

# Effect of colon cancer and surgical resection on skeletal muscle mitochondrial enzyme activity in colon cancer patients: a pilot study

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## Abstract

**Background** Colon cancer (CC) patients commonly suffer declines in muscle mass and aerobic function. We hypothesised that CC would be associated with reduced muscle mass and mitochondrial enzyme activity and that curative resection would exacerbate these changes.

**Methods** We followed age-matched healthy controls and CC patients without distant metastasis on radiological imaging before and 6 weeks after hemi-colectomy surgery. Body composition was analysed using dual energy X-ray absorptiometry. Mitochondrial enzyme activity and protein concentrations were analysed in vastus lateralis muscle biopsies.

**Results** In pre-surgery, there were no differences in lean mass between CC patients and age-matched controls (46.1±32.5 vs. 46.1±37.3 kg). Post-resection lean mass was reduced in CC patients (43.8±30.3 kg,  $P<0.01$ ). When comparing markers of mitochondrial function, the following were observed: pyruvate dehydrogenase (PDH) activity was lower in

CC patients pre-surgery ( $P<0.001$ ) but normalized post-resection and cytochrome c oxidase and pyruvate dehydrogenase E2 subunit protein expression were lower in CC patients pre-surgery and not restored to control values post-resection ( $P<0.001$ ). Nuclear factor kappa-B, an inflammatory marker, was higher in CC patients pre-surgery compared to controls ( $P<0.01$ ), returning to control levels post-resection.

**Conclusion** Muscle mass was affected by surgery rather than cancer per se. PDH activity was however lower in cancer patients, suggesting that muscle mass and mitochondrial enzyme activity are not inextricably linked. This reduction in mitochondrial enzyme activity may well contribute to the significant risks of major surgery to which CC patients are exposed.

**Keywords** Cancer · Muscle · Mitochondria · Pyruvate dehydrogenase

## 1 Introduction

Colorectal cancer (CRC) is a common condition with approximately 600,000 new cases per annum worldwide [1]. Over the past 40 years, CRC has increased in prevalence by over 40 % [2] accounting for 42,000 deaths between 2001 and 2003 in England and Wales [3]. CRC is now the third most common cancer and second leading cause of cancer-related death in the UK [4].

CRC patients may present at the clinic with cachexia syndrome [5, 6]; 35 to 60 % of CRC patients show some degree of muscle wasting and 28 % lose >5 % of their body weight in the 6 months preceding diagnosis [7]. Moreover, CRC patients also frequently display symptoms of chronic

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fatigue and report reductions in physical capacity [8, 9]. Indeed, 60–96 % of (former) cancer patients complain of fatigue during and/or after treatment [8, 9], and although the extent of fatigue decreases gradually in disease-free survivors, 30 % of cancer survivors still report serious complaints of fatigue 3 years after completion of medical treatment [8, 9].

Despite advances in adjuvant and neo-adjuvant treatments, surgical resection remains the mainstay of curative treatment. However, surgical resection itself places additional metabolic demands upon the body (including skeletal muscle) which can exacerbate symptoms related to the initial cancer burden [10]. Although various researchers have followed changes in muscle mass and markers of muscle protein breakdown and synthesis in the immediate post-operative period [10–12], to our knowledge, there are no studies exploring the effect of colon cancer (CC) on indices of cellular skeletal muscle oxidative capacity in patients both before and after surgery, compared to healthy age-matched volunteers.

In addition to the simple loss of muscle, skeletal muscle pathophysiology is a feature of a number of chronic conditions including cancer, chronic obstructive pulmonary disease (COPD), diabetes and congestive heart failure [13–16], representing a major contribution to adverse outcomes in these conditions. In these instances, skeletal muscle has been shown to display corresponding morphological and biochemical changes [13–15, 17–21]. For instance, in the skeletal muscle of COPD patients, mitochondrial content/density and the activities of the mitochondrial enzymes cytochrome c oxidase and succinate dehydrogenase are reduced with associated declines in exercise capacity and increased symptoms of fatigue [14, 16]. Adenosine triphosphate (ATP) production is also impaired in these individuals through a reduced ability of pyruvate dehydrogenase (PDH) to convert pyruvate to acetyl-CoA, a process which may be mediated by pyruvate dehydrogenase kinase isozyme 4 (PDK4), the enzyme responsible for inactivating PDH. Although these processes are well documented in conditions such as COPD, little is known about the effect of cancer on such vital pathways in skeletal muscle.

