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1 **REPRODUCIBILITY OF NIRS ASSESSMENT OF MUSCLE OXIDATIVE CAPACITY IN SMOKERS**
2 **WITH AND WITHOUT COPD**

3
4 Alessandra Adami, Robert Cao, Janos Porszasz, Richard Casaburi, Harry B. Rossiter

5
6 Rehabilitation Clinical Trials Center, Division of Respiratory and Critical Care Physiology and
7 Medicine, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Torrance, CA,
8 USA

9
10 RUNNING HEAD: Non-invasive assessment of muscle oxidative capacity in COPD

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12 CORRESPONDING AUTHOR:

13 Harry B. Rossiter
14 Rehabilitation Clinical Trials Center
15 Division of Respiratory and Critical Care Physiology and Medicine
16 Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center
17 1124 W Carson St
18 CDCRC building
19 Torrance, CA, 90502
20 USA
21 E-mail: hrossiter@ucla.edu

22
23
24 AUTHORS' E-MAIL ADDRESSES:
25 Adami Alessandra: aadami@labiomed.org
26 Cao Robert: rcao@labiomed.org
27 Janos Porszasz: porszasz@ucla.edu
28 Richard Casaburi: casaburi@ucla.edu

29

30 **ABSTRACT**

31 Low muscle oxidative capacity contributes to exercise intolerance in chronic obstructive pulmonary
32 disease (COPD). Near-infrared spectroscopy (NIRS) allows non-invasive determination of the
33 muscle oxygen consumption ($m\dot{V}O_2$) recovery rate constant (k), which is proportional to oxidative
34 capacity assuming two conditions are met: 1) exercise intensity is sufficient to fully-activate
35 mitochondrial oxidative enzymes; 2) sufficient O_2 availability. We aimed to determine
36 reproducibility (coefficient of variation, CV; intraclass correlation coefficient, ICC) of NIRS k
37 assessment in the *gastrocnemius* of 64 participants with (FEV_1 $64\pm 23\%$ predicted) or without COPD
38 (FEV_1 $98\pm 14\%$ predicted). 10-15s dynamic contractions preceded 6min of intermittent arterial
39 occlusions (5-10s each, ~ 250 mmHg) for k measurement. k was lower ($P < 0.05$) in COPD
40 ($1.43\pm 0.4\text{min}^{-1}$; $CV=9.8\pm 5.9\%$, $ICC=0.88$) than controls ($1.74\pm 0.69\text{min}^{-1}$; $CV=9.9\pm 8.4\%$; $ICC=0.93$).
41 Poor k reproducibility was more common when post-contraction $m\dot{V}O_2$ and deoxygenation were
42 low, suggesting insufficient exercise intensity for mitochondrial activation and/or the NIRS signal
43 contained little light reflected from active muscle. The NIRS assessment was well tolerated and
44 reproducible for muscle dysfunction evaluation in COPD.

45
46

47 **KEY WORDS**

48
49 Skeletal muscle; Mitochondria; Exercise intolerance; Oxygen consumption; Kinetics; Quality-control

50

51 1. INTRODUCTION

52 Chronic obstructive pulmonary disease (COPD) is characterized by dyspnea on exertion, with
53 subsequent reduced exercise tolerance and quality of life. Skeletal muscle dysfunction is a systemic
54 consequence of COPD that also contributes to increased morbidity and mortality in this population
55 (Agustí et al., 2003; Casaburi et al., 2001; Decramer et al., 2008; Maltais et al., 2000, 2014; Nici,
56 2000; Vogiatzis and Zakyntinos, 2012; Wouters et al., 2002). Morphological and structural
57 skeletal muscle alterations in COPD are especially prevalent in the locomotor muscles, and include
58 atrophy and weakness, loss of type I fibers, loss of muscle oxidative capacity and mitochondrial
59 dysfunction, among others (Allaire et al., 2004; Coronell et al., 2004; Couillard and Prefaut, 2005;
60 Engelen et al., 2000; Gosker et al., 2002, 2007; Maltais et al., 2014; Picard et al., 2008; Whittom et al.,
61 1998). Amelioration of these muscular alterations contributes to the substantial benefits of
62 pulmonary rehabilitation in COPD patients (Maltais et al., 2014).

63
64 The prevalence and progression of the loss of muscle oxidative phenotype in relation to disease
65 severity is still unclear, and this is partly because measurement of muscle oxidative capacity
66 requires an invasive biopsy or complex ^{31}P magnetic resonance spectroscopy assessments. In
67 review, Meyer et al. (2013) showed that low muscle oxidative capacity and increased reactive
68 oxygen species production was evident in skeletal muscle across all spirometric stages of COPD
69 disease severity. Furthermore, Natanek et al. (2013) showed wide heterogeneity in quadriceps type
70 I fiber expression in 114 COPD patients evenly distributed across GOLD stages 2-4. These findings
71 demonstrate that muscle oxidative capacity appears to be highly variable across disease severity,
72 which underscores the need for simple methods to assess changes in muscle oxidative capacity in
73 COPD patients independent from systemic effects of the disease.

74
75 We aimed to address this using a non-invasive method based on near-infrared spectroscopy (NIRS;
76 Motobe et al., 2004; Ryan et al., 2012). This technique provides measurement of the recovery rate
77 constant (k) of muscle oxygen consumption ($m\dot{V}\text{O}_2$), isolated from influences of circulatory or
78 pulmonary function, and which is directly related to muscle oxidative capacity in single muscle
79 fibers ($r^2=0.77$; Wüst et al., 2013). Muscle k can be assessed by NIRS during ~6 minutes of recovery
80 from brief contractions, using a series of intermittent arterial occlusions (5-10 s each); during
81 occlusions, the rate of decline in the muscle tissue saturation index (TSI) is directly proportional to
82 $m\dot{V}\text{O}_2$. This technique has been validated in young healthy subjects against phosphocreatine

83 recovery kinetics and quadriceps muscle biopsy (Ryan et al., 2013, 2014). It has also been used to
84 assess muscle oxidative capacity in spinal cord injury (Erickson et al., 2013), amyotrophic lateral
85 sclerosis (Ryan et al., 2014) and chronic heart failure (Southern et al., 2015), among other
86 conditions. However, to our knowledge, this technique has not been applied in COPD where muscle
87 morphologic adaptations such as fat infiltration, fibrosis, inflammation, increased subcutaneous
88 adipose, loss of type I fibers and mitochondrial density (Maltais et al., 2014) may hamper NIRS
89 measurement of muscle oxidative capacity.

90

91 The method relies on two competing assumptions: 1) that exercise is sufficiently intense to
92 maximally activate mitochondrial oxidative enzymes and elicit a sufficient increase in $m\dot{V}O_2$
93 (Korzeniewski and Rossiter, 2015; Wüst et al., 2011, 2013); 2) that O_2 delivery is not limiting to k
94 (Haseler et al., 2004). This latter condition is especially important in COPD where poor systemic O_2
95 delivery, muscle capillary rarefaction and brief arterial occlusions may combine to reduce TSI below
96 some critical threshold, thereby slowing $m\dot{V}O_2$ recovery kinetics. Test-retest reliability (intraclass
97 correlation coefficient, ICC) of k in healthy subjects ranges from 0.26 to 0.68 (Ryan et al., 2012;
98 Southern et al., 2014), and whether reliable measurements are possible in COPD is currently
99 unknown. This is particularly important in relation to the expected effect magnitude of oxidative
100 capacity loss in COPD (~10-50%; Meyer et al., 2013). Therefore, we aimed to determine the
101 reliability of NIRS assessment of *gastrocnemius* muscle oxidative capacity in smokers with and
102 without COPD. We hypothesized that test-retest variability in k would be sufficiently low to allow
103 NIRS estimates of oxidative capacity to a useful method to detect COPD-related loss. Secondly, we
104 aimed to identify correlates of high variability in repeated k measurement, if it occurred. These
105 correlates may provide a basis for quality control of the NIRS muscle assessment.

