovascular or respiratory parameters at baseline even before and after the 6MWT, except for heart rate – see Tables 1 and 2. Of note, no patient had oxygen desaturation after the 6MWT. Also, no significant difference was observed regarding antihypertensive agent classes or use of erythropoietin-stimulating agents.
3.3. Changes in pulmonary function test and pulmonary muscle strength

To check the effectiveness of our RMT program, we performed pulmonary function test and assessed pulmonary muscle strength before and after the protocol was applied. After the 8 weeks of study protocol, the RMT group had an increment in all spirometry and respiratory muscle strength parameters (maximal inspiratory and expiratory pressures) in comparison with the control group—see Table 2.

3.4. Changes in functional capacity

Compared to patients in the control group, intervention patients had a significant reduction in heart rate and mean blood pressure both at rest and after the 6MWT—see Table 2 for detailed p values. No significant difference was observed in SpO₂. Although permitted by the protocol study, there was no change in the prescribed BP-lowering agents from beginning to end of the study protocol. Also, in the RMT group, there was both a significant reduction in Borg scale and an increment in the 6-min walk distance (between-group difference at study end adjusted for baseline: 123.7 m; 95% CI, 82.1 to 165.2; p < 0.001) when compared with the control group (all parameters and between-group difference at study end adjusted for baseline are shown in Table 2).

3.5. Changes in oxidative stress biomarkers

No significant between-group difference was detected in plasma malondialdehyde - between-group difference at study end adjusted for baseline: 0.69 nmol/mL; 95% CI, −0.49 to 1.87; p = 0.243.

3.6. Change in endothelium biomarkers

No significant between-group difference was detected in relation to endothelium-activation related biomarkers. ICAM-1 had a between-group difference at study end adjusted for baseline: −26.2 ng/mL; 95% CI, −132.5 to 80.0; p = 0.620 and VCAM-1 had a mean reduction of 112.9 ng/mL; 95% CI, −221.9 to 447.8; p = 0.498. However, those assigned to RMT had significantly lower plasma syndecan-1 levels during study follow-up (between-group difference at study end adjusted for baseline: −84.5 ng/mL; 95% CI, −148.1 to −20.9; p = 0.011) — Fig. 2. Also, there was a significant reduction in the levels of angiotensin-2 (between-group difference at study end adjusted for baseline: −480 pg/mL; 95% CI, −1003.7 to −97.0; p = 0.034).

3.7. Changes in plasma endothelin-1 levels

To explore the mechanisms mediating blood pressure reduction during the study protocol, we performed a post-hoc analysis of plasma endothelin-1 levels. We observed a significant reduction in the RMT group when compared with the control group - between-group difference at study end adjusted for baseline: −8.4 pg/mL; 95% CI, −14.3 to −2.4; p = 0.015, see Fig. 2. Also, there was a correlation between mean blood pressure reduction and reduction in endothelin-1 levels (r = 0.487, p = 0.001).

4. Discussion

In our study, we first evaluated, in a controlled trial, the short-term effects of an 8-week respiratory muscle-training program in patients undergoing maintenance hemodialysis. Patients submitted to breathing against pressure threshold loading had significant improvement in pulmonary function, respiratory muscle strength, functional capacity, and blood pressure. Moreover, RMT significantly decreased plasma concentrations of syndecan-1 - a biomarker of endothelial glycoalyx derangement - and endothelin-1 - a potent vasoconstrictor. In the primary intention-to-treat analysis, there was no significant effect of RMT on levels of plasma endothelial activation biomarkers (VCAM-1 and ICAM-1) or stress oxidative biomarker (MDA).

Patients undergoing maintenance hemodialysis have decreased pulmonary function [22,23]. In addition to interdialytic fluid accumulation and potential increase in pulmonary capillary permeability, respiratory muscle impairment can explain this reduced pulmonary function [24]. The effects of muscle respiratory training on the rehabilitation of patients with COPD or other lung diseases are largely known [25,26]; however, there is insufficient data about the effects on...
In our study, the training group had significant improvement in almost all parameters related to pulmonary function/nergy, functional capacity and blood pressure. We found 3 other controlled trials evaluating RMT in dialysis patients [27-29]. All of them disclosed better results in respiratory muscle strength, pulmonary function and functionality. Only one of these studies evaluated biomarkers [29] and there was a reduction in C-reactive protein levels, a marker of inflammation, in the respiratory training group.

