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**Title:** Acute high intensity interval exercise reduces colon cancer cell growth

**Running Title:** Acute HIIE and colon cancer cell growth

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Compliance with ethical standards

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Table of contents category: Exercise
Abstract:

Purpose: Physical activity is associated with significant reductions in colorectal cancer mortality. However, the mechanisms by which exercise mediates this antioncogenic effect are not clear. Methods: Colorectal cancer survivors completed acute (n=10) or chronic (n=10) exercise regimes. An acute high intensity interval exercise session (HIIE; 4x4 min at 85-95% peak heart rate) was completed with serum samples collected at baseline, and 0 and 120 minutes post-exercise. For the ‘chronic’ intervention, resting serum was sampled before and after 4 weeks (12 sessions) of HIIE. The effect of serum on colon cancer cell growth was evaluated by incubating cells (CaCo-2 and LoVo) for up to 72 hours and assessing cell number. Results: Serum obtained immediately following HIIE, but not 120 minutes post-HIIE, significantly reduced colon cancer cell number. Significant increases in serum interleukin-6 (p=0.023), interleukin-8 (p=0.036) and tumour necrosis factor-α (p=0.003) were found immediately following acute HIIE. At rest, short-term HIIE training did not promote any changes in cellular growth or cytokine concentrations. Conclusions: The acute effects of HIIE and the cytokine flux may be important mediators of reducing colon cancer cell progression. Repetitive exposure to these acute effects may contribute to the relationship between exercise and improved colorectal cancer survival.
1.0 Introduction

Increased physical activity has been associated with significant reductions in mortality for survivors of colorectal cancer (1, 2). The relationship may in part be explained by exercise-induced changes in systemic levels of various mechanistic host pathways (e.g. oxidative stress, metabolic hormones, sex hormones), to create a less carcinogenic environment (1, 3).

The direct effect of exercise-induced changes in serum can be examined by incubation with cancer cells in vitro, allowing the correlation of systemic changes following exercise with changes in cancer cell growth. Exercise training has been shown to significantly reduce breast and prostate cancer cell growth following exercise training, concomitant with reductions in insulin-like growth factor 1 (IGF1) and insulin (4-7). However recent data have challenged this notion of chronic adaptations to exercise being the only systemic mechanisms by which exercise may benefit cancer survivors. Dethlefsen et al. (8) showed that whilst long-term exercise training did not alter breast cancer cell growth, acute exercise suppressed growth and was associated with elevation of several cytokines [interleukin (IL)-6, IL-8, tumour necrosis factor-α (TNF-α)]. These findings present an important distinction between the roles of acute and chronic exercise on cancer cell growth, a relationship that is yet to be explored in colorectal cancer survivors. Conceptually, it is important to demarcate that the beneficial effects of exercise reflect the cumulative effect of regular alterations in systemic factors in response to acute bouts of exercise. Additionally, transient changes expressed acutely following exercise (e.g. catecholamines, immune responses, cytokines/myokines) have been suggested to have an equally important suppressive effect on cancer cell growth (9). Whilst acute benefits of exercise on cancer cell growth have been demonstrated (8, 9), the time course of these growth-suppressive effects have not been determined. Exploring and contrasting these responses between acute exercise and exercise training can inform how targeted exercise interventions may improve long-term outcomes for colorectal cancer survivors.

High intensity interval exercise (HIIE) may be a particularly strong stimulus for promoting changes in colon cancer cell growth. Previous findings have demonstrated a strong relationship between the effects of systemic changes in cytokines following exercise (8, 10, 11), with an increased cytokine response (particularly IL-6) following
higher versus lower intensity exercise (12). Additionally, higher intensity exercise may promote more favourable changes in metabolic variables such as IGF1, insulin and glucose due to the increased volume of metabolically active skeletal muscle recruited during high intensity exercise (13, 14). The primary aim of this pilot study was to evaluate the effect of exercise serum on colon cancer cell growth, following acute and short-term HIIE training in colorectal cancer survivors. It was hypothesised that serological changes after both acute and chronic HIIE would lead to reductions in growth in colon cancer cells.

