Mobilizing serum factors and immune cells through exercise to counteract age-related changes in cancer risk

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ABSTRACT

An increasing body of evidence suggests that age-related immune changes and chronic inflammation contribute to cancer development. Recognizing that exercise has protective effects against cancer, promotes immune function, and beneficially modulates inflammation with ageing, this review outlines the current evidence indicating an emerging role for exercise immunology in preventing and treating cancer in older adults. A specific focus is on data suggesting that muscle-derived cytokines (myokines) mediate anti-cancer effects through promoting immunosurveillance against tumourigenesis or inhibiting cancer cell viability. Previous studies suggested that the exercise-induced release of myokines and other endocrine factors into the blood increases the capacity of blood serum to inhibit cancer cell growth in vitro. However, little is known about whether this effect is influenced by ageing. Prostate cancer is the second most common cancer in men. We therefore examined the effects of serum collected before and after exercise from healthy young and older men on the metabolic activity of androgen-responsive LNCaP and androgen-unresponsive PC3 prostate cancer cells. Exercise-conditioned serum collected from the young group did not alter cell metabolic activity, whereas post-exercise serum (compared with pre-exercise serum) from the older men inhibited the metabolic activity of LNCaP cancer cells. Serum levels of candidate cancer-inhibitory myokines oncostatin M and osteonectin increased in both age groups following exercise. Serum testosterone increased only in the younger men post-exercise, potentially attenuating inhibitory effects of myokines on the LNCaP cell viability. The data from our study and the evidence in this review suggest that mobilizing serum factors and immune cells may be a key mechanism of how exercise counteracts cancer in the older population.

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INTRODUCTION

The global increase in the elderly population is associated with growing challenges both for individuals and health care systems (5, 25, 108). The incidence and prevalence of most cancers increase with ageing (9, 64, 70), and this has major health implications. It is therefore important to improve the understanding of the efficacy of lifestyle-based strategies for preventing and treating cancer with advancing age. Epidemiological and observational evidence shows that regular physical activity and exercise[1] protect against the development of some of the most common cancers (such as breast, colon and endometrial cancer), and reduce the risk of disease recurrence and mortality (1, 64, 70, 74). The protective effects of exercise against cancer are proposed to revolve around changes in the endocrine and immune systems (56, 58, 73). Notably, age-related alterations in the immune system may contribute to the increased cancer risk in the older population (43). Recognizing the increasing body of evidence suggesting immune benefits of exercise especially in older adults (34, 116), this notion has important implications for the role of exercise immunology in the protection against cancer with ageing.

Recent experimental data have also shown that blood serum collected after exercise from mice (57), healthy younger adults (105), and cancer patients and survivors (32, 33), inhibits cancer cell growth *in vitro.* These effects of exercise-conditioned serum are likely due to the exercise-induced secretion of endocrine factors by skeletal muscle – termed myokines – and/or other tissues into the blood (32, 33, 57, 105). Muscle-derived endocrine factors that may mediate cancer-inhibitory effects of exercise through controlling cancer cell metabolism and growth kinetics include, for example, irisin, osteonectin (also known as secreted protein acidic and rich in cysteine, or SPARC), and oncostatin M (OSM) (57, 93). Furthermore, certain myokines, including interleukin (IL)-6, IL-7 and IL-15, have immune regulatory effects (56, 91, 93), which might have important consequences for counteracting carcinogenesis. For example, IL-6 regulates the exercise-induced redistribution of cytotoxic natural killer (NK) cells from the blood circulation into tumours, thereby decreasing tumour growth (95). The data suggesting a myokine-mediated ‘crosstalk’ of skeletal muscle with immune and cancer cells, provide a novel conceptual framework that links active skeletal muscle and immune function with exercise-associated protection against cancer (34, 58). However, so far, only very little information is available on how ageing influences these muscle-immune- and muscle-cancer cell-interactions.

Prostate cancer is the second most common cancer in males, and, in particular, a type of cancer that becomes more common as men age (1). The epidemiological evidence for a preventive effect of physical activity specifically against prostate cancer is limited (1, 64, 70, 74). However, epidemiological data suggest that physical activity is associated with a 38% reduction in the relative risk of cancer-specific mortality in individuals diagnosed with prostate cancer (74). Observational studies have also shown an exercise-dependent reduction in the risk of disease recurrence for prostate cancer (58, 63). Moreover, a study by Rundqvist et al. has demonstrated that serum collected from 10 healthy men (aged between 18 and 37 years) after one hour of cycling inhibited the growth of prostate cancer cells (LNCaP) *in vitro*, as compared with serum obtained in resting conditions (105). Nevertheless, there are no data available on whether ageing influences the exercise-induced changes in the capacity of serum to inhibit the viability of cancer cells in general, and prostate cancer cells in particular. Expanding upon the work by Rundqvist et al. (105), the primary aim of the current study was to compare how serum collected pre- and post-exercise from younger and older men affects prostate cancer cell growth. We hypothesised that acute exercise would improve the capacity of serum to inhibit prostate cancer cell growth in younger and older men, as compared with serum collected in resting conditions. As another extension to the study by Rundqvist et al. (105), we used two different prostate cancer cell lines, androgen-responsive LNCaP and androgen-unresponsive PC3 cells, for incubation with the serum. Androgen signalling plays an important role in normal male physiology and prostate cancer (29, 106). Considering that systemic levels of androgens, such as testosterone, change in response to exercise (79, 125) and with ageing (53, 109), we used these two cell lines to gain insights into potential androgen-related mechanisms. In addition, we assessed whether there are age-related differences in the exercise-induced changes in the serum concentrations of testosterone and candidate immune-regulatory or cancer-inhibitory cytokines including myokines IL-6, IL-15, irisin, osteonectin, and OSM.

In addition to our original contribution, we have outlined the current evidence on the role of exercise-induced changes in serum myokine/cytokine concentrations and the immune system in counteracting cancer in the older population. Several reviews on the impact of exercise on immune function in the context with either ageing (34, 116) or cancer (56) are available. A recent review by Hojman et al. has provided an updated status of research related to the molecular mechanisms underlying the link between exercise and cancer prevention and treatment (58). To present a contemporary view, this review combines and integrates available data on how myokines, cytokines and the cellular immune system mediate protective exercise effects against cancer specifically with advancing age, with the major focus on human studies.

METHODS

Ethical approval
The study was approved by the Queensland University of Technology Human Research Ethics Committee (ethics no. 1500000881). A total of 22 healthy males volunteered to participate and all participants provided written, informed consent before their inclusion in the study.

Study participants
Twelve healthy males aged 20 to 33 years (young age group) and ten healthy males aged 60 to 73 years (old age group) were recruited for this project. Before the enrollment into the study, all participants were assessed by a medical physician and were classified as clinically healthy. This medical entrance examination included a standard medical history questionnaire, height, weight, body mass index (BMI), and blood pressure measurements. The participants also completed a physical activity questionnaire. Both age groups had a

normal BMI of 18.5–25 kg/m^2. Exclusion criteria included any evidence of acute or chronic diseases such as cancer, or heart, lung, nerve or muscle disease and diabetes. Further exclusion criteria included training for and participation in any competitive sports events, smoking and the use of blood-thinning medication, and other drugs such as anti-inflammatory medicines or statins. All participants performed an incremental exercise test on a cycle ergometer (LODE Excalibur Sport, LODE BV, Groningen, The Netherlands) to determine their maximum oxygen uptake ($\dot{V}O_{2\ max}$). After a warm-up of 5 minutes, the test commenced at a power output of 25 W, with increments of 25 W/min until cadence dropped below 60 rpm, or voluntary exhaustion occurred. Gas analysis during the test was performed using a TrueOne2400 metabolic cart (ParvoMedics, East Sandy, UT, USA), while heart rate was monitored by telemetry (Vantage NV, Polar, Finland). The participants' anthropometric and physiological characteristics are summarized in Table 1.

Table 1. *Anthropometric and physiological characteristics of the study participants*

	Total group (n=22)	Young age group (n=12)	Old age group (n=10)	P value
Anthropometric characteristics				
Age (years)	44.05 ± 18.46	28.2 ± 2.6	63.1 ± 6.9	0.000*
Height (m)	1.80 ± 0.06	1.80 ± 0.08	1.80 ± 0.03	0.923
Weight (kg)	76.9 ± 6.5	74.1 ± 6.2	80.15 ± 5.6	0.025*
BMI (kg/m^2)	23.8 ± 2.2	22.8 ± 2.1	24.9 ± 1.92	0.024*
Physical activity/exercise level				
Duration (min/week)		243 ± 193	492 ± 432	0.047*
Physiological characteristics				
$\dot{V}O_{2\ max}$ (L/min)	3.20 ± 0.6	3.27 ± 0.61	3.12 ± 0.69	0.602
$\dot{V}O_{2\ max}$ (mL/kg/min)	41.7 ± 8.1	44.1 ± 7.3	38.9 ± 8.4	0.148
METs at $\dot{V}O_{2\ max}$	12.0 ± 2.4	12.6 ± 2.1	11.1 ± 2.6	0.180

Data are presented as mean ± SD. BMI, body mass index; $\dot{V}O_{2\ max}$, maximum oxygen uptake; METs; metabolic equivalents. * Significantly different between age groups.

Exercise protocol

On the morning of the exercise trial, the participants were provided with a standardized breakfast. This standardized breakfast consisted of four high-fiber low-sugar cereal biscuits (983 kJ, 0.9 g fat, 44.2 g carbohydrates, 8.2 g protein) and 200 mL milk (538 kJ, 6.8 g fat, 10.2 g carbohydrates, 6.6 g protein). The participants were asked to have their breakfast 2 to 2.5 h before the exercise trial. Furthermore, the participants were required not to exercise in the 24 h preceding the exercise trial and to refrain from any caffeine and alcohol consumption during this time frame. As in the study by Rundqvist et al. (105), the exercise trial consisted of 20 min of cycling at a work rate corresponding to 50% of their $\dot{V}O_{2\ max}$, before the work rate was increased to a work rate corresponding to 65% of their $\dot{V}O_{2\ max}$ for an additional 40 min. Immediately before and immediately after exercise, approximately 25 mL of blood was collected through a 21-gauge butterfly needle inserted into an antecubital vein. Blood was collected into vacutainers (Becton Dickinson Biosciences, San Jose, CA, USA), containing either ethylenediamine tetraacetic acid (EDTA; for hematological analysis) or a clot activator (CAT; for serum analysis and serum incubation with the cell lines). For serum analysis and serum incubation experiments, blood samples were centrifuged at 1500 × g for 10 min at 22 °C, within 20 minutes of collection. Serum was aliquoted and stored at –80 °C until analysis or use for cell culture experiments.

