

1 **Muscle weakness precedes atrophy during cancer cachexia and is associated with muscle-**
2 **specific mitochondrial stress**

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38 **Abstract**

39 Muscle weakness and wasting are defining features of cancer-induced cachexia. Mitochondrial
40 stress occurs before atrophy in certain muscles, but distinct responses between muscles and across
41 time remains unclear. We aimed to determine the time-dependent and muscle-specific responses
42 to Colon-26 (C26) cancer-induced cachexia in mice. At 2 weeks post-inoculation, the presence of
43 small tumours did not alter body or muscle mass but decreased force production in the quadriceps
44 and diaphragm. Pyruvate-supported mitochondrial respiration was lower in quadriceps while
45 mitochondrial H₂O₂ emission was elevated in diaphragm. At 4 weeks, large tumours corresponded
46 to lower body mass, muscle mass, and cross-sectional area of fibers in quadriceps and diaphragm.
47 Force production in quadriceps was unchanged but remained lower in diaphragm vs control.
48 Mitochondrial respiration was increased while H₂O₂ emission was unchanged in both muscles vs
49 control. Mitochondrial creatine sensitivity was compromised in quadriceps. These findings
50 indicate muscle weakness precedes atrophy in quadriceps and diaphragm but is linked to
51 heterogeneous mitochondrial alterations. Eventual muscle-specific restorations in force and
52 bioenergetics highlight how the effects of cancer on one muscle do not predict the response in
53 another muscle. Exploring heterogeneous responses of muscles to cancer may reveal new
54 mechanisms underlying distinct sensitivities, or resistance, to cancer cachexia.

55 **Introduction**

56 Cancer-induced cachexia is a multifactorial syndrome characterized, in part, by a loss of skeletal
57 muscle mass that cannot be fully reversed by conventional nutritional support (1). This condition
58 leads to progressive reductions in functional independence and quality of life (2) . Such declines
59 in muscle mass also reduce tolerance to anticancer therapies and overall survivability (3, 4), and
60 is associated with increased hospitalization time (5). 20-80% of cancer patients are thought to
61 develop cachexia depending on the type and stage of cancer (6). However, the time-dependent
62 relationship between muscle atrophy and weakness remains unclear, as does the degree to which
63 this relationship may vary between muscle types. Exploring the natural divergence of muscle
64 responses to cancer may be an opportunistic approach to identify distinct mechanisms underlying
65 muscle weakness and wasting during cancer cachexia.

66

67 Contemporary theories posit that muscle wasting during cachexia is induced by circulating factors
68 generated during cancer which trigger protein degradation and loss of myofibrillar proteins
69 through various mechanisms (4, 7, 8). However, recent literature suggests skeletal muscle
70 mitochondria are also subject to damage during cancer cachexia (9–11) and may be direct
71 contributors to either muscle weakness or atrophy. Current literature suggests oxidative
72 phosphorylation is impaired in the soleus, gastrocnemius and plantaris muscle of tumour-bearing
73 mice, while reactive oxygen species (ROS) - in the form of mitochondrial H₂O₂ emission (mH₂O₂)
74 - can be increased or decreased depending on the muscle and duration of cancer (9, 11, 12). This
75 suggests cellular mechanisms contributing to muscle loss during cancer cachexia may be more
76 complicated than previously believed. Moreover, in the Lewis lung carcinoma (LLC) xenograft
77 mouse model, certain indices of skeletal muscle mitochondrial dysfunction preceded the onset of
78 muscle atrophy, suggesting mitochondria may be a potential therapeutic target (12). This theory
79 was supported by subsequent studies reporting positive effects of the mitochondria-targeting
80 compound SS-31 in preventing certain indices of cachexia in some but not all muscles of the C26
81 xenograft mouse model (13, 14). However, given the multifactorial contributions to cachexia
82 during cancer, it seems likely the relationship between mitochondria and myopathy may differ
83 between muscle type and throughout cancer progression.

84

85 Indeed, skeletal muscle mitochondria are known to be highly adaptable to metabolic stressors and
86 can super-compensate during an energy crisis (15, 16). In this light, the available literature does
87 not provide sufficient information to predict the extent to which cancer will affect individual
88 muscles, particularly in relation to their underlying mitochondrial responses to the systemic stress
89 of this disease. Understanding the time-dependent nature of unique mitochondrial signatures
90 during cancer-induced cachexia might better inform the development of mitochondrial therapies
91 that have so far yielded disparate results across various muscle types in the C26 cancer mouse
92 model (13, 14).

93

94 In this study, we compared the time-dependent relationship of muscle dysfunction and
95 mitochondrial bioenergetic responses to cancer between locomotor (quadriceps) and respiratory
96 muscles (diaphragm). In so doing, we employed a careful consideration of mitochondrial substrate
97 titration protocols modeling key parameters governing mitochondrial bioenergetics *in vivo*.
98 Similar assay design considerations have been essential for identifying precise mitochondrial
99 bioenergetic contributions to cellular function in our previous research (17–20). Using the C26
100 tumour-bearing mouse model, we reveal muscle weakness precedes atrophy in quadriceps and
101 diaphragm. Energetic insufficiencies were more pronounced in quadriceps whereas mitochondrial
102 redox stress was more evident in diaphragm, yet both muscles showed a delayed correction, if not
103 super-compensation, as cancer progressed. These findings demonstrate the effects of cancer on
104 one muscle do not necessarily predict the response in another muscle type. Moreover, the
105 heterogeneous muscle-specific and time-dependent mitochondrial relationships to cancer may
106 represent an opportunity for informing a more targeted approach to developing mitochondrial
107 therapies to improve muscle health in this debilitating disorder.

108 **Results**

109 **C26 tumour-bearing mice show progressive reductions in body weight and muscle mass**

110 Body weights were reduced 4 weeks after subcutaneous implantations of C26 cells (Figure 1A),
111 while tumour-free body weights progressively decreased beginning at 3 weeks to a net loss of 27%
112 by 4 weeks (Figure 1B, C) at a time of substantial tumour growth (Figure 1D). Tumours grew to
113 ~0.2g at 2 weeks and ~2.2g at 4 weeks (Figure 1E). C26 spleen mass (marker of inflammatory
114 stress) was not different from PBS at 2 weeks but was significantly greater at 4 weeks (Figure 1F).
115 The mass of specific muscles was similar between C26 and PBS at 2 weeks (Figure 1G). At 4
116 weeks soleus (SOL) mass was similar between C26 and PBS while lower muscle masses were
117 observed in C26 for extensor digitorum longus (EDL; -23%), plantaris (PLA; -20%), tibialis
118 anterior (TA; -26%), gastrocnemius (GA; -21%) and quadriceps (QUAD; -29%) vs PBS (Figure
119 1H).

120

121 **Force production is reduced prior to atrophy in quadriceps and diaphragm but eventually** 122 **returns to normal in quadriceps**

123 At 2 weeks, C26 muscle force was lower in quadriceps (Figure 2A) and diaphragm (Figure 2B)
124 relative to PBS as a group main effect. In the quadriceps, there was an interaction whereby C26 at
125 2 weeks produced less force at 80Hz, 100Hz and 120Hz compared to both PBS groups and the
126 C26 at 4 weeks (not shown). By 4 weeks, quadriceps force in C26 was not different compared to
127 PBS (Figure 2A). In contrast, diaphragm force remained lower relative to PBS control mice at 4
128 weeks (Figure 2B).

129

130 In both quadriceps and diaphragm, fibre CSA was similar between C26 and PBS groups for
131 specific MHC isoforms (Figure 3A-D) and when pooling all MHC isoforms (data not shown) at 2
132 weeks. However, at 4 weeks, quadriceps muscle exhibited lower CSA in pooled fibres (-40%,
133 $p < 0.05$, data not shown) with specific reductions in MHC IIX (-32%) and MHC IIB (-49%) but
134 not the MHC IIA isoform (Figure 3E, F) vs PBS. MHC I-positive fibres were not detected in the
135 quadriceps (Figure 3B, F). At 4 weeks, diaphragm muscle also showed lower CSA in pooled fibres
136 (-31%, $p < 0.05$, data not shown) which mirrored changes in MHC I (-28%), MHC IIA (-21%),
137 MHC IIB (-30%) and MHC IIX (-35%) vs PBS at 4 weeks (Figure 3G, H).

138

139 **Mitochondrial electron transport chain protein contents are reduced in quadriceps but do**
140 **not change in diaphragm by 4 weeks of tumour development**

141 At 2 weeks, electron transport chain (ETC) subunit contents in both muscles were unchanged in
142 C26 relative to PBS controls (Figure 4A, B). At 4 weeks, C26 showed lower contents in subunits
143 of complex I (-31%), complex II (-18%), complex IV (-37%), complex V (-11%) and total ETC
144 subunit content (-22%; Figure 4C) relative to PBS that were significant or approached significance.
145 ETC subunit contents did not change in diaphragm at 4 weeks relative to PBS (Figure 4D).

146

147 **Mitochondrial respiratory control by ADP is greater in both muscles by 4 weeks of tumour**
148 **development despite early reductions in the quadriceps**

149 We determined if the central role of ADP in stimulating respiration was impaired in both
150 quadriceps and diaphragm at 2 and 4 weeks after subcutaneous implantations of C26 cancer cells.
151 We stimulated complex I with NADH generated by pyruvate (5mM) and malate (2mM) across a
152 range of ADP concentrations to challenge mitochondria with a spectrum of metabolic demands.
153 The ADP titrations were repeated with (+Creatine) and without (-Creatine) 20mM creatine to
154 model the two main theoretical mechanisms of energy transfer from mitochondria to cytosolic
155 compartments that utilize or bypass mitochondrial creatine kinase (mtCK) respectively (Figure 5).
156 Briefly, the +Creatine system stimulates mitochondria to export phosphocreatine (PCr) whereas
157 the -Creatine condition drives ATP export.