In addition to the dysregulation outlined above, in states of inflammation and ischaemia, the inflammatory cytokine nuclear factor kappa-B (NF $\kappa$ B) is upregulated playing an important role in modulating cellular responses [22]. Although there is a large body of evidence supporting the upregulation of NF $\kappa$ B in the rapid loss of muscle protein seen *in vitro* and in animal models of cancer [23], only recently has evidence emerged suggesting that NF $\kappa$ B is upregulated in the skeletal muscle of patients with gastric cancer [24].

The aim of this study was therefore to determine the effect of CC on muscle mass and indices of mitochondrial enzyme expression/activity and muscle inflammation before

and after surgery. We hypothesised that CC would be associated with increased cellular inflammation, reduced muscle mass and reduced mitochondrial enzyme activity and that these changes would be exacerbated by curative resection, despite removal of tumour burden.

## 2 Materials and methods

### 2.1 Subject characteristics

We recruited two groups of subjects consisting of healthy volunteers (70.7 $\pm$ 1.6 years old, four males, four females, body mass index (BMI) 26.2 $\pm$ 1.0 kg m<sup>-2</sup>) and patients with colon cancer (62.5 $\pm$ 8.3 years old, four males, four females, BMI 27.6 $\pm$ 1.6 kg m<sup>-2</sup>) presenting to colorectal out-patients, excluding those with distant metastasis on pre-operative staging. The healthy volunteers were asked to undergo a single acute study, and the CC patients were studied pre-operatively and 6 weeks post-resection (open hemicolectomy) prior to any chemotherapy regime. All CC patients underwent uncomplicated recoveries following hemicolectomy. Before beginning the study, all subjects were screened using a medical questionnaire, physical examination and resting ECG. Exclusion criteria for patients and controls were metabolic, respiratory or cardiovascular disorders or any other contraindications to a healthy status. As a condition of entry to the study, all subjects had normal blood chemistry and were normotensive (BP <140/90). All subjects gave their written, informed consent to participate in the study. The study was approved by the local NHS REC committee and the University of Nottingham Ethics Committee and complied with the Declaration of Helsinki.

### 2.2 Acute studies

Subjects were instructed to refrain from unaccustomed exercise for 72 h and from alcohol and caffeine for 24 h before study days. All subjects fasted from 2100 hours the night before, with water *ad libitum*, and reported to the laboratory at 0900 hours. Body composition was measured by dual-energy X-ray absorptiometry (Lunar Prodigy II, GE Medical Systems), and a single muscle biopsy of vastus lateralis was taken using the conchotome technique under post-absorptive conditions at the beginning of the acute study.

### 2.3 Immunoblotting

Muscle biopsies (~10–20 mg) were homogenised with scissors in ice-cold extraction buffer (10  $\mu$ l/mg<sup>-1</sup>) containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 0.5 mM activated sodium orthovanadate (all from Sigma-Aldrich, Poole, UK) and a complete protease

inhibitor cocktail tablet (Roche, West Sussex, UK). Homogenates were rotated on a Vibramax for 10 min at 4°C then centrifuged at  $10,000 \times g$  for 10 min at 4°C, before recovery of supernatants representing sarcoplasmic fractions. Bradford assays were used to determine sarcoplasmic protein concentrations after which samples were standardised to  $1 \mu\text{g}/\mu\text{l}^{-1}$  by dilution with Laemmli loading buffer in order to measure relative protein concentrations of pan actin, myosin, NF $\kappa$ B, pyruvate dehydrogenase E2 subunit (PDH-E2), PDK4 and cytochrome c oxidase. Samples were mixed and heated at 95°C for 5 min before 15  $\mu\text{g}$  of protein/lane was loaded on to Criterion XT Bis–Tris 12 % SDS-PAGE gels (Bio-Rad, Hemel Hempstead, UK) for electrophoresis at 200 V for ~60 min. Gels were equilibrated in a transfer buffer (25 mM Tris, 192 mM glycine, 10 % methanol) for 30 min before proteins were electroblotted on to 0.2- $\mu\text{m}$  PVDF membranes (Bio-Rad) at 100 V for 30 min. After blocking with 5 % low-fat milk in Tris-buffered saline (TBS-T) and 0.1 % Tween-20 (both from Sigma-Aldrich, Poole, UK) for 1 h, membranes were rotated overnight with a primary antibody (PDK4, PDH-E2, cytochrome c oxidase, Abcam, UK; pan actin, myosin, NF $\kappa$ B, Sigma-Aldrich, UK) against the aforementioned targets at a concentration of 1:2,000 at 4°C. Membranes were washed ( $3 \times 5$  min) with TBS-T and incubated for 1 h at room temperature with HRP-conjugated anti-rabbit secondary antibody (New England Biolabs, UK), before further washing ( $3 \times 5$  min) with TBS-T and incubation for 5 min with ECL reagents (enhanced chemiluminescence kit, Immun-Star, Bio-Rad). Blots were imaged and quantified by assessing peak density after ensuring bands were within the linear range of detection using the ChemiDoc XRS system (Bio-Rad, Hemel Hempstead, UK). Protein concentration was corrected for loading anomalies to eukaryotic translation initiation factor 4E or glyceraldehyde-3-phosphate dehydrogenase dependent upon the expected size of the target protein.