2.0 Methods

2.1 Participants
Male colorectal cancer survivors were recruited for this trial. Additionally participants were ≥1 month since last completed radiation therapy, chemotherapy or surgical treatment, aged ≥40 years old (mean ages: acute trial = 66.9 ± 8.4 years; short-term training = 64.9 ± 6.0 years), not medicated with insulin or any insulin sensitising agents and free of any conditions that may prevent safe completion of the exercise demands of the study. Participants were required to obtain physician consent for participation in the program, and were individually screened via a medical history form and interview with the investigators to determine eligibility. Full details of participant recruitment have been reported elsewhere (15). This study was approved by the Human Research Ethics Committee of The University of Queensland and informed consent was obtained from all participants. No participants died before the experiments were concluded.

2.2 Experimental models of exercise
Participants completed either an acute session of HIIE or short-term HIIE training prescription. The HIIE 38 minute session commenced with a 10 minute warm up at 50-70% peak heart rate (HRpeak) before 4x4 minute bouts of cycling at 85-95% HRpeak were completed. Three minutes of active recovery separated each exercise bout. The short-term training program involved repeating the HIIE protocol three times a week for four weeks. Further details of the safety and feasibility of this intervention are reported elsewhere (15).
2.3 Serum collection and analysis

During the acute exercise sessions, venous blood was sampled at baseline (fasted), immediately post- (0 minutes) and 120 minutes post-exercise. Following fasting/resting blood sampling, participants consumed a light liquid meal replacement of 0.5 g.kg\(^{-1}\) of Sustagen Sport\(^\circledR\) (Nestle Australia, Sydney, Australia) mixed with 300 ml of water; they then rested for 30 minutes before exercising. For the training intervention, fasting blood was sampled between 3 and 7 days before (pre-intervention testing) and following the intervention (post-intervention testing) at approximately the same time of day. At each time point, blood (20 ml) was collected from an antecubital vein. Samples were allowed to clot at room temperature (~30 minutes), were centrifuged at 8500 rpm for 10 minutes (900 x g), frozen at -80°C and stored for later analysis. Assays using serum samples were partially blinded by allocating samples with sequential number codes prior to analysis.

2.4 Culture of colon cancer cell lines

The effects of exercise were investigated in two human colon cancer cell lines (CaCo-2 and LoVo) purchased from Cell Bank Australia (Westmead, Australia). These two colon cancer cell lines were chosen for their different genetic features in critical genes related to colorectal cancer: CaCo-2 are \(TP53\) mutant while LoVo cells are \(KRAS\) mutant and wild type for the other gene (16). Cells were cultured in a mycoplasma-free tissue culture environment in Eagle’s Minimum Essential Medium (CaCo-2) or RPMI-1640 medium (LoVo), supplemented with 10% foetal bovine serum (FBS), 1% glutamine and 1% penicillin-streptomycin. Cells were incubated at 37°C in 5% CO\(_2\) and were routinely passaged at approximately 80% confluence.

2.5 Cell number assay

Cell number was assessed using the alamarBlue\(^\circledR\) assay (Thermo Fisher Scientific, Waltham, MA). Cells were counted using an automated cell counter (TC20 Automated Cell Counter, Biorad, Hercules, CA), after which four replicate wells of CaCo-2 cells were seeded at approximately 2x10\(^3\) cells.well\(^{-1}\) and LoVo cells at 1x10\(^4\) cells.well\(^{-1}\) in black, clear-bottom 96-well plates (Corning Incorporated, Corning, NY). Cells were not incubated in the outer ring of wells (middle 60 wells only) to minimise the influence of evaporation on differences in fluorescence across the plate (17) and quadruplicates were arranged in a two-by-two arrangement to minimise the effect of row- or column-
specific variability. Serum samples from each individual were used on the same plate to negate inter-plate variability for comparisons within individuals. Cells were seeded in 100μL of normal culture medium with 10% FBS for 24 hours to allow for attachment, then aspirated and replaced with 100μL of medium containing 10% serum from individual participants instead of FBS. Separate plates were then incubated for 24, 48 or 72 hours. Fluorescence was then measured using a microplate reader (FLUOstar Optima, BMG Labtech, Ortenberg, Germany) at an excitation of 540 nm and emission of 590 nm. The median of four replicates was used for analysis, with background fluorescence subtracted from the value for each well and then normalised to the fluorescence of control cells grown in 10% FBS instead of participant serum. The intra-assay coefficient of variation for this assay based on four replicates was 4.3% for CaCo-2 and 4.6% for LoVo cells.

