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1 **Exercise training reverses extrapulmonary impairments in smoke-exposed mice**

2
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16 **Running title:** Smoke-induced extrapulmonary impairments

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23 **Abstract**

24 **Purpose:** Cigarette smoking is the main risk factor for chronic obstructive pulmonary disease and
25 emphysema. However, evidence on the extrapulmonary effects of smoke exposure that precede
26 lung impairments remains unclear at present, as are data on non-pharmacological treatments such
27 as exercise training.

28 **Methods:** Three groups of mice including control (n=10), smoking (n=10), and smoking with 6
29 weeks of high-intensity interval treadmill running (n=11) were exposed to 20 weeks of fresh air or
30 whole-body cigarette smoke. Exercise capacity (peak oxygen uptake) and lung destruction
31 (histology) were subsequently measured, while the heart, peripheral endothelium (aorta), and
32 respiratory (diaphragm) and limb (extensor digitorum longus and soleus) skeletal muscles were
33 assessed for *in vivo* and *in vitro* function, *in situ* mitochondrial respiration, and molecular
34 alterations.

35 **Results:** Smoking reduced body weight by 26% ($P<0.05$) without overt airway destruction
36 ($P>0.05$). Smoking impaired exercise capacity by 15% while inducing right ventricular dysfunction
37 by ~20%, endothelial dysfunction by ~20%, and diaphragm muscle weakness by ~15% (all
38 $P<0.05$), but these were either attenuated or reversed by exercise training ($P<0.05$). Compared to
39 controls, smoking mice had normal limb muscle and mitochondrial function (cardiac and skeletal
40 muscle fibers), however diaphragm measures of oxidative stress and protein degradation were
41 increased by 111% and 65% ($P<0.05$), but these were attenuated by exercise training ($P<0.05$).

42 **Conclusions:** Prolonged cigarette smoking reduced exercise capacity concomitant with functional
43 impairments to the heart, peripheral endothelium, and respiratory muscle that preceded the
44 development of overt emphysema. However, high-intensity exercise training was able to reverse
45 these smoke-induced extrapulmonary impairments.

- 46 **Key words:** Diaphragm; Skeletal muscle; Endothelium; High-intensity interval training;
- 47 Mitochondrial function; COPD

48 **Introduction**

49 Cigarette smoking is the main risk factor for chronic obstructive pulmonary disease (COPD). This
50 is characterized not only by airway destruction and airflow limitation but also extrapulmonary
51 impairments to the heart (12, 25), peripheral vasculature (6, 27), and skeletal muscles (2, 3, 18, 24),
52 which collectively exacerbate the key symptom of exercise intolerance. Interestingly, initial
53 evidence suggests that smokers at risk of COPD may also suffer extrapulmonary impairments
54 similar to those demonstrated in smokers with COPD (11, 31). However, data detailing the
55 extrapulmonary effects of prolonged smoke exposure in the period preceding COPD remain
56 unclear at present, as are data on potential favorable therapeutic interventions. For example, it still
57 remains unknown whether smoke-induced extrapulmonary impairments in the heart, endothelium,
58 and respiratory and limb skeletal muscle occur in parallel as a direct consequence to cigarette
59 smoke (i.e., primary effect) or as an indirect consequence following the subsequent development
60 of emphysema (i.e., secondary effect) (2, 3, 7, 13). In addition, data on whether prolonged smoke
61 exposure induces skeletal muscle dysfunction in the lower limbs remains controversial, with some
62 (24, 28) but not all (7, 34) studies reporting impairments. Further, whether smoking directly induces
63 respiratory (i.e., diaphragm) fiber contractile dysfunction remains unknown, despite the diaphragm
64 playing a direct role in the pathogenesis of dyspnea in smokers (11) and also not being affected by
65 disuse (as is common for the lower limb muscles (7)). As such, more evidence is needed to clarify
66 the role of cigarette smoke on respiratory and limb skeletal muscle contractile function.

67
68 Interestingly, physical inactivity has recently been demonstrated to exacerbate the extrapulmonary
69 impairments associated with prolonged cigarette smoking (7), while exercise training has been
70 shown to reverse smoke-induced cardiac dysfunction in mice (15). Yet whether exercise training
71 can also reverse numerous smoke-induced systemic impairments related not only to cardiac

72 function but also to the peripheral endothelium as well as respiratory and locomotor skeletal
73 muscles remains unknown. In this study, therefore, we exposed mice to cigarette smoke followed
74 by the intervention of high-intensity exercise training and used an integrative approach by
75 combining measures of the lung and exercise capacity in parallel with multiple-extrapulmonary
76 organs. We hypothesized that cigarette exposure would impair cardiac, endothelial, respiratory and
77 limb muscle function prior to the development of overt emphysema, and that exercise training
78 would be able to attenuate such impairments.

79

80

81

82 **Materials and Methods**

83 *Animals and smoke exposure*

84 Ten week old A/JOLAHsd female mice (Harlan Laboratories, UK) were used in this study, as
85 approved by the Norwegian Animal Research Authority and in accordance with ACSM's animal
86 care standards. This study lasted a total of 26 weeks: 0-20 weeks mice were exposed to either fresh-
87 air or cigarette smoke; 20-26 weeks mice performed exercise training or remained sedentary; week
88 26 mice were sacrificed. Briefly, mice were randomly separated into three groups to receive either:
89 (1) fresh air (control mice; n=10); (2) cigarette smoke (smoking mice; n=10); and (3) cigarette
90 smoke and high-intensity interval exercise training (smoking+HIIT mice; n=11). The exposure
91 period consisted of 6 hours per day for 5 days a week and this was achieved by placing mice in a
92 chamber connected to a smoking device (TE-10, Teague Enterprises, California, US), which
93 provided fresh air or cigarette smoke (Research Cigarettes 3R4F, University of Kentucky,
94 Lexington, US). The total particulate matter (TPM) was 100 mg/m³ for the first 2 weeks and 200
95 mg/m³ for the remaining exposure time (a level of 250 TPM has been suggested to be equivalent
96 to humans smoking around 15 packs per day (21)), which was monitored weekly and measured
97 with gravimetric method as previously described (20). The exposure period was terminated after
98 20 weeks and the exercise training intervention was then performed.