Hematological analysis

Pre- and post-exercise whole blood collected in vacutainer tubes with EDTA was used for assessment of the hematological profile (including concentrations of total leukocytes and leukocyte subpopulations) using an automated hematology analyzer (Ac T diff 2, Beckman Coulter, Brea, CA, USA). Before data acquisition, the instrument accuracy and precision were checked using the 4C-ES Cell Control (Beckman Coulter). The collected blood samples were gently mixed before placing the tube into the analyzer.

Cell culture

Two commonly used prostate cancer cell lines from the American Type Culture Collection (ATCC), PC3 (ATCC® CRL-1435™) and LNCaP (ATCC® CRL-1740™), were used in this study. Both cell lines differ in their biological characteristics, with PC3 cells demonstrating stronger invasiveness compared to LNCaP cells *in vitro*. LNCaP cells are androgen-responsive, unlike PC3 cells, which are not responsive to androgens (72). Both cell lines were cultured in 75-cm^2 flasks using phenol red-free Roswell Park Memorial Institute medium-1640 (RPMI-1640) supplemented with 10% (v/v) fetal bovine serum (FBS), 0.1 mg/mL streptomycin, 100 units/mL penicillin.

Cellular metabolic activity assay

The Alamar blue assay® (ThermoFisher, Waltham, MA, USA) was used to assess the effect of pre- and post-acute exercise serum on the metabolic activity, an indirect measure of the cellular viability, of the prostate cancer cells. This assay is a widely used to evaluate the metabolic function and cellular health of cultured cells (102). Specifically, it incorporates

an oxidation-reduction indicator that fluoresces and changes color in response to chemical reduction of growth medium resulting from cell growth. As the cultured cells grow, their metabolic activity results in a chemical reduction of this indicator. Continued growth maintains a reduced environment, whereas inhibition of growth maintains an oxidized environment. In 400 µL of growth medium, LNCaP and PC3 cells were seeded (at cell densities of 5.82 x 10^3 cells/cm^2 and 2.91x 10^3 cells/cm^2 for the LNCaP and PC3 cells, respectively) into individual wells of 48-well plates and incubated at 37 °C with 5% CO_2 for 24 h. On the following day, the cells were serum-starved for 3 h. After serum starving, 200 µL of RPMI 1640 medium supplemented with 5% FBS and 5% each participants' rest or exercise serum were added to each well plate in triplicate. Cells were returned to the incubator at 37 °C with 5% CO_2 for another 96 h, with the medium refreshed at the 48 h-time point. At the 96 h-endpoint, Alamar blue was added to each well at a final concentration of 8% (v/v). The plates were incubated at 37 °C for another 4 h and from each well, 100 µL of medium were transferred to a 96-well black plate in duplicate. Fluorescence intensity (excitation 544 nm, emission 590 nm) was detected using the POLARstar OPTIMA plate reader (BMG, Labtech, Ortenberg, Germany). After the Alamar blue assay, the cell monolayers were washed twice with PBS to remove residual Alamar blue reagent, and then stored at –80 °C for at least 48 h before the PicoGreen assay was performed.

DNA content assay for determining cell number

In addition to assessing the metabolic activities of prostate cancer cells, the cell number and cellular proliferation were measured indirectly by using a DNA quantification assay. This was achieved by using the PicoGreen reagent (ThermoFisher, Waltham, MA, USA) in conjunction with a DNA standard curve according to the manufacturer's instruction. After thawing the frozen plates, 0.5 mg/mL proteinase K (Invitrogen™) in phosphate-buffered EDTA (PBE) was added into each well and the plates were incubated at 37 °C with 5% CO_2 overnight. The detached cells were thoroughly resuspended and incubated at 56 °C for another 8 h. Subsequently, the cells were centrifuged (Microfuge 18, Beckman Coulter) at 2000 rpm for 5 min, and the supernatant containing DNA was diluted (1:50) in PBE. The DNA standard curve was prepared with lambda DNA standard using a dilution series, consisting of 1000, 500, 250,125, 62.5, 31.25, 15.625 ng/mL). The standards and samples were plated in triplicates of 100 µL into black 96-well plates. PicoGreen working solution (100 µL) was added to each sample or standard, and the plates were incubated for 5 to 10 min at room temperature and protected from light. The fluorescent signals were detected using a POLARstar OPTIMA fluorescence plate reader (excitation 485 nm emission 520 nm).

Serum testosterone analysis

Serum concentrations of total testosterone were assessed by electrochemiluminescence using an automated immunoassay analyser (Cobas E411, Roche diagnostics, Indianapolis, IN, USA) and native reagents (Elecsys Testosterone II, Roche diagnostics, Indianapolis, IN, USA). The manufacturer-reported measuring range is 2.5–1500 ng/dL. Analysis was performed with technical duplication, resulting in a coefficient of variation of 1.3 %. Due to difficulty to collect sufficient blood volumes from some of the participants, it was not possible to analyze serum testosterone from the whole study population. Samples from 10 young participants and seven old participants were included in the testosterone analysis.

Serum cytokine/myokine analysis

A Milliplex Myokine immunoassay kit (Millipore Corp., Billerica, MD, USA) was used to simultaneously measure serum levels of five known myokines, including IL-6, IL-15, irisin, osteonectin (also known as SPARC), and OSM (catalogue number: HMYOMAG-56K), as previously described (45). The assay was performed according to the manufacturer's instructions and all samples were run in duplicate. Briefly, 25µL of the provided standards, controls or blanks were added to the appropriate wells. In addition, 25 µL of each thawed serum aliquot was diluted 1:2 in assay buffer and then added to the sample wells prior to the addition of another 25 µL of assay buffer. A 25 µL aliquot of the provided serum matrix was added to wells containing standards, controls and blanks. A final 25 µL volume of working solution containing multiple microbeads, labelled with specific antibodies against each of the aforementioned factors were then added into each well and allowed to incubate overnight on a plate shaker at 4 °C. The plate was then washed twice with 200µL Milliplex wash buffer and the beads were incubated for 1 h at room temperature in 25 µL of detection antibodies. A 25 µL aliquot of Streptavidin-Phycoerythrin was then added to each well and allowed to incubate for 30 min at room temperature with agitation. The plate was then washed twice more, 150 µL of drive fluid was added to each well and the plates were then incubated for 5 min on a plate shaker. The plate was read on Millipore Magpix System (Millipore), and data was analysed using Milliplex Analyst software (V5.1, Millipore). Mean inter-assay coefficient of variation for the five factors analyzed were as follows: IL-6: 2.4 %; IL-15: 1.9 %; irisin: 2.3 %; osteonectin: 2.9%; OSM: 2.1%. Minimum detectable concentrations (minDC) and sensitivity (i.e., minDC+2SD; indicated in brackets) were as follows: IL-6: 0.53 pg/mL (0.9 pg/mL); IL-15: 0.73 pg/mL (5 pg/mL); irisin: 224.42 pg/mL (281 pg/mL); osteonectin: 3.62 ng/mL (7.5 ng/mL); OSM: 0.96 pg/mL (6 pg/mL).

Statistical analysis

Statistical analyses were conducted using SPSS version 25 (IBM, New York, USA) and GraphPad Prism version 7 (GraphPad Software, Inc., San Diego, CA). To compare the means between two unpaired groups, normality was tested using the Shapiro-Wilk test. If the data followed a Gaussian distribution, a Student T-test was performed; otherwise, the non-parametric Mann-Whitney test was performed. To compare among the two cell lines treated with pre- and post-exercise serum, a two-way repeated-measures ANOVA was performed, in conjunction with Sidak's multiple comparisons test. A paired T-test was used to compare the blood leukocyte, serum testosterone concentrations and cytokine/myokine concentrations pre- and post-exercise. Furthermore, a two-way repeated measures ANOVA was conducted to determine interaction effects of exercise and age on serum concentrations of testosterone and cytokines/myokines. Statistical significance was set at a $P<0.05$.

RESULTS

Exercise-induced changes in total leukocyte counts and leukocyte subpopulations in the blood circulation

Total circulating leukocyte counts, granulocyte and lymphocyte counts increased significantly ($P<0.05$) in both age groups after the exercise trial. No differences were detected in the magnitude of these changes between the two age groups. All changes in leukocyte subpopulations are summarized in Table 2.

Effects of exercise on the metabolic activity of prostate cancer cells

For the young age group, there was no significant difference in the metabolic activity of LNCaP and PC3 cells treated with pre- versus post-exercise serum (Figure 1). For the old age group, there was a significant reduction in LNCaP cell metabolic activity when cells were treated with post-exercise serum compared with pre-exercise serum ($P<0.01$). For the PC3 cell line, there was no significant difference in the metabolic activity when cells were treated with pre- versus post-exercise serum from both the young and older group.

Effects of exercise on prostate cancer cell numbers

As a proxy indicator of cell growth/proliferation, there was no difference in the total amount of DNA for LNCaP cells and PC3 cells treated with pre- versus post-exercise serum (Figure 2). There were no exercise age interaction effects for the total amount of DNA.

Table 2. *Blood concentrations of leukocytes and subpopulations before and after the exercise trial in both age groups*

	Young age group (n=12)			Old age group (n=10)		
	Pre-exercise	Post-exercise	P value	Pre-exercise	Post-exercise	P value
WBC ($x10^9$/L)	5.45 ± 1.24	7.42 ± 1.31*	<0.001	6.47 ± 1.7	8.44 ± 2.77*	0.004
LY ($x10^9$/L)	1.69 ± 0.41	2.35 ± 0.83*	0.004	1.86 ± 0.31	2.32 ± 0.56*	0.019
MO ($x10^9$/L)	0.39 ± 0.18	0.48 ± 0.12	0.098	0.38 ± 0.18	0.40 ± 0.17	0.629
GR ($x10^9$/L)	3.36 ± 0.98	4.60 ± 1.22*	0.002	4.25 ± 1.51	5.70 ± 2.44*	0.022

Data are presented as mean ± SD. WBC, white blood cells; LY, lymphocytes; MO, monocytes; GR, granulocytes.
* Significantly different from pre-exercise.