158

159 In both the -Creatine and +Creatine conditions, pyruvate/malate-supported ADP-stimulated
160 respiration normalized per mass of fibre bundles (not corrected for ETC subunit content) was lower
161 2 weeks after C26 implantations in the quadriceps compared to PBS with a main effect across all
162 ADP concentrations with or without creatine (Figure 6A, B). The general reduction in respiration
163 for C26 normalized per mass of fibre bundle was also seen when data were normalized to ETC
164 subunit content (Figure 6C, D). This suggests respiratory control was reduced within mitochondria
165 due to an inherent property of the ETC not related to ETC abundance. We also evaluated if creatine
166 sensitivity was altered in the C26 tumour-bearing muscle by calculating the +Creatine/-Creatine
167 respiratory ratio. This creatine sensitivity index is a measure of the ability of creatine to stimulate
168 respiration by accelerating matrix ADP/ATP cycling and represents coupling of the creatine kinase
169 system to ATP generation (Figure 5), particularly at sub-maximal ADP concentrations (21, 22)

170 which we have reported previously (17, 18). In quadriceps, creatine sensitivities at 100 μ M and
171 500 μ M ADP were unchanged at 2 weeks in C26 vs PBS at this time point (Figure 6E, F).
172 Collectively, these findings indicate respiration was reduced to similar extents in both -Creatine
173 and +Creatine conditions of energy exchange between mitochondria and cytoplasmic
174 compartments.

175
176 These early decrements in quadriceps respiration in C26 at 2 weeks were reversed by 4 weeks.
177 This apparent compensation was seen in both -Creatine and +Creatine conditions. Specifically,
178 respiration was similar to PBS control mice at 4 weeks when normalized per mass of fibre bundle
179 (Figure 6G, H) and higher than controls when normalized to ETC subunit protein content (Figure
180 6I, J) despite reductions in ETC content as described above (Figure 4C). These findings suggest
181 mitochondria in quadriceps are highly plastic and can super-compensate by upregulating their
182 responsiveness to ADP to levels exceeding PBS controls. Additionally, at 4 weeks, quadriceps
183 mitochondrial creatine sensitivity was impaired in C26 relative to PBS when considering
184 respiration normalized to ETC subunit content given the ratio did not exceed a value of 1.0 which
185 indicates that creatine could not stimulate respiration above the level elicited by ADP alone (Figure
186 6L). Thus, while C26 cancer strongly increased ADP-stimulated respiration by 4 weeks (Figure
187 6I), it compromised the coupling of creatine kinase energy transfer, suggesting that this system did
188 not contribute to restored force at this time point (Figure 2A).

189
190 In the diaphragm, respiration was similar between C26 and PBS at 2 weeks (Figure 7A-F) but was
191 greater in C26 vs PBS at 4 weeks in both the -Creatine and +Creatine conditions (Figure 7G-J).
192 This upregulation by 4 weeks occurred despite no changes in ETC subunit content as noted above
193 (Figure 4D) which suggests mitochondria increase their responsiveness to ADP through
194 mechanisms that may be independent of mitochondrial content. No changes in creatine sensitivity
195 were observed in C26 vs PBS at 4 weeks (Figure 7K, L) suggesting that coupling of creatine kinase
196 to ATP generation was maintained, in contrast to impaired creatine sensitivity seen in the
197 quadriceps as noted above. Lastly, there was a significant interaction whereby respiration was
198 greater in C26 vs PBS at 5000 μ M and 7000 μ M ADP when normalized per mass of fibre bundles
199 (Figure 7G, H) and at all ADP concentrations except 25 μ M and 100 μ M when normalized to total
200 ETC subunit content (Figure 7I, J).

201

202 These alterations were specific to pyruvate/malate-supported ADP-stimulated respiration as there
203 were no changes in respiration in response to glutamate (further NADH-generation) and succinate
204 (FADH₂) generation when comparing C26 to PBS at either time point (Figure S1, S2). By 2 weeks
205 of C26, diaphragm showed a decrease in State II respiration (no ADP, supported by
206 pyruvate/malate; Figure S1, S2) which is generally used as a marker of respiration driven by proton
207 leak into the matrix from the inner membrane space through various sites that are not coupled to
208 ATP synthesis (23). However, State II respiration was greater than control by 4 weeks in both
209 muscles suggesting greater uncoupling at occurs as cancer progresses (Figure S2). Lastly, changes
210 in respiration noted above did not result in changes to the phosphorylation of AMPK in C26
211 relative to PBS at either 2- or 4-week timepoints (Figure 8); albeit increases in AMPK and the p-
212 AMPK/AMPK ratio were trending in the C26 (4wk) group in the quadriceps.

213

214 **H₂O₂ emission is increased in diaphragm early during tumour development and restored to**
215 **normal by 4 weeks but is unaffected in quadriceps**

216 We stimulated complex I with pyruvate (10mM) and malate (2mM) to generate NADH in the
217 absence of ADP to elicit mH₂O₂ emission and determined ADP's ability to attenuate this emission
218 as occurs naturally during oxidative phosphorylation (see schematic representation, Figure 5). At
219 2 weeks following C26 implantations, quadriceps mH₂O₂ emission was similar to PBS controls
220 under maximal emission conditions (no suppression by ADP, State II; Figure 9A, C) and during
221 suppression by ADP (Figure 9B, D). By 4 weeks of C26 growth, quadriceps mH₂O₂ was lower
222 than PBS in both maximal and ADP-suppressive states (Figure 9E, F). However, when mH₂O₂
223 was normalized to total ETC subunit content, no differences were observed between C26 and PBS
224 (Figure 9G, H). This finding suggests eventual decreases in quadriceps mH₂O₂ by 4 weeks were
225 related to decreased ETC subunit content as shown in Figure 4. Due to limited tissue availability,
226 pyruvate-supported mH₂O₂ was assessed only in the +Creatine condition.

227

228 In contrast to the lower mH₂O₂ in quadriceps, diaphragm mH₂O₂ was greater in C26 mice at 2
229 weeks relative to PBS in the presence of ADP despite no change in maximal mH₂O₂ (Figure 9I,
230 J). This finding reveals C26 causes early elevations in diaphragm mH₂O₂ that are likely due to a
231 specific impairment in the ability of ADP to attenuate H₂O₂ emission. Moreover, when mH₂O₂

232 emission was normalized to total ETC subunit content at 2 weeks, maximal mH_2O_2 emission
233 remained unchanged (Figure 9K), while the higher emissions in the presence of ADP did not reach
234 significance (Figure 9L) but mirrored patterns observed when normalized to wet mass of tissue as
235 noted above. At 4 weeks, there were no differences in diaphragm mH_2O_2 under maximal or
236 submaximal (presence of ADP) conditions using either normalization approach (Figure 9M-P)
237 suggesting diaphragm mitochondria are plastic and can eventually restore mH_2O_2 to normal levels.
238
239 Succinate-supported mH_2O_2 emission generally did not change in either muscle in C26 vs PBS at
240 either time point (Figure S3). This finding suggests reverse electron flux to Complex I from
241 Complex II (23) was not altered by C26 cancer, and the responses mentioned above using
242 pyruvate/malate reveal a specific alteration in mH_2O_2 emission supported by forward electron flux
243 through Complex I.

244 **Discussion**

245 Certain indices of skeletal muscle mitochondrial dysfunction have been associated with cancer
246 cachexia in various mouse models (11–13, 24, 25), but the time- and muscle-dependent
247 relationship remains unclear. Here, we demonstrate how quadriceps and diaphragm have both
248 shared and distinct time-dependent responses to cancer in the C26 colon carcinoma mouse model
249 of cancer cachexia. First, weakness was observed prior to atrophy in both muscles, yet an eventual
250 increase in force production to control levels occurred only in quadriceps. Second, atrophy in most
251 fibre types was preceded by altered mitochondrial bioenergetics but the specific relationship
252 differed between muscles with decreases in respiration occurring in quadriceps in contrast to
253 elevated mH_2O_2 emission in diaphragm. Third, both muscles upregulated mitochondrial
254 respiration supported specifically by pyruvate and malate substrates at 4 weeks which may reflect
255 a hormetic adaptation to maintain energy homeostasis during cachexia. Likewise, the diaphragm
256 restored Complex I-supported mitochondrial H_2O_2 emission to normal lower levels by 4 weeks
257 which demonstrates the transient nature of this potential redox pressure.

258

259 Collectively, these findings suggest muscle weakness can occur before atrophy during C26 cancer,
260 and this progression is related to dynamic time-dependent changes in mitochondrial bioenergetics
261 that are unique to each muscle.

262

263 **Mitochondrial bioenergetic alterations and skeletal muscle force reductions precede**
264 **skeletal muscle atrophy**

265 Work from *Brown* et al. suggested mitochondrial degradation and dysfunction precedes muscle
266 atrophy in the LLC xenograft mouse model of cancer cachexia (12). In this study, atrophy markers
267 occurred after earlier indices of mitochondrial degeneration in comparator muscles (flexor
268 digitorum brevis and plantaris) including mitochondrial degradation, respiratory control ratios and
269 H_2O_2 emission. The findings of the present study support this proposal with a comparison of
270 atrophy, mitochondrial respiration and mH_2O_2 emission within the same muscle types, namely
271 quadriceps and diaphragm. These findings also extend the proposal by showing muscle-specific
272 mitochondrial alterations occur concurrent to muscle weakness and before atrophy. Specifically,
273 early decreases in respiratory kinetics in quadriceps were not seen in diaphragm suggesting that
274 more oxidative muscle might avoid such respiratory decrements. Conversely, early increases in

275 mH₂O₂ emission seen in diaphragm did not occur in quadriceps. These relationships suggest
276 targeted therapies to counter mitochondrial alterations during cancer cachexia should consider the
277 specific bioenergetic function that is altered at precise timepoints in each muscle type.

278
279 This relationship between early mitochondrial stress prior to atrophy in both muscles becomes
280 further complex when considering force production. Muscle weakness occurred at 2 weeks in both
281 muscles before atrophy which highlights a shared pattern in the progression of muscle dysfunction
282 during cancer. While the purpose of this investigation was not to address other mechanisms
283 regulating force production, reduced fibre sizes cannot be an explanation given atrophy did not
284 occur until after weakness was first observed. However, the distinct mitochondrial signatures in
285 both muscles at 2 weeks could guide additional questions. For example, in the quadriceps, the early
286 reductions in force were associated with early decreases in mitochondrial respiratory control by
287 ADP. When force production was restored to control levels by 4 weeks, respiration actually
288 increased above control levels when normalized to ETC subunit content. This dynamic relationship
289 is intriguing and suggests early quadriceps weakness might be due to impairments in mitochondrial
290 energy provision that is nonetheless plastic and capable of adapting – possibly as a hormetic
291 response to the earlier respiratory deficiency – to correct this weakness through super-
292 compensations in energy supply.