99

100 *Exercise training and exercise capacity*

101 High-intensity interval training (HIIT) was performed on a treadmill (25° incline) 5 times per week
102 over 6 weeks. Each session consisted of 10 intervals (4 min at ~90 % peak work rate) separated by
103 2 min recovery (~60% peak work rate). Exercise capacity was assessed in all mice by measuring
104 peak oxygen uptake ($\dot{V}O_{2\text{peak}}$) in ml/min via a closed metabolic chamber, as previously described
105 (17).

106
107 *In vivo cardiac function and hemodynamics*

108 Echocardiography was performed as previously described (15). Briefly, in the week before
109 sacrifice mice were anesthetized with 2 % isoflurane adjusted to maintain similar heart- and
110 respiration rates between animals. The echocardiography system with a 24 MHz probe was used
111 to determine both right and left ventricular function (Vevo 2100 Visual Sonics, Toronto, Canada).
112 Right ventricular systolic function was assessed by measuring the tricuspid annular plane systolic
113 excursion (TAPSE), which was defined as the differences in systolic and diastolic displacement of
114 the right ventricular base, as obtained from the apical four chamber view in B-mode. This included
115 the M-mode cursor being oriented to the junction of the tricuspid valve plane in the right ventricular
116 free wall and through the apex. Right ventricular systolic velocity (RVS') was obtained from the
117 apical four chamber view using tissue Doppler from the lateral wall close to the tricuspid inflow.
118 Pulmonary acceleration time (PAP), defined as the onset of pulmonary flow to the peak flow, was
119 measured using pulsed-wave Doppler in the short-axis view at the level of the aortic valve. In
120 addition left ventricular function was assessed using B-mode images in the parasternal short axis
121 view to calculate left ventricular ejection fraction (LVEF), while left ventricular fractional
122 shortening (LVFS) was calculated in M-mode. *In vivo* hemodynamics of the right ventricular
123 systolic pressure was also determined as previously described (33). Briefly, immediately prior to
124 sacrifice the mice were anesthetized with 2 % isoflurane under spontaneous breathing and the
125 external jugular vein was dissected free and a 1.2 F pressure catheter inserted to determine right
126 ventricular systolic pressure (Scisense pressure catheter FTH-1211B-0018, Scisense Inc, Canada).
127 Data were acquired with Advantage PV System ADV500 (Transonic Inc, Canada). Body
128 temperature was measured with a rectal probe, which was kept constant at 37.5°C during the
129 experiment. The animal was observed until stable readings before recording. The animals were

130 killed immediately following the recordings by removal of the heart. Data were analyzed with
131 LabChart version 7 (ADInstruments, UK). Right ventricular systolic pressure was used an index
132 of pulmonary hypertension, which is usually developed during emphysema.

133

134 *Lung emphysema*

135 The right lung was inflated by instilling a 4 % formaldehyde phosphate buffered solution via the
136 trachea at a pressure of 25 cm H₂O. It was maintained to fixate like this for a minimum of 15 min
137 before ligating the right main bronchus and putting the whole specimen in formaldehyde solution.
138 Later, it was paraffin-embedded, sectioned, and stained with hematoxylin, eosin and saffron. Light
139 microscopy was then used to identify suitable areas free from artefacts and 4-8 images per animal
140 were selected on a random basis. A reference grid was placed on images in order to count the points
141 of intercepts between the grid lines and alveolar walls, followed by dividing this number by the
142 total length of all grid lines, in order to determine the mean linear intercept length (L_m), as
143 previously detailed (10).

144

145 *In vitro skeletal muscle function*

146 A diaphragm strip, the soleus, and the extensor digitorum longus (EDL) were isolated and
147 stimulated across a range of frequencies for measurement of *in vitro* isometric contractile function,
148 as previously described (5). Muscle force (N) was normalized to muscle cross-sectional area (cm²)
149 by dividing muscle mass (g) by the product of L_o (cm) and estimated muscle density (1.06) (8),
150 which allowed specific force in N/cm² to be calculated.

151

152 *In vitro peripheral vascular endothelial function*

153 To provide an index of vascular function, aortic rings (n=4) were dissected from mice and mounted
154 in a buffer-filled (in mmol/L: 118 NaCl, 25 NaHCO₃, 4.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 2.5 CaCl₂,
155 5.5 glucose) organ bath between a hook and a force transducer, as previously described (1). After
156 30 min of equilibrium, the maximal constriction was achieved by adding KCl (final concentration
157 100 mmol/L) to the buffer. After several rinses, aortic rings were pre-constricted by adding
158 increasing concentrations of phenylephrine (Sigma, Taufkirchen Germany; 10⁻⁹ to 10⁻³ mol/L) to
159 around 70 % of maximal KCl constriction. Relaxation to increasing concentrations of acetylcholine
160 (Sigma, Taufkirchen Germany; 10⁻⁹ to 10⁻² mol/L) and sodium nitroprusside (Sigma; 10⁻⁹ to 10⁻²
161 mol/L) were recorded until no further changes were observed in order to determine endothelial-
162 dependent and endothelial-independent vasodilation, respectively.

163

164 *In situ mitochondrial respiration*

165 Mitochondrial respiration was measured *in situ* from permeabilized cardiac and diaphragm fibers
166 using high resolution respirometry (Oxygraph-2k: Oroboros Instruments, Innsbruck, Austria), as
167 previously described (5). Dissected fibers were immediately placed in ice-cold BIOPS (2.77 mM
168 CaK₂EGTA buffer, 7.23 mM K₂EGTA buffer, 20 mM imidazole, 20 mM taurine, 50 mM 2-(N-
169 morpholino)ethanesulfonic acid hydrate, 0.5 mM dithiothreitol, 6.56 mM MgCl₂·6H₂O, 5.77 mM
170 Na₂ATP and 15 mM Na₂PCr (pH 7.1)) and under a microscope carefully dissected in 2 ml BIOPS
171 at 4°C and permeabilized in saponin (50 µg/ml) for 30 min. Fiber bundles were then transferred to
172 2 ml ice cold mitochondrial respiration medium (MiR05; 0.5 mM EGTA, 3mM MgCl₂·6H₂O,
173 60mM Lactobionic acid, 20mM taurine, 10mM KH₂PO₄, 20mM HEPES, 110mM D-Sucrose, 1
174 g/L BSA essentially fatty acid free, pH 7.1) and gently agitated for 10 minutes at 4°C before placed
175 in a 2 mL oxygraph chamber incubated in MIR05 at 37°C oxygenated at ~300-400 µM (Oxygraph-
176 2k, Oroboros, Innsbruck, Austria). All experiments were performed in duplicates. A protocol