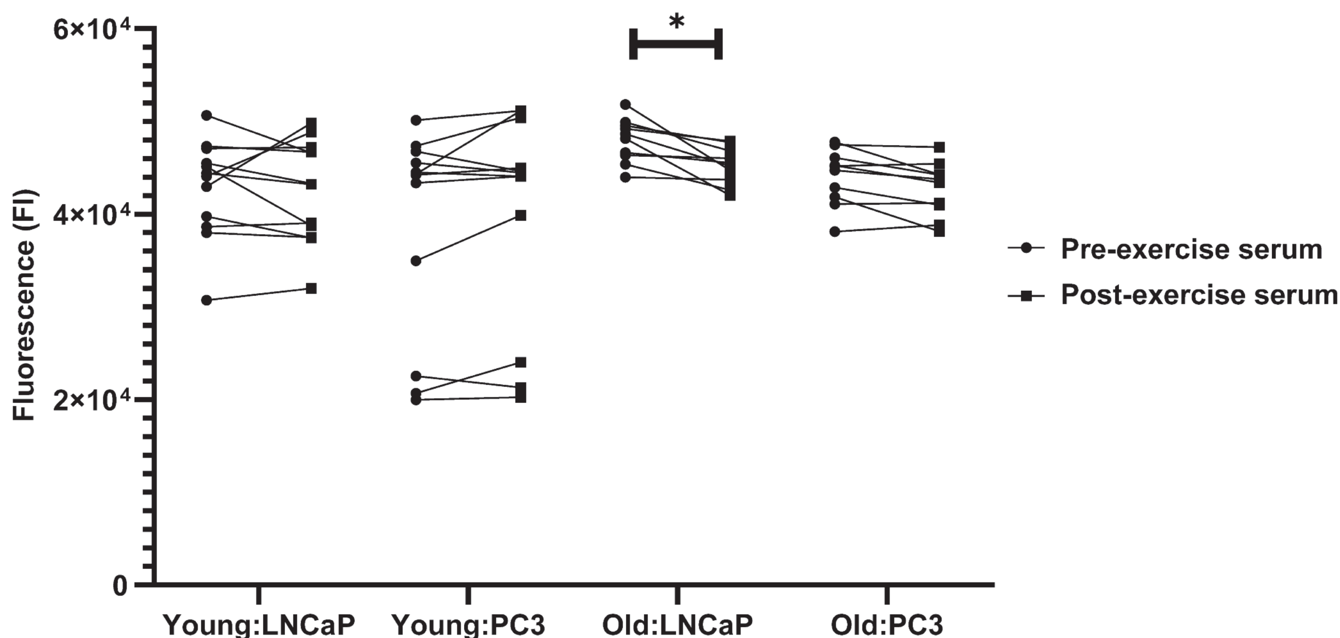

Fig. 1. Effects of exercise on the metabolic activity of LNCaP and PC3 prostate cancer cells after incubation with serum collected from the young and older men, as assessed by using the Alamar blue assay.

Metabolic activity results measured with Alamar blue reagent for LNCaP and PC3 cells treated with pre- or post-exercise human serum, separated based on the age group (young n=12, old n=10) and cell types (LNCaP, PC3). Values shown are the averages of the technical replicates for cells treated with each participant's pre- (circle) or post- (square) exercise serum. Each connected set of dots represent a different individual. A two-way repeated measure ANOVA with Sidak's multiple comparisons test was used to compare between the pre- and post-exercise response for each cell line within young or old age groups. *, $P<0.05$

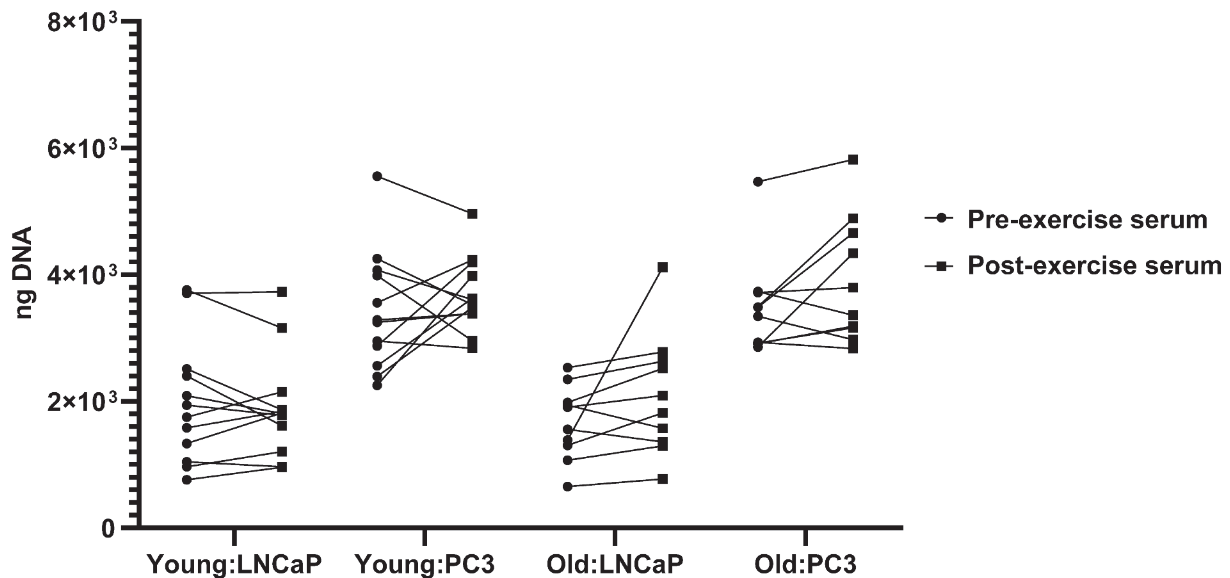

Fig. 2. Effects of exercise on numbers of LNCaP and PC3 prostate cancer cells after incubation with serum collected from the young and older men, as assessed by using the PicoGreen assay.

Combined cellular DNA results using the Alamar blue reagent for LNCaP and PC3 cells treated with pre- or post-exercise human serum, separated based on the age group (young n=12, old n=10) and cell types (LNCaP, PC3). Values shown are the averages of the technical replicates for cells treated with pre- (circle) or post- (square) exercise serum. Each connected set of dots represent an individual. A two-way repeated measure ANOVA with Sidak's multiple comparisons test was used to compare between the pre- and post-exercise response for each cell line within young or old age groups.

Exercise-induced changes in serum testosterone concentrations

Serum testosterone concentration increased from pre- to post-exercise in the young group (P<0.01), but not in the old group (shown in Figure 3). Results of the two-way repeated measures ANOVA on the serum testosterone data showed that there was neither an age effect nor an interaction effect of exercise and age.

Exercise-induced changes in serum concentrations of candidate cytokines/myokines

Serum concentrations of osteonectin and OSM increased in both young and older men from pre- to post-exercise (*P*<0.05). Significant effects of exercise for the total study group (P<0.001) as well as for the young (P<0.05) and the older group (P<0.01) were evident for both osteonectin and

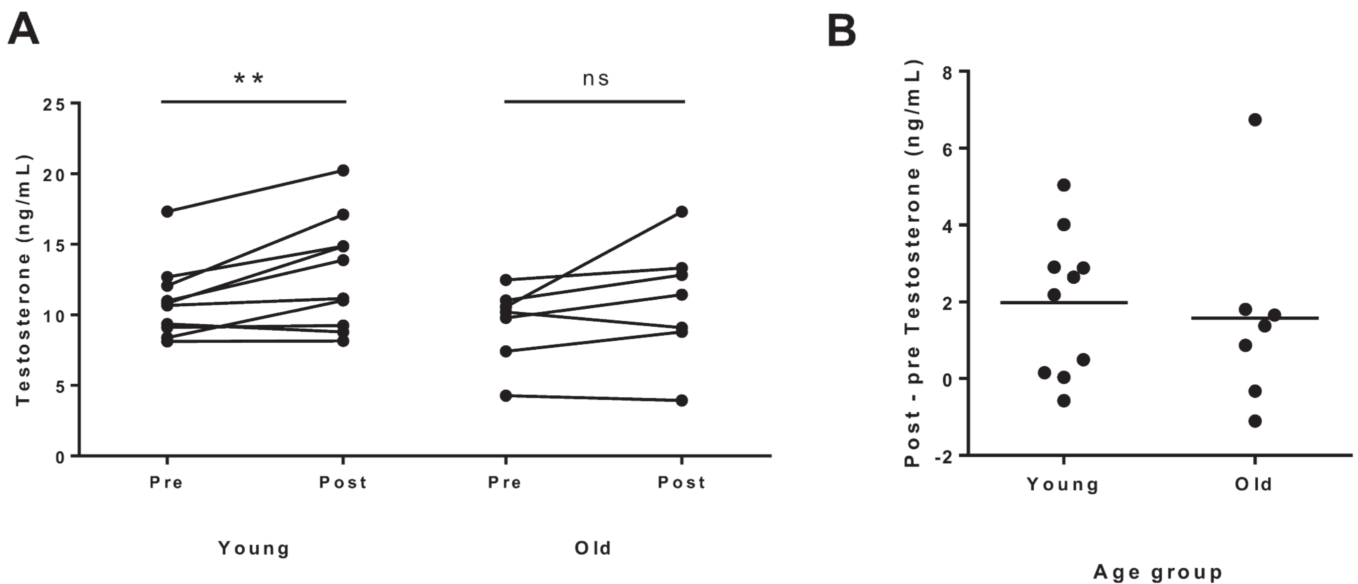

Fig. 3. Changes in the serum concentrations of testosterone from pre- to post-exercise in the young and older adults.

Univariate scatterplots of the level of testosterone (A) in pre- or post-exercise serum samples from each participant grouped on their age group (young n = 10, old = 7). Each connected set of dots represent a different individual. Fig. 3B shows the change in the testosterone levels post-exercise for each individual. A two-way repeated measures ANOVA was used to compare between the pre- and post-exercise response for each age group and interaction effects of exercise and age. A paired T-test showed that testosterone increased in the young group. ns = non-significant (*P*>0.05); **, *P*<0.01.