2.6 Cell death assay

Levels of phosphatidylserine externalization were determined using binding of Annexin-V, a common feature of apoptotic cells. Briefly, CaCo-2 and LoVo cells were seeded in 60 mm dishes at a density of 7.5 x 10⁴ cells and 2 x 10⁵, respectively, in 4 ml of their respective growth media. Cells were allowed to attach for 24 hours prior to media replacement with 10% patient serum for 72 hours. After harvesting via trypsinisation, cells were resuspended in 50 μL of Annexin-V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂) and placed on ice for 5 minutes. Cells were then stained with 5 μL.ml⁻¹ Annexin-V Alexa Fluor® 488 (Thermo Fisher Scientific, Waltham, MA) in a further 50 μL of buffer and incubated for 15 minutes in the dark. To distinguish necrotic cells, propidium iodide (Sigma Aldrich, St. Louis, MO) was added at a concentration of 1 μg.ml⁻¹ in 400 μL of buffer before being analysed on a C6 Accuri flow cytometer (BD Biosciences, San Jose, CA). For an apoptotic positive control, cells were incubated with 1 μM staurosporine for 68 hours. Flow cytometry data analysis was conducted using the FlowJo™ software (Flow Jo LLC, Ashland, OR). Following doublet discrimination, population gating based on single-colour and healthy controls to determine healthy (Annexin-V negative, PI negative), early apoptotic (Annexin-V positive, PI negative) or late apoptotic/necrotic (Annexin-V positive or negative, PI positive).
2.7 Systemic marker analyses

Systemic concentrations of IL-6, IL-8, and TNF-α were measured using a high-sensitivity magnetic bead-based multiplex assay (R&D, Minneapolis, MN) and a Magpix® system (Merck Millipore, Billerica, MA). IGF1 was analysed using an enzyme linked immunosorbet assay (Quantikine, R&D Systems, Minneapolis, USA). Insulin and glucose were measured using a Cobas e 411 analyser (Roche Diagnostics, Mannheim, Germany) and a Randox RX Daytona+ analyser (Randox Laboratories Limited, Crumlin, United Kingdom), respectively. The coefficient of variation for IL-6, IL-8, TNF-α, IGF1, insulin and glucose were 3.1%, 1.8%, 2.9%, 2.1%, 1.1% and 0.8%, respectively.

2.8 Statistical Analyses

Data were analysed unblinded using the SPSS statistical software package (version 23.0, SPSS, Inc., Chicago, IL). For experiments with multiple factors, outcomes were assessed using linear mixed modelling analysis. The combination of sampling time point and incubation time (24, 48 or 72 hours) were treated as fixed factors, with participants treated as a random factor with individual intercepts. Normality of the resulting model residuals were assessed using the Shapiro-Wilk test and via inspection of histogram and quintile-quintile plots. Data found to be positively skewed (insulin, IGF1) were analysed with a generalised linear mixed model specifying a gamma distribution and log-link as previously described (18). Changes in levels of apoptosis were determined using independent samples t-tests. Paired samples t-tests and the Wilcoxon Signed Rank test were used for analysis of systemic markers pre- and post-training. Bonferroni adjustments were made to account for multiple pairwise comparisons within each analysis. Effect sizes (ES) were calculated using the Cohen’s d statistic to describe the mean difference in relative cell number (19). An alpha level of 0.05 was used for statistical significance.

3.0 Results

3.1 Effect of acute and short-term HIIE on colon cancer cell number

Details of the participants in the acute (n=10) and short-term training (n=10) exercise experiments are presented in Table 1. Compared to baseline serum, incubation with serum obtained immediately after HIIE exercise cessation significantly reduced CaCo-2 cell number after 24 (ES=-1.3, p=0.002), 48 (ES=-1.7, p<0.001) and 72 (ES=-1.1,
Incubation of LoVo cells with serum collected immediately post-exercise also reduced cell number at 24 (ES=-1.2), 48 (ES=-0.8) and 72 (ES=-1.1) hours, with significant decreases relative to baseline at 24 (p=0.001) and 72 (p=0.032) hours. There was no significant difference (p≥0.05) between sera collected at baseline and 120 minutes post-exercise across the incubation times in either cell line.