177 evaluating both leak (L) and oxidative phosphorylation (P) respiration states in complex I (C_I) and
178 complex I+II (C_{I+II}) was assessed by addition of malate (2mM) and glutamate (10mM), followed
179 by saturating ADP (5mM), and then succinate (10mM), respectively. All measurements were
180 normalized to muscle wet weight and are presented as $\text{pmol O}_2\text{sec}^{-1}\text{mg}^{-1}$. In addition, the
181 respiratory control ratio (RCR) was calculated as the ratio of maximal C_I ($P C_I$) to leak state ($L C_I$)
182 respiration, and also a substrate control ratio (SRC) was calculated as $P C_I$ to $P C_{I+II}$.

183

184 *Tissue analyses*

185 Fiber typing and cross sectional-area analyses were performed on diaphragm sections, as
186 previously described (5). Protein expression of MuRF1, MAFbx, NADPH oxidase (subunit
187 gp91^{phox}), myosin heavy chain, and eNOS (phosphorylated and acetylated) was assessed by western
188 blot, while enzyme activity of NADPH oxidase, catalase, and superoxide dismutase was quantified
189 in a photometric manner, as previously described (5). All data are presented as fold change relative
190 to sham.

191

192 *Statistical analyses*

193 Data are presented as mean \pm SEM. Between-group differences in terms of physical characteristics,
194 cardiac function, $\dot{V}O_{2\text{peak}}$, mitochondrial function, protein expression, and enzyme activity were
195 assessed by one-way ANOVA (Bonferroni post hoc) or non-parametric Kruskal-Wallis (Dunn's
196 post hoc). Endothelial function (group X concentration) as well as force-frequency (group X
197 frequency) and fatigue protocols (group X time) from each skeletal muscle were assessed by two-
198 way repeated measures ANOVA with Bonferroni post hoc analysis. Statistical significance was
199 accepted as $P < 0.05$.

200

201 **Results**

202 Smoke exposure in mice reduced body weight by 26 % ($P < 0.05$; Fig. 1A) and impaired exercise
203 capacity by 15 % ($P < 0.05$; Fig. 1B), however the latter was normalized following exercise training
204 ($P > 0.05$; Fig. 1B). In addition, in line with our hypothesis cigarette smoke exposure did not cause
205 the development of overt emphysema, as demonstrated by no discernable alveolar destruction (i.e.,
206 mean linear intercept remained unchanged; $P > 0.05$; Fig. 1C) and by the absence of pulmonary
207 hypertension (i.e., right-ventricular systolic pressures were unchanged; $P > 0.05$; Fig. 1D).
208 Similarly, no differences were found between groups in terms of tibia length or skeletal muscle wet
209 weights ($P > 0.05$).

210

211 *Smoke-induced extrapulmonary impairments*

212 *Heart:* Heart weight remained unaltered following cigarette smoking ($P > 0.05$). In addition,
213 while echocardiography revealed that left ventricular function was not impaired by smoking
214 ($P > 0.05$; Table 1), right ventricular systolic function was reduced compared to controls by ~20 %
215 as assessed by TAPSE ($P < 0.05$; Table 1). To determine whether mitochondrial dysfunction may
216 underlie this impairment, we further assessed mitochondrial respiration in fibers from the right and
217 left ventricle, but found no differences ($P > 0.05$; Fig. 2A-B).

218 *Peripheral endothelium:* The effect of smoking on peripheral endothelial function was
219 assessed from isolated aortic rings for both endothelial-dependent and -independent vasodilation
220 (i.e., relaxation). Stimulation of aortic rings with increasing concentrations of acetylcholine
221 revealed that smoking was associated with impaired endothelial-dependent vasodilation ($P < 0.05$;
222 Fig. 3A), with smoke-exposed mice achieving only 70 ± 7 % of their maximal dilation compared to
223 control mice values of 91 ± 3 % ($P < 0.01$). In contrast, smoke exposure did not impair endothelium-
224 independent vasodilation ($P > 0.05$; Fig. 3B), with maximal vasodilation values of 100% achieved

225 in both groups ($P>0.05$). To determine potential mechanisms underlying these functional
226 impairments, we further assessed protein expression of phosphorylated eNOS and acetylated eNOS
227 in the aorta but found no differences between control and smoke-exposed mice ($P>0.05$).

228 *Respiratory and limb skeletal muscles:* Compared to controls, diaphragm fiber bundles
229 from smoking mice were weaker by an average of $15\pm 2\%$ (range: 11 - 19%; $P<0.05$; Fig. 4A),
230 which occurred at the lower and more clinically-relevant frequencies of 1, 15, 30, and 50 Hz.
231 However, no differences were discerned during the fatigue protocol between groups. In contrast to
232 the diaphragm, smoking had no adverse effect on limb muscle contractile function for either the
233 glycolytic EDL muscle (Fig. 4B) or oxidative soleus muscle (Fig. 4C), or on relative force during
234 the fatigue protocol ($P>0.05$; data not shown). As these data suggested the diaphragm was more
235 susceptible to cigarette smoke-induced impairments, we focused thereafter on this specific skeletal
236 muscle. Histological analyses of the diaphragm between control and smoke-exposed mice revealed
237 no structural changes in terms of fiber cross-sectional area (fast and slow twitch: 1077 ± 102 vs.
238 $1222\pm 82\ \mu\text{m}^2$ and 895 ± 123 vs. $877\pm 76\ \mu\text{m}^2$, respectively; $P>0.05$) or fiber proportions (fast and
239 slow twitch: 92 ± 1 vs. $92\pm 1\%$ and 8 ± 1 vs. $8\pm 1\%$, respectively; $P>0.05$). In contrast, while the
240 protein expression of NADPH oxidase, a marker of oxidative stress, was unaltered between groups
241 ($P>0.05$; 5A), the more functional measure of enzyme activity was increased by 111 % ($P<0.05$;
242 Fig. 5B). Similarly, the protein expression of MAFbx (a key marker of protein degradation) was
243 also increased by 65 % in smokers compared to controls ($P<0.05$; 5C). In contrast, however, the
244 protein expression of MuRF1 (another marker of protein degradation) was not different between
245 groups ($P>0.05$; 5D), as was the case for the contractile protein myosin heavy chain (1.00 ± 0.17 vs.
246 0.90 ± 0.15 ; $P>0.05$). We further assessed whether mitochondrial dysfunction could be contributing
247 to the diaphragm muscle weakness following smoke exposure, however *in situ* mitochondrial
248 respiration of permeabilized fibers was not different between groups ($P>0.05$; Fig. 2C).