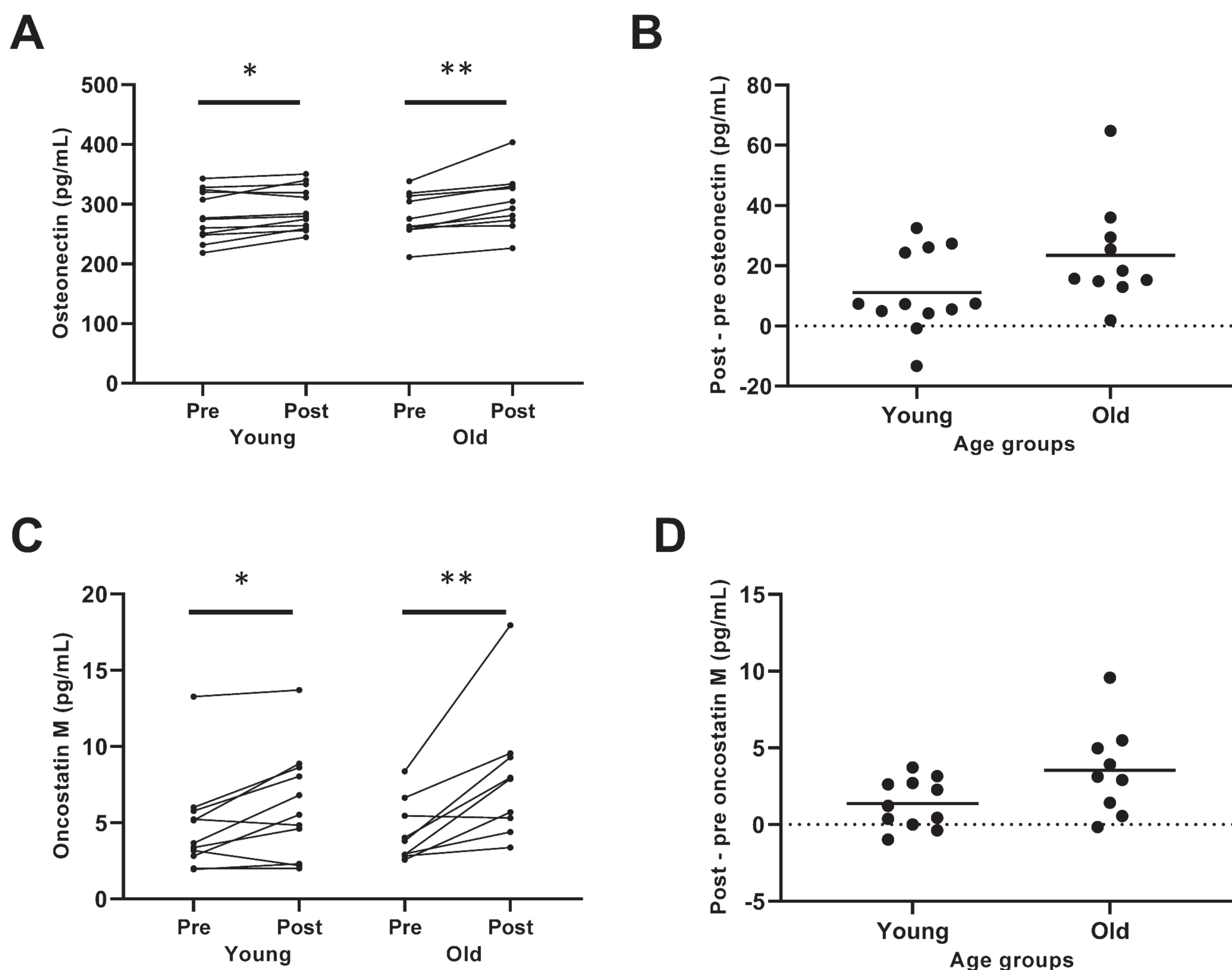

Fig. 4. Changes in the serum concentrations of osteonectin (SPARC) and oncostatin M (OSM) from pre- to post-exercise in the young and older adults.

Univariate scatterplots of the level of osteonectin (A) and OSM (C) in pre- or post-exercise serum samples from each participant grouped on their age group (young n=12, old n=10). Each connected set of dots represent a different individual. The change in the osteonectin and OSM levels post-exercise for each individual are represented (B, D), with the mean depicted as a horizontal line. A two-way repeated measures ANOVA was used to compare between the pre- and post-exercise response for each age group and interaction effects of exercise and age. *; *P*<0.05; **, *P*<0.01.

OSM. There was no effect of age or interaction effect of exercise and age. The pre- to post-exercise changes in the serum osteonectin and OSM concentrations in both age-groups are shown in Figure 4. Serum levels IL-6, IL-15 and irisin were not consistently detectable in all samples or did not change after exercise (data not shown).

DISCUSSION

The most important finding of this study was that, compared with pre-exercise serum, serum collected from older men after a single bout of exercise reduced the metabolic activity of (androgen-responsive) LNCaP prostate cancer cells *in vitro*. By contrast, no such effect was observed after treatment of the cancer cells with pre- versus post-exercise serum collected from young men. Furthermore, there was no difference between the effects of pre- versus post-exercise serum on the total amount of DNA of LNCaP and PC3 prostate cancer cells. Exercise elicited an increase in the serum levels of osteonectin (also known as SPARC) and oncostatin (OSM) in both age groups, while serum testosterone concentrations increased only in the young adults following the exercise trial.

Prostate cancer is the second most common cancer in men and the incidence of the disease increases with advancing age (1). For examining whether acute exercise influences the capacity of serum from young versus older men to modify prostate cancer cell viability *in vitro*, we used two commonly used prostate cancer cell lines, LNCaP and PC3 cells. A major difference between these prostate cancer cells lines is that unlike the PC3 cell line, the LNCaP cell line is responsive to androgens, including testosterone and testosterone metabolites (28). The androgen signalling-axis plays a central role in both prostate physiology and in the pathogenesis of prostate cancer (29, 106). Androgens, particularly testosterone, likely contribute to prostate cancer growth during advanced stages of the disease (20, 100). However, contrary to the more traditional concept that high serum testosterone levels could be a risk fac-

tor for prostate cancer, more recent evidence suggests that high levels or variations in serum concentrations of androgens (including testosterone) within a 'normal' physiological range are not associated with an increased risk of developing prostate cancer (37, 78, 100, 101, 136). Experimental and epidemiological data indicate a complex relationship between systemic concentrations of androgens and the growth versus the differentiation of a prostate tumour (100, 101). A variety of mechanisms, including pre- and post-receptor regulation and intra-tumoural androgen synthesis, contribute to the complexity of dysregulated androgen signalling in prostate tumourigenesis (29). Furthermore, various cytokines and growth factors, such as IL-6, IL-8, epidermal growth factor receptor and insulin-like growth factor 1 (IGF-1), have been implicated in the crosstalk with androgen receptors in prostate cancer (29). Blood testosterone concentrations in young, healthy men have been reported to either increase or decrease after acute endurance exercise, dependent on the exercise duration and intensity (79, 125). Data also suggest that regular endurance training can help to counteract the age-related decline in basal systemic testosterone concentrations in middle-aged and older men (53, 109). In our study, serum testosterone concentrations increased in response to the exercise trial only in the young participants, but not in the older adults. The lack of an acute response of testosterone to exercise in the older men might reflect a reduced capacity to produce or secrete testosterone with age, as was suggested by a previous study (3).

With regards to the serum effects on prostate cancer cells, in our study, post-exercise serum (compared with pre-exercise serum) from the older men inhibited the metabolic activity of androgen-responsive LNCaP (but not that of androgen-unresponsive PC3) prostate cancer cells. Contrary to previous findings by Rundqvist et al. (105), exercise did not influence the capacity of serum from younger men to affect the viability or metabolism of either of the prostate cancer cells. Various biological mechanisms might have played a role in the discrepancy between the findings of these studies. For example, age-dependent interactive effects of androgens with cytokines and growth factors on the LNCaP prostate cancer cells following exercise may be a potential mechanism contributing to the differences in the effects of post-exercise serum from young versus older men. As a possible explanation, the exercise-induced increase in serum testosterone in the young adults might have influenced the cell metabolic activity and 'masked' inhibitory effects of certain cytokines or myokines. Different assays used to assess prostate cancer cell growth as well as different incubation conditions might also account in part for the disparity between the studies.

Another important observation of our study was that serum levels of two candidate cancer-inhibitory myokines, OSM and osteonectin, increased in both younger and older men following exercise. The outcome of a previous study has suggested that OSM might suppress cancer cell growth (57). By incubating a human mammary cancer cell line (MCF-7) with serum collected from mice after exercise, Hojman et al. showed that the exercise-conditioned serum inhibited the proliferation and increased caspase activity of the cancer cells (57). Additional mechanistic investigations indicated that incubating these cells with recombinant OSM inhibited cell proliferation and induced apoptosis, whereas adding anti-OSM-antibodies to the cell media reduced the induction of caspase activity (57). Moreover, OSM was upregulated in the mouse muscle and increased in the serum after exercise, suggesting that OSM is a myokine (57). Although OSM displays contrasting roles in cancer (dependent, at least partly, on the investigated tissue compartment) (60), the findings by Hojman et al. suggest that OSM is a possible candidate myokine mediating an inhibitory effect on mammary cancer cell growth (57). Our data tend to support the concept that OSM is a candidate factor that inhibits the viability or metabolism of cancer cells. However, considering that the metabolic activity of LNCaP prostate cancer cells only decreased after incubation with post-exercise serum from the older group, the present study points toward a context-dependent nature of such effects. Such context-dependent effects might relate to the differential serum testosterone responses between the two age groups, interactions between OSM and testosterone or other androgens, and the different physiology (e.g., androgen responsiveness) of the two different prostate cancer cell lines.