106

107 **2. MATERIALS AND METHODS**

108 **2.1 Participants**

109 Both smoking (Montes de Oca et al., 2008) and COPD (Maltais et al., 2014) have each been
110 implicated in COPD-associated muscle dysfunction. Therefore, to account for the independent
111 influence of smoking history, we sought current and former smokers with at least 10 pack-year
112 smoking history to volunteer: 32 COPD patients (GOLD stage 1-4, defined by the criteria for the
113 Global initiative for Chronic Obstructive Lung Disease) and 28 participants with normal spirometry
114 (CON) (Table 1). This was an ancillary study of COPDGene (ClinicalTrials.gov Identifier

115 NCT00608764), for which a complete list of inclusion and exclusion criteria is given in Regan et al.
116 (2010). Participants were informed about the procedures and risks associated with the study, and
117 gave written informed consent. The study was approved by the Institutional Review Board of Los
118 Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, in accordance with the
119 Declaration of Helsinki.

120

121 **2.2 Protocol**

122 Each participant visited the laboratory once, during which NIRS muscle oxidative capacity and a
123 spirometry tests were performed.

124

125 *2.2.1 NIRS muscle oxidative capacity test.* A wireless, portable, continuous-wave, spatially-resolved
126 spectroscopy (SRS) NIRS device (PortaMon, Artinis, The Netherlands) was used to measure relative
127 concentrations of *deoxy*-hemoglobin and *deoxy*-myoglobin (here termed HHb for simplicity) and
128 *oxy*-hemoglobin and *oxy*-myoglobin (HbO_2) in the tissues ~ 1.5 cm beneath the probe (interoptode
129 distance was 3 cm). From these measurements relative changes in total hemoglobin and myoglobin
130 ($\text{THb} = \text{HHb} + \text{HbO}_2$) and the Hb difference ($\text{Hb}_{\text{diff}} = \text{HbO}_2 - \text{HHb}$) were calculated. In addition, the
131 tissue saturation index (TSI, %) was measured using the SRS approach (using interoptode distances
132 of 2-3 cm) (Ferrari et al., 2004).

133

134 A modified NIRS protocol based on Ryan et al. (2012) was used. The participant lay supine and the
135 NIRS probe was wrapped in plastic film, placed longitudinally on the belly of the right medial
136 *gastrocnemius*, and secured with an elastic bandage. A 13 x 85 cm rapid-inflation pressure-cuff
137 (SC12D, Hokanson, USA) was placed on the proximal thigh of the same leg and attached to an
138 electronically-controlled rapid cuff-inflator (E20, Hokanson, USA). A pad was placed under the ankle
139 such that the lower leg and NIRS probe was suspended above the bed. During the ~ 30 min
140 assessment, the participant was asked to relax and refrain from moving the leg except when
141 instructed.

142

143 Initially, the participant was familiarized with the execution of cyclical plantar-flexion/relaxation
144 exercise at ~ 1 Hz, to activate the medial *gastrocnemius* against a manually applied resistance, and
145 with the rapid-cuff inflation procedures. Repeated cuff inflations from low (~ 50 mmHg) to high
146 (~ 250 mmHg) pressures were performed during this familiarization phase. Arterial occlusion was

147 determined from a tolerated cuff-pressure within the range of 230-300 mmHg (236±17 mmHg) that
148 resulted in HHb rise, HbO₂ fall and approximately constant THb over ~15-20 s.

149
150 The measurement protocol began after 2-3 min of rest, where baseline TSI and SpO₂ at a fingertip
151 (Rad-5 Pulse Oximeter MasimoSET®, Masimo Co., Irvine, CA) were measured over 2 min.
152 Subsequently, after having removed the pulse oximeter, the participant was instructed to execute
153 10-12 cycles of plantar-flexion exercise, followed immediately by arterial occlusion until a steady-
154 state in TSI was reached (mean duration ~90 s; Figure 1). The cuff was then instantly deflated and
155 muscle reoxygenation was recorded until a steady-state was reached (typically ~3 min). This
156 procedure (the physiologic normalization, PN) identified the functional range of TSI under resting
157 conditions from TSI_{min} at the end of the sustained arterial occlusion to TSI_{max} at the peak of the
158 reactive hyperemia (Figure 1). Finally, the participant performed two oxidative capacity
159 assessments. These consisted of: 1) cyclical plantar-flexion exercise to desaturate the muscle to a
160 target of 50% of the PN amplitude (typically 10-15 s of contractions) (Hamaoka et al., 2007; McKully
161 et al., 1994; Motobe et al., 2004; Ryan et al., 2012) (Figure 1); 2) a series of intermittent arterial
162 occlusions (AO; 5 occlusions for 5 s, and 10 for 10 s, each separated by 5-20 s recovery). A single
163 oxidative capacity assessment lasted ~6 minutes. The second repetition was conducted once a
164 resting steady state was re-established (typically ~1 min).

165
166 At the end of the procedure the skinfold at the NIRS site was measured to estimate adipose tissue
167 thickness (ATT, mm) (Lange Skinfold Caliper, Beta Technology Inc., Santa Cruz, CA).

168
169 *2.2.2 Spirometry.* Approximately 15 minutes before spirometric testing, participants inhaled two
170 puffs of metered dose albuterol sulfate (ProAir HFA, Teva Respiratory, Horsham, PA, USA).
171 Spirometry was performed in accordance with the American Thoracic Society guidelines (Miller et
172 al., 2005) using a dual beam Doppler ultrasound-based spirometer (EasyOne Pro, Ndd Medical,
173 Zürich, Switzerland) (Regan et al., 2010). FEV₁ and forced vital capacity (FVC) were measured from
174 the greatest FEV₁ and FVC over up to eight maximum expiratory maneuvers, where the greatest two
175 measurements were within 150 mL.

176
177 **2.3 Analyses**

178 *2.3.1 NIRS oxidative capacity test.* During the repeated oxidative capacity tests, for each intermittent
179 arterial occlusion the negative slope of TSI (%.s⁻¹) was fitted by a linear function to estimate relative
180 m $\dot{V}O_2$ (Figure 2A,C). Note that during occlusion the rate of deoxygenation (the negative slope of TSI)
181 is inversely proportional to m $\dot{V}O_2$, and is therefore reported below as a positive value (%.s⁻¹). The
182 m $\dot{V}O_2$ exponential recovery rate constant (k , min⁻¹) was estimated using non-linear least-squares
183 regression (Figure 2B,D) (OriginPro v8.6, OriginLab Co., Northampton, USA) (Wüst et al., 2013).