249

250 *Effects of high-intensity exercise training in smoke-exposed mice*

251 Six weeks of exercise training normalized the smoke-induced reduction in exercise capacity (Fig.
252 1B), while also improving left ventricular systolic function (i.e., ejection fraction and fractional
253 shortening) when compared to sedentary smoke-exposed mice ($P<0.05$; Table 1). In addition,
254 exercise essentially reversed the smoke-induced impairment of TAPSE, suggesting right
255 ventricular function was normalized ($P<0.05$; Table 1), while also reversing peripheral endothelial
256 dysfunction such that maximal endothelium-dependent dilation was normalized to control values
257 ($P>0.05$; Fig. 3A). In line with this, we found the protein expression of phosphorylated eNOS to
258 be increased in the aorta of smoke-trained mice compared to both control and smoke-exposed
259 sedentary mice ($P<0.05$). Importantly, smoke-induced diaphragm contractile dysfunction was also
260 reversed by exercise training to similar values of that observed in control mice ($P>0.05$; Fig. 4A),
261 which was also the case for both the enzyme activity of NADPH oxidase ($P>0.05$; Fig. 5B) and the
262 protein expression of MAFbx ($P>0.05$; Fig. 5C). Nevertheless, no differences were subsequently
263 found between control, smoking, and smoke-trained mice in terms of the anti-oxidative enzyme
264 activity of superoxide dismutase (1.00 ± 0.09 vs. 1.22 ± 0.22 vs. 1.30 ± 0.23 , respectively; $P>0.05$) or
265 catalase (1.00 ± 0.13 vs. 1.01 ± 0.13 vs. 0.83 ± 0.12 , respectively; $P>0.05$). Furthermore, exercise
266 training had no influence on the expression of other proteins (e.g., myosin heavy chain, MuRF1,
267 NADPH oxidase), on fiber properties (i.e. cross-sectional area and type), or on mitochondrial
268 function (Fig. 2C; all $P>0.05$).

269

270 **Discussion**

271 The main findings of this study include: 1) cigarette smoking induced functional impairments to
272 the heart, peripheral endothelium, and respiratory muscle, which occurred in the absence of overt
273 emphysema and these likely converged to reduce exercise capacity; and 2) high-intensity exercise
274 training reversed these smoke-induced extrapulmonary impairments in line with normalizing
275 exercise capacity. Collectively, therefore, our findings may have important clinical implications as
276 they suggest that cigarette smokers in the absence of emphysema likely develop simultaneous
277 extrapulmonary impairments to the heart, peripheral endothelium, and diaphragm that exacerbate
278 exercise intolerance (11, 31), but such maladaptations could be reversed by the non-
279 pharmacological intervention of exercise training.

280

281 *Smoke exposure and skeletal muscle function*

282 Skeletal muscle dysfunction is a key component of COPD (19) and is associated with numerous
283 adaptations (e.g., fiber atrophy, mitochondrial dysfunction, and increased ROS) (2, 3, 18, 24), yet
284 numerous studies have yielded contradictory results with some (24, 28), but not all (7, 34), showing
285 contractile impediments in the lower limbs. In these previous studies, however, overt emphysema
286 had either developed (7, 24) or was not reported (18, 28), leaving it difficult to discriminate whether
287 the cigarette smoke or emphysema was the causal factor underlying skeletal muscle dysfunction.
288 Our study indicates that cigarette exposure in the absence of emphysema does not induce limb
289 muscle contractile dysfunction, as supported by findings in both highly oxidative (i.e. soleus) and
290 glycolytic (i.e., EDL) muscles. In addition, muscle disuse may have also confounded previous
291 studies where limb muscle dysfunction was reported, as recent data demonstrated physical
292 inactivity combined with smoke-induced emphysema significantly exacerbates limb muscle
293 impairments (7). In the current experiments, therefore, we also assessed the main muscle of

294 respiration, the diaphragm, which is not only constantly active (i.e., thus attenuating any potential
295 confounding factors associated with disuse) but also is thought to play a direct role in the
296 pathogenesis of dyspnea (a key symptom in smokers even without overt COPD (11)). In the present
297 study, we provide novel evidence that prolonged smoking induces diaphragm contractile
298 dysfunction that is independent of lung structural damage, with fiber bundles ~15-20 % weaker
299 compared to control mice. These data suggest, therefore, a greater sensitivity of respiratory rather
300 than locomotor striated muscle to cigarette smoke-induced dysfunction. Furthermore, this loss of
301 function was not due to muscle atrophy as force was normalized to muscle density, which suggests
302 fiber weakness induced by smoking was the result of intracellular impairments (i.e., contractile
303 dysfunction).

304

305 While diaphragm muscle weakness in emphysema has previously been observed in diaphragm
306 fibers from humans (23) and animals (32), we are the first to provide direct evidence that cigarette
307 smoke exposure can lead to diaphragm fiber weakness in the period preceding lung structural
308 damage. The mechanisms underlying the smoke-induced diaphragm muscle weakness may be
309 related to an increase in oxidative stress and protein degradation, as NADPH oxidase activity (but
310 not protein content) and MAFbx protein expression was increased in the diaphragm of smoking
311 mice, but these were attenuated in the smoke-exercise trained mice in parallel with normalized
312 contractile function. These findings compliment previous studies, which also reported increased
313 levels of oxidative stress and oxidative protein modifications in the diaphragm following prolonged
314 cigarette smoking in mice (2, 3). However, contrary to data from the soleus muscle (29),
315 mitochondrial dysfunction was not present in diaphragm fibers following prolonged smoke
316 exposure, which suggests a low oxidative phosphorylation capacity was unlikely mediating the
317 smoke-induced respiratory muscle weakness. Taken together, therefore, our data support a role for

318 cigarette smoking as a causal mechanism in diaphragm muscle weakness, potentially mediated by
319 elevated oxidative stress levels and activation of proteolytic pathways, which likely predisposes
320 many smokers to respiratory dysfunction in the period preceding emphysema (11).