Notably, our study appears to be the first showing an exercise-induced increase in blood serum levels of osteonectin in both young and older humans. A previous study by Aoi et al. has identified this matricellular protein as a myokine secreted by skeletal muscle contractions into the blood circulation in mice and healthy young men (6). Using a colon cancer mouse model, it was shown that regular exercise inhibited colon tumourigenesis in wild-type mice but not in osteonectin-null mice, suggesting an anti-tumourigenic effect of osteonectin (6). Similar as for OSM, the data from the present study do not reveal any apparent effects of the serum osteonectin response to exercise and the viability and metabolic activity of the prostate cancer cells after treatment with pre- to post-exercise serum. Osteonectin is a multifunctional protein that regulates cell-cell and cell-matrix interactions in a cell type- and context-dependent manner (123). Future research may, therefore, focus on factors that influence the effects of osteonectin, for example, on different (cancer) cell types and in different tissue environments.

In both age groups, the exercise trial induced changes in blood leukocyte concentrations, i.e., increases in total leukocytes, lymphocytes and granulocytes (including neutrophils). These changes in the cellular immune system are characteristic for moderate to more intense endurance exercise (90, 129). On the contrary, serum levels IL-6, IL-15 and irisin were not detectable or did not change immediately after exercise. As one possible explanation, the assay used might have lacked the sensitivity to detect potentially minimal serum changes in these myokines. For example, available human data on changes in the plasma/serum concentrations of IL-15 following acute exercise suggest that increased IL-15 levels are small (88, 122). Furthermore, while there has originally been some controversy about the specificity and methods used to measure irisin (4, 22), irisin has meanwhile been identified in human plasma by mass spectrometry (59). However, the reported increases in circulating irisin levels after exercise training have also been relatively small (59). Considering that these myokines only increase to such small extents, we might have missed peak concentrations, in case IL-15 and irisin increased in a slightly delayed manner after exercise. With regards to IL-6, the lack of a detectable increase in the serum after exercise was somewhat surprising. Most (but not all) studies that have assessed systemic IL-6 responses to acute exercise have shown increases in IL-6 plasma or serum concen-

trations, dependent on the combination of mode, intensity and duration of exercise (as discussed below) (22, 88, 92). The few studies that did not find an increase in plasma or serum IL-6 levels mostly involved cycling at a more moderate intensity (22, 69). It remains possible that the combination of mode (i.e., cycling), intensity and duration (20 min at 50% $\dot{V}O_{2\ max}$ followed by 40 min at 65% $\dot{V}O_{2\ max}$) in this study was not sufficient to elicit a detectable systemic IL-6 response.

It is also worth mentioning that, taking into account the age-associated decline of $\dot{V}O_{2\ max}$ (50), the fitness level of the older study participants based on their $\dot{V}O_{2\ max}$ was relatively high (i.e., 38.95 ± 8.4 mL/kg/min; mean ± SD), as compared with the younger participants (44.1 ± 7.3 mL/kg/min). This somewhat higher training status of the older individuals, relative to the young group, may have contributed to the observed effect of post-exercise serum on the LNCaP cells. Higher physical activity levels and regular exercise training might influence serum concentrations of cytokines, myokines and growth factors both at rest and following acute exercise (22). Dependent on what serum factors are considered, training might induce a greater or a smaller acute response to exercise. For example, as discussed below, there is some evidence suggesting that long-term training might attenuate the pro-inflammatory cytokine response to a single bout of exercise in older animals and humans (75, 85).

A limitation of the current study was the lack of data on whether the treatment with pre- and post-exercise serum also affects the metabolism and viability of normal prostate cells. Originally, we planned to compare the effects of human serum on prostate cancer cells versus non-tumourigenic human prostatic epithelial cells (i.e., the RWPE-1 cell line; ATCC® CRL-11609™). However, due to technical problems with the culturing and serum treatment of this normal prostate cell line, we were not able to provide evidence on effects on these cells. The findings by Rundqvist et al. suggested that the growth-inhibitory effect of exercise-conditioned serum from healthy men was specific for prostate cancer cells, but did not influence the growth of fibroblasts (105).

Together, our findings contribute important new information on the effect of a single bout of exercise on the capacity of serum, collected from young versus older men, to modify the metabolic activity of androgen-responsive prostate cancer cells. These data provide tentative support of the notion that acute exercise elicits beneficial effects against the viability of prostate cancer cells specifically in older men. Additional investigations are required to substantiate the impact of ageing on these effects, and to further investigate mechanisms contributing to the differential responses of the androgen-responsive versus the androgen-unresponsive prostate cancer cells. Future research may also focus on examining potential interactive effects of androgens with key candidate cancer-inhibitory cytokines/myokines such as OSM and osteonectin. Furthermore, as discussed in our review below, another emerging aspect that warrants further investigation is the exercise-dependent interplay of serum factors with the cellular immune system.

Mechanisms linking age-related changes of the immune system to cancer development

Ageing, immune function, and cancer development

The incidence and prevalence of most cancers, in particular breast, colon and prostate cancer, increase with ageing (9, 64, 70). Various age-associated molecular, cellular and physiological changes have been proposed to contribute to the increased cancer risk with advancing age (9). At a cellular level, for example, underlying ageing processes such as increased oxidative stress, macromolecular damage, genomic instability, cellular senescence, and dysregulated cellular growth and differentiation might affect the development and growth of cancer (9, 18, 41, 49). Over the past years, there has been increasing research interest in the putative role that age-related alterations in the immune system play in the increased cancer incidence and prevalence in older adults (34, 43, 68). Advanced age is associated with remodelling and dysregulation of the immune system, which is commonly referred to as 'immunosenescence' (34, 68, 87). The age-associated decreased immune competence likely results from lifelong exposure to antigens and pathogens (particularly latent cytomegalovirus or CMV infection), intrinsic changes in immune cells and genetic predisposition (40, 68). Bone marrow ageing and thymic atrophy also contribute to the age-related impairment in the development and function of immune cells, such as T- and B-cells (7, 67). Longitudinal studies in octo- and nonagenarians have shown that age-related changes in various robust immune variables are associated with clinical outcomes, including frailty and mortality (2, 119, 130, 131). Based on the results of these studies, a cluster of immunological variables that are predictive of mortality and accepted as 'hallmarks' of human immune ageing has been established (68, 87). Referred to as the 'Immune Risk Profile' (IRP) (68, 87), this cluster includes variables indicative of T-cell senescence, such as an inversion of the peripheral blood $CD4^+$ to $CD8^+$ T-cell ratio (as discussed below). A causal relationship between the decline in the normal functioning of the immune system with ageing and cancer has not been established (43, 44). However, various age-related immune changes may contribute to cancer development and progression in older adults (43, 44).

The potential link of 'immunosenescence' with cancer development

The phenotypic and functional changes in both the innate and the adaptive immune systems with ageing and the physiological mechanisms underlying these changes have been comprehensively covered elsewhere (7, 43, 67, 68, 111). This review focuses instead on some of the age-associated alterations in the immune system that have been suggested to play important roles for cancer development. An increasing body of evidence from clinical epidemiology and experimental studies in animal models support the notion that a fully functioning immune system is central for counteracting carcinogenesis (49, 73). In a process that is termed immunosurveillance, both the innate and adaptive cellular arms of the immune system track, recognize and eliminate antigens and abnormal cells (43, 49). Effective immunosurveillance recognizes and eliminates premalignant lesions and cancer cells before the formation of a clinically recognizable tumour (43). Among the key components of immunosurveillance against cancer development are cytotoxic immune cells, specifically natural killer (NK) cells and CD8+ T-cells (44). Natural killer cells are cytotoxic effector cells of the innate immune system that, under normal physiological conditions, can recognize and eradicate tumour cells without prior antigen exposure (116). Age-related changes in the phe-

notypic and functional characteristics of these cells are therefore likely to contribute to the survival of cancer cells and increased cancer risk with ageing (43, 44, 68). With regards to NK cells, ageing is associated with a phenotypic redistribution of NK cell subsets characterized by decreased numbers of more immature and more cytotoxic $CD56^{hi}$ NK cell subtypes, coupled with increased numbers of more secretory $CD56^{low}$ NK cell subtypes (19, 44, 111). Furthermore, ageing is accompanied by an impairment in the NK cell cytotoxicity at the single cell level (54). These alterations may impair the efficacy of NK cells to control transformed cells with advancing age (44, 68). In addition to an inverted $CD4^+$ to $CD8^+$ T-cell ratio, key features of T-lymphocyte senescence include: decreased proportions of naïve T-cells (34); increased proportions of T-cells expressing surface markers associated with a late differentiated, exhausted and senescent phenotype (34, 68, 116, 118); increased secretion of pro-inflammatory cytokines by T-cells (7, 34); an imbalance in helper T cell phenotypes, with a shift toward type 2 (Th2) > type 1 (Th1) cells (43, 116); decreased T-cell proliferative responses (7, 34, 68, 116); and decreased $CD8^+$ T-cell cytotoxicity (34, 44). Together, these age-related alterations are indicative of a restricted repertoire and functional capacity of T-cells (43, 116). All of these changes might contribute to an impaired immune response against cancer development, but the decreased killing functions of $CD8^+$ T-cells is specifically a central aspect in this context (44).

Ageing is also associated with impairments in Toll-like receptor (TLR) signalling (103, 111, 127). As a critical component of cellular innate immune function, TLRs recognize pathogen- and damage-associated molecular patterns (PAMPs and DAMPs, respectively). They also link innate and adaptive immune responses (24, 62, 111). By sensing of danger and damage signals originating for example from pre-malignant lesions, TLRs are important for an effective immunosurveillance against carcinogenesis (43, 133). Age-associated decreases in TLR functions may therefore not only contribute to the impairment in immune responses to infectious diseases and vaccination, but also to a decreased capacity to recognize and eliminate abnormal and transformed cells in the elderly (127).

Other aspects of immune ageing that might contribute to a reduced immunosurveillance against cancer development include impaired dendritic cell functions (potentially contributing to a decreased presentation of tumour antigens to T-cells) (21, 44, 68); an increase in immunosuppressive immune cells such as regulatory T cells and myeloid-derived suppressor cells (which might suppress anti-tumour T-cell responses) (43, 44, 48); and decreased effector functions of neutrophils and monocytes/macrophages (43, 55, 111). Regarding the age-dependent dysregulation of innate immunity, a more general characteristic is that innate immune cells are already activated in the basal state, whereas their responses to additional stimulation are impaired (44, 111). In accordance with the presence of a stronger inflammatory milieu (as discussed below), ageing is associated a shift in monocyte subpopulations towards more pro-inflammatory phenotypes, and an increased production of pro-inflammatory cytokines by monocytes (8, 30, 44, 111).