184

185 *2.3.2 NIRS quality control.* Low test-retest variability (>1 SD) was used as the quality control
186 criterion. For those tests with reproducibility outside 1 SD, the potential for limitations in O₂
187 delivery and/or contraction-induced activation of mitochondrial oxidative phosphorylation during
188 the oxidative capacity test were investigated to assess for physiologic contributors to test-retest
189 variability. To determine a value of TSI during the sustained occlusion in the PN phase where the
190 decline in TSI began to slow (a deflection in TSI; Figure 1B), a linear regression was applied from
191 the onset of the sustained AO up to a point just before TSI deviated from linearity. This was
192 investigated as a potential marker for the onset of O₂ delivery limitation to m $\dot{V}O_2$ during arterial
193 occlusion. The lowest TSI (TSI_{LOW}) reached during each oxidative capacity test was recorded (both
194 as an absolute muscle saturation and relative to the PN) and compared with the TSI deflection point
195 (Figure 1). The increase in m $\dot{V}O_2$ during contractions was estimated from the greatest m $\dot{V}O_2$
196 recorded during the oxidative capacity test, and expressed in absolute units (%.s⁻¹) and as a fold-
197 change above the steady-state resting m $\dot{V}O_2$ (measured at the end of the oxidative capacity test): a
198 small increase or fold-change in m $\dot{V}O_2$ may indicate insufficient contractile stimulus for
199 mitochondrial oxidative phosphorylation and result in a low k .

200

201 *2.3.3 Statistics.* A Student's paired t test was used to identify differences between COPD and CON. A
202 Bland-Altman analysis for repeated measurements was used to assess the agreement between the
203 two m $\dot{V}O_2$ recovery k assessments (Bland and Altman, 1999). Coefficient of variation (CV) and
204 intraclass correlation coefficient (ICC) were used to assess within-subject test-retest reproducibility.
205 Variables correlated with the difference between repeated-measures of k (Δk) were sought by
206 Spearman univariate linear regression analysis. Significant differences were accepted at $P \leq 0.05$.
207 Results are presented as mean \pm SD, unless otherwise specified. A Shapiro-Wilk's test ($P \geq 0.05$) and
208 visual inspection of the histograms, Q-Q plots and box plots were performed to determine normal
209 distribution of k values for both COPD and CON groups (COPD, $P > 0.45$; CON, $P > 0.06$). Statistical

210 analyses were performed using Prism v6.0f (GraphPad, San Diego, CA, USA) and SPSS v20 (IBM,
211 Chicago, IL, USA).

212

213 **3. RESULTS**

214 **3.1 Participant characteristics**

215 Four COPD patients were unable to successfully complete the NIRS muscle protocol: two could not
216 tolerate the sustained arterial occlusion for the PN, and the $m\dot{V}O_2 k$ could not be confidently
217 resolved in one repeat of two other COPD patients. These 4 COPD patients were excluded from
218 further analysis. Results are reported from 28 COPD and 28 normal spirometry CON participants.
219 Two COPD patients required nasal cannula O_2 during the visit (at 3-4 L.min⁻¹). Participant
220 characteristics are shown in Table 1. CON were younger than COPD (60 ± 7 vs. 65 ± 8 years, $P <$
221 0.05).

222

223 **3.2 Muscle near-infrared spectroscopy**

224 **3.2.1 Resting muscle.** Resting muscle TSI and ATT did not differ between COPD patients and CON
225 (Table 1).

226

227 **3.2.2 Physiologic normalization (PN).** In all COPD patients, PN ranged from a minimum of 22 % TSI to
228 a maximum of 77 % TSI, with a mean range (max - min) of 32 ± 9 %. This was not different ($P >$
229 0.05) than CON: PN ranged 19 to 81 %, with a mean of 32 ± 11 %.

230

231 **3.2.3 Muscle oxidative capacity ($m\dot{V}O_2 k$).** A total of 112 $m\dot{V}O_2$ recovery kinetics assessments were
232 performed for the study. On average, there was no difference between repeated k measurements
233 within COPD or CON participants (Table 2). The individual test-retest reliability was not different
234 between COPD (CV = 9.9%, ICC = 0.88) and CON (CV = 9.9%, ICC = 0.93) (Table 2, Figure 3). Power
235 analyses (G*Power 3.1; Faul et al, 2007) revealed a $1 - \beta = 0.81$ for comparison of k between groups
236 (the primary outcome). In all participants, Bland-Altman limit of agreement analysis revealed low
237 mean bias (-0.03 min^{-1}), and 95% confidence intervals of $-0.58, 0.64 \text{ min}^{-1}$ (Figure 4). We could
238 detect no order effect between repeats of k measurement ($P = 0.24$; 1-tailed t-test). On average,
239 $m\dot{V}O_2 k$ was ~25% lower in COPD than CON (Table 2) and was diminished at all GOLD stages: CON,
240 $1.74 \pm 0.71 \text{ min}^{-1}$ (n=28); GOLD 1, $1.45 \pm 0.36 \text{ min}^{-1}$ (n=7); GOLD 2, $1.48 \pm 0.37 \text{ min}^{-1}$ (n=13); GOLD 3,
241 $1.22 \pm 0.32 \text{ min}^{-1}$ (n=5); and GOLD 4, $1.54 \pm 0.41 \text{ min}^{-1}$ (n=3).

242

243 *3.2.4 NIRS test quality control.* During the oxidative capacity test, the lowest TSI (TSI_{LOW}) in both
244 repeats was typically achieved within the first or second AO (e.g., see Figure 1). In both COPD and
245 CON, TSI_{LOW} averaged ~47% absolute (Table 3), equivalent to ~32% and ~29% of the PN range
246 respectively. On average, the TSI deflection point occurred at 46 ± 9 % in COPD and 46 ± 11 % in
247 CON. This meant that, typically (66% of tests), TSI_{LOW} was greater than TSI deflection point. In 38
248 tests (27%) TSI_{LOW} was below TSI deflection point.

249

250 In COPD, the peak $\dot{m}\dot{V}O_2$ during the oxidative capacity test was 1.38 ± 0.59 % \cdot s⁻¹ and 1.51 ± 0.88 % \cdot s⁻¹
251 respectively for the first and second repetitions (equivalent to a 14 ± 7 and 16 ± 13 fold increase
252 above the recovery steady-state, 0.12 ± 0.12 % \cdot s⁻¹). Peak $\dot{m}\dot{V}O_2$ values during the oxidative capacity
253 test in CON were 1.71 ± 1.89 % \cdot s⁻¹ and 1.49 ± 1.17 % \cdot s⁻¹ respectively for the first and second repeat,
254 equivalent to a 18 ± 12 and 16 ± 11 fold increase above resting (0.10 ± 0.05 % \cdot s⁻¹), and were not
255 different compared with COPD ($P = 0.68$).

256

257 Variables predictive of poor reproducibility were sought as potential quality control indices for the
258 NIRS oxidative capacity test. Univariate linear regression analysis revealed that variability in
259 repeated k measurements, assessed from the difference between the two k values (Δk), was
260 positively correlated with k ($r^2 = 0.17$; $P \leq 0.001$): meaning faster $\dot{m}\dot{V}O_2$ kinetics were related to
261 greater variability of measurement. However, other variables hypothesized to explain variability in k ,
262 including those expected to contribute to limitations in $\dot{m}\dot{V}O_2$ activation, O₂ delivery, or NIRS signal
263 sensitivity, such as minimum TSI during PN and TSI_{LOW}, age and resting TSI, PN maximum value and
264 $\dot{m}\dot{V}O_2$ fold change, did not show a strong association with Δk ($P > 0.10$).