321

322 *Smoke exposure and cardiovascular function*

323 Following prolonged smoke exposure cardiac dysfunction is known to develop, with right
324 ventricular function particularly compromised (25), while peripheral vascular endothelial
325 dysfunction is also manifest (27). Our study confirmed that right ventricular function is reduced
326 and peripheral endothelial function is impaired following cigarette exposure, which occurred in the
327 absence of overt emphysema. In addition, we also confirmed previous findings that right
328 ventricular dysfunction can be reversed by exercise training (15). In order to better understand the
329 mechanisms of these smoke-induced cardiac impairments, we assessed mitochondrial function in
330 cardiac fibers. However, and in contrast to a previous study reporting impaired respiration in fibers
331 from the left ventricle (30), we found no differences between groups in either the left or right
332 ventricle, which suggests mitochondrial dysfunction is unlikely a key mechanism of smoke-
333 induced right ventricular dysfunction, at least in relation to the present animal model. In terms of
334 the peripheral endothelium, we provide novel data that high-intensity exercise training can reverse
335 smoke-induced peripheral vascular impairments, as endothelium-dependent vasodilation in aortic
336 rings was normalized to control values following exercise training. We also found a significant
337 increase in the protein expression of phosphorylated eNOS in smoke-trained mice, which may help
338 explain how exercise mediated its benefits, as smoking is well known to reduce NO bioavailability
339 and eNOS activity (4). However, why endothelial dysfunction was only observed at the higher
340 rather than the lower acetylcholine concentrations in smoke-exposed mice is unclear, but it may be
341 related to differences in membrane receptor sensitivity or density. Nevertheless, that data collected

342 from both animals (27) and humans (16, 26) show a similar trend after smoke exposure supports
343 the physiological relevance of the present findings.

344
345 If we take an integrative approach, it could be speculated that diaphragm muscle weakness induced
346 by smoke-exposure is mediated, in part, by upstream impairments related to cardiac and peripheral
347 endothelial dysfunction that limit blood flow to induce local hypoxia, with the latter suggested as
348 a key mechanism underpinning skeletal muscle dysfunction in COPD (19). Hypoxia has been
349 shown in diaphragm fiber bundles to potentiate ROS production which, in turn, directly impair
350 subsequent force generation (22). As such, our overall findings of impaired central and peripheral
351 vascular function coupled with diaphragm muscle weakness in concert with elevated markers of
352 ROS suggest hypoxia could be a putative mechanism for the smoke-induced respiratory muscle
353 weakness.

354
355 *Animal model of cigarette smoke exposure*

356 The uncertainty related to the role of cigarette smoke exposure on extrapulmonary impairments
357 had previously been clouded by the fact that emphysema was usually developed secondary to
358 cigarette exposure (2, 3, 7, 13) or that lung indices of emphysema were not measured (18, 28). In
359 the current study we selected a period of 20 weeks for smoke exposure so animals would not
360 develop overt emphysema, which is generally documented after 24 weeks in mice (9, 13).
361 Furthermore, the A/J mouse strain we studied is moderately susceptible to lung destruction
362 following smoke exposure (21), but not as sensitive (e.g., C57BL/6J) or resistant as other strains
363 (e.g., NZWLac/J) (14, 35). Collectively, therefore, the present data support our objective and
364 reinforce the clinical translation of our data, as prolonged smoke exposure resulted in no signs of
365 emphysema, while numerous multi-organ impairments converged concomitant with an impaired

366 exercise capacity of ~20%, with the latter value similar to that reported in patients at high risk of
367 developing emphysema (11). Nevertheless, we acknowledge the possibility our mouse model may
368 have still induced lung injury and airflow limitation despite the absence of emphysema
369 histologically. As such, the addition of lung functional measurements including measuring airway
370 resistance would have provided further clarity on this issue. Furthermore, as the present study
371 assessed female mice only, it also remains unclear whether the observed multi-organ impairments
372 would have been reduced or even exacerbated in males exposed to the same amount of cigarette
373 smoke.

374

375 *Conclusion*

376 High-intensity exercise training reversed cigarette smoke-induced extrapulmonary impairments to
377 the heart, the peripheral vascular endothelium, and the diaphragm as well normalizing exercise
378 capacity, which occurred before the onset of overt emphysema. These findings suggest, therefore,
379 that cigarette smokers in the absence of emphysema likely develop extrapulmonary impairments
380 that exacerbate exercise intolerance, but exercise training can reverse these detrimental
381 consequences.

382 **Acknowledgments**

383 None

384

385

386 **Conflict of interest**

387 The authors have no conflicts of interest to disclose in relation to professional relationships with
388 companies or manufacturers who will benefit from the results of the present study. The authors
389 state that the results of the present study do not constitute endorsement by ACSM. The authors
390 declare that the results of the study are presented clearly, honestly, and without fabrication,
391 falsification, or inappropriate data manipulation

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488 **Figure Legends**

489 Fig. 1 Body weight (A), exercise capacity (B), mean linear intercept (Lm; C) and right
490 ventricular systolic pressure (RVSP; D) of control, smoking, and smoking mice that performed
491 high-intensity interval training (HIIT). That lung destruction (C) or pulmonary hypertension (D)
492 did not differ between groups supports the contention that overt emphysema was not developed in
493 smoke-exposed mice. *P<0.05 vs. Control.

494
495 Fig. 2 *In situ* mitochondrial respiration states and the respective control ratios in
496 permeabilized fibers of the left ventricle (A), right ventricle (B), and diaphragm (C), as assessed
497 from control, smoking, and smoking mice that performed high-intensity interval training (HIIT).
498 Respiration states were assessed, in turn, including: C_I leak, complex I leak respiration following
499 addition of malate and glutamate; C_I, complex I phosphorylated state following addition of ADP;
500 C_{II}, complex II phosphorylated state following addition of succinate. No significant differences
501 were found between groups for all measures.