293
294 In contrast, the diaphragm weakness seen at 2 weeks might be linked to an early redox pressure
295 given elevated mH₂O₂ emission was observed. This observation is consistent with prior
296 observations of early and transient increases in H₂O₂ emission in diaphragm in the LLC mouse
297 model of cancer cachexia (24). However, while we did not observe lower respiration in the
298 diaphragm, the increased respiration seen at 4 weeks in this muscle is surprising. The explanation
299 for this increase is not apparent but might suggest an earlier energetic deficiency occurred outside
300 of our selected time points, but this is speculative and would require additional examination.

301
302 Overall, while the precise mechanism of lower muscle force is not apparent at 2 weeks in both
303 muscles, the possible contributions of mitochondria could be related more to an early energy crisis
304 in quadriceps vs a redox stress in diaphragm that, as noted above, also preceded the eventual
305 atrophy of each muscle (Figure 10). Additional insight could be gained by extending the current

306 investigation's focus on fibre type-specific cross sectional area responses to cancer by comparing
307 a wider spectrum of fibre types with regards to mitochondria-atrophy relationships.

308

309 **Perspectives on the potential for mitochondrial hormesis in quadriceps and diaphragm**

310 The findings of lower respiration and increased mH_2O_2 emission at 2 weeks is consistent with prior
311 reports at various time points and muscle in the LLC, C26 and peritoneal carcinosis mouse models
312 (11–13, 24, 25). To our knowledge, the eventual increase in pyruvate-supported respiration seen
313 in both quadriceps and diaphragm in the present study is novel, while, the attenuation of mH_2O_2
314 emission seen in the diaphragm is consistent with past reports in the plantaris (12) and diaphragm
315 (24) in the LLC mouse model of cachexia. As noted above, mitochondrial respiratory control by
316 ADP increased above control in both muscles despite a stress being observed earlier only in
317 quadriceps. We questioned whether this early reduction in respiration represented an energy crisis
318 triggering compensatory signaling through the energy sensor AMPK – a pathway that triggers
319 compensatory mitochondrial biogenesis or upregulation of substrate-specific oxidation (26). We
320 did not observe an effect of cancer on AMPK phosphorylation at either time point (Figure 8),
321 although there was a trend in the quadriceps at 4 weeks of tumour bearing whereby AMPK content
322 was increased. Nonetheless, these results do not rule out the potential for AMPK activation at other
323 time points. There are also multiple feedback control systems linking metabolic stress to
324 respiratory control that are independent of AMPK which might be considered in future
325 investigations (27).

326

327 The design of substrate titration protocols lends insight into the specific mechanisms by which
328 respiration and mH_2O_2 become altered during cancer. For example, as pyruvate/malate was used
329 as the substrates to generate NADH to stimulate complex I-supported respiration, future
330 investigations might consider the potential for cancer to upregulate pyruvate dehydrogenase
331 activity, albeit maximal activity given saturating pyruvate concentrations were used. Also, the
332 consistent increase in respiration across a wide spectrum of ADP concentrations by 4 weeks in
333 both muscles suggests mitochondrial responsiveness to a wide range of metabolic demands may
334 have been enhanced such that key regulators of matrix ADP/ATP cycling could be considered for
335 future directions (Figure 5). ADP was also more effective at attenuating mH_2O_2 (23) in quadriceps
336 by 4 weeks (Figure 9) which supports this possibility. Collectively, these findings suggest cancer

337 disrupts mitochondrial bioenergetics by specifically desensitizing mitochondria to ADP in both
338 muscles.

339

340 Mitochondrial creatine metabolism appeared to be less capable of adapting in quadriceps by 4
341 weeks (Figure 6L) suggesting mitochondrial creatine kinase-dependent phosphate shuttling is
342 more affected in this muscle than diaphragm which showed no such deficiency. In fact, the
343 creatine-independent (-Creatine) system showed homogeneous plasticity by upregulating in both
344 muscles by 4 weeks while the creatine-dependent system upregulated only in the diaphragm. These
345 findings suggest mitochondrial creatine metabolism may be disrupted in quadriceps muscle during
346 cancer which may impact energy homeostasis given the importance of this system in certain
347 muscles (21).

348

349 In general, the diaphragm appeared to be superior to quadriceps with respect to maintaining
350 mitochondrial ETC content markers and respiratory control by ADP at 2 weeks with evidence of
351 super-compensation in respiratory function at 4 weeks. Furthermore, reductions in ETC protein
352 contents were observed in quadriceps at 4 weeks after C26 implantation whereas no changes were
353 observed in diaphragm. This resilience of diaphragm appears to be a unique finding given prior
354 reports have also shown lower mitochondrial protein markers from various pathways and muscle
355 types in LLC and *APC*^(Min/+) mouse models of cancer cachexia (10). While the mechanisms for this
356 muscle heterogeneity remain unclear, one possibility relates to muscle contractile activity.
357 Diaphragm constantly contracts *in vivo* whereas quadriceps is used only during locomotion. As
358 mitochondrial content and substrate oxidation are regulated by contractile activity (27), future
359 directions might consider whether the diaphragm holds a special mitochondrial ‘resistance’ to
360 cancer with respect to energy homeostasis which might support the growing notion of chronic
361 contractile activity in improving muscle health during cancer (28).

362

363 In conclusion, this investigation reports muscle weakness precedes atrophy of quadriceps and
364 diaphragm in the C26 colon carcinoma mouse model of cancer cachexia. This progression was
365 associated with heterogenous muscle-specific and time-dependent mitochondrial responses in both
366 muscles. Specifically, an early energetic stress (impaired respiratory control by ADP) was more
367 apparent in quadriceps in contrast to a mitochondrial redox pressure in diaphragm. These early

368 mitochondrial stressors were seemingly corrected as cancer progressed despite the development
369 of atrophy in both muscles and a unique increase in force production in quadriceps. Moreover,
370 C26 cancer caused a unique impairment in the coupling of the mitochondrial creatine kinase
371 system to ATP generation in quadriceps whereas this system was not affected in diaphragm. This
372 dynamic plasticity across time demonstrates how the effects of cancer on one muscle may not
373 predict the response in another muscle type. The findings also highlight how understanding
374 heterogeneity may identify mechanisms that determine whether a given muscle might be sensitive,
375 or resistant, to cancer cachexia.

376

377

378 **Methods**

379 **Animal Care**

380 48 eight-week-old male CD2F1 mice were purchased from Charles River (Massachusetts, USA).
381 Upon arrival, mice were housed and given a minimum of 72 h to acclimatize before cancer
382 implantations. All mice were provided access to standard chow and water ad libitum as differences
383 in food intake has been shown to not impact the C26 model of cancer cachexia (29). Mice were
384 monitored daily for general well-being, tumour ulcerations and tumour size. If mice demonstrated
385 signs of extreme distress, mice would be sacrificed as soon as possible, however, this was never
386 required.

387

388 **C26 Cell Culture and Tumour Implantation**

389 C26 cancer cells (Purchased from NCI – Frederick, MD USA) were plated at passage 2-3 in T-75
390 flasks in DMEM supplemented with 10% foetal bovine serum plus 1% penicillin and streptomycin.
391 Once confluent, cells were trypsinized, counted and diluted in PBS. C26 cells (5×10^5) suspended
392 in 100 μ L sterile PBS were implanted subcutaneously to both flanks of mice at 8 weeks of age
393 (11). For control, mice received identical subcutaneous injections of 100 μ L sterile PBS and aged
394 for 2 weeks (**PBS (2wk); n= 8**) and 4 weeks (PBS (4wk); n= 16). Tumours developed for 14-17
395 days (C26 (2wk); n= 8) and 26-29 days (C26 (4wk); n= 16). Tumours were measured daily,
396 recording the length and width of tumours with digital calipers using the following formula to
397 obtain tumour volume (volume of a sphere): $(4/3 * \pi * (\text{length}/2) * (\text{width}/2)^2)$ in accordance with
398 York University Animal Care Committee guidelines. The same investigator was responsible for

399 measuring tumour sizes throughout the length of the study as preliminary work demonstrated that
400 tumour size measurements can vary between individuals (data not shown; CV – 7.2% between 3
401 individuals, CV – 1.3 within designated individual).

402

403 **Surgery Procedure**

404 Quadriceps, soleus, plantaris, gastrocnemius, tibialis anterior, extensor digitorum longus and
405 spleen were quickly collected under isoflurane anesthesia prior to euthanasia. Tissues were
406 weighed and snap-frozen in liquid nitrogen and stored at -80°C. Quadriceps and diaphragm
407 muscles were placed in BIOPS containing (in mM) 50 MES Hydrate, 7.23 K₂EGTA, 2.77
408 CaK₂EGTA, 20 imidazole, 0.5 dithiothreitol, 20 taurine, 5.77 ATP, 15 PCr, and 6.56 MgCl₂·6 H₂O
409 (pH 7.1) to be prepared for mitochondrial bioenergetic assays. Quadriceps from one leg and
410 diaphragm strips were harvested for mitochondrial bioenergetic assays while the quadriceps from
411 the contracted leg and a separate diaphragm strip were used for force measurements. The
412 diaphragm strip used for force measurements was cut within 30 seconds of the entire diaphragm
413 being placed in BIOPS prior to transferring the strip to Ringer's solution as noted below.

414

415 ***In Situ* Quadriceps Force and *In Vitro* Diaphragm Force**

416 *In situ* force production for quadriceps muscle was partially adapted from previous literature (30).
417 Mice were anesthetized with isoflurane and shaved of all hair on their hindlimb. An incision was
418 made above the patella to expose the femoral tendon which was then tightly secured with suture.
419 Once the knot was in place, the tendon was carefully severed, and the suture was attached to an
420 Aurora Scientific 305C muscle lever arm with a hook (Aurora, Ontario, Canada). The knee was
421 secured with a vertical knee clamp immobilizing the knee joint with a 27G needle. Contraction of
422 the quadriceps was controlled through percutaneous stimulation of the femoral nerve anterior to
423 the hip joint. Optimal resting length (L_o) was determined using single twitches (pulse width =
424 0.2ms) at varying muscle lengths. Once L_o was established, force as a function of stimulation
425 frequency was measured during 8 isometric contractions at varying stimulation frequencies (1, 20,
426 40, 60, 80, 100, 120, 140 Hz). The quadriceps muscle was then weighed and used for normalization
427 of force production.