265

266 We investigated the characteristics of poorly-reproducible tests where Δk exceeded 1 SD (equal to
267 the mean effect size for COPD; $\Delta k > 0.3$ min⁻¹): thirteen participants (5 COPD, 8 CON) exceeded this
268 variability threshold. These unreliable tests were characterized by a low $\dot{m}\dot{V}O_2$ (TSI = $1.15 \pm$
269 0.44 % \cdot s⁻¹) and poor exercise deoxygenation (e.g. a high TSI_{LOW} value of 53.5 ± 5.8 %). Six of these
270 participants had large adipose layer (ATT = 8.3 ± 3.0 mm) and six had high skin melanin, each likely
271 limiting the volume of the muscle interrogated by the NIRS probe. In all these 13 participants, the
272 lowest TSI during the test (TSI_{LOW}) was below the TSI deflection point, suggesting that low O₂
273 availability was not associated with muscle oxidative capacity assessment reliability. Excluding tests

274 on the basis of $\Delta k > 0.3 \text{ min}^{-1}$ improved k measurement reliability (CV = 7.0±4.3%, ICC = 0.98, n =
275 43).

276

277 4. DISCUSSION

278 This is the first study to measure locomotor muscle oxidative capacity (from $m\dot{V}O_2$ recovery rate
279 constant, k) in a large group of smokers with or without COPD, using a non-invasive, relatively
280 simple, short-duration assessment by NIRS. Fifty-six out of 60 participants (93%) tolerated the NIRS
281 assessment and returned interpretable results. Overall these data showed: there was no mean bias
282 between test-retest repeats of *gastrocnemius* k measurement by NIRS in both COPD patients and
283 age-similar smokers without airflow obstruction; that individual test-retest reproducibility was
284 high (CV = 9.9%, ICC = 0.9); and that k averaged 25 % less in COPD compared to smokers with
285 normal spirometry. Despite known muscle morphologic adaptations including increased fat and
286 fibrotic infiltration, inflammation, loss of type I fibers and mitochondrial density (Maltais et al.,
287 2014), our findings support that the NIRS assessment is a reliable method to detect COPD-related
288 loss of muscle oxidative capacity.

289

290 We also aimed to identify correlates of tests with reproducibility that lay outside 1 SD of the
291 distribution of all tests, as potential features for quality control. 13 of 56 participants (5 COPD, 8
292 CON) showed a high variability in k ($\Delta k > 0.3 \text{ min}^{-1}$, which was the mean effect size of COPD). Poor
293 reproducibility was not associated with presence of COPD. Poor reproducibility was explained
294 principally by a small increase in $m\dot{V}O_2$ and only modest deoxygenation during contractions. This
295 suggests that insufficient contractile stimulus for mitochondrial activation and/or that the NIRS
296 signal contained little light reflected from active muscle (large adipose layer in n=6, and high skin
297 melanin content in n=6), may contribute to poor test quality. These findings indicate that a poor
298 quality tests are related to an insufficient increase in $m\dot{V}O_2$ during contractions, and not to the
299 presence of an O_2 delivery limitation, as might be anticipated in COPD.

300

301 4.1 Muscle oxidative capacity in COPD

302 We found that k in the *gastrocnemius* skeletal muscle was, on average, 25 % less (range ~12-30%)
303 in COPD than smokers of a similar age but without pulmonary obstruction. This average is
304 consistent with the ~10-50% lower muscle oxidative enzyme activity (e.g. citrate synthase) or
305 oxidative capacity observed in *quadriceps* biopsy samples from COPD patients compared with

306 controls (Meyer et al., 2013), suggesting that the NIRS test provides a relevant non-invasive
307 alternative to invasive biopsy assessments (Ryan et al., 2014). It is of note that k in our control
308 group was 25% lower than non-smoking control participants in other studies using the same NIRS
309 methods (non-smoker *gastrocnemius* $k = \sim 2 \text{ min}^{-1}$ equivalent to an oxidative capacity of ~ 250
310 $\text{pmol}\cdot\text{s}^{-1}\cdot\text{mg dry weight}^{-1}$; Ryan et al., 2014). However, those studies almost exclusively included
311 young participants aged 24-27 years, while our smokers ranged 49-77 years. Whether the
312 apparently low muscle oxidative capacity in our study relates to the effects of long-term smoking, or
313 alternatively to age or physical inactivity remains to be determined.

314

315 While our study was not sufficiently powered to detect differences across disease severity, one
316 outlier of the three GOLD 4 patients ($k = 2.12 \text{ min}^{-1}$) likely skewed a general trend for a progressive
317 decline in oxidative capacity across GOLD severity classifications 2-4. Even so, the distribution of k
318 values among the 28 COPD patients suggests that low k may occur at any GOLD stage, and therefore
319 muscle dysfunction may not be solely associated with the disease severity in COPD (Maltais et al.,
320 2014; Wagner, 2006). The precise etiologies resulting in loss of muscle oxidative capacity in COPD
321 awaits further research. Nevertheless, the 93% tolerability and good test-retest agreement of the
322 NIRS test in COPD prove the feasibility and reliability of this approach for discriminating patients
323 with poor muscle function i.e. *gastrocnemius* k value lower than the 2 min^{-1} reference for a healthy
324 adult. Because the NIRS test is non-invasive, it may provide the opportunity for muscle assessment
325 in large-cohort studies, which are needed to better identify the complex multifactorial etiology of
326 muscle oxidative dysfunction in COPD.

327

328 4.2 Variables influencing reproducibility of the NIRS muscle oxidative capacity test

329 The NIRS assessment of muscle oxidative capacity relies on the observed linear proportionality
330 between the recovery rate constant (k) of cellular $\dot{V}\text{O}_2$ and cellular oxidative capacity i.e. the $\dot{V}\text{O}_{2\text{max}}$
331 of the muscle cells investigated (Wüst et al., 2013). The primary predictor in our study of poor k
332 reproducibility was a high value for k ($r^2 = 0.17$; $P \leq 0.001$). This is likely a simple reflection of the
333 limited ability to accurately model the recovery rate constant with a limited number of $\text{m}\dot{V}\text{O}_2$
334 measurements when $\text{m}\dot{V}\text{O}_2$ kinetics are rapid. Nevertheless, this does not limit the ability of the test
335 to detect abnormally low muscle oxidative capacity, which is the primary aim for studies of COPD
336 patients or other conditions of chronic inactivity or disease.

337

338 The relationship between cellular recovery k and oxidative capacity in muscle is predictable based
339 on first order rate reaction kinetics (Voet and Voet, 2004), as long as O_2 concentration in muscle
340 mitochondria remains non-limiting. However, recent studies suggest that control of oxidative
341 phosphorylation in skeletal muscle in humans is not first order, and that allosteric or 'each step'
342 activation of mitochondrial oxidative pathways is required to activate the enzymes limiting cellular
343 $\dot{V}O_{2max}$ (Korzeniewski and Rossiter, 2015; Wüst et al., 2011). Thus, accurate measurement of
344 oxidative capacity by NIRS relies on competing demands to achieve a sufficiently high level of
345 muscle activity and $m\dot{V}O_2$ to release mitochondrial oxidative enzyme regulation, but to limit muscle
346 activity to a sufficiently low level such that muscle mitochondrial O_2 delivery does not become a
347 limiting variable. Failure to meet either of these conditions would result in an erroneously low
348 measurement of muscle oxidative capacity by NIRS.