#### 2.4 Pyruvate dehydrogenase activity

PDH enzyme activity was measured using a PDH Enzyme Activity Microplate Assay Kit (MitoSciences, USA). The protocol was followed according to manufacturer's instruction. In brief, 10 mg of vastus lateralis muscle was homogenised in 100  $\mu\text{l}$  phosphate-buffered saline. Sample protein concentration was measured using the Bradford assay before detergent was added to the sample to solubilise intact functional PDH. The samples were then incubated on ice for 10 min before centrifuging at  $10,000 \times g$  for 10 min at 4°C. Sample supernatant was then diluted with buffer to achieve a dilution of 0.2  $\mu\text{g}/200 \mu\text{l}$ ; 200  $\mu\text{l}$  of solubilised, diluted sample was added to each well of the microplate before the microplate was incubated for 3 h prior to washing with a stabiliser. Finally, assay solution was added to the plate

before reading the absorbance of each well at 450 nm using a kinetic programme with 60 s between reads (Multiskan Ascent plate reader, Ascent software V 2.6, Thermo Scientific, UK).

#### 2.5 Statistical analysis

Results are reported as means  $\pm$  SEM. Variables were analysed with one-way ANOVA to assess significant differences between the groups. Post-hoc analyses were performed with Tukey's method, and  $P < 0.05$  was the accepted level of statistical significance.

### 3 Results

#### 3.1 Body composition

Patients with CC were matched for age with healthy controls. BMI was not significantly different between the two groups ( $26.2 \pm 1.0 \text{ kg m}^{-2}$ , healthy controls, and  $27.6 \pm 1.6 \text{ kg m}^{-2}$ , colon cancer patients) and did not change significantly after surgery in the cancer patients. There were no significant differences in whole body ( $46.1 \pm 3.7$  vs.  $46.1 \pm 3.3$  kg) or appendicular lean mass ( $20.7 \pm 1.6$  vs.  $19.4 \pm 1.5$  kg) between the healthy controls and the cancer patients before surgery although the cancer patients did have significantly lower whole body ( $43.8 \pm 3.0$  kg,  $P < 0.01$ ) and appendicular ( $18.0 \pm 1.4$  kg,  $P < 0.05$ ) lean mass post-resection (Table 1).

#### 3.2 Immunoblotting

Protein expression of NF $\kappa$ B, a marker of muscle inflammation, was significantly higher in pre-operative cancer patients compared to the healthy control subjects ( $1.42 \pm 0.32$  vs.  $0.50 \pm 0.07$ ,  $P < 0.01$ ). Post-resection levels of NF $\kappa$ B were reduced to a value not significantly different to that of the healthy controls ( $0.75 \pm 0.13$  vs.  $0.50 \pm 0.07$ , Fig. 1). Protein expressions of both pan actin and total myosin were not significantly different between the healthy controls and cancer patients either before or after resection, with neither surgery or cancer having any significant effect on the protein expression of either of these targets (pan actin,  $0.45 \pm 0.06$  vs.  $0.46 \pm 0.08$  and  $0.56 \pm 0.12$ ; total myosin,  $0.98 \pm 0.08$  vs.  $0.75 \pm 0.12$  and  $0.74 \pm 0.07$ ). PDH-E2 protein expression was significantly higher in the healthy controls than in the cancer patients both before and after resection ( $2.14 \pm 0.20$  vs.  $0.89 \pm 0.08$  and  $0.80 \pm 0.12$ ,  $P < 0.001$ ) with no significant difference between values in the cancer patients pre- and post-operatively (Fig. 2). Protein expression of PDK4 was not significantly different between the healthy controls and cancer patients either before or after resection ( $1.36 \pm 0.08$