428

429 *In vitro* force production for diaphragm muscle was partially adapted from previous literature (31).
430 Briefly, the diaphragm strip used for force production was placed in a petri dish of ~25°C Ringer's
431 solution containing (in mM): 121 NaCl, 5 KCl, 1.8 CaCl₂, 0.5 MgCl₂, 0.4 NaHPO₄, 24 NaHCO₃,
432 5.5 glucose and 0.1 EDTA; pH 7.3 oxygenated with 95% O₂ and 5% CO₂. Diaphragm strips were
433 cut from the central region of the lateral costal hemidiaphragm. Silk suture was tied to the central
434 tendon as well the ribs, and the preparation was transferred to an oxygenated bath filled with
435 Ringer solution, maintained at 25°C. The suture secured to the central tendon was then attached to
436 a lever arm while the suture loop secured to the ribs was attached to a force transducer. The
437 diaphragm strip was situated between flanking platinum electrodes driven by a biphasic stimulator
438 (Model 305C; Aurora Scientific, Inc., Aurora, ON, Canada). Optimal L_o was determined using
439 twitches (pulse width = 0.2ms) at varying muscle lengths. Once L_o was established, force as a
440 function of stimulation frequency was measured during 10 isometric contractions at varying
441 stimulation frequencies (1, 10, 20, 40, 60, 80, 100, 120, 140, 200 Hz). Force production was
442 normalized to the calculated cross-sectional area (CSA) of the muscle strip (m/l*d) where m is the
443 muscle mass, l is the length, and d is mammalian skeletal muscle density (1.06mg.mm³).

444

445 **Mitochondrial Bioenergetic Assessments**

446 *Preparation of Permeabilized Muscle Fibres.* The assessment of mitochondrial bioenergetics was
447 performed as described previously in our publications (17, 19, 32). Briefly, the quadriceps and
448 diaphragm from the mouse was removed and placed in BIOPS. Muscle was trimmed of connective
449 tissue and fat and divided into small muscle bundles (~1.2 – 3.7 mg wet weight for quadriceps and
450 0.6 – 2.1 mg for diaphragm). Each bundle was gently separated along the longitudinal axis to form
451 bundles that were treated with 40 µg/mL saponin in BIOPS on a rotor for 30 min at 4°C. Following
452 permeabilization, the permeabilized muscle fibre bundles (PmFB) for respiration assessments
453 were blotted and weighed in ~ 1.5mL of tared pre-chilled BIOPS (muscle relaxing media) to ensure
454 PmFB remained relaxed and hydrated rather than exposed to open air. Wet weights were used
455 given small pieces of muscle can detach during respirometry assessments, albeit greatly reduced
456 by blebbistatin (described below). Mean ± SEM wet weights (mg) were 2.4 ± 0.07 for quadriceps
457 and 1.3 ± 0.04 for diaphragm. The remaining PmFB for mH₂O₂ were not weighed at this step as
458 this data was normalized to fully recovered dry weights taken after the experiments. All PmFB
459 were then washed in MiRO5 on a rotator for 15 minutes at 4°C to remove the cytoplasm. MiRO5

460 contained (in mM) 0.5 EGTA, 10 KH₂PO₄, 3 MgCl₂•6H₂O, 60 K-lactobionate, 20 Hepes, 20
461 Taurine, 110 sucrose, and 1 mg/ml fatty acid free BSA (pH 7.1).

462

463 *Mitochondrial Respiration.* High-resolution O₂ consumption measurements were conducted in 2
464 mL of respiration medium (MiRO5) using the Oroboros Oxygraph-2k (Oroboros Instruments,
465 Corp., Innsbruck, Austria) with stirring at 750 rpm at 37°C. MiRO5 contained 20 mM Cr to
466 saturate mitochondrial creatine kinase (mtCK) and promote phosphate shuttling through mtCK or
467 was kept void of Cr to prevent the activation of mtCK (33) as described in Figure 5. For ADP-
468 stimulated respiratory kinetics, our previously published procedures to stimulate complexes I and
469 II-supported respiration were employed (17–19). 5 mM pyruvate and 2 mM malate were added as
470 complex I-specific substrates (via generation of NADH to saturate electron entry into complex I)
471 followed by a titration of sub-maximal ADP (25, 100 and 500 μM) and maximal ADP (up to 5000
472 μM in the presence of Cr or 30000 μM in the absence of Cr). 25 μM and 100 μM are close to low
473 and high points of previous estimates of free ADP concentrations in human skeletal muscle in
474 resting and high intensity exercise states and therefore allow the determination of mitochondrial
475 responsiveness to a physiological spectrum of low to high energy demands (34–38). Saturating
476 [ADP] were different depending on the muscle and presence or absence of creatine in the
477 experimental media. Mitochondrial respiration was normalized to mass of fibre bundles as well as
478 total ETC subunit contents to evaluate whether changes in respiration per mass were due to
479 alterations in mitochondrial content or intrinsic mitochondrial respiratory responses.

480

481 Kmapp for creatine to ADP was not established as we have observed that many permeabilized
482 fibers from past studies do not fit Michaelis-Menten kinetics with these assay conditions (low to
483 modest R²). Creatine accelerates matrix ADP/ATP cycling at submaximal [ADP] and lowers the
484 Kmapp for ADP in some muscles (21, 33). Therefore, in order to evaluate mitochondrial creatine
485 sensitivity, 100 and 500 μM ADP were used to calculate a creatine sensitivity index. Following
486 the ADP titration, cytochrome *c* was added to test for mitochondrial membrane integrity. Finally,
487 succinate (20 mM) was then added to saturate electron entry into Complex II.

488

489 All experiments were conducted in the presence of 5 μM blebbistatin (BLEB) in the assay media
490 to prevent spontaneous contraction of PmFB, which has been shown to occur in response to ADP

491 at 37°C that alters respiration rates (33, 39). Polarographic oxygen measurements were acquired
492 in 2 second intervals with the rate of respiration derived from 40 data points and expressed as
493 pmol/s/mg wet weight. PmFB were weighed in ~1.5 mL of tared BIOPS to relax muscle as noted
494 above.

495
496 *Mitochondrial H₂O₂ Emission (mH₂O₂)*. mH₂O₂ was determined spectrofluorometrically
497 (QuantaMaster 40, HORIBA Scientific, Edison, NJ, USA) in a quartz cuvette with continuous
498 stirring at 37°C, in 1 mL of Buffer Z supplemented with 10 µM Amplex Ultra Red, 0.5 U/ml
499 horseradish peroxidase, 1mM EGTA, 40 U/ml Cu/Zn-SOD1, 5 µM BLEB and 20mM Cr. Buffer
500 Z contained (in mM) 105 K-MES, 30 KCl, 10 KH₂PO₄, 5 MgCl₂ • 6H₂O, 1 EGTA, and 5mg/mL
501 BSA (pH 7.4). State II mH₂O₂ (maximal emission in the absence of ADP) was induced using the
502 Complex I-supporting substrates (NADH) pyruvate (10mM) and malate (2mM) to assess maximal
503 (State II, no ADP) mH₂O₂ as described previously (18). Following the induction of State II mH₂O₂,
504 a titration of ADP was employed to progressively attenuate mH₂O₂ as occurs when membrane
505 potential declines during oxidative phosphorylation (Figure 5). After the experiments, the fibers
506 were rinsed in double deionized H₂O, lyophilized in a freeze-dryer (Labconco, Kansas City, MO,
507 USA) for > 4h and weighed on a microbalance (Sartorius Cubis Microbalance, Gottingen
508 Germany). The rate of mH₂O₂ emission was calculated from the slope (F/min) using a standard
509 curve established with the same reaction conditions and normalized to fibre bundle dry weight.

510

511 **Western Blotting**

512 A frozen piece of quadriceps and diaphragm from each animal was homogenized in a plastic
513 microcentrifuge tube with a tapered Teflon pestle in ice-cold buffer containing (mm) 20 Tris/HCl,
514 150 NaCl, 1 EDTA, 1 EGTA, 2.5 Na₄O₇P₂, 1 Na₃VO₄, 1% Triton X-100 and PhosSTOP inhibitor
515 tablet (Millipore Sigma, Burlington, MA, USA) (pH 7.0) as published previously (40). Protein
516 concentrations were determined using a bicinchoninic acid (BCA) assay (Life Technologies,
517 Carlsbad, CA, USA). 15-30 µg of denatured and reduced protein was subjected to 10-12% gradient
518 SDS-PAGE followed by transfer to low-fluorescence polyvinylidene difluoride membrane.
519 Membranes were blocked with Odyssey Blocking Buffer (LI-COR, Lincoln NE, USA) and
520 immunoblotted overnight (4°C) with antibodies specific to each protein. A commercially available
521 monoclonal antibody was used to detect electron transport chain proteins (rodent OXPHOS

522 Cocktail, ab110413; Abcam, Cambridge, UK, 1:250 dilution), including V-ATP5A (55kDa), III-
523 UQCRC2 (48kDa), IV-MTCO1 (40kDa), II-SDHB (30 kDa), and I-NDUFB8 (20 kDa).
524 Commercially available polyclonal antibodies were used to detect AMP-activated protein kinase α
525 (AMPK α) (rabbit, CST, 2532; 62kDa; 1:1000) and Phospho-AMPK α Thr172 (P-AMPK) (rabbit
526 CST, 2535, 62kDa; 1:500) as used previously (40).

527

528 After overnight incubation in primary antibodies, membranes were washed 3x5 minutes in TBST
529 and incubated for 1 hour at room temperature with the corresponding infrared fluorescent
530 secondary antibody (LI-COR IRDye 680nm or 800nm) at a dilution previously optimized (1:20
531 000). Immunoreactive proteins were detected by infrared imaging (LI-COR CLx; LI-COR) and
532 quantified by densitometry using ImageJ. All images were normalized to Amido Black total
533 protein stain (A8181, Sigma) using the entire lane corresponding to each sample.