'Inflammageing' and cancer

Another central feature of ageing that is, in part, affected by immunosenescence is chronic low-level inflammation, also termed 'inflammageing' (34, 40). Inflammation is an integral component of the innate immune response to infectious pathogens and tissue damage, but the resolution of inflammation is central for restoring homeostasis (16, 89, 129). Inflammageing describes a state of systemic inflammation that is unresolved and associated with a two- to four-fold increase in the circulating levels of inflammatory mediators such as cytokines (e.g., tumour necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6) and acute-phase proteins (e.g., C-reactive protein (CRP)) in older individuals (15, 16, 40). The exact aetiology underlying this age-associated chronic inflammation is incompletely understood (40). Potential mechanisms that contribute to unresolved inflammation with ageing include accumulating macromolecular and cell damage, dysfunctional mitochondria, cellular senescence, an imbalance in the gut microbiota, and the accumulation of visceral fat (16, 40, 91). Each of these sources activates a network of inflammatory pathways. In addition, as described above, the dysregulation of components of the innate and adaptive immune systems, such as increases in basal immune cell activation and pro-inflammatory cytokine production, play an important role in chronic inflammation with advancing age (16, 40, 68, 111). Although a causal relationship has not been established, there is evidence suggesting that chronic low-grade inflammation is a major contributor to the pathology of several age-related conditions and chronic diseases, including cancer (16, 40, 91, 131). Chronic inflammation may contribute to cancer development by enhancing oxidative stress and by altering the transcriptional regulation of cytokines, oncogenes and tumour suppressor genes (40, 44). Unresolved inflammation also supplies or activates various bioactive molecules within the tumour microenvironment that promote tumour growth (49). These molecules include growth factors that enhance proliferative signalling; anti-apoptotic factors that suppress (cancer) cell death; extracellular matrix-modifying enzymes that facilitate angiogenesis, invasion, and metastasis; and signalling factors that activate the epithelial-mesenchymal transition (a cellular program that broadly regulates invasion and metastasis) (49).

Exercise training improves or preserves the normal functioning of various components of the immune system in older adults (116). Furthermore, acute exercise produces an anti-inflammatory environment in the body, and exercise training mediates additional anti-inflammatory effects in the long-term (91, 129). In the following sections, we discuss available evidence whereby exercise may contribute to counteracting cancer development through modulating immune function and inflammation in general, and in particular in the ageing population.

The emerging role of exercise immunology in cancer prevention and survival

Epidemiological evidence linking physical activity and exercise with a decreased cancer risk

There is strong evidence from epidemiological research showing that physical activity reduces the risk and improves the survival for several cancers, including some of the most common cancers (1, 64, 70, 74). A recent systematic review of 45 reports comprising hundreds of epidemiologic studies with several million study participants has indicated that physical activity is associated with an approximate 10 to 20 per cent

reduction in the relative risk for cancers of the breast, colon, endometrium, bladder, stomach, oesophagus (adenocarcinoma) and kidney (74). Notably, the levels of physical activity that were associated with this risk reduction corresponded with the amount of physical activity recommended in the US 2018 Physical Activity Guidelines Advisory Committee Scientific Report (i.e., at least 150 to 300 minutes of moderate-intensity aerobic activity plus muscle-strengthening activity at least two days each week (110)) (74). Furthermore, a dose-response relationship between physical activity and specific cancer risk was evident for several cancers, with the strongest evidence for a dose-dependent reduction in the risk for breast and colon cancers (74). Data from randomized clinical trials also support the beneficial effects of physical activity for cancer primary prevention and the reduction in the risk of disease recurrence (42, 63). Potential mechanisms underlying the benefits of physical activity or exercise for protection against cancer include exercise-dependent reductions in cancer risk factors such as sex hormones, metabolic hormones, and pro-inflammatory factors, as well as improved immune function (73). Over the past few years, there has been increasing evidence in support of the concept that the exercise-dependent regulation of the immune system and inflammation plays a central role in counteracting cancer (56, 58). Recent experimental studies have also provided important mechanistic information in agreement with this concept (95).

Exercise-induced effects on the cellular immune system that might counteract cancer

Changes in the number and composition of blood leukocytes are one of the most prominent effects of acute exercise bouts on the immune system, most likely as a result of hemodynamic shear stress and in response to catecholamines, glucocorticoids and cytokines (90, 94, 129). Being an integral part of the physiological stress response to exercise, these changes in the cellular immune system likely reflect a redeployment of specific immune cell subtypes, for example, out of the blood to peripheral tissues that require enhanced immunosurveillance following physical stress (90). Importantly, acute exercise preferentially mobilizes immune cells with potent effector functions, including NK cells and $CD8^+$ T-cells, both of which are highly cytotoxic against tumours (as discussed above) (90, 115). A recent preclinical study by Pedersen et al. demonstrated how important the exercise-induced mobilisation and redistribution of NK cells are for counteracting tumour incidence and progression (95). Using a model of voluntary wheel running in mice, this study showed that six weeks of exercise training reduced tumour onset and growth across different tumour models by at least 60 per cent, as compared with non-exercising control conditions (95). Additional mechanistic experiments within this study suggested that these protective effects resulted from the exercise-dependent mobilization of NK cells, their infiltration into the tumours and the subsequent destruction of tumour cells (95). Exercise also appears to "prime" effector functions of immune cells, thereby enhancing immunosurveillance (90, 115). As shown by a study in healthy cyclists, NK cells present in the blood during exercise recovery, as compared with resting conditions, are more efficient killers of various cancer cell lines *in vitro* (12). However, it is poorly understood how exercise mediates tumour-killing NK cell functions.

Furthermore, the mobilisation, redistribution, transcriptional reprogramming and functional changes of neutrophils following acute bouts of exercise are among the most pronounced exercise-induced effects on the cellular immune system (80, 90, 129). Neutrophils and their heterogeneity are suggested to play an important role in cancer (81). Additional research may, therefore, focus on whether the beneficial effects of exercise against cancer are mediated through regulation of neutrophils (56).

Experimental evidence linking myokines with cancer protection

The concept that skeletal muscle is an endocrine organ provides another important mechanistic basis for linking exercise immunology with cancer protection (34, 56, 91). Contracting muscle is capable of producing and releasing several hundred cytokines and other peptides with autocrine, paracrine and endocrine effects, referred to as myokines (88, 91). Several myokines have immune regulatory functions, including, for example, IL-6, IL-7 and IL-15 (56, 91, 93). Other muscle-derived endocrine factors, such as OSM, irisin, and osteonectin (also known as secreted protein acidic and rich in cysteine (SPARC), may mediate cancer-inhibitory effects of exercise more directly by controlling growth kinetics of cancer cells (57, 93) (discussed below).

The first identified and most studied myokine is IL-6 (92). The magnitude of the exercise-induced increase in plasma IL-6 is related to exercise duration, intensity, the muscle mass involved during exercise, and muscle glycogen levels (92). In addition to its potent metabolic effects, IL-6 likely also mediates some of the anti-inflammatory and immune-regulatory effects of exercise (93, 120, 121, 129). IL-6 is commonly used as a marker of inflammatory status and associated with various age-related pathologies that have a strong chronic inflammatory component (40, 131). However, the transient response of IL-6 and other cytokines to exercise is markedly different from the cytokine cascade induced in pathological situations (92, 93, 129). For example, in contrast to inflammatory responses to sepsis or chronic low-grade inflammation, the IL-6 production by contracting muscles does not involve the activation of the pro-inflammatory nuclear factor- pathway. Nor does muscle-derived IL-6 stimulate the production of the pro-inflammatory cytokine TNF-α (92, 97). Under physiological conditions such as during exercise, muscle-derived IL-6 has metabolic and anti-inflammatory effects (92, 97). The acute increase in plasma IL-6 in response to exercise is followed by increases in the anti-inflammatory cytokines IL-1 receptor antagonist (IL-1ra) and IL-10 (79, 80, 97). Experimental data support a direct role of IL-6 in these anti-inflammatory effects. For example, it has been shown that infusion of IL-6 at concentrations corresponding to levels obtained after exercise enhances plasma levels of IL-1ra and IL-10 and inhibits TNF-α production in healthy humans (120, 121). Muscle-derived IL-6 also contributes to the exercise-induced redeployment of leukocytes (121). The study by Pedersen et al. (described above) showed that exercise-induced increases in IL-6 played a central role in regulating NK cell trafficking and NK cell-dependent tumour control (43).

Interleukin-7 has also been identified as a myokine (51). It is also produced in the thymus and is essential for the development and survival of T-cells and NK cells (107, 113, 128).

Another myokine with immune-regulatory properties is IL-15 (91). In addition to its metabolic effects, IL-15 is an important proliferative and activating factor for T-cells and NK cells (107, 128). In a clinical trial, IL-15 administration positively affected lymphocyte homeostasis and immune responses in cancer patients (26). However, at present there is only little information available on whether IL-7 and IL-15 responses to exercise have clinical implications (35).

In addition to these observed effects of myokines on immune cell activity, some experimental studies suggest that specific myokines affect cancer cell viability and tumour growth kinetics in a more direct manner (58). In a preclinical study by Hojman et al. (57), post-exercise blood serum collected from mice inhibited mammary cancer cell proliferation and induced apoptosis of these cells *in vitro* (29). Further mechanistic experiments suggested that OSM might be a key candidate myokine mediating the observed inhibitory effects on cancer cell growth (57).

Moreover, osteonectin has been identified as another novel myokine (6, 23). It was shown that osteonectin inhibits colon cancer cell growth *in vitro* and reduces colon tumourigenesis in exercising mice (6). Both the transcription and translation of osteonectin was downregulated in skeletal muscle of old sedentary mice as compared with young sedentary mice, suggesting an impact of ageing on the expression of osteonectin (6). In an experimental model, enhanced growth of pancreatic tumours has been observed in osteonectin-null mice (14). Osteonectin is involved in both physiological processes such as development and tissue remodelling, and pathological conditions such as cancer (123). Considering that osteonectin is also expressed by other tissues (including neoplastic tissues) and that it might also favour tumourigenesis (123), additional investigations examining factors that influence the function of osteonectin (e.g., different microenvironments) might help to better explain its contrasting roles in cancer.