**Table 1** Body composition data for healthy controls and cancer patients pre- and post-resection

	Healthy controls	Cancer patients Pre-surgery	Cancer patients Post-surgery
Age (years)	70.7±1.6	62.5±8.3	62.7±8.3
BMI (kg m <sup>-2</sup> )	26.2±1.0	27.6±1.6	26.9±1.4
Total lean mass (kg)	46.1±3.7*	46.1±32.5*	43.8±3.0
Appendicular lean mass (kg)	20.7±1.6*	19.4±1.5*	18.0±1.4

Values are means ± SEM. Analysis was done via ANOVA with Tukey's post-analysis

\* $P < 0.01$  (vs. cancer patients post-surgery)

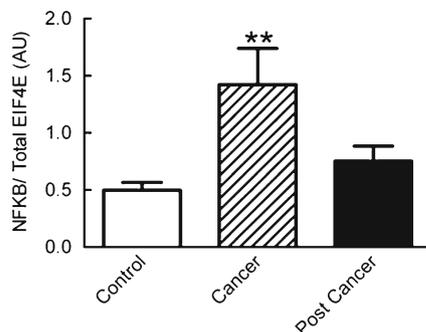
vs.  $1.35 \pm 0.08$  and  $1.37 \pm 0.06$ ). Cytochrome c oxidase protein expression was significantly higher in the healthy controls than in the cancer patients both before and after resection ( $1.26 \pm 0.22$  vs.  $0.56 \pm 0.09$  and  $0.42 \pm 0.07$ ,  $P < 0.01$  and  $P < 0.001$ , respectively) with no significant difference between the cancer patients pre- and post-operatively (Fig. 3).

### 3.3 Pyruvate dehydrogenase enzyme activity

PDH enzyme activity was significantly lower in pre-operative cancer patients compared to the healthy controls ( $0.16 \pm 0.06$  vs.  $1.00 \pm 0.11$ ,  $P < 0.001$ ). Post-resection PDH enzyme activity in the cancer patients was restored to values not different to that of the healthy controls ( $1.00 \pm 0.15$  vs.  $1.00 \pm 0.11$ , Fig. 4).

## 4 Discussion

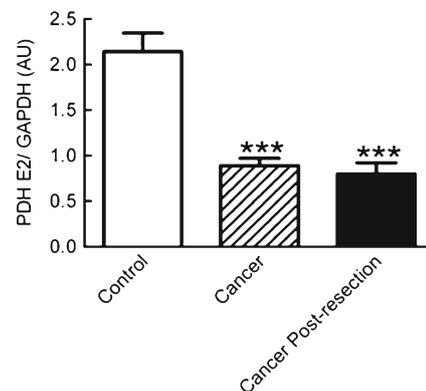
Although surgery represents the only cure for CC, it is still associated with a 30-day mortality rate of approximately 4 % [25]. Adequate skeletal muscle mass and function (i.e., aerobic capacity) have been associated with the incidence and severity of complications in the post-operative period and may ensure survival following major surgical resection [26]. In this study, we have demonstrated that although lean body mass in healthy controls and CC patients was not significantly different, indices of mitochondrial



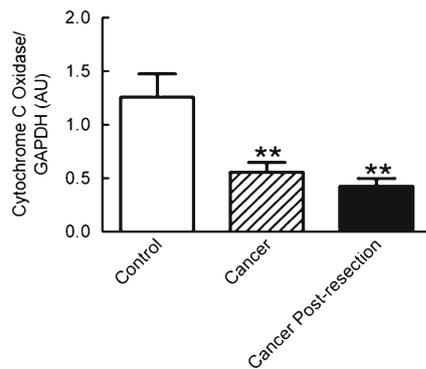
**Fig. 1** Protein expression of NFκB in healthy controls and cancer patients before and after resection. Values are means ± SEM for eight control subjects and eight cancer patients. \*\* $P = 0.007$ . Analysis was done via ANOVA with Tukey's post-analysis

enzyme activity in skeletal muscle are lower in patients with colon cancer prior to curative surgical resection compared to healthy age-matched controls. Using a longitudinal study design, we investigated the effects of CC and subsequent surgical resection (in comparison to a healthy age-matched control group) on muscle mass and indices of mitochondrial function. Specifically, we have demonstrated that protein concentrations of the mitochondrial enzymes PDH and cytochrome c oxidase (COX) and the activity of PDH are lower in the vastus lateralis of individuals with CC, despite there being no measurable cachexia. Moreover, PDH activity, but not PDH and COX protein concentrations, returned to control levels 6 weeks after resection, despite significant declines in muscle mass. Finally, depressions in PDH activity and the subsequent post-surgery 'normalisation' inversely reflected the protein concentration of the pro-inflammatory transcription factor, NFκB.