349

350 Using single muscle fibers from the frog suspended in a medium containing a high O_2 concentration,
351 Wüst et al. (2013) observed that, unlike poorly-oxidative fibers, $m\dot{V}O_2$ recovery k of highly-oxidative
352 fibers was dependent on the frequency of stimulation of the preceding contractions. A contractile
353 protocol sufficient to elicit $\sim 50\%$ $\dot{V}O_{2max}$ was required to release oxidative enzyme regulation to
354 allow recovery k to become proportional to cellular oxidative capacity. The duration required of this
355 contractile protocol was not assessed. The human medial *gastrocnemius*, the site of NIRS probes in
356 our study, expresses a mixed fiber type distribution and contains both poorly and highly-oxidative
357 muscle fibers. Therefore, a low $m\dot{V}O_2$ response during muscle contractions would be predictive of a
358 poor quality assessment and result in an erroneously low oxidative capacity. Consistent with this
359 notion, in 13 of our participants the NIRS test results were poorly reproducible and in these there
360 was a tendency ($P = 0.20$) for a low exercise-induced increase in $m\dot{V}O_2$. This highlights the
361 importance of ensuring high-intensity muscle contractions during the NIRS oxidative capacity
362 assessment for validity of the test.

363

364 As stated previously, another condition that must be satisfied for validity of the NIRS test is non-
365 limiting mitochondrial O_2 delivery. Using ^{31}P magnetic resonance spectroscopy to determine
366 *gastrocnemius* PCr recovery kinetics from plantar flexion exercise (a proxy for $m\dot{V}O_2$ k), Haseler et
367 al. (2004) showed in young sedentary subjects that PCr recovery was not limited by O_2 delivery
368 under normoxic conditions. However, during hypoxic gas breathing, PCr recovery kinetics were
369 slowed. It should be pointed out that the duration of exercise was 6 min, and therefore $m\dot{V}O_2$ and

370 muscle deoxygenation were likely far greater than those observed in our study where exercise was
371 limited to ~15 s. Nevertheless, were TSI to be driven below some limiting value, $m\dot{V}O_2$ recovery k
372 may become limited by O_2 delivery *in vivo*, which would result in an erroneously low k . While the
373 brief exercise in the NIRS test does not strain central cardiac or pulmonary limits for O_2 delivery,
374 age- or disease-related chronic adaptations, such as muscle capillary rarefaction, inflammation,
375 anemia or reduced muscle myoglobin (Maltais et al., 2014), have the potential to limit muscle
376 mitochondrial O_2 concentration in COPD and therefore invalidate the NIRS assessment. However,
377 we found that the lowest value of TSI measured during the NIRS assessment (TSI_{LOW}; typically
378 reached during the 1st or 2nd post-exercise arterial occlusion) was not related to poor test-retest
379 reliability of k . This suggests that outlying low k values are not consequent to O_2 delivery limitation,
380 at least down to TSI_{LOW} values of ~30% of the physiologic range.

381 382 *4.3 Quality control of the muscle NIRS oxidative capacity assessment*

383 In part, the beauty of the NIRS test of muscle oxidative capacity is that it relies on $m\dot{V}O_2$ kinetics, and
384 therefore does not require calibrated measurements. For this reason, one aim of this study was to
385 identify variables that could be used as markers of quality control. We proposed to identify features
386 within any tests that showed poor reproducibility. The strongest correlate of variability in repeated
387 k measurements (Δk) was the value of k itself, which does not provide a basis for quality control. To
388 our surprise, however, we found no correlation between Δk and proposed quality assessments (e.g.
389 increase in $m\dot{V}O_2$ increase during contractions or low TSI during occlusions). This may reflect the
390 overall strong test-retest reproducibility in COPD patients and controls. We therefore identified 13
391 participants in whom variability exceeded 1 SD ($\Delta k > 0.3 \text{ min}^{-1}$). Of these 13, there was a high
392 prevalence of large ATT, high skin melanin content, low increase in $m\dot{V}O_2$ and a small exercise-
393 induced deoxygenation. While these features alone do not form the basis of quality control, they
394 highlight that patient physical characteristics limiting reflected light from active muscle tissue are
395 likely partly responsible for reducing reproducibility of k measurements. Alternative NIRS systems,
396 such as high-power time-resolved (TRS) NIRS, allow deeper penetration into muscle during rest and
397 exercise (Okushima et al., 2015), and therefore may increase the reliability of $m\dot{V}O_2$ recovery kinetic
398 assessment in these patients.

399
400 Based on our findings and experience, a few considerations emerge to inform quality control of the
401 NIRS oxidative capacity test. First, the current best method of quality control is to perform the

402 measurement twice in the same visit. We propose that poorly-reproducible tests, where $\Delta k > 0.3$
403 min^{-1} , be repeated to reduce the influence of outlying results, and repeated measurements averaged.
404 Careful attention should be made to the NIRS probe placement, to ensure that a muscle region is
405 chosen that both minimizes the skinfold under the probe and maximizes the sampling of active
406 muscle during contractions and recovery. Doppler ultrasound, skinfold calipers, muscle palpation
407 during contraction and/or surface EMG may help to identify optimal NIRS probe placement.

408

409 Exercise stimuli that result in a small increase $\dot{m}\dot{V}O_2$ and small reduction in saturation were
410 associated with poor test reliability. Therefore, our data suggest that the risk to NIRS test validity of
411 under-stimulating the muscle during dynamic contractions is greater than the risk of O_2 limiting
412 deoxygenation caused by contractions that are too intense or sustained. Thus, ensuring that the
413 exercise-induced desaturation reaches a value of $\sim 30\text{-}50\%$ of the physiologic range (PN) helps in
414 test quality assurance. This can be confirmed in real time by monitoring the TSI response to
415 contractions and adjusting the intensity and/or duration of exercise (i.e. extending the duration
416 beyond the $\sim 10\text{-}15$ s we used here) to achieve the desaturation target. This, of course, requires
417 knowledge in the PN range prior to the oxidative capacity test, which is a modification to the
418 protocol of Ryan et al. (2012) where the PN range measurement is performed last. This has the
419 concern that a period of ischemia (even briefly) may affect mitochondrial function and therefore
420 influence the assumptions inherent in the measurement of oxidative capacity from $\dot{m}\dot{V}O_2$ recovery
421 kinetics e.g. an ischemic preconditioning effect (Crisafulli et al., 2011). Our findings, however, that
422 muscle k in older controls ($\sim 1.75 \text{ min}^{-1}$) and COPD ($\sim 1.45 \text{ min}^{-1}$) were consistent with that
423 predicted from biopsy studies (Meyer et al., 2013) suggest that brief ischemia does not harm the
424 validity of the test.

425

426 *4.4 Clinical implications*

427 Low muscle oxidative capacity is associated with exercise intolerance in COPD and therefore may
428 contribute to reduce physical activity and quality of life in these patients (Maltais et al., 2014; Meyer
429 et al., 2013). We studied the *gastrocnemius* muscle as a primary locomotor muscle for walking, and
430 which is also extensively activated during standing and in sway. Rehabilitation (cycling and
431 walking) is known to ameliorate oxidative capacity deficits in quadriceps (Maltais et al., 1996) and
432 is associated with a reduction of dyspnea and leg fatigue symptoms during exercise in COPD.
433 Therefore, reliable measurements of skeletal muscle structure and function, independent of disease-

434 related impairments in pulmonary function or muscle blood flow, are of crucial importance to
435 monitor the peripheral consequences of COPD. This reliable, non-invasive, short-duration, relatively
436 inexpensive and well-tolerated assessment of oxidative capacity in COPD muscle may also enable a
437 better targeting to therapeutic strategies to improve physical activity, exercise tolerance and quality
438 of life in these patients.