In general, several human studies have shown that blood serum collected after acute exercise from healthy men (105), breast cancer patients (32), and colorectal cancer survivors (33) inhibits the growth of prostate cancer cells (105), breast cancer cells (32), and colon cancer cells (33). These data support the conceptual framework that exercise-induced changes in the blood, likely through the secretion of molecular factors by skeletal muscle and/or other tissues, contribute to the protective effects of exercise against cancer development (58). Although these findings are promising, to date, only a limited number of potential cancer-inhibitory myokines and other 'exercise factors' have been identified. In addition to identifying these factors, future research may also examine whether myokines play a role in the exercise-dependent enhancement of certain functions of the cellular immune system, such as the cytotoxicity of NK cells against tumours.

Multiple health benefits of exercise are mediated through long-term adaptation of physiological systems, such as skeletal muscle or the cardiovascular system, following regular physical training (39, 52). Notably, it has been suggested that the transient, but repetitive release of 'anti-oncogenic' molecular factors into the blood following single bouts of exercise may be at least as important for tumour control as chronic changes in resting blood concentrations of 'risk factors' such as pro-inflammatory cytokines (32, 33, 56, 58). This notion agrees with the data from some of the aforementioned *in vitro* serum incubation studies. Serum collected after acute exercise inhibited tumour growth kinetics, while serum obtained in resting conditions following longer-term exercise training did not (32, 33).

Exercise-dependent regulation of inflammation and anti-inflammatory effects in the context with cancer protection

An increasing body of evidence suggests that the anti-inflammatory effects of exercise are of major importance for health (39, 91, 129). Considering the association of cancer with low-grade chronic inflammation (as described above), it is therefore likely that exercise-associated anti-inflammatory effects also play an important role for counteracting cancer (56, 91, 129). It is widely recognized that regular exercise exerts its anti-inflammatory effects by producing an anti-inflammatory environment with each bout of exercise, and through a reduction of visceral fat (the latter of which is pro-inflammatory in nature, e.g., by secreting pro-inflammatory cytokines and adipokines) (91, 93, 97, 129). The anti-inflammatory effects of single exercise bouts are, at least partially, mediated by the release of myokines, such as IL-6, into the blood circulation (92, 93, 97). As described above, the IL-6 release from contracting skeletal muscle has anti-inflammatory effects, e.g., by stimulating the production of IL-1ra and IL-10 by blood mononuclear (immune) cells (92, 97, 121).

Numerous studies have investigated the systemic responses of cytokine and other inflammatory mediators to exercise (88, 129). Dependent on various factors, including exercise duration and intensity and the extent of exercise-induced muscle damage, exercise induces a transient increase in cytokines/myokines (e.g., IL-1ra, IL-6, IL-8, IL-10) and acute-phase proteins (e.g., CRP) in the circulation (79, 80, 88, 129). While skeletal muscle cells within contracting muscles are the main contributors to the exercise-induced increase in circulating IL-6, other potential cellular sources of circulating cytokines include cells within the microvasculature, fibroblasts and leukocytes (88). The changes of cytokines and acute-phase proteins in the circulation following exercise likely are an important part of the body's response to the exercise-associated physiological demands (88). Under non-pathophysiological conditions, even a more pronounced immune-endocrine, cytokine and stress response to a single bout of strenuous exercise is a tightly coordinated and dynamic process that is followed by counter-regulatory and anti-inflammatory mechanisms (90, 129). Furthermore, regular exercise training and a high degree of physical fitness appear to attenuate the systemic inflammatory response to acute exercise (65), which might reflect adaptive mechanisms for counteracting 'overshooting' inflammation. In the context with cancer, such robust, but rapidly resolved systemic inflammatory responses to repeated bouts of exercise might enhance the clearance of transformed cells and nascent tumours (56). Moreover, chronic exercise training might 'shape' acute inflammatory responses in a manner that facilitates the protection from carcinogenesis (56). In a rodent model, the acute inflammatory response to a liver carcinogen was attenuated and more rapidly resolved after voluntary wheel running for six weeks, leading to a faster and more effective clearance of damaged liver cells as compared with less active mice (11).

In contrast to the transient response of inflammatory mediators to acute exercise, data from cross-sectional and interven-

tion studies suggest that regular physical training is associated with lowered systemic levels of inflammation markers in resting conditions (42, 47, 84, 91, 97). For example, a randomized controlled trial that involved 400 previously inactive, healthy women aged 50 to 74 years, showed reduced basal blood concentrations of CRP and IL-6 after a year-long exercise training (42). Moreover, a stronger reduction of CRP and IL-6 was observed with increased weekly exercise time (42).

Collectively, there is some evidence suggesting that the cumulative effect of repeated responses of cytokines/myokines to acute exercise along with the regulation of inflammation by regular exercise are important mechanisms that contribute to counteracting tumour growth and progression (32, 33, 56, 58). Long-term exercise training likely also contributes to a reduced risk of cancer development by suppressing chronic inflammation (58, 91).

Beneficial effects of exercise on the ageing immune system and the potential role of these effects in counteracting cancer with advancing age

Immune-protective and anti-inflammatory effects of exercise in older adults

Accumulating evidence suggests that regular exercise or physical activity improves immune function in the older population (34, 116). A causative link between exercise-associated benefits for immune health and the risk of chronic disease, especially cancer, has not been established to date (34, 126). Nevertheless, cross-sectional data show that several of the immune biomarkers that are key components of the IRP cluster (e.g., T-cell responsiveness to mitogens, naïve/memory T-cell ratio) are positively displayed in physically active compared with sedentary elderly individuals (83, 114, 116, 118). In agreement with the concept that exercise has immune benefits in older adults, prospective population-level studies have shown that regular physical training reduces the risk of infection, as compared with sedentary behaviour (34, 86). Data from clinical trials indicating that increased physical activity enhances the efficacy of vaccinations in aging humans also support this concept (135). Furthermore, as discussed above, data from both cross-sectional and intervention studies suggest that regular exercise is associated with lowered systemic levels of inflammation markers in healthy individuals and in populations at-risk of diseases associated with low-grade inflammation, such as the elderly (42, 47, 84, 91, 97). In a recent animal study, Nilsson et al. observed that lifelong aerobic exercise alleviated systemic inflammation, including key drivers of the cytokine cascade and tumour progression (TNF-α, IL-6, and IL-1β), and protected against several different types of cancer in naturally-aged mice (85). The extent to which these immune-protective and anti-inflammatory effects of exercise contribute to a decreased cancer risk in older humans is unknown (34, 126). However, various components of the immune system that improve with exercise in older adults, such as increased cytotoxic functions of NK cells and $CD8^+$ T cells, play important roles for immunosurveillance against cancer (34, 44).

Exercise-induced effects on the cellular immune system in older adults

Some, but not all, studies investigating the effects of regular physical training on NK cell number and function in healthy, older individuals reported benefits of exercise (17, 83, 116, 134). Compared with their sedentary, less fit counterparts, elderly women with a high aerobic capacity were shown to have superior NK cell cytolytic activity, despite similar NK cell numbers in the blood (83). *In vitro* NK cell cytotoxicity against a target cell line (human leukemic K562 cells) was higher in a group of sedentary men and women (aged 650.8 years) after six months of aerobic exercise training (134), but not in postmenopausal women (aged 50–75 years) after a 12-month intervention involving aerobic exercise training (17). Furthermore, 12 weeks of moderate aerobic exercise training did not improve NK cell function in previously sedentary women aged 67–85 years (83). Only a few studies have investigated the effects of exercise on NK cells in cancer patients. For example, 15 weeks of aerobic exercise training increased NK cell cytotoxic activity in postmenopausal breast cancer survivors (aged 596 years) (38), while eight weeks of combined aerobic and resistance exercise did not alter NK cell cytotoxicity against K562 cells in breast cancer patients (aged 35–72 years) (82). Among other influencing factors, the mixed results among these studies are likely due to differences in exercise doses and modes, the duration of the training interventions, and the diversity of the initial health and fitness status of the participants. Of note, comparable reductions in tumour volume with exercise were observed in adult and old mice in the aforementioned experimental study by Pedersen et al. (95), suggesting a similar capacity of exercise to enhance tumour-killing NK cell functions in both age groups. More research is needed to examine under which conditions (particularly concerning the individuals' health status) exercise training is most efficient for augmenting cytotoxicity of NK cells against cancer cells. Additional translational research is also required to verify the interplay of exercise and ageing on these 'anticancer' functions of NK cells in humans. Further benefits of exercise training on the innate immune system in older adults include lower monocyte subpopulations displaying a more pro-inflammatory phenotype ($CD14^+CD16^+$) (124), and improved neutrophil migratory dynamics (10). In general, however, relatively little is known about the influence of exercise training on innate immune cells, especially neutrophils, with ageing (116).

With regards to T-cells, cross-sectional data are relatively consistent in showing greater T-cell proliferative responses and lower proportions of senescent T-cells in physically active, as compared to sedentary older adults (76, 83, 116, 118). A cross-sectional study involving 102 healthy males aged 18–61 years has demonstrated that aerobic fitness ($\dot{V}O_{2\,max}$ was associated with a lower age-related accumulation of senescent $CD8^+$ T-cells (118). Detailed analysis of the data from this study suggests that the relative fitness status of individuals might have a more pronounced effect on phenotypic shifts in blood T-cells than ageing *per se* (118). A study by Duggal et al. assessed phenotypic characteristics of immune cells in 125 older cyclists (55–79 years) who had maintained a high level of physical activity for much of their adult lives, as compared with 75 age-matched, healthy individuals who were not involved in regular exercise (35). An accumulation of senescent T-cells was observed in both the sedentary and the active older adults, yet the masters cyclists displayed a higher proportion of naïve T-cells and recent thymic emigrants (indicative of a better-preserved thymus output), higher serum levels of IL-7

and lower serum levels of IL-6 under resting conditions (35). Contrary to findings in cross-sectional studies, results from longitudinal studies examining T-cell mediated-immunity in older individuals are less consistent (17, 83, 116, 134). Features of T-cell senescence were either unchanged (17, 83, 134) or improved in previously sedentary healthy elderly individuals following training interventions (134). In postmenopausal breast cancer survivors, T-cell proliferation responses were increased after a 15-week aerobic training intervention (38). Furthermore, a recent study in pre-diabetic adults (aged 57.0 ± 5.2 years) showed that 3 weeks of regular endurance exercise training stimulated the production and mobilization of naïve T-cells, while terminally differentiated effector memory cells might have disappeared by apoptosis (98). These findings agree with the concept that regular exercise mobilizes 'older' functionally exhausted/senescent lymphocytes to undergo apoptosis and create 'vacant space' for the expansion of newly functional T-cells (77, 114). Together, these findings suggest that the benefits of exercise training might be greatest in individuals who already display more profound impairments within the peripheral T-cell compartment (e.g., cancer survivors) at the beginning of an exercise intervention (116).