Previous studies analysing changes in gene expression in the skeletal muscle of tumour-bearing mice showed a reduced expression of a number of genes involved in encoding the key proteins in mitochondrial energy production. Amongst these, two genes encoding the pyruvate dehydrogenase complex were shown to be reduced in tumour-bearing mice compared to healthy controls [27]. Pyruvate dehydrogenase is crucial in enabling carbohydrate (CHO) to enter the tricarboxylic acid cycle and for achieving higher work intensities [28]. At intensities below about 75 % of



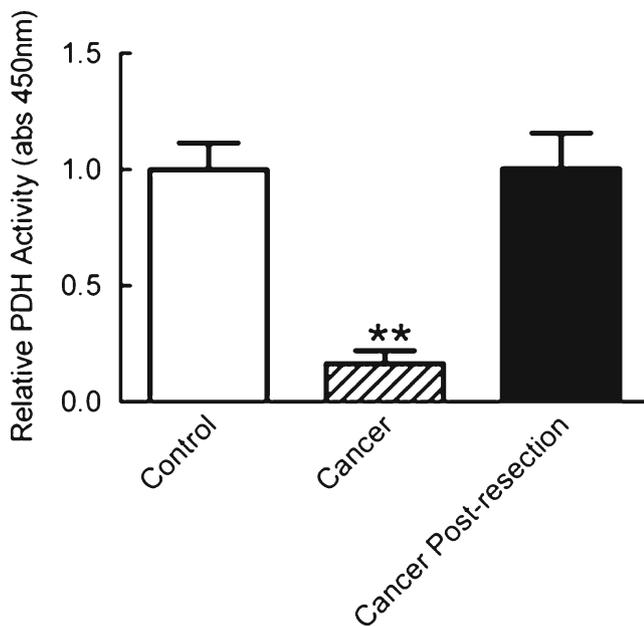
**Fig. 2** Protein expression of pyruvate dehydrogenase E2 subunit in healthy controls and cancer patients before and after resection. Values are means ± SEM for eight control subjects and eight cancer patients. \*\*\* $P < 0.001$ , vs. healthy controls. Analysis was done via ANOVA with Tukey's post-analysis



**Fig. 3** Protein expression of cytochrome c oxidase in healthy controls and cancer patients before and after resection. Values are means  $\pm$  SEM for eight control subjects and eight cancer patients.  $**P=0.001$ , vs. healthy controls. Analysis was done via ANOVA with Tukey's post-analysis

maximum, energy is generated within skeletal muscle by the oxidation of free fatty acids and CHO, whereas at higher workloads, CHO becomes the primary fuel source [29]. Reductions in exercise capacity and early onset of anaerobic metabolism and lactic acidosis on exercising have been shown to be predictors of increased mortality after major surgery [30–33]. Additionally, in human studies, several investigations have shown that a number of pathologies such as COPD and type II diabetes can result in limitations of skeletal muscle oxidative capacity [14–16, 18–21].

Our findings suggest that the capacity of PDH for oxidative CHO disposal is significantly lower in colon cancer



**Fig. 4** Pyruvate dehydrogenase activity in 10 mg of muscle from healthy controls and cancer patients before and after resection. Values are means  $\pm$  SEM for eight control subjects and eight cancer patients.  $**P<0.001$ , vs. healthy controls. Analysis was done via ANOVA with Tukey's post-analysis

before tumour resection, albeit returning to normal 6 weeks post-resection. One possible mechanism for the reductions in PDH activity would be through a reduction in PDH protein expression. However, PDH protein expression was lower in cancer patients both prior to and following hemicolectomy surgery compared to controls. Although protein abundance may not return to normal so soon after tumour resection, these results indicate that factors other than PDH protein expression are responsible for the diminution seen in PDH activity before surgery. Moreover, protein concentrations of PDK-4, the kinase primarily responsible for phosphorylating and inactivating PDH in skeletal muscle [13, 34, 35], did not follow PDH activity, instead acting similarly to PDH protein showing lower abundance regardless of tumour burden. This suggests that in the presence of colon cancer, PDH dysfunction is not mediated through reductions in PDH or increases in PDK-4.