439

440 *4.5 Additional considerations*

441 Our approach focused on biological variability, in that test-retest precision of k was assessed in
442 smokers with and without COPD with the same day and session, and the NIRS probe was not
443 removed from the muscle between repeated tests. Southern et al. (2013) assessed the test-retest
444 variability between consecutive days in young healthy participants, an approach that included the
445 combined effects of both methodological and biological variability. This may partly explain why
446 variability for k assessment in our study (ICC range 0.88-0.93; CV = 9.9 %) was higher than
447 Southern et al. (2013) (ICC range 0.26-0.59; CV = 10.6 %). In addition, Southern et al. (2013)
448 assessed reliability in 15 participants whereas our study investigated 56. This difference also
449 contributes to the greater ICC found in our study. Nevertheless, our data show that the approach is
450 reliable in COPD and older controls where muscle quality is reduced compared with young subjects.

451

452 Furthermore, Southern et al. (2013) found greater day to day test-retest variability of the NIRS
453 oxidative capacity test when using self-metered exercise (specifically, exercise against elastic
454 resistance bands) compared with monitored exercise using a custom-built plantar flexion
455 ergometer. We were specifically interested in assessing a pragmatic approach to the NIRS oxidative
456 capacity test using self-metered exercise in order to assess the applicability of the assessment in the
457 clinical setting without the requirement for additional specialized equipment. We used a manually-
458 applied resistance to plantar flexion administered by the same researcher in all participants. The
459 intensity of the 10-15 contractions was assessed indirectly by feel and also monitored in the TSI
460 trace in real time. This meant that the operator could instruct the participant to alter the intensity
461 or duration of contractions during the test itself to achieve an 'optimal' deoxygenation signal (and,
462 by implication, $m\dot{V}O_2$ response). We believe that this approach might have advantages over delivery
463 of a standardized metered exercise dose in patients with chronic disease where there is wide
464 variability in O_2 delivery and O_2 utilization responsiveness. Our high ICC values for k support the

465 suggestion, and that our pragmatic approach provides a reliable assessment of muscle oxidative
466 capacity suitable for clinical research or routine assessment in COPD patients.

467

468 *4.6 Limitations*

469 There are few limitations to report for this study. The CON group were slightly, but significantly
470 younger than the COPD patients (Table 1). It is possible that the younger age in CON may contribute
471 to the mean effect size of k in COPD (-0.3 min^{-1}). Nevertheless, the average difference in age was
472 small (5 years on an average age of 65 years), and is not the only variable that affects muscle
473 oxidative capacity. For example, physical activity, occupation, drug therapies and comorbidities are
474 expected to play a significant role in the etiology of loss of muscle oxidative capacity in COPD
475 (Maltais et al., 2014; Wagner, 2006).

476

477 Two COPD patients used nasal cannula O_2 delivery during the NIRS assessment. However, these
478 participants' data lay well within the range of the group as a whole (Δk was -0.05 and -0.13 min^{-1}).
479 We could identify no reason additional to treat these data differently from the group. In addition,
480 while baseline arterial O_2 saturation was measured by pulse oximetry, we found no influence of
481 SpO_2 on the reproducibility of k . The brief single leg plantar-flexion contractions were insufficient to
482 alter SpO_2 and we found no differences in muscle HbO_2 among the 13 participants with poor
483 reproducibility of k and the rest of the group. We therefore believe that arterial oxygenation did not
484 influence our results. However, this should be monitored in future studies to ensure that the
485 assumption of constant SpO_2 is met.

486

487 Finally, we did not assess oxidative capacity from muscle biopsy in this study in relation to NIRS
488 measures in these COPD patients. By their nature, NIRS assessment and muscle biopsy sample
489 different muscle regions, complicating direct comparison. Assessment of quadriceps oxidative
490 capacity by NIRS and biopsy was previously established in healthy young subjects ($r > 0.6$; Ryan et
491 al., 2014). Validation of accuracy of the NIRS assessment of muscle oxidative capacity in COPD
492 therefore awaits further investigation.

493

494 **5. CONCLUSION**

495 We found that a non-invasive NIRS-based assessment of oxidative capacity of *gastrocnemius* muscle
496 was well tolerated and reliable in middle-aged to elderly smokers with or without COPD. Our data

497 were consistent with direct assessment of muscle citrate synthase activity or oxidative capacity
498 from biopsy studies (Meyer et al., 2013) in that *gastrocnemius k* (a direct correlate of muscle
499 oxidative capacity) was 25% lower in COPD than in smoker controls without pulmonary obstruction.
500 We found high test-retest reliability of the NIRS oxidative capacity test in both COPD (CV = 9.9%;
501 ICC = 0.88) and CON groups (CV = 9.9%; ICC = 0.93). Our attempts to identify objective markers of
502 NIRS test quality were less successful: nevertheless, performance of repeated assessments in the
503 same visit can identify outlying results, and these were associated with small $\dot{m}\dot{V}O_2$ and
504 deoxygenation responses during dynamic contractions, and participants with a large skinfold or
505 high skin melanin. Together these data suggest that poor-quality assessments occur when the
506 exercise stimulus is insufficient for mitochondrial activation and/or the NIRS signal contains little
507 light reflected from active muscle. Low post-contraction TSI was unrelated to NIRS test reliability,
508 suggesting that O_2 supply is sufficient for NIRS test validity at least down to TSI of ~30% of the
509 individuals physiologic range. Therefore it is recommended to err towards a more intense exercise
510 rather than the maintenance of a high muscle oxygenation to optimize NIRS assessment of muscle
511 oxidative capacity. Our findings support the reliability of non-invasive muscle oxidative capacity
512 assessment by NIRS in COPD, which may be helpful to track the efficacy of interventions in COPD
513 such as pulmonary rehabilitation that are designed to redress skeletal muscle dysfunction.

514

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517 members of Rehabilitation Clinical Trials Center at Los Angeles Biomedical Research Institute for
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519

520 **CONFLICTS OF INTEREST**

521 No conflicts to declare.

522

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526

527

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- 647

648 **FIGURE CAPTIONS**

649

650 **Figure 1: Tissue saturation index (TSI, %) changes during the NIRS muscle assessment.** Panel
651 A. Protocol phases, and the parameter calculated from the analyses of TSI signal changes, are
652 indicated at the bottom of the graph. Grey shading indicates brief dynamic plantar-flexion exercise.
653 AO = arterial occlusion; *max* and *min* = highest and lowest TSI values during the Physiologic
654 Normalization (PN) phase; TSI_{LOW} = lowest saturation value reached during oxidative capacity
655 assessments (for further details see *Methods*). Panel B. Expansion of panel A to illustrate the linear
656 regression to determine the deflection point of muscle TSI (arrow) during the sustained arterial
657 occlusion (AO). Grey shading indicates brief dynamic plantar-flexion exercise.