Incubation with serum collected at rest (3-7 days) following the 4-week HIIE training resulted in no significant differences on either CaCo-2 (p=0.223) or LoVo (p=0.375) cells when compared to treatment with serum from the pre-intervention control conditions. Consideration of cancer site (colon and rectal) had no influence (p≥0.05) on the in vitro colon cancer cell number assays.

3.2 Effect of exercise serum on colon cancer cell apoptosis

A reduction in cell number may be due to increased apoptosis, as previously reported with exercise serum treatment (20). Therefore, serum incubation experiments were also performed to determine the effect of exercise serum on colon cancer cell apoptosis. Compared to cells incubated with serum collected prior to acute HIIE, there were no significant differences in the proportion of apoptotic (Annexin-V positive, PI negative) CaCo-2 (-0.04%, 95% CI -0.24-0.17%; p=0.702) or LoVo cells (0.64%, 95% CI -0.92-2.20%; p=0.395) following 72 hours of incubation with serum collected immediately post-HIIE (Figure 2). There were no significant differences (p≥0.05) in the proportion of healthy cells or any other measures of cell death from this assay.

3.3 Changes in serological factors

Immediately following acute HIIE, significant increases were observed for TNF-α (+0.7pg.ml\(^{-1}\), +15.2%, p=0.003), IL-6 (+0.30pg.ml\(^{-1}\) +44.8%, p=0.023), IL-8 (+2.3pg.ml\(^{-1}\) +24.7%, p=0.036), and insulin (+3.1pmol.L\(^{-1}\), +38.8%, p=0.023). The concentration of TNF-α, IL-6 and IL-8 returned to baseline levels at 120 minutes post-exercise (p≥0.05), with insulin significantly lower than baseline at 120 minutes (-2.1pmol.L\(^{-1}\), p=0.001).

No significant differences in TNF-α (p=0.765), IL-6 (p=0.338), IL-8 (p=0.074), IGF1 (p=0.725), insulin (p=0.976) or glucose (p=0.138) were observed between resting serum collected at baseline and after four weeks of HIIE.
4.0 Discussion

By combining exercise with in vitro cell function assays, the present study indicates that systemic changes following acute HIIE suppress the growth of colon cancer cells. Acute HIIE promoted transient increases in systemic cytokine concentrations (IL-6, IL-8 and TNF-α) immediately following exercise, which abated by two hours post-exercise in concordance with the changes in cell number. Despite likely inducing repetitive up-regulation of these acute growth suppressive effects of HIIE immediately following each session, short-term HIIE training was not associated with changes in cell number or common metabolic factors related to the risk of colorectal cancer when measured at rest. These data provide support for the benefits of transient systemic changes following HIIE; repeated acute benefits resulting from regular exercise may favour a systemic profile less conducive to colon cancer cell growth.

To the best of our knowledge, this is the first study to show that the acute serological changes immediately following HIIE are associated with a reduction in colon cancer cell growth. The distribution of apoptotic cells in either cell line following incubation with pre-exercise serum or serum immediately-post HIIE was not significantly different, suggesting that the observed reductions in overall cell number are likely mediated via reductions in cellular proliferation rather than increased rates of apoptosis. Rundqvist et al. (21) also showed that reductions in prostate cancer cell viability following post-acute exercise serum incubation were due to the inhibition of proliferation, with no changes in levels of apoptosis. Dethlefsen et al. (8) reported a reduction in breast cancer cell viability following incubation with serum obtained immediately after a two-hour exercise session (resistance and high intensity aerobic exercise). Similar to the present study, significant increases in TNF-α, IL-6 and IL-8 were reported immediately post-exercise, with increases in, epinephrine, and norepinephrine also observed (8). This study extends these findings to show that the transient suppression of cancer cell growth had subsided by two hours post-exercise, coinciding with the return of TNF-α, IL-6 and IL-8 to baseline levels at this time point. These findings tend to suggest that the transient increases in cytokine concentrations immediately following HIIE in colorectal cancer survivors may be important mechanisms contributing to the observed growth suppression effect in colon cancer cells.
Recent data on tumour progression have provided evidence that the exercise-induced acute cytokine response is an important mechanism underscoring the anticarcinogenic effects of exercise (10, 11). Pederson et al. (11) showed that exercise-mediated increases in natural killer (NK) cell mobilisation (catecholamine mediated) and redistribution (IL-6 mediated), substantially reduced tumour incidence following exercise in a murine model. However in both the present study and that of Dethlefsen et al. (8), the absence of NK cells in culture following acute exercise serum-replacement suggests that reductions in tumour cell viability according to this catecholamine-NK cell-cytokine mechanism must reflect the downstream cellular consequences of effector cytokine release (such as TNF-α) following exercise rather than direct NK cell mediated apoptosis (10, 22-24).