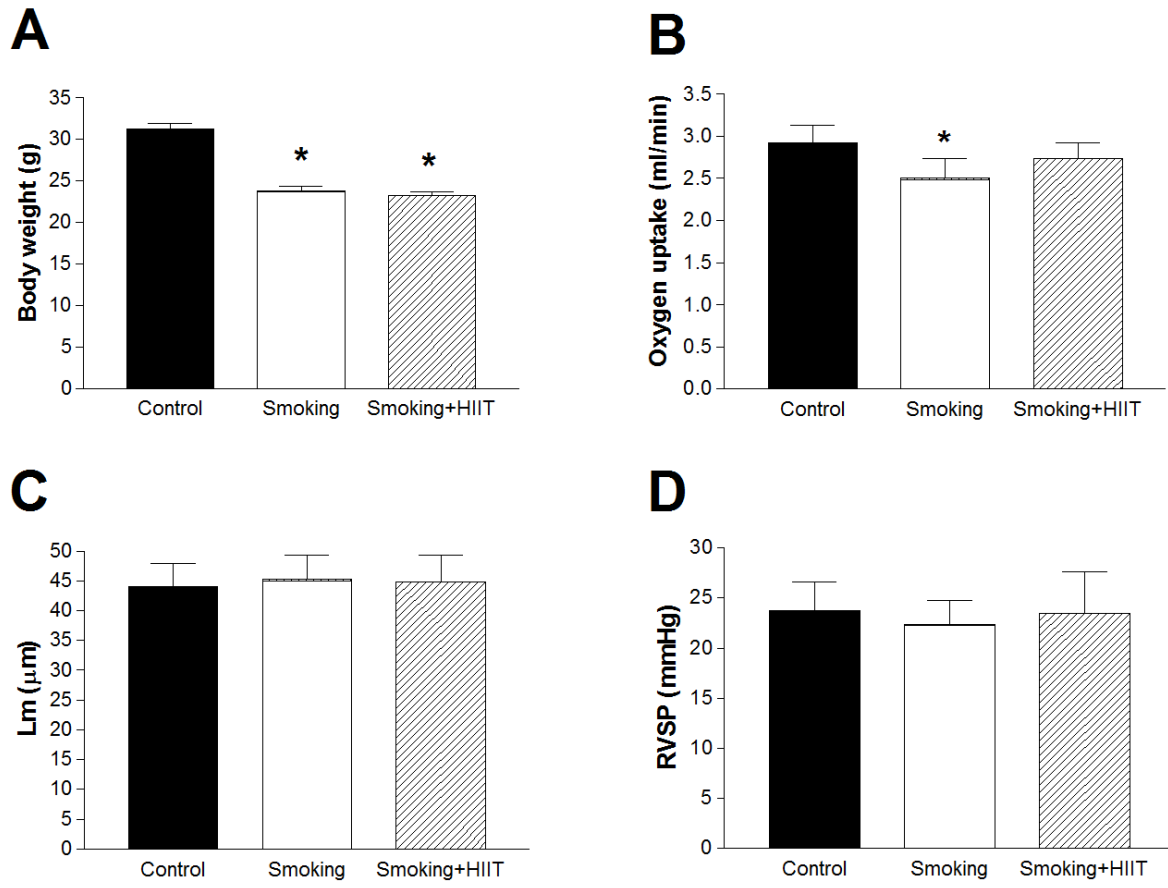
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503 Fig. 3 Peripheral *in vitro* endothelial-dependent (A) and -independent (B) function in
504 response to increasing acetylcholine and sodium nitroprusside (SNP) concentrations, as assessed
505 in isolated aortic rings from control, smoking, and smoking mice that performed high-intensity
506 interval training (HIIT). *P<0.05 vs. Control and Smoking+HIIT; #P<0.05 vs. Smoking+HIIT

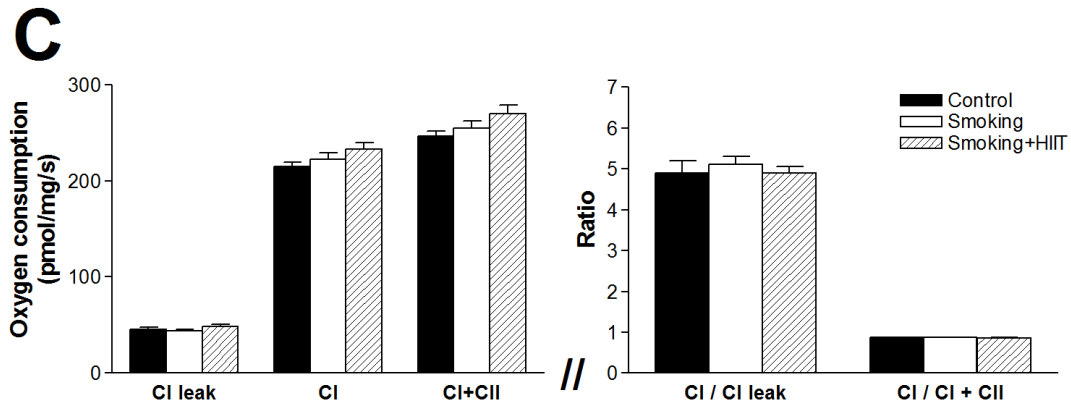
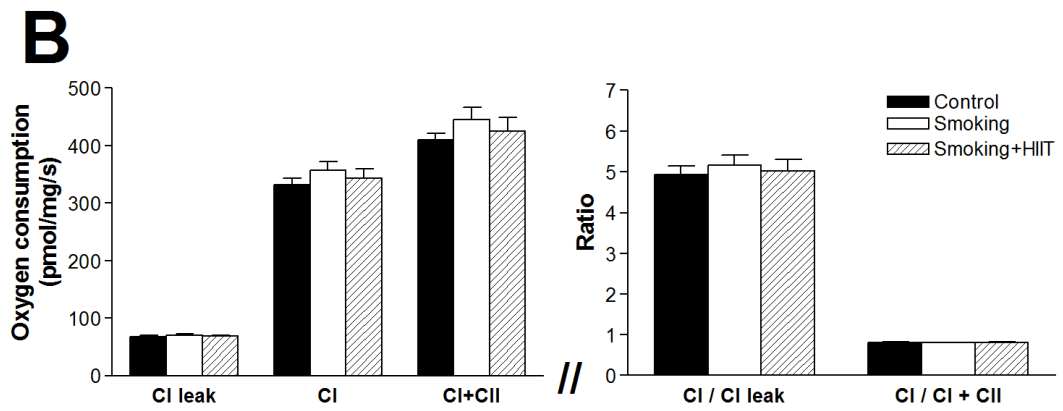
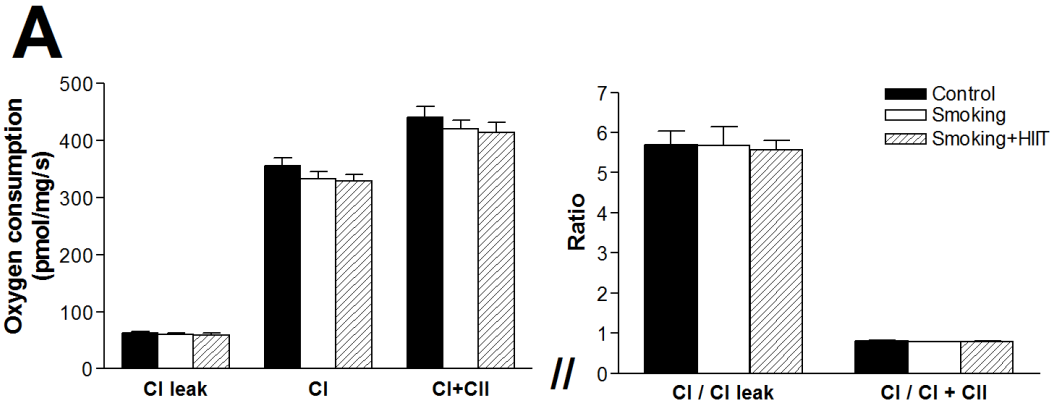
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508 Fig. 4 *In vitro* respiratory and lower limb skeletal muscle contractile function assessed
509 across a range of stimulation frequencies in the diaphragm (A), EDL (B), and soleus (C) for control,
510 smoking, and smoking mice that performed high-intensity interval training (HIIT). *P<0.05 vs.
511 Smoking.