658

659 **Figure 2: Representative COPD (A, B) and control (C, D) participants' responses during the**
660 **muscle oxidative capacity assessment.** Panels A and C show the TSI profiles during dynamic
661 exercise, and intermittent arterial occlusion during recovery. Panels B and D show the calculated
662 muscle $\dot{V}O_2$ recovery profiles and kinetic fit (dashed line). The letters (a-e) are given to illustrate
663 how the corresponding $m\dot{V}O_2$ value is derived from respective TSI negative slopes during
664 intermittent occlusions. The grey area (EX) indicates the brief dynamic exercise. τ (sec) is the $m\dot{V}O_2$
665 time constant determined by non-linear least-squares regression. k , is the rate constant, which is
666 linearly related to muscle oxidative capacity ($k = (1/\tau) \cdot 60, \text{min}^{-1}$).

667

668 **Figure 3: Muscle oxidative capacity (k) test-retest analyses.** Comparison of muscle k inferred from
669 the two repetitions of muscle $\dot{V}O_2$ recovery kinetics in COPD ($n = 28$) and controls, CON ($n = 28$).
670 Continuous line is the linear regression. Dotted line is the line of identity ($x = y$).

671

672 **Figure 4: Bland-Altman plot of the agreement between repeated measurements of muscle $\dot{V}O_2$**
673 **recovery rate constant (k).** Closed symbols are COPD patients ($n = 28$) and open symbols are controls
674 (CON, $n = 28$). Horizontal dashed lines indicate the 95% limits of agreement (range $-0.58, 0.64 \text{ min}^{-1}$).

675

676

677

678 **TABLES**

679

680 **Table 1:** Participant characteristics

	COPD	CON
Characteristics		
N.	28	28
Age (yrs)	65 (± 8)	60 (± 7) *
Weight (kg)	76 (± 15)	79 (± 17)
Height (cm)	171 (± 11)	170 (± 8)
BMI (kg/m^2)	26 (± 5)	27 (± 5)
Gender (M/F)	17/11	16/12
Race (AA/NHW)	5/23	15/13
FVC (L)	3.3 (± 0.9)	3.5 (± 0.8)
FEV ₁ (L)	1.8 (± 0.7)	2.8 (± 0.6)
FEV ₁ %pred	63.9 (± 23.4)	97.9 (± 13.6) *
SpO ₂ (%)	97 (± 1.6)	98 (± 1.1)
GOLD stage N. (1/2/3/4)	7/13/5/3	
Resting Muscle Characteristics		
Saturation (TSI) (%)	66 (± 6)	68 (± 5)
ATT (mm)	2.3 (± 1.9) ¶	2.8 (± 1.9) ¶

Data are mean (\pm SD). CON = controls; BMI = body mass index; M = male; F = female; AA = African American; NHW = Non Hispanic White; FVC = forced vital capacity; FEV₁ = forced expiratory volume in 1st second; SpO₂ = arterial oxygen saturation; TSI = tissue saturation index; ATT = adipose tissue thickness

* $p \leq 0.01$ vs. COPD patients; ¶ = COPD n=21; ¶ = CON n=18

681

682

683

684 **Table 2:** Reproducibility and coefficient of variation (CV) of *gastrocnemius* muscle oxidative
 685 capacity assessed by near-infrared spectroscopy, in smokers with and without COPD.

686

	<i>k</i> (min⁻¹)		CV	ICC	1st vs. 2nd rep
	1st rep	2nd rep			
COPD	1.45 (±0.38)	1.42 (±0.36)	9.85	0.88	N.S.
CON	1.75 (±0.70) *	1.72 (±0.70)	9.94	0.93	N.S.

Data are mean (±SD). CON = controls; *k* = proportional to muscle oxidative capacity;
 CV = coefficient of variation; ICC = intraclass correlation coefficient.

* $p \leq 0.05$ vs. COPD patients

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690 **Table 3:** Near-infrared spectroscopy tissue saturation index (TSI) variables of the *gastrocnemius*
 691 during post-contraction intermittent arterial occlusion. Mean and range of the saturation nadir
 692 (TSI_{LOW}, %) and relative peak muscle oxygen consumption ($m\dot{V}O_2$, % $\cdot s^{-1}$) during repeated tests in
 693 smokers with and without COPD.
 694

	1st repetition		2nd repetition		CV	
	TSI _{Low}	$m\dot{V}O_2$	TSI _{Low}	$m\dot{V}O_2$	TSI _{Low}	$m\dot{V}O_2$
	%	% s^{-1}	%	% s^{-1}	%	%
COPD	47.5	1.38	46.7	1.51	3.92	16.11
	(30.4 – 65.9)	(0.66 – 3.18)	(26.2 – 65.2)	(0.37 – 3.74)		
CON	47.4	1.71	42.2	1.49	5.25	17.65
	(6.5 – 67.8)	(0.20 – 10.60)	(12.6 – 69.8)	(0.20 – 6.55)		

Data are mean (range). CON = controls; TSI_{LOW} = lowest value reached by tissue saturation index during the arterial occlusions series; $m\dot{V}O_2$ = muscle oxygen consumption

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Figure 1

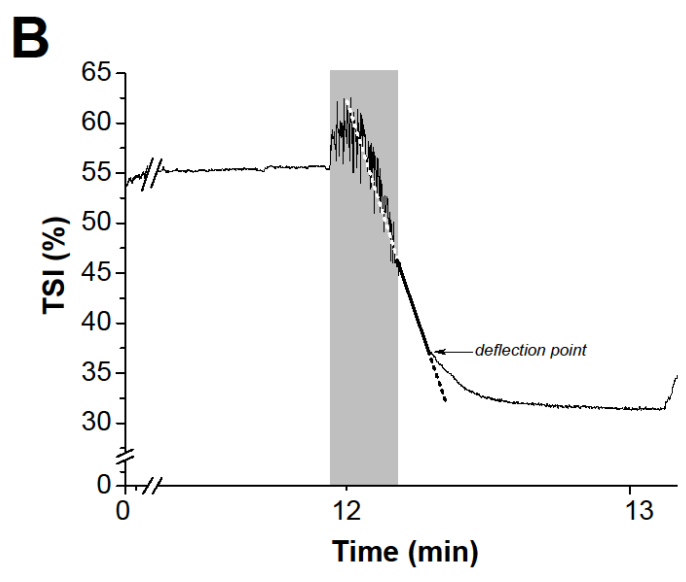
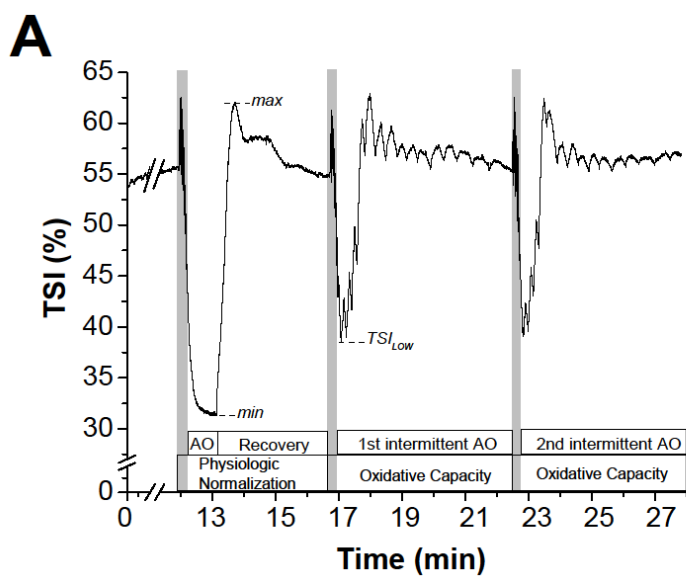