One possible alternative mechanism by which PDH activity is lowered in colon cancer may relate to our observations surrounding the pro-inflammatory transcription factor NF $\kappa$ B. Indeed, previous researchers have postulated that lipopolysaccharide-induced endotoxaemia in rats may lead to a reduction in pyruvate dehydrogenase activity [36], while NF $\kappa$ B activity has been shown to be increased in the muscle of cancer patients [24] and implicated in the rapid weight loss seen in these individuals [37]. In our study, NF $\kappa$ B expression was higher in CC patients pre-resection than in control subjects, with a return to normal 6 weeks after resection. These variations in NF $\kappa$ B expression closely reflected changes in PDH activity, indicating a possible association between a low PDH activity and the ongoing inflammatory milieu within the skeletal muscle of CC patients.

As with PDH protein, cytochrome c oxidase was also less abundant in the skeletal muscle of CC patients pre- and post-operatively when compared to that of healthy controls. Due to its position at the end of the electron transport chain, deficiencies in cytochrome c oxidase expression and function may have a profound impact on oxidative ATP generation and health, with deficiencies being described in a number of conditions including encephalomyopathies, Leigh syndrome, lactic acidosis and hypertrophic cardiomyopathies; all of which can be fatal [38]. Although we did not specifically assay the activity of cytochrome c oxidase, its low expression suggests that in skeletal muscle, its activity may also be lower and oxidative ATP production depressed in CC, leading to deficits in skeletal muscle energy production. These findings indicate that oxidative energy production within the mitochondrion is not only limited by an inability of CHOs to enter the tricarboxylic acid cycle through reductions in PDH expression and activity but also through limitations in cytochrome c oxidase expression and oxidative ATP generation in the mitochondrial inner membrane.

Colon cancer and major surgical operations are both frequently associated with losses of skeletal muscle mass which may confer an increased risk of perioperative complications and mortality [10, 26, 37, 39]. In this study, pre-resection CC patients and age-matched controls demonstrated no significant differences in lean mass or BMI. Although this is at odds with much of the literature published in this field, this present study contains a select group of patients without distant metastasis in the earlier stages of CRC and this may well account for our inability to show marked changes in whole body lean mass between controls and patients pre-operatively. However, 6 weeks post-resection whole-body lean mass was reduced in CC patients ( $46.1 \pm 3.3$  vs.  $43.8 \pm 3.0$  kg,  $P < 0.01$ ), although this was not paralleled by decreases in expression of the structural proteins myosin or actin. This finding mirrors the observations of previous researchers [10] and may be due to a combination of the trauma and catabolic effect of surgery [40] as well as patient inactivity and/or reduced dietary intake in the post-operative period [41]. There is strong evidence that muscle mass is closely correlated with maximal aerobic performance in health and that maximal loss of muscle occurs 14 days post-surgery [10, 42–44]. Our findings however suggest that although mitochondrial oxidative capacity is beginning to normalise 6 weeks post-resection, muscle mass is still diminished compared to pre-operative values. These findings will have contrasting effects on global aerobic performance, implying that in CC muscle mass may not be as tightly linked to maximal aerobic performance as in health.

In this study, we have shown that in CC indices of human skeletal muscle oxidative capacity are lower than in healthy age-matched controls, with reductions in PDH and cytochrome c oxidase expression and PDH activity. These findings are similar to the changes seen in mitochondrial oxidative capacity in other systemic disease states. In conjunction with the reduction in muscle mass in the post-operative period reported by us and other researchers [10], these cellular changes may well contribute to the significant risks of major surgery to which CC patients are exposed.

## 5 Study limitations

We acknowledge that there are limitations to our elected study design. Firstly, this is a pilot study presenting preliminary findings following a small group of patients through the perioperative period; therefore, larger studies need to be undertaken to confirm the changes observed in measured markers of inflammation and cellular metabolic function. Second, although groups were matched with no statistically significant difference in age between patients and controls, controls were on average 8 years older. We accept that this

increase in age could be expected to be associated with a reduced muscle mass in the control population and may in itself result in derangements of inflammatory and cellular metabolic function. Finally, patients were studied for a second time 6 weeks after colorectal surgery. Although all made uncomplicated progress in the post-operative period, it could be argued that the inflammatory response to surgery had not completely subsided at this time point and that the second study would have been better undertaken at a later post-operative time point.

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**Conflict of interest** We have no conflict of interest to declare.

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