Furthermore the hypothesised role of inflammatory cytokines is somewhat perplexing given that chronic inflammation (including increases in C reactive protein, IL-6 and TNF-α) is a hallmark of cancer and is associated with an increased risk of incidence (9). This presents an interesting juxtaposition between the proposed anti-carcinogenic effects of IL-6, IL-8 and TNF-α following acute exercise and the role of chronic elevations in these markers in cancer development. The precise mechanisms by which these factors may influence cellular growth in vivo remain unclear at this point. Future research is needed to confirm the relative temporal changes in these cytokines and establish if a causal relationship exists with the antioncogenic properties of acute exercise, as well as investigating the role of additional immune cells in the response to exercise (9). It remains possible that these markers may not influence cellular progression in vivo but may be markers of the release of other effector mediators following this catecholamine-NK cell-cytokine sequence that directly influence cellular outcomes.

Surprisingly, yet in agreement with previous reports (8), short-term HIIE training did not promote similar growth reductions to acute HIIE. Several factors may explain this finding. Previous reported reductions in prostate (7, 20) and breast (4) cancer cellular growth following an intervention (exercise and/or dietary modification) were associated with significant decreases in IGF1 and insulin, and increases in IGFBP1. IGFBP1 can sequester IGF1, preventing initiation of tumour promoting intracellular signalling and is associated with improved insulin resistance (25, 26). The lack of
change in IGF1 or insulin observed in the present study may therefore explain the absence of changes in response to incubation with serum following short-term HIIE training. At baseline, participants in this cohort had notably low levels of IGF1 relative to age-referenced normative values (27). This may be partly explained by the duration since diagnosis and treatment of participants included in this study (Table 1). IGF1 and insulin have been shown to promote tumour progression (25, 28, 29), and systemic overexpression of these factors has been associated with an increased risk of colorectal cancer (30, 31). Therefore the absence of these risk factors at baseline in the present cohort may have supressed the potential for improvements following exercise training.

Acute excursions in factors following exercise (e.g. cytokines) and chronic changes in biomarkers (e.g. metabolic markers) at rest are two separate mechanisms that may contribute to improvements in cancer prognosis with increased levels of physical activity (1, 8, 9). Suppressed cell growth following acute HIIE warrants further investigation that assesses the translatability into clinically meaningful improvements for colorectal cancer survivors and to evaluate the mechanisms specific to the biology of in vivo tumours by which this may occur. Additionally, whether the acute HIIE response differs between sedentary and trained individuals remains to be determined but may provide further insight into how long-term exercise programs may improve colorectal cancer outcomes. Further research is also warranted to understand the effects of time elapsed from diagnosis and the effects of treatment on colon cancer cell growth.

Finally, given the transient nature of the changes in cell number and cytokines following exercise, which had abated at two hours post-exercise, the importance of engagement in and adherence to regular physical activity cannot be overstated. It is unlikely that a single bout of exercise would produce a sufficient volume of circulating factors in such a short post-exercise duration to contribute to improvements in prognosis. However as part of a long-term exercise program, the repetitive induction of acute changes in cytokines and the eventual accumulation of chronic changes in other risk factors for colorectal cancer (e.g. IGF axis) may explain the relationship between exercise and improvements in cancer prognosis (9).
4.1 Conclusions and implications