534

535 **Immunofluorescence Analysis**

536 Quadriceps (vastus intermedius & vastus lateralis) and diaphragm muscle samples embedded in
537 O.C.T medium (Fisher Scientific) were cut into 10- μ m- thick sections with a cryostat (HM525
538 NX, Thermo Fisher Scientific, Mississauga, ON, Canada) maintained at -20°C. Muscle fibre type
539 was determined as previously described (41), with minor modifications. All primary antibodies
540 were purchased from the Developmental Studies Hybridoma Bank (University of Iowa), and
541 secondary antibodies were purchased from Invitrogen (Burlington, ON, Canada). Briefly, slides
542 were blocked with 5% goat serum (Sigma Aldrich) in PBS for 1 hour at room temperature. Next,
543 slides were incubated with primary antibodies against myosin heavy chain (MHC) I (BA-F8; 1:25),
544 MHC IIA (SC-71; 1:1000) and MHC IIB (BF-F3; 1:50) for 2 hours at room temperature.
545 Afterwards, slides were washed 3x in PBS for 5 minutes and then incubated with secondary
546 antibodies (MHCI; Alexa Fluor 350 IgG2b; 1:1000), (MHCIIa; Alexa Fluor 488 IgG1; 1:1000),
547 (MHC Iib; Alexa Fluor 568 IgM; 1:1000) for 1 hour at room temperature. Slides were then washed
548 3x in PBS for 5 minutes and mounted with ProLong antifade reagent (Life Technologies,
549 Burlington, ON, Canada). Images were acquired the next day using EVOS FL Auto 2 Imaging
550 System (Invitrogen, Thermo Fisher Scientific, Mississauga, ON, Canada). Individual images were
551 taken across the entire cross section and then assembled into a composite image. 20-30 muscle
552 fibers per fiber type were selected randomly throughout the cross section and traced with ImageJ

553 software to assess CSA after calibrations with a corresponding scale bar. Muscle fibers that
554 appeared black were recorded as MHC IIX (41).

555

556 **Statistics**

557 Results are expressed as mean \pm SEM. The level of significance was established at $P < 0.05$ for
558 all statistics. The D'Agostino – Pearson omnibus normality test was first performed to determine
559 whether data resembled a Gaussian distribution. Western blot results for proteins in the electron
560 transport chain subunit complexes I, IV and V in quadriceps failed normality as did proteins in
561 complexes I, II, IV and V for diaphragm. In addition, quadriceps and diaphragm delta glutamate
562 respiration failed normality and were analyzed using a non-parametric Mann-Whitney t-test. All
563 other data passed normality. A two-tailed unpaired t-test was used to compare C26 to PBS within
564 each time point with respect to muscle mass, fibre cross-sectional area, and remaining western
565 blots. Mitochondrial respiration, mH_2O_2 and force-frequency were analyzed using a two-way
566 ANOVA with factors of timepoint (2 vs 4 week) and treatment (C26 vs PBS) followed by
567 Benjamini, Krieger and Yekutieli's post-hoc analysis when a main affect was obtained to identify
568 a significant interaction between groups (GraphPad Prism Software 8.4.2, La Jolla, CA, USA)
569 (42).

570

571 **Study Approval**

572 All experiments and procedures were approved by the Animal Care Committee at York University
573 (AUP Approval Number 2019-10) in accordance with the Canadian Council on Animal Care.

574

575 **Author Contributions**

576 L.J.D., C.A.B, M.R.C., N.P.G. and C.G.R.P. contributed to the rationale and study design. L.J.D.,
577 C.A.B., S.G. and C.G.R.P. conducted all experiments and/or analyzed all data. C.G.R.P. and L.J.D.
578 wrote the manuscript. All authors contributed to the interpretation of the data and manuscript
579 preparation. All authors have approved the final version of the manuscript and agree to be
580 accountable for all aspects of the work. All persons designated as authors qualify for authorship,
581 and all those who qualify for authorship are listed.

582

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592
593

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713

714

715 **Figure Legends**

716 **Figure 1** The effects of C26 colon cancer cells implantation on body size, tumour size, muscle
717 mass and force. Analysis of CD2F1 mice with subcutaneous C26 implantations or with PBS were
718 performed. Body weights (*A*, *n*=8-16) and tumour-free body weights (*B*, *n*=8-16) were analyzed
719 every week (2[&] mice were measured at a 14-17 day window and 4[&] mice were measured on a 26-
720 29 day window). Percent change in tumour free body weights were analyzed from day 0 to end
721 point (*C*, *n*=8-16). *In vivo* tumour volume measurements were made using calipers (*D*, *n*=16).
722 Tumour mass (*E*, *n*=7-16) and spleen mass (*F*, *n*=8-16) measurements were also completed.
723 Evaluation of hindlimb muscle wet weights were made in the 2-week cohort (*G*, *n*=8) and 4-week
724 cohort (*H*, *n*=16). Results represent mean ± SEM; # *P*<0.05 PBS(2wk) vs C26(2wk); * *P*<0.05
725 PBS(4wk) vs C26(4wk).

726
727 **Figure 2** The effects of C26 colon cancer on quadriceps and diaphragm force production. *In situ*
728 quadriceps force production was assessed using the force frequency relationship (*A*, *n*=6-14) and
729 *in vitro* diaphragm force production was also measured using the force frequency relationship (*B*,
730 *n*=6-12). Results represent mean ± SEM; # *P*<0.05 PBS(2wk) vs C26(2wk); * *P*<0.05 PBS(4wk)
731 vs C26(4wk); \$ *P*<0.05 C26 (2wk) vs C26 (4wk).

732
733 **Figure 3** Evaluation of quadriceps and diaphragm fibre-type atrophy in skeletal muscle from C26
734 tumour-bearing mice. Analysis of fibre histology on MHC isoforms of PBS and C26 mice was
735 performed. Cross-sectional area of MHC stains was evaluated in the quadriceps (*A*, *n*=8; *B*,
736 representative image, magnification x 20) and diaphragm at 2 weeks of tumour bearing (*C*, *n*=6;
737 *D*, representative image, magnification x 20). The same was completed for the quadriceps (*E*, *n*=9;
738 *F*, representative image, magnification x 20) and diaphragm at 4 weeks of tumour bearing (*G*, *n*=9;
739 *H*, representative image, magnification x 20). Results represent mean ± SEM; * *P*<0.05 PBS (4wk)
740 vs C26(4wk).

741
742 **Figure 4** Muscle-specific changes in markers of oxidative phosphorylation in C26 tumour-bearing
743 skeletal muscle. Protein content of electron transport chain components were quantified at in the
744 quadriceps (*A*, *n*=8) and diaphragm at 2 weeks (*B*, *n*=8) and 4 weeks in both muscles respectively

745 *C, D n=12*). *E*, representative image for quadriceps and *F*, representative image for diaphragm.
746 Results represent mean \pm SEM; * $P < 0.05$ PBS (4wk) vs C26 (4wk).

747

748 **Figure 5** Schematic representation of energy homeostasis in low metabolic (left) vs. high
749 metabolic (right) demand states. When ADP is low, less ATP is produced. A concomitant
750 accumulation of $[H^+]$ in the inner membrane space (IMS) increases membrane potential ($\Delta\Psi$),
751 attenuates H^+ pumping, induces premature electron slip and generates superoxide ($O^{\bullet-}$) which is
752 dismutated to H_2O_2 by manganese superoxide dismutase (MnSOD; top left). Only Complex I-
753 derived superoxide is displayed. When ADP is high, more ATP is produced as $[H^+]$ diffuse from
754 the IMS to the mitochondrial matrix through ATP synthase. The decrease in $\Delta\Psi$ lowers premature
755 electron slip, generating less $O^{\bullet-}$ and H_2O_2 (top right). ADP generated by ATPases throughout the
756 cell enter the matrix through the voltage dependent anion channel (VDAC) on the outer
757 mitochondrial membrane (OMM) and the adenine nucleotide translocase (ANT) on the inner
758 mitochondrial membrane (IMM; bottom left). Creatine accelerates matrix ADP/ATP cycling and
759 ATP synthesis by reducing the diffusion distance of the slower diffusing ADP and ATP while
760 shuttling phosphate to the cytoplasm through rapidly diffusing phosphocreatine which is used by
761 cytosolic creatine kinase (cCK) to recycle local ATP to support the activity of various ATPases.
762 Rapidly diffusing creatine returns to the IMS to be re-phosphorylated by mitochondrial creatine
763 kinase (mtCK). Non-ATPase sites of ATP hydrolysis are not displayed but also contribute to net
764 metabolic demand (kinases, and other ATP-dependent processes). The net effect of metabolic
765 demand (global ATP hydrolysis) on matrix ADP/ATP cycling is displayed under the context of
766 creatine independent (-Creatine) and creatine dependent (+Creatine) conditions. Figure adapted
767 from *Aliev et al.*, 2011, *Guzun et al.*, 2012, *Wallimann et al.*, 2011 and Nicholls 2013(23, 43–45).
768 Created with BioRender.com

769

770 **Figure 6** Complex I-supported mitochondrial respiration in quadriceps muscle of C26 tumour-
771 bearing mice. ADP-stimulated (State III) respiration, supported by complex I supported (NADH)
772 substrates pyruvate (5mM) and malate (2mM), was assessed in the absence (-Creatine) and
773 presence (+Creatine) of 20mM creatine at a range of $[ADP]$ until maximal respiration was
774 achieved to model a spectrum of metabolic demands. Respiration was assessed in the quadriceps
775 normalized to bundle size at 2 weeks (*A, B*) and normalized to ETC subunit content (*C, D*) to

776 permit comparisons of intrinsic mitochondrial respiratory responses in each group. Creatine
777 sensitivity was assessed by calculating the +creatine/-creatine ratio (*E, F*) given creatine normally
778 increases ADP-stimulated respiration. The same measurements were completed at 4 weeks (*G-L*).
779 Results represent mean \pm SEM; n=8-16; # $P<0.05$, PBS(2wk) vs C26(2wk); * $P<0.05$, PBS(4wk)
780 vs C26(4wk).