Effects of ageing on systemic cytokine responses to exercise

Relatively little is known about the interplay of ageing and regular physical training on the systemic changes of cytokines and myokines in response to acute exercise. In a naturally ageing mouse model, acute exercise elicited a lower pro- and anti-inflammatory cytokine response in young and old aerobically trained mice, compared with sedentary old mice (85). This might reflect a protective adaptation associated with long-term exercise training to prevent excessive inflammation (85). Available data in older humans are varied. In an earlier human study that investigated the relation of ageing and muscle-derived IL-6, healthy older men released the same amount of IL-6 from skeletal muscle as healthy young men at the same relative exercise intensity (96). This suggests that ageing skeletal muscle maintains its capacity to produce and release IL-6 into the circulation in response to exercise. In a recent study, differential systemic cytokine changes were reported in young and middle-aged non-athletes and masters athletes (aged 53.1±8.8 years) following an incremental test to exhaustion (75). Blood IL-4 and IL-6 concentration increased only in the young adults, while an increase in IL-1 concentration was observed only in the masters athletes (75). Moreover, although the concentrations of all other assessed cytokines did not change in response to exercise in any of the three groups, the TNF-α/IL-10 ratio (used as a proxy marker for the overall pro- and inflammatory cytokine balance) increased only in untrained middle-aged adults (75). Another study observed no measurable circulatory IL-6 and IL-10 response to either moderate- and higher-intensity exercise in healthy older adults with either lower or higher levels of physical fitness (132). Collectively, while there is some evidence suggesting that long-term exercise training may attenuate the pro-inflammatory response to exercise with advancing age, available data do not enable us to make more definitive conclusions about the impact of ageing on the systemic cytokine response to exercise. More research is warranted comparing these responses between older and younger adults and between older trained and untrained adults.

Methodological considerations of *in vitro* cancer cell culture assays

There are important methodological considerations for *in vitro* cell culture studies. Traditional studies in which cancer cells are cultured as two-dimensional (2D) monolayers on rigid, plastic surfaces cannot fully represent the complexity of the tumour microenvironment (71). *In vivo*, cancer cells interact not only with neighboring cancer cells, but also other cell and tissue types such as fibroblasts, adipocytes, the surrounding vasculature, as well as cells from the immune system (13). Three-dimensional (3D) cell cultures, such as tumour spheroids, are better able to recapitulate *in vivo* cellular physiology including cell-cell and cell-matrix interactions, nutrient transport properties, and oxygen and nutrient diffusion gradients (36, 99). Gene and protein expression profiles of 3D cultures are better able to recapitulate *in vivo* profiles than 2D cultures (61). Furthermore, within a 3D tumour spheroid configuration cancer cells are more reflective of the *in vivo* situation. The cancer cells form spheroids which then develop a necrotic core and an outer proliferative zone (104) and respond to stimuli differently in comparison to 2D culture systems. For example, the cancer cell response to chemotherapy, growth factors and interaction with the extracellular matrix are more physiologically relevant in 3D as compared to 2D models (36, 99). With these points in mind, future studies investigating the response of cancer cells to exercise may consider utilizing 3D cell culture systems for a more physiologically relevant *in vitro* model.

Evidence from *in vitro* or *in vivo* studies demonstrates that exercise does not completely eliminate cancer (31, 95, 105). Rather, pre-incubating cancer cells with serum collected from individuals post-exercise decreases the ability of those cancer cells to form tumours *in vivo* (i.e., the tumour take rate) or colony-forming ability in *in vitro* studies. Furthermore, pre-incubation of cancer cells with post-exercise serum slows tumour growth in *in vivo* cancer models. Culturing cancer cells in the presence of post-exercise serum decreased cancer cell growth in *in vitro* models of several cancers (31, 33, 57, 95, 105, 117, 126). As discussed above, previous studies have suggested a link between myokines and modulation of the tumour microenvironment. However, *in vitro* serum incubation studies generally only represent the effects of a single 'snapshot' in time, such as a specific time point following exercise. As such, they cannot replicate the cumulative effect of repeated acute exercise responses that may lead to tumour growth control *in vivo* (58).

Hojman et al. postulated that exercise may have a more potent effect on cancer metastatic potential, as opposed to direct control of cancer cell viability (58). In addition, exercise is reported to normalize tumour vasculature, alter the systemic immunological profile and enhance tissue-immune cell surveillance (66). Future research may therefore also focus on the effects of exercise on the tumour microenvironment, rather than only on the malignant cells themselves.

Exercise can influence the tumour microenvironment by affecting tumour metabolism, nutrient and growth factor signaling, autophagy, lactate levels, angiogenesis and the localized immune response (66). Generally, cancerous cells escape detection by suppressing the immune response within the tumour microenvironment. Recognizing the beneficial effects of exercise on immunosurveillance (as discussed above),

future investigations may, therefore, focus on this aspect by using 3D models. The immunosurveillance effects of activated NK and T cells have already been modelled in 3D spheroid culture systems, but not in the context of exercise. Co-culture studies of allogenic $CD8^+$ T and NK cells, stimulated with IL-15, and patient-derived colorectal cancer spheroids demonstrated that these immune cells can infiltrate into the cancer spheroids and concomitantly increase their expression of activation markers (CD25 and CD107a), leading to increased cancer cell apoptosis (27). Sherman et al. used a 3D lung carcinoma spheroid model to demonstrate that NK cells could migrate towards a chemotactic gradient, infiltrate the tumour spheroids and cause cancer cell destruction (112). Moreover, serum from participants engaged in acute exercise activated NK cells, leading to increased NK cell cytotoxic activity directed against lymphoma and multiple myeloma cells *in vitro* (12). Together, this suggests that exercise serum could be used to study immune cell infiltration and activity directed against cancer cells using 3D spheroid culture models.

CONCLUSION AND FUTURE DIRECTIONS

Emerging data suggest that the immune system and the 'crosstalk' of contracting skeletal muscle with immune and cancer cells through myokines mediate some of the protective effects of exercise against the development and progression of cancer. Recognizing the increased cancer risk with advancing age, more research is needed for verifying these effects in experimental and clinical settings in older humans.

In particular, more well-controlled, long-term studies measuring molecular and cellular markers combined with clinical outcomes are required to determine the immune-protective and anti-carcinogenic effects of regular exercise training in the older population. Another aspect that warrants further investigation is whether there are 'dose-dependent' exercise effects in this context. As discussed above, some evidence suggests dose-response relationships between physical activity levels and the risk for certain cancers (i.e., a decreased cancer risk with increased exercise 'doses') (74). Data from a previous meta-analysis showed that even former elite athletes, including elite endurance athletes (i.e., a population group with exceptionally high exercise loads), have a lower risk of cancer mortality than the general population (46). There is also strong epidemiological and clinical evidence that supports the widely recognized notion that the numerous health benefits of regular exercise outweigh potential negative effects of even very strenuous exercise training (such as a transient dysfunction of certain immune components (90, 129)). Future research may focus on determining whether there is an optimal range of training loads that confers the most benefits for immune function, health, and cancer protection, and whether hypothetical dose-response benefits are age-specific and change over the lifespan. Additional investigations are also required for identifying which exercise modes (e.g., endurance- or resistance exercise, or a combination of both) are most efficient in enhancing anti-carcinogenic and anti-tumorigenic effects of the cellular immune system and cytokines/myokines especially in older adults.

Future research could also take advantage of 'omics' technologies (e.g., blood plasma proteomics) and advanced bioinformatics methods (e.g., network-driven methods) to identify 'anti-cancer' candidate factors as well as linkages of such factors with functional and clinical outcomes. Furthermore, using 3D cell culture platforms will be a key extension upon previous investigations that used 2D cell culture models for examining how blood serum collected before and after exercise interventions influences the viability of cancer cells *in vitro*. Such 3D cell culture models more realistically mimic the *in vivo* tumour microenvironment, as compared with 2D structures. This is especially important with regards to the interactions of cancer cells with exercise-induced cytokines, myokines and other serum factors. Another emerging aspect that warrants further investigation is the exercise-modulated interplay between serum factors and the cellular immune system. In the light of data suggesting that exercise enhances anti-tumourigenic functions of immune cells, such as cytotoxic NK cells (12, 115), investigating whether such tumour-killing immune cell functions are affected by exercise-induced changes in the serum, will provide important mechanistic information in this regard.

The evidence in this review suggests that mobilizing cancer-inhibitory and immune-regulatory serum factors and immune cells may be a key mechanism of how regular exercise mediates its protective effects against cancer in the older population. Collectively, this is a promising and important area of research within the domain of exercise immunology. It is likely that advancing the understanding of the role of immune-endocrine responses to exercise for counteracting carcinogenesis will expand a variety of potential targets for preventing and treating cancer, and help to define more efficient exercise prescriptions especially for older adults.

AUTHOR CONTRIBUTIONS

O.N., J.M.P., J.M.H., T.J.P., and E.W.T. conceived and designed this research project; J.H.H., J.McG., G.M.M., P.D.-G., and L.R. performed experiments and contributed to the acquisition of the research data; J.H.H., J.McG., J.M.P. and O.N. analysed and interpreted the research data; J.H.H. and J.M.P. prepared figures; O.N., J.H.H., and J.McG. drafted this manuscript; O.N., J.McG., G.M.M., J.M.H., T.J.P., E.W.T., and J.M.P. edited and critically revised this manuscript. All authors approved the final manuscript version.

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