The present study demonstrates that the serological changes associated with acute HIIE transiently reduce colon cancer cell number. There are several limitations worthy of comment. As this was a pilot study, the findings are limited by a small sample size that should be considered when drawing conclusions. Despite the small sample size, post-hoc power analysis based on cell viability effect sizes following incubation for 24, 48 or 72 hours with serum immediately following acute HIIE achieved a power of between 87.1 – 99.7% for CaCo-2 and between 61.6 – 92.0% for LoVo cells. The encouraging within-group changes provides support for the design of larger, well-powered trials implementing this type of analysis to investigate differences between prescriptions of exercise to more adequately assess these outcomes. Secondly the assay used in the present trial does not account for the in vivo environment in which tumour cells survive, which limits conclusions regarding whether the observed systemic effects were sufficiently large to directly influence tumour cells in vivo. Whilst the present trial has demonstrated distinct systemic cytokine (TNF-α, IL-6 and IL-8) responses between acute and chronic exercise, it is possible that other contributory mechanisms may underlie the observed reductions in colon cancer cell growth. Given the pleiotropic effects of exercise, which influences a multitude of systems, we cannot conclude that the observed changes in colon cancer cell growth were due to any one series of factors (32). Finally, the lack of a non-exercising control is a limitation that should be considered when drawing conclusions from these results.

Notwithstanding these limitations, the acute serological changes following exercise in the present trial and others (8, 11) tend to suggest that the exercise-induced cytokine changes may constitute an important mechanism that contributes to the changes observed in colon cancer cell growth. Therefore even without inducing chronic changes in various systemic factors associated with colorectal cancer risk, the repetitive induction of the exercise-induced cytokine flux associated with HIIE training may translate into a more favourable systemic profile. Given the apparent importance of the transient serological responses to acute exercise, this may be an important mechanism contributing to the relationships observed between physical exercise and cancer mortality.
Figures

**Fig. 1** Cell number following incubation of (A) CaCo-2 and (B) LoVo cells with serum from either baseline (pre-exercise), 0 or 120 minutes post-high intensity interval exercise, or (C) CaCo-2 and (D) LoVo cells with serum collected at rest (fasting) either pre- or post-exposure to short-term high intensity interval exercise training (baseline and post-intervention). Cells were incubated for 24, 48 or 72 hours. Data presented as mean and 95% CI
References

Additional information

Competing interests

The authors declare that they have no conflicts of interest.

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This study was funded by The University of Queensland HABS/MABS Collaboration Seeding Grant, Sports Medicine Australia Research Foundation and The University of Queensland Graduate School International Travel Award.
<table>
<thead>
<tr>
<th>Table 1: Baseline participant characteristics</th>
<th>Acute Exposure</th>
<th>Short-term Exposure</th>
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<tr>
<td>n</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Age (years)</td>
<td>66.9 ± 8.4</td>
<td>64.9 ± 6.0</td>
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<tr>
<td>Body mass (kg)</td>
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<tr>
<td>ĊO₂peak (ml.kg⁻¹.min⁻¹)</td>
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<td>23.2 ± 3.9</td>
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<tr>
<td>Cancer History</td>
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<tr>
<td>Colon cancer [n (%)]</td>
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<td>8 (80)</td>
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<tr>
<td>Rectal cancer [n (%)]</td>
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<td>2 (20)</td>
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<tr>
<td>Time since diagnosis (years)</td>
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<td>3.6 ± 1.1</td>
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<tr>
<td>Time since treatment (years)</td>
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<tr>
<td>Cancer Stage [n (%)]</td>
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<tr>
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<td>1 (10)</td>
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<tr>
<td>II A</td>
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<td>Exercise Intensity</td>
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<td>Heart rate (% HRpeak): HIIE</td>
<td>84.1 ± 1.8</td>
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<tr>
<td>Power Output (% PPO): HIIE</td>
<td>64.2 ± 4.6</td>
<td>94.2 ± 9.3</td>
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Continuous variables are presented as mean ± SD; Nominal variables are presented as n (%)

HIIE: high intensity interval exercise; HRpeak: peak heart rate; PPO: peak power output; ĊO₂peak: peak oxygen consumption
First Author Biography

James Devin is an early career researcher who received his PhD from the University of Queensland. James also completed a Bachelor of Exercise and Sports Science, majoring in Clinical Exercise Physiology, and concurrently works as a Clinical Exercise Physiologist. His research interests focus on exercise oncology for colorectal cancer patients and survivors aiming to better understand the mechanistic link between exercise and colorectal cancer progression. His work ultimately aims to contribute to the growing body of evidence for how exercise can be used to improve health outcomes for people affected by colorectal cancer.