781

782 **Figure 7** Complex I-supported mitochondrial respiration in diaphragm muscle of C26 tumour-
783 bearing mice. ADP-stimulated (State III) respiration, supported by complex I supported (NADH)
784 substrates pyruvate (5mM) and malate (2mM), was assessed in the absence (-Creatine) and
785 presence (+Creatine) of 20mM creatine at a range of [ADP] until maximal respiration was
786 achieved to model a spectrum of metabolic demands. Respiration was assessed in the diaphragm
787 normalized to bundle size at 2 weeks (*A, B*) and normalized to ETC subunit content (*C, D*) to
788 permit comparisons of intrinsic mitochondrial respiratory responses in each group. Creatine
789 sensitivity was assessed by calculating the +creatine/-creatine ratio (*E, F*) given creatine normally
790 increases ADP-stimulated respiration. The same measurements were completed at 4 weeks (*G-L*).
791 Results represent mean \pm SEM; n=8-16; * $P<0.05$, PBS(4wk) vs C26(4wk)

792

793 **Figure 8** Muscle-specific changes in markers of growth in C26 tumour-bearing skeletal muscle.
794 Protein content of AMPK α and P-AMPK α were quantified at in the quadriceps at 2 weeks (*A*,
795 n=8) and 4 weeks (*B*, n=12). Markers were also quantified at in the diaphragm at 2 weeks (*C*, n=8)
796 and 4 weeks (*D*, n=12). *E*, representative image for quadriceps and *F*, representative image for
797 diaphragm. Results represent mean \pm SEM.

798

799 **Figure 9** Complex I stimulated mH₂O₂ emission in quadriceps and diaphragm muscle of C26
800 tumour bearing mice. At 2 and 4 weeks, quadriceps mH₂O₂ emission supported by pyruvate
801 (10mM) and malate (2mM) (NADH) was assessed under maximal State II (no ADP) conditions in
802 the presence of 20mM creatine (*A, E*) and under a range of [ADP] to model metabolic demand (*B*,
803 *F*). These measures were also normalized to total ETC subunit content (*C, D, G, H*) to permit
804 comparisons of intrinsic mitochondrial respiratory responses in each group. These measures were
805 repeated in the diaphragm (*I-P*). Results represent mean \pm SEM; n=8-16; # $P<0.05$, PBS(2wk) vs
806 C26(2wk); * $P<0.05$, PBS(4wk) vs C26(4wk)

807

808 **Figure 10** Summary of the time-dependent and muscle-specific adaptations to C26 xenografts in
809 CD2F1 mice. At 2 weeks, early impairments in force generating capacity are associated with
810 reductions in mitochondrial pyruvate/malate-supported ADP-stimulated respiration in quadriceps
811 and elevated mH_2O_2 emission in diaphragm. These distinct mitochondrial responses precede
812 atrophy in both muscles by 4 weeks. At this time, quadriceps and diaphragm responses to C26
813 become heterogeneous. The restoration of force generating capacity in quadriceps in spite of
814 atrophy is not observed in the diaphragm even though both muscles demonstrate apparent
815 compensatory increases in mitochondrial ADP-stimulated respiration. The mitochondrial
816 responses to cancer are more diverse in quadriceps than diaphragm, with increases in respiration
817 by 4 weeks occurring as a potential compensation for reductions in mitochondrial electron
818 transport chain markers. Mitochondrial creatine metabolism is impaired in quadriceps by 4 weeks.
819 Created with BioRender.com

820

821 **Supplemental Figure Legends**

822 **Figure S1** Multiple substrate evaluation of oxygen consumption in quadriceps permeabilized
823 muscle fibre bundles. Oxygen consumption was evaluated in the absence of creatine at 2 weeks
824 and 4 weeks post C26 implantation or PBS injections in permeabilized muscle fibres when
825 stimulated with glutamate (*A, B*), succinate (*E, F*) and pyruvate/malate (*I, J*). This was repeated in
826 the presence of 20mM Creatine (*C, D, G, H, K, L*). Results represent mean \pm SEM; n=8-16; #
827 $P<0.05$ PBS (2wk) vs C26 (2wk); * $P<0.05$ PBS (4wk) vs C26 (4wk)

828

829 **Figure S2** Multiple substrate evaluation of oxygen consumption in diaphragm permeabilized
830 muscle fibre bundles. Oxygen consumption was evaluated in the absence of creatine at 2 weeks
831 and 4 weeks post C26 implantation or PBS injections in permeabilized muscle fibres when
832 stimulated with glutamate (*A, B*), succinate (*E, F*) and pyruvate/malate (*I, J*). This was repeated in
833 the presence of 20mM Creatine (*C, D, G, H, K, L*). Results represent mean \pm SEM; n=8-16; #
834 $P<0.05$ PBS (2wk) vs C26 (2wk); * $P<0.05$ PBS (4wk) vs C26 (4wk).

835

836 **Figure S3** Succinate stimulated mH_2O_2 emission in quadriceps and diaphragm muscle of C26
837 tumour bearing mice. At 2 and 4 weeks, quadriceps mH_2O_2 emission supported by succinate

838 (10mM) (FADH₂) was assessed under maximal State II (no ADP) conditions in the absence of
839 creatine (*A, E*) and in the presence of 20mM Creatine (*C, G*). State III (range of [ADP] to model
840 metabolic demand) was also assessed in the absence of creatine (*B, F*) and in the presence of 20mM
841 creatine (*D* and *H*). These measures were repeated in the diaphragm (I-P). Results represent mean
842 ± SEM; n=7-16; # *P*<0.05, PBS (2wk) vs C26 (2wk); * *P*<0.05, PBS (4wk) vs C26 (4wk).

Figure 1

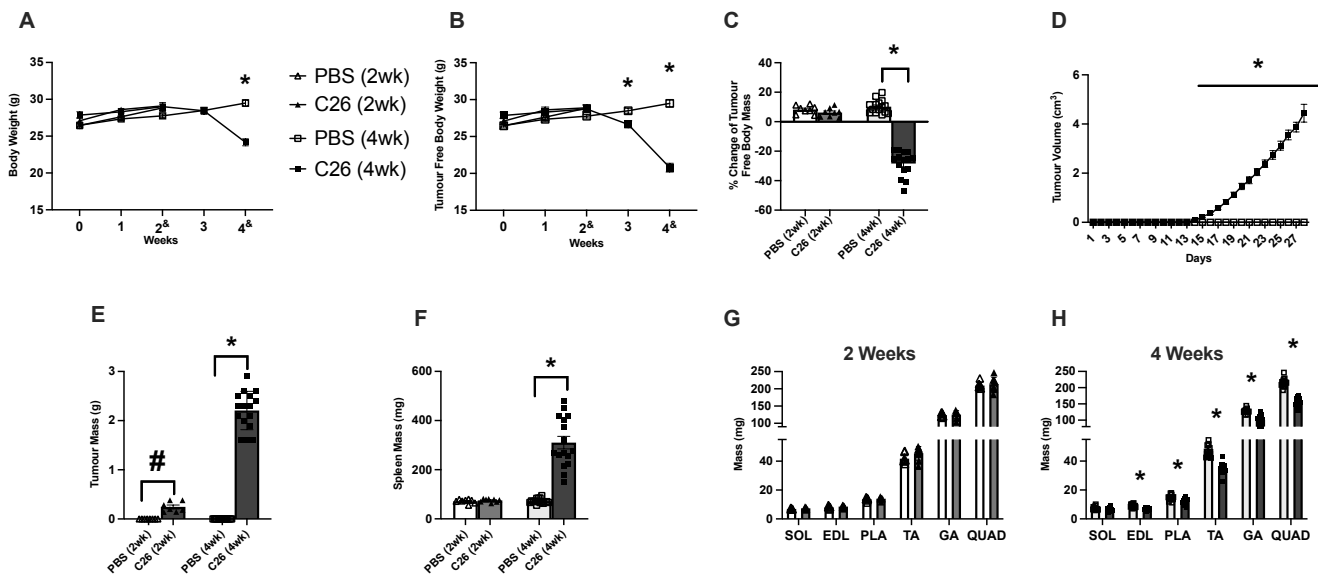
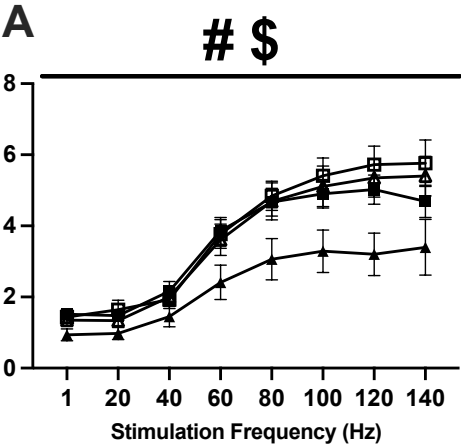