We have chosen several endothelium-related biomarkers as endpoints. To the best of our knowledge, there is only one other study evaluating the effects of RMT on endothelium and it showed a negative result: in patients with heart failure, inspiratory muscle training led to improvement in dyspnea and exercise tolerance, but had no effect on heart rate variability or endothelial function [30]. In the aforementioned study, endothelial function was evaluated only by endothelium-dependent vasodilation (a function closely related to endothelial cell function). This is in agreement with our results, as we did not find any populations without primary lung disease.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Intervention Group</th>
<th>Paired t-test</th>
<th>Control Group</th>
<th>Paired t-test</th>
<th>Mean Between-group difference</th>
<th>p§</th>
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<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
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<td>Mean ± SD</td>
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<tr>
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<td>Follow up</td>
<td>Baseline</td>
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<td><strong>Respiratory Parameters</strong></td>
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<td>MBP(cm/H2O)</td>
<td>79.7 ± 22.3</td>
<td>105.5 ± 21.9</td>
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<td>80.4 ± 29.9</td>
<td>66.2 ± 28.4</td>
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<td>FVC (l/min)</td>
<td>74.1 ± 23.5</td>
<td>97.2 ± 24.6</td>
<td>&lt; 0.001</td>
<td>65.4 ± 23.5</td>
<td>59.1 ± 24.1</td>
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<td>FVC (%) predicted</td>
<td>85.8 ± 28.2</td>
<td>92.3 ± 30.3</td>
<td>0.03</td>
<td>81.8 ± 26.4</td>
<td>77.3 ± 26.3</td>
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<td>FEV1 (%) predicted</td>
<td>75.8 ± 22.2</td>
<td>89.1 ± 25.1</td>
<td>0.02</td>
<td>78.5 ± 25.4</td>
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<td><strong>Functional Capacity (6MW)</strong></td>
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<td>Distance 6MWT (m)</td>
<td>380.5 ± 108.0</td>
<td>459.0 ± 103.0</td>
<td>&lt; 0.001</td>
<td>388.5 ± 99.7</td>
<td>338.5 ± 87.1</td>
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<td>MBPbe (mm/hg)</td>
<td>108.3 ± 19.0</td>
<td>102.3 ± 14.5</td>
<td>0.03</td>
<td>103.3 ± 18.3</td>
<td>107.3 ± 12.8</td>
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<tr>
<td>MBPaf (mm/hg)</td>
<td>116.2 ± 27.4</td>
<td>105.6 ± 18.0</td>
<td>0.01</td>
<td>108.3 ± 14.9</td>
<td>113.6 ± 9.0</td>
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<td>HRbe (rpm)</td>
<td>84.7 ± 70.0</td>
<td>76.5 ± 14.3</td>
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<td>73.4 ± 13.4</td>
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<td>HRaf (rpm)</td>
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<td>86.1 ± 17.0</td>
<td>&lt; 0.001</td>
<td>81.3 ± 12.7</td>
<td>86.9 ± 12.8</td>
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<td>18.1 ± 2.3</td>
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<td>15.6 ± 1.7</td>
<td>16.2 ± 1.2</td>
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<td>RRaf (ipm)</td>
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<td>20.3 ± 2.6</td>
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<td>18.5 ± 2.7</td>
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<td>SpO2be (%)</td>
<td>97.5 ± 0.6</td>
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<td>SpO2af (%)</td>
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<td>0.56</td>
<td>95.9 ± 0.7</td>
<td>96.2 ± 1.2</td>
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</table>

MIP: maximal inspiratory pressure; MBP: Maximal expiratory pressure; FVC: Forced vital capacity; FEV1: Forced expiratory volume in the first second; 6MW: 6 min walk test; MBPbe: mean blood pressure before 6MW; MBPaf: mean blood pressure after the 6MW; HRbe: heart rate at rest before the 6MW; HRaf: Heart rate after the 6MW; BORGbe: modified BORG scale score before the 6MW; BORGaf: modified BORG score after the 6MW; RRbe: respiratory rate at rest before the 6MW; RRaf: respiratory rate after the 6MW. Mean between-group difference is the comparison of within-group changes between intervention and control groups (intervention – control) after adjusting for baseline covariates using ANCOVA. *p < 0.05 for t-test comparison of baseline parameters between intervention and control groups. §p for mean between-group difference.