512

513 Fig. 5 Protein expression (A) and enzyme activity (B) of NADPH oxidase as well as
514 protein expression of MAFbx (C) and MuRF1 (D) in the diaphragm from control, smoking, and
515 smoking mice that performed high-intensity interval training (HIIT). Representative blots for each
516 protein are also provided in the upper panel, which were normalized to the loading control GAPDH
517 for control (c), smoking (s), and smoking mice that performed HIIT (H). *P<0.05 vs. Control and
518 Smoking+HIIT; #P<0.05 vs. Control.

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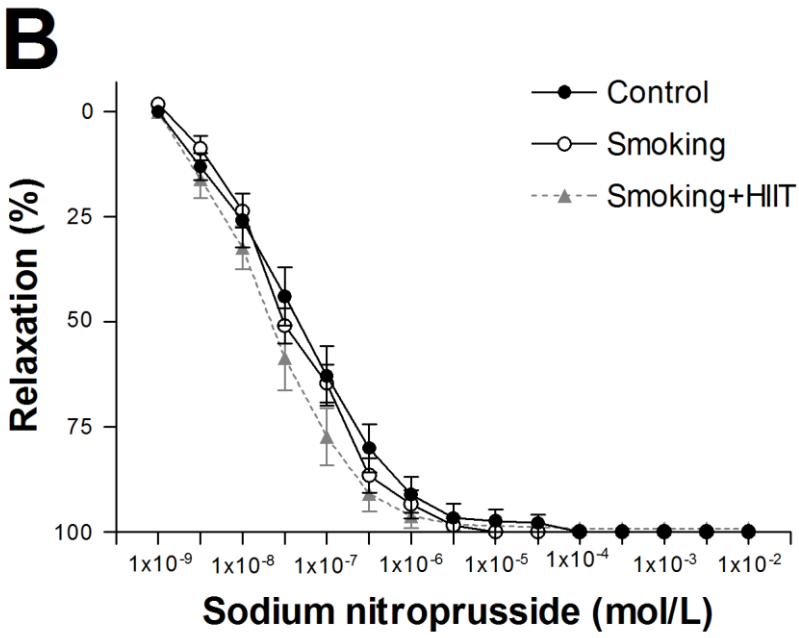
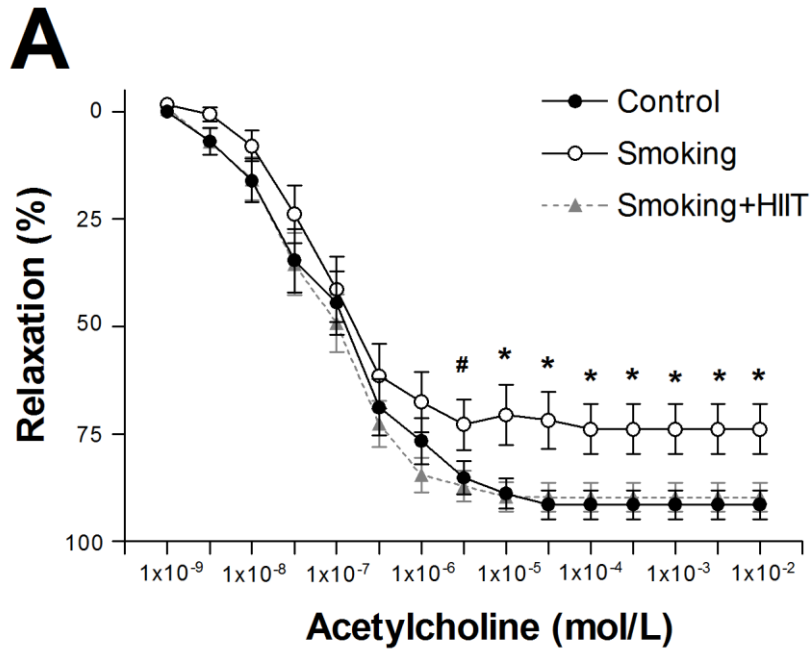