Figure 2

Quadriceps



Diaphragm

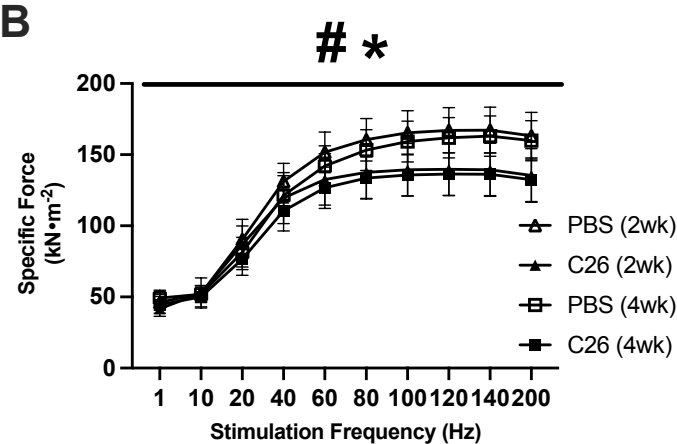


Figure 3

2 week

4 week

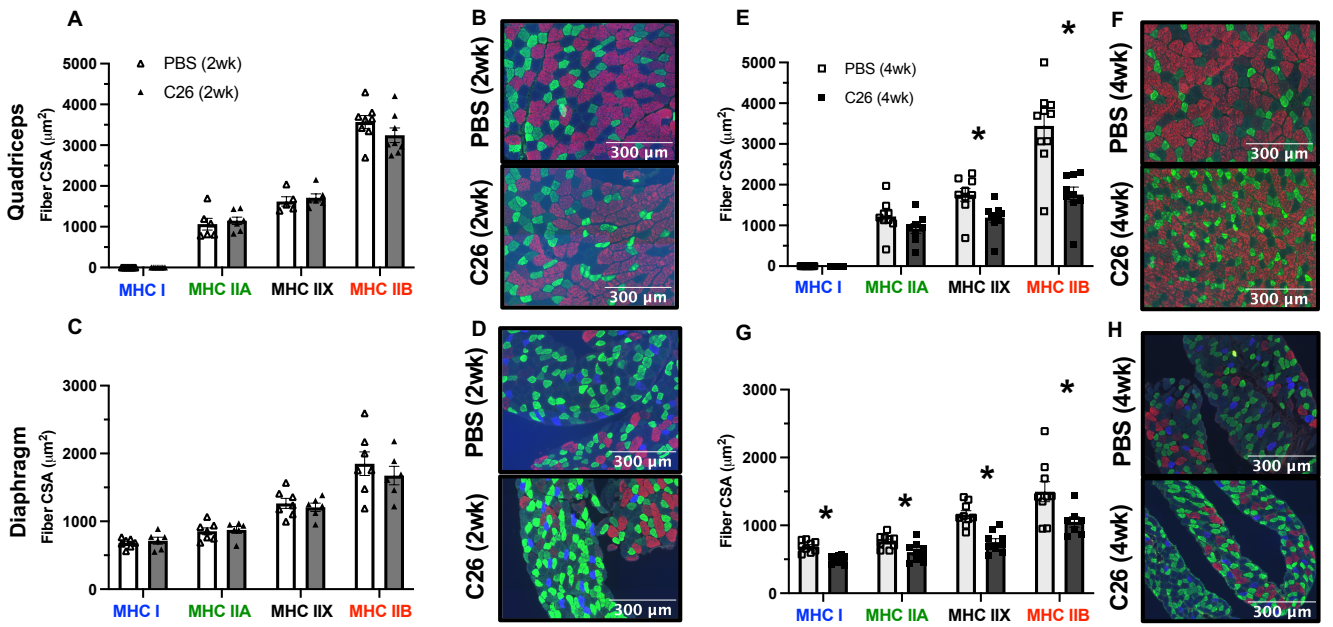


Figure 4

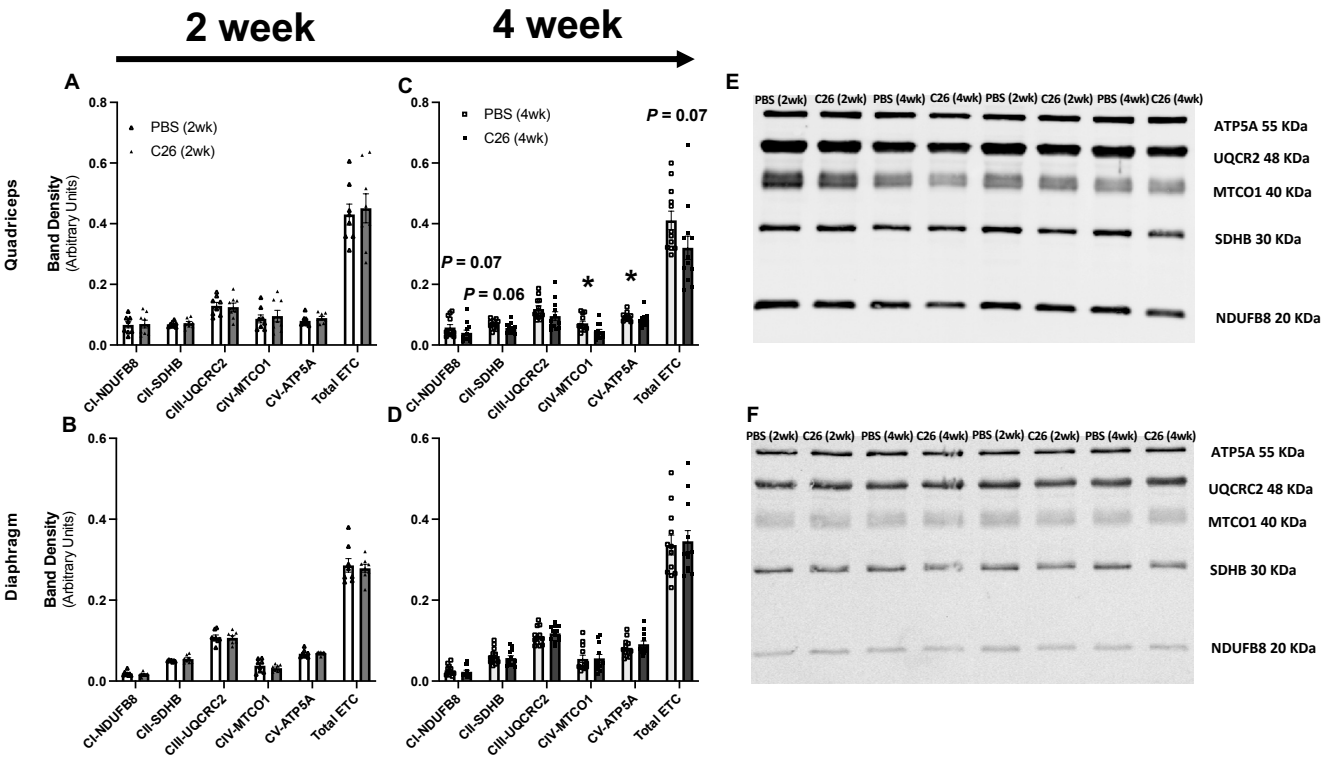
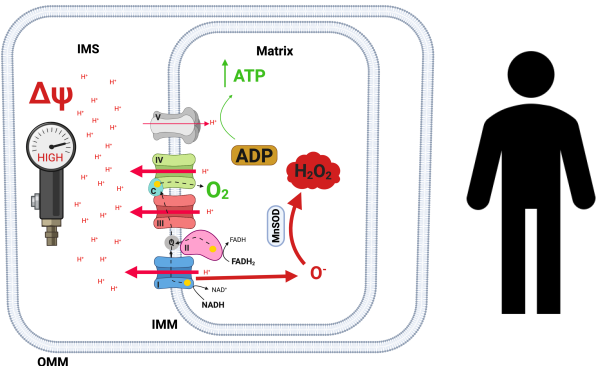
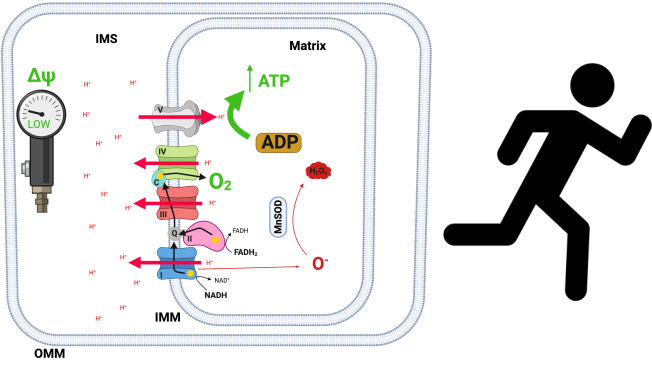


Figure 5

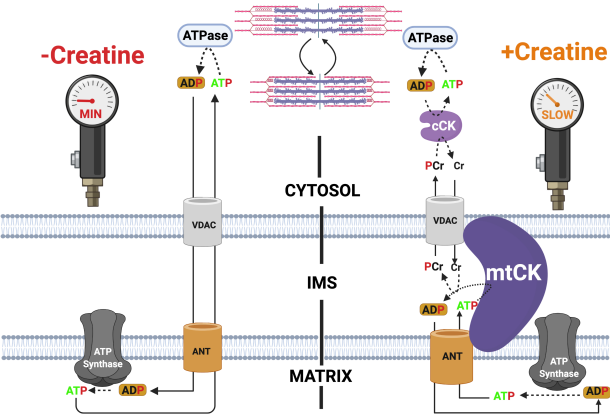
Low Metabolic Demand (Low ADP)



High Metabolic Demand (High ADP)



ATP/ADP Import/Export (Low ADP)



ATP/ADP Import/Export (High ADP)