Fig. 2. Mean change in plasma syndecan 1, intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion protein 1 (VCAM-1), angiopoietin-2, endothelin-1 concentrations from baseline to week 8 of follow-up, by treatment group. Values are in means of percentage from baseline adjusted for baseline measurements, age, sex, body mass index, dialysis vintage, cause of end-stage renal disease and comorbidities. *P < 0.05 for comparison of mean change from baseline between groups, exact p values in the text.
significant differences in endothelial activation biomarkers (VCAM and ICAM) as discussed below. However, we demonstrated a clear reduction in several other biomarkers: the first one is syndecan-1, a biomarker of endothelial glycocalyx derangement. Damage to the endothelial glycocalyx, which helps maintain vascular homeostasis, heightens vascular lature sensitivity to atherogenic stimuli. It has been previously demonstrated that hemodialysis patients have damaged endothelial glycocalyx and, thus, limiting such damage can reduce cardiovascular events in this population [13]. The mechanisms through which RMT improves endothelial glycocalyx are unknown. However, circulating noradrenaline and adrenaline correlates with endothelial glycocalyx injury [31] and one possible explanation is that RMT induces a reduced sympathoadrenal activation, supported by a significant reduction of at rest heart rate in the intervention group, thus limiting this damage. However, this explanation remains to be clarified.

We also evaluated angiopoietin-2, a natural antagonist of angiopoietin-1, which leads to aberrant neovascularization and endothelial abnormalities [32]. Moreover, angiopoietin-2 has been recently identified as a prognostic biomarker of cardiovascular events and mortality in chronic kidney disease patients [33] and those with subclinical cardiovascular disease [34]. Together with the syndecan-1 findings, the reduced levels of angiopoietin-2 in patients submitted to training permit us to speculate that RMT has a great potential to have a positive impact on cardiovascular disease, a highly prevalent condition in hemodialysis patients.

Another important finding in the present study is the considerable reduction in blood pressure after RMT. To better investigate this point, we analyzed serum levels of endothelin-1, a potent vasoconstrictor that is increased in hemodialysis patients [35]. Here, we demonstrated a near 50% reduction of endothelin-1 in the training group. Interestingly, we disclosed a significant association between reductions in endothelin-1 levels and mean blood pressure. Supporting our findings, another group disclosed that patients undergoing maintenance hemodialysis with high levels of endothelin-1 have poor pulmonary function in comparison with those with lower levels [36]. These findings support our suggestion that RMT will help achieve better intra- and interdialytic blood pressure control through endothelin-1 reduction.

Although there is a scarcity of data about RMT in other patients without primary lung disease, the beneficial effects of resistive/aerobic training on endothelium function in normal individuals [37–39] are well established and, in parallel, there is a growing body of evidence about exercise (not specifically RMT) in hemodialysis patients [40–45]. Recent studies on aerobic exercise in CKD (in patients undergoing regular dialysis or not) have shown controversial findings about its effects on endothelium [41,42,45]. Taken together, it seems exercise only results in better endothelium parameters after a long-term performance [42]. We did not detect any significant differences in endothelial activation biomarkers (VCAM and ICAM). It is possible that by maintaining RMT for long-term periods can lead to reduction in such biomarkers. However, we cannot rule out the possibility that RMT have beneficial effects only on endothelial glycocalyx (here, evaluated by syndecan-1 levels) and on the production/release of angiopoietin-2 by endothelial cells.

Although no protocol has been evaluated about the effects of exercise on syndecan-1 and angiopoietin-2 levels, one study has demonstrated a reduction in their serum levels after an exercise training program in non-dialytic CKD patients [41]. Although some parallels can be identified between aerobic/resistive exercise and RMT, it is possible that RMT benefits are unique and the findings about syndecan-1 and angiopoietin-2 cannot be extrapolated to other exercise protocols.

Our study has several limitations. First, because of inherent difficulties related to RMT, our study was not blinded. This can, at least in part, explain the deterioration of many respiratory and functional capacity-related parameters in the control group. The maximal values obtained for some of these parameters are partly dependent on patient motivation, so they are subject to bias influence. However, the main findings of our research are the improvement in biomarkers, which are less subject to such bias. Second, we excluded patients with a previous clinical diagnosis of chronic respiratory disease, but we did not perform a complete pulmonary assessment regarding gas exchange and other respiratory parameters and patients with pulmonary disease could have been included in the study. However, it is unlikely that patients with undiagnosed pulmonary disease had been unequally allocated between the 2 groups and biased our results. Thirdly, as stated above, longer follow-up studies are necessary to evaluate if these effects are sustained over time and to evaluate the possible effects of long-term training on other parameters, including hard clinical endpoints. Also, we did not evaluate other parameters related to endothelial function, such as pulse wave velocity and flow-mediated dilatation.

In conclusion, a short-term RMT program improves respiratory and functional capacity. Also, there was a significant reduction in biomarkers of endothelial glycocalyx derangement (syndecan-1) and angiopoietin-2 (a mediator of angiogenesis and a destabilizer of endothelial cells). Finally, better blood pressure control was attained during training and it was associated with a reduction in endothelin-1 levels.

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Conflict of Interest: The authors declare no conflicts of interest.

Trial registration

www.ClinicalTrials.gov; study number: NCT 03041155.

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