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532 Fig. 2

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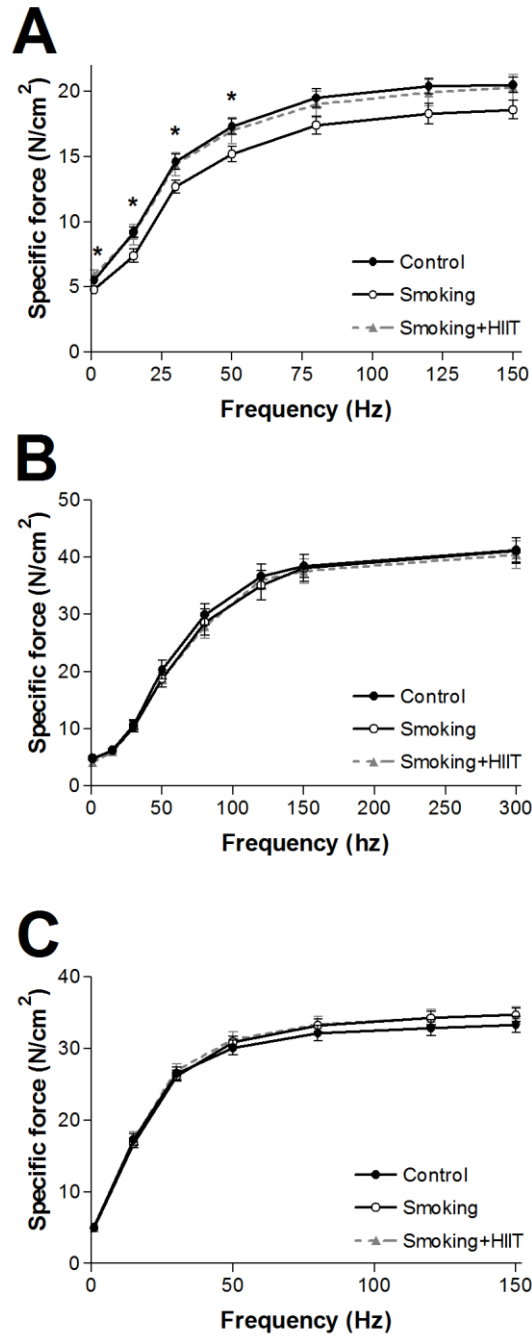


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536 Fig. 3

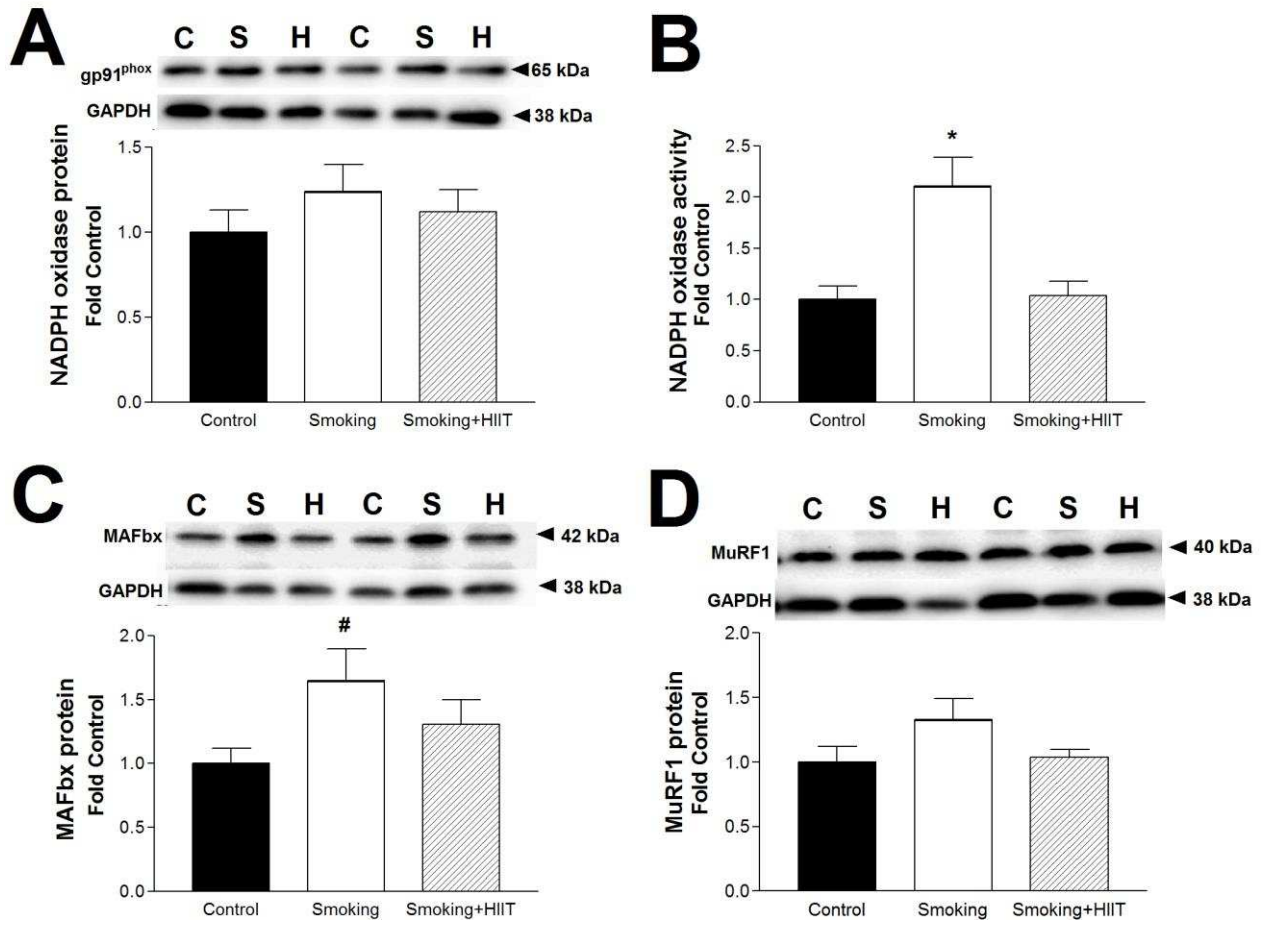
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539 Fig. 4

540 Fig. 4



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542

543 Fig. 5.