Figure 1

Figure 2

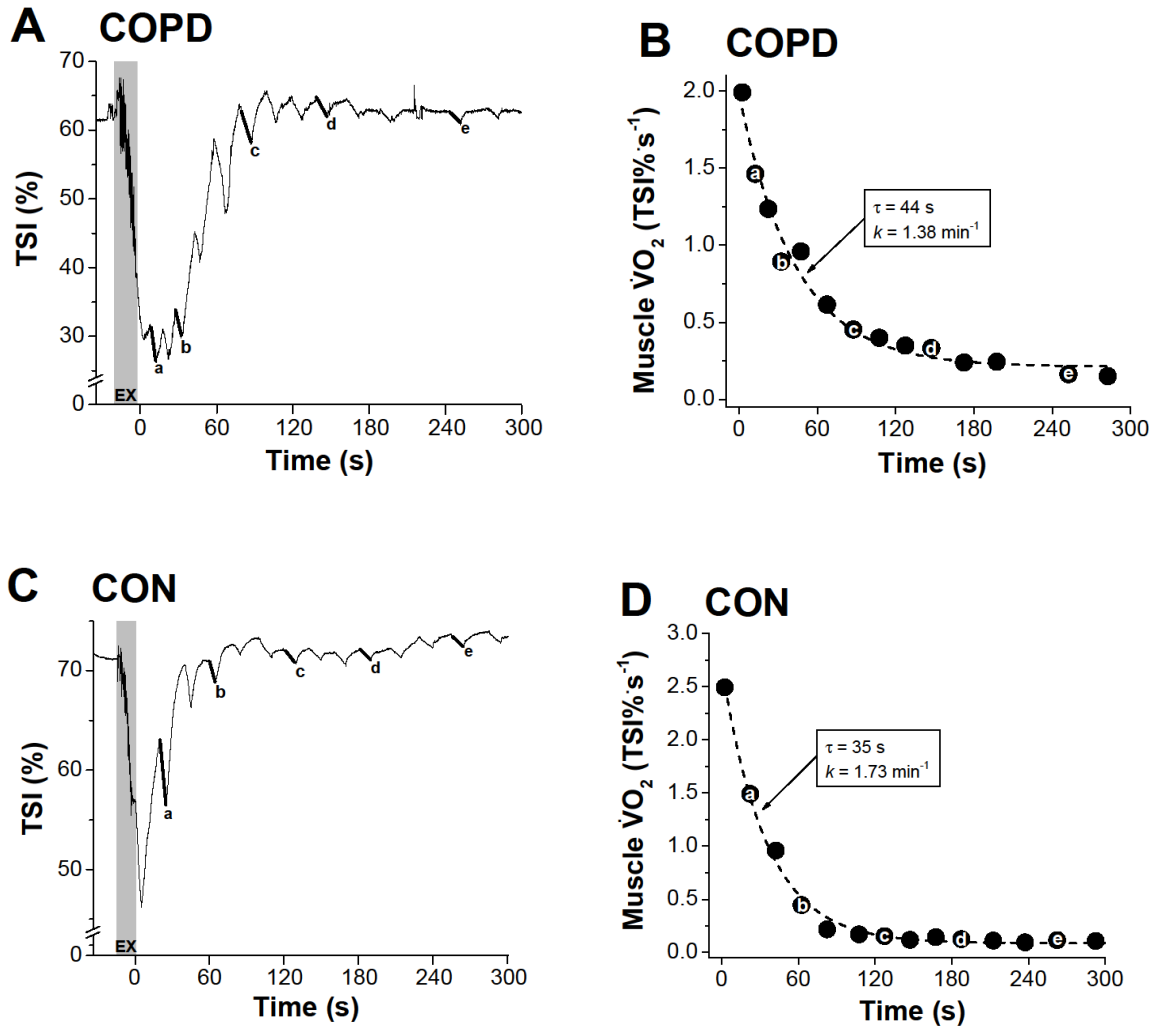


Figure 2

Figure 3

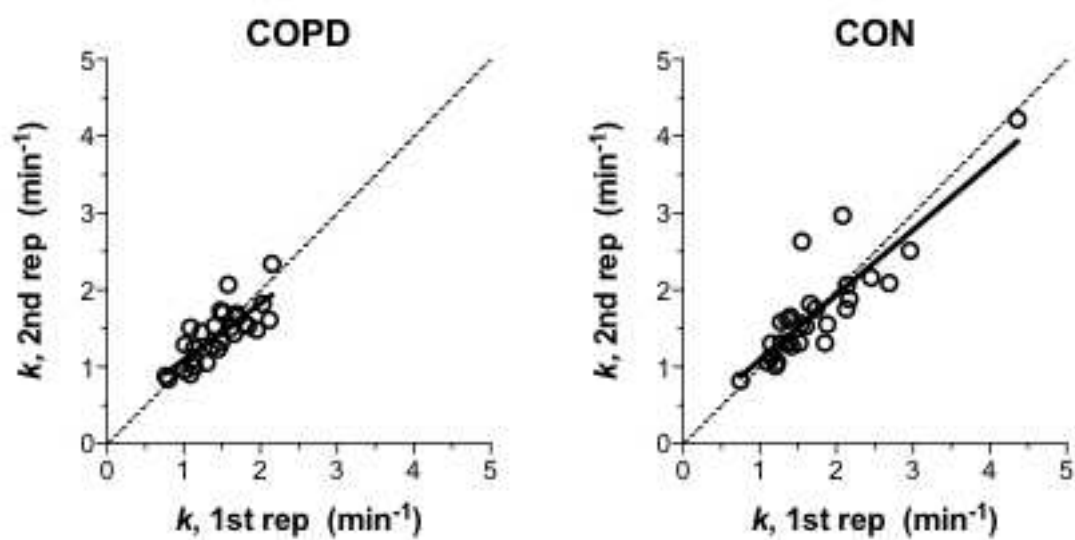


Figure 3

Figure 4

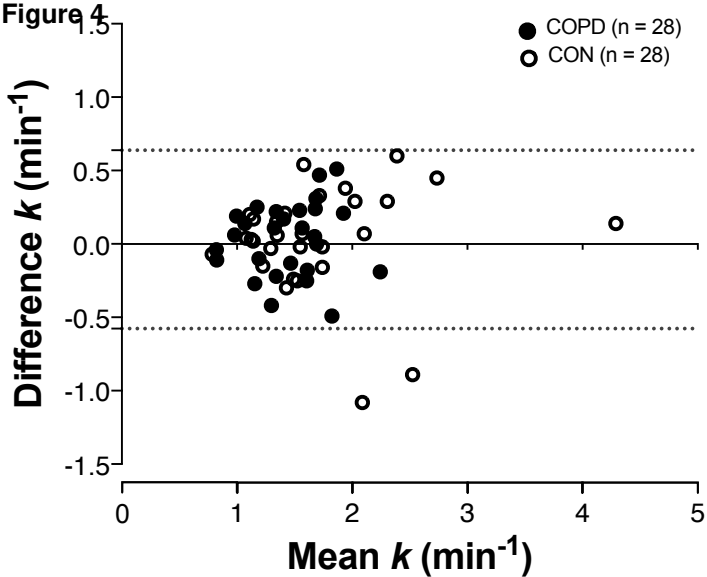


Figure 4