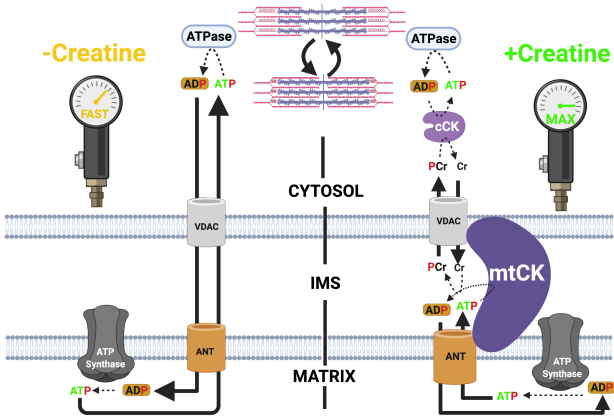


Figure 6

Quadriceps ADP-Stimulated Mitochondrial Respiration

2 week

4 week

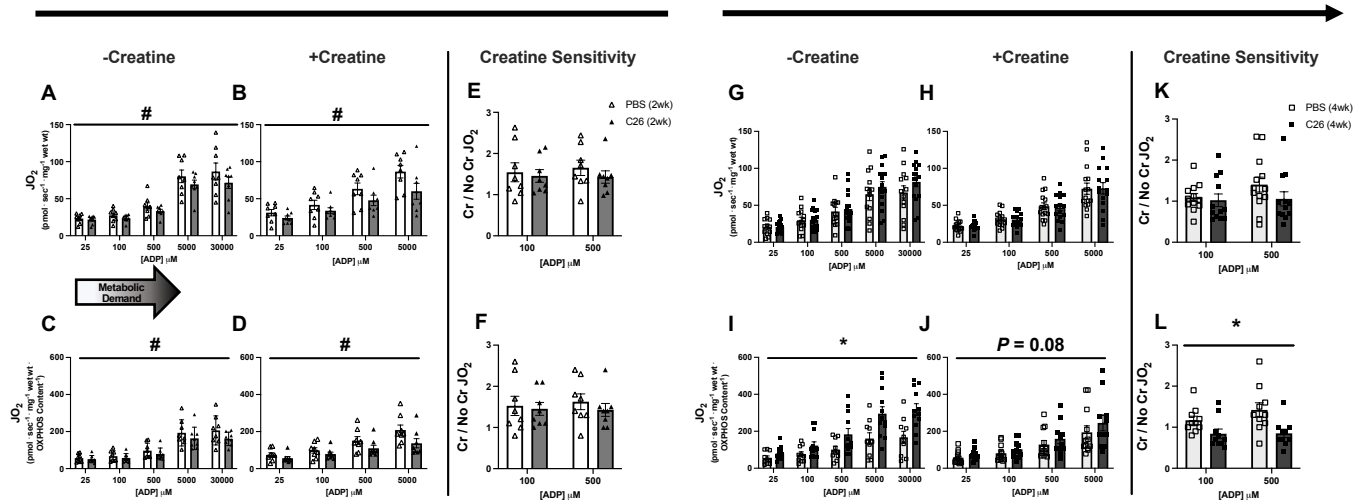


Figure 7

Diaphragm ADP-Stimulated Mitochondrial Respiration

2 week

4 week

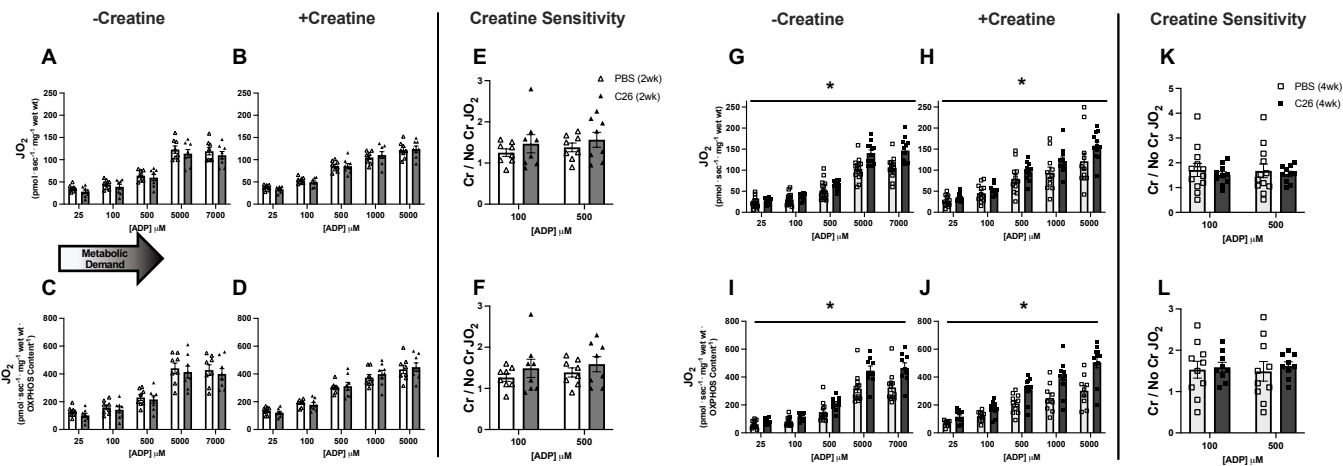


Figure 8

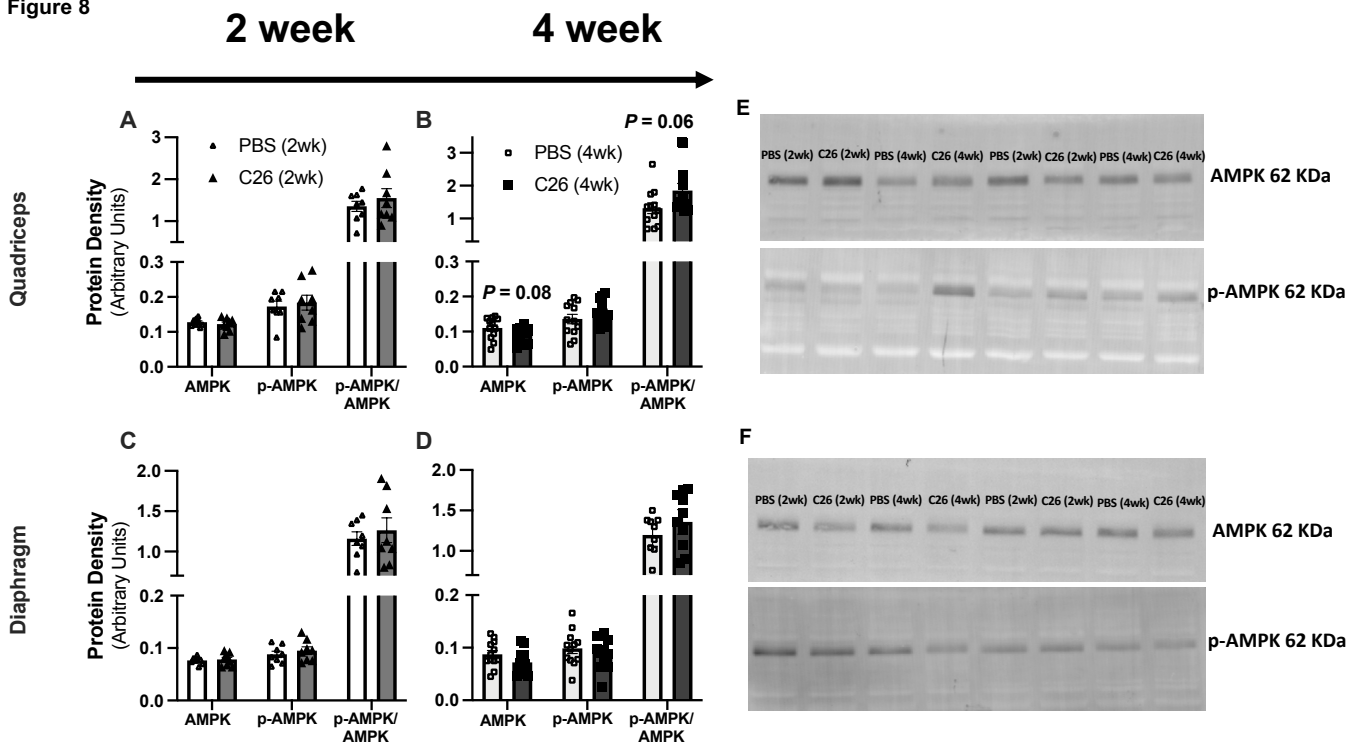


Figure 9

Complex I Stimulated H_2O_2

Quadriceps

Diaphragm

2 week

4 week

2 week

4 week

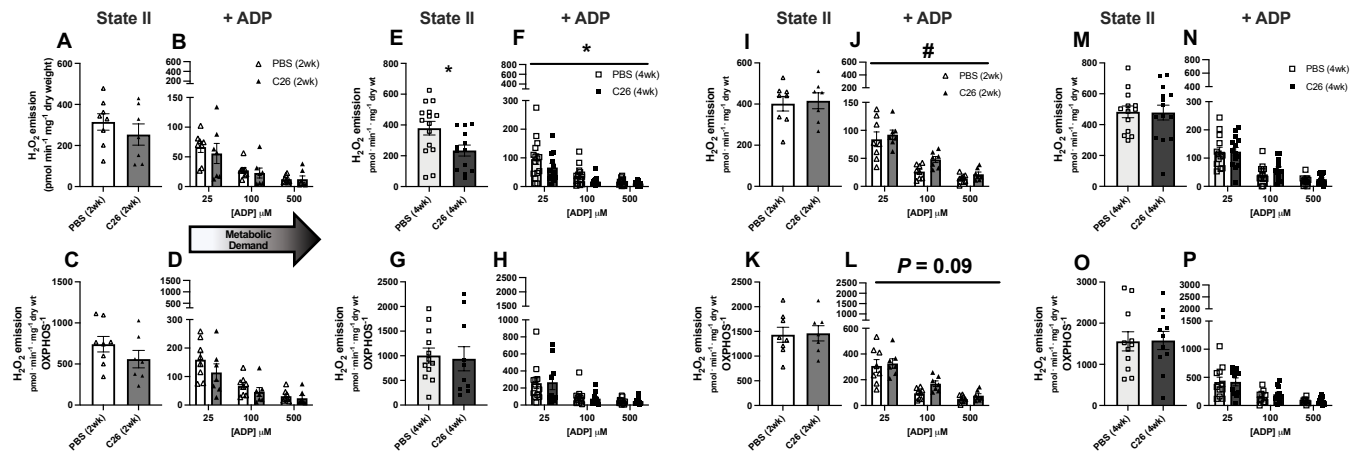


Figure 10

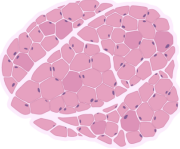
QUADRICEPS

2 WEEKS



4 WEEKS

Fibre Area =



Force - Low



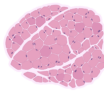
↓ Control by ADP
= Cr Sensitivity
= H₂O₂
= Content



↑ Control by ADP
↓ Cr Sensitivity
= H₂O₂
↓ Content

Conclusion

Fibre Area ↓



Force - Normal



- Muscle weakness **PRECEDES** atrophy
- Early deficits in pyruvate-stimulated respiration **PRECEDE** atrophy
- Compensatory increases in pyruvate-stimulated respiration are coupled to **RESTORATION** of force in spite of atrophy
- **CREATINE INDEPENDENT** ATP synthesis up-regulates to a greater degree than the creatine dependent system

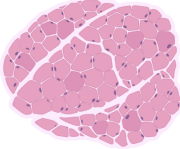
DIAPHRAGM

2 WEEKS



4 WEEKS

Fibre Area =



Force - Low



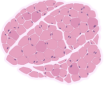
= Control by ADP
= Cr Sensitivity
↑ H₂O₂
= Content



↑ Control by ADP
= Cr Sensitivity
= H₂O₂
= Content

Conclusion

Fibre Area ↓



Force - Low



- Muscle weakness **PRECEDES** atrophy
- Early deficits in pyruvate-stimulated H₂O₂ emission **PRECEDE** atrophy
- Compensatory increases in pyruvate-stimulated respiration occur despite **NO CHANGE** in force
- **BOTH** creatine dependent and independent ATP synthesis systems up-regulate to similar degrees