Voluntary exercise influences metastatic organotropism in a murine colorectal cancer model

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Abstract

Background  Physical activity is associated with a lower risk of colorectal cancer (CRC) and CRC-specific mortality. However, evidence for a causal relationship between physical activity and disease progression is lacking. Here, we have used CRC organoids to create a novel mouse model for spontaneous metastasis formation to multiple organs. We have used this model to assess the influence of voluntary exercise on disease progression.

Methods  Collagen-embedded murine colorectal tumour organoids were transplanted into the livers of immunocompetent C57Bl/6 mice using microsurgery. Voluntary exercise in tumour-bearing mice was modelled by offering running wheels continuously (n = 12) or 3 h/day (n = 12) versus no wheel access (n = 12). Running wheel revolutions were cumulatively measured every 30 min and physical activity was continuously monitored by infrared cameras. Food intake was monitored throughout the experiment and body composition was assessed with echoMRI. Animals were sacrificed 14 weeks after tumour initiation. Tumour load was quantified by EpCAM immunohistochemistry staining. Systemic inflammation parameters were assessed in blood plasma by a multiplex immunoassay.

Results  Tumour growth was initiated by implantation of CRC organoids into the livers of immunocompetent mice. The resulting tumours spontaneously formed distant metastases to non-implanted liver lobes and to the lungs. Mice with access to the running wheels for 3 h/day ran relatively short distances (2.3 ± 0.3 km/night; 221 ± 29 km total distance) with relatively high intensity (wheel revolutions/h). Mice with continuous access to the running wheels ran significantly longer distances (6.6 ± 3.0 km/night; 600 ± 290 km total distance) with a significantly lower intensity. Both exercise groups showed increased lean body mass, and decreased fat mass and body weight compared with tumour-bearing control mice. Food intake was unaffected by exercise or tumour growth. Primary tumour growth was not significantly affected by exercise or tumour growth. However, mice with continuous wheel access (long distance-lower intensity group) displayed increased lung metastasis and decreased liver metastasis formation, when compared with the sedentary control group. Short distance-higher intensity exercise did not affect metastasis formation. Analysis of blood cytokine levels revealed that mice with continuous wheel access displayed signs of systemic inflammation.

Conclusions  These results suggest that exercise has the potential to influence the patterns and extent of metastasis in CRC, and that the degree and intensity of exercise are likely to be important variables. Confirmation of these results in additional preclinical models with or without systemic treatment is warranted.

Keywords  Exercise oncology; Running; Colorectal cancer; Metastasis; Organotropism; Inflammation
Introduction

Epidemiological evidence suggests that physical activity is associated with a lower risk of developing cancer, including colorectal cancer (CRC), and with a lower risk of cancer-specific mortality. Increased exercise in cancer patients and survivors has a positive impact on quality of life, cancer-related fatigue, physical fitness and psychological distress. Exercise may help cancer patients cope with and recover from treatment and may even improve their survival. However, the mechanisms linking physical activity with tumour progression and patient survival are poorly defined and may involve local effects on tumour metabolism, the tumour microenvironment, and immune recognition, as well as changes in whole-body physiology.

Mechanistic studies on cancer progression and metastasis formation depend, for a large part, on the use of pre-clinical mouse models. Such models have also been applied to study how exercise, either voluntary or forced, influences metastatic progression. However, the outcome of these studies is markedly heterogeneous: in some studies exercise was associated with reduced metastasis formation, while in others it was either unaffected or even stimulated. Several factors may contribute to this apparent lack of consensus, including the very heterogeneous nature of the disease models and exercise protocols used. Of particular importance is the fact that metastatic cancer is often induced by direct injection of tumour cells into the blood circulation, thereby completely bypassing all early steps of the metastatic cascade. Any effect of exercise on these early steps will therefore be missed in such model systems. In addition, the patterns of metastasis in cancer patients often involve multiple organs, in particular the liver and the lungs in CRC. However, models in which tumour cells are injected directly into the vasculature usually cause colonization of a single organ, thereby precluding the measurement of exercise effect(s) on the patterns of metastasis (i.e. metastatic organotropism).

Exercise interventions in mice can be modelled in a forced or voluntary manner. While forced exercise can lead to elevated levels of stress, voluntary exercise is less stressful. In breast cancer models, forced exercise increases the release of stress-related hormones such as corticosterone, resulting in changes in proliferation, migration and invasion potential, thereby promote tumour progression. Hence, forced exercise-induced stress may be a confounding factor when assessing the effects of exercise on metastatic tumour progression.

In the current study, we aimed to generate a model for spontaneous metastatic CRC involving multiple organs, and assessed the effect of aerobic voluntary exercise on body composition, systemic inflammation and metastasis patterns. Based on the available epidemiological evidence, we hypothesized that an increase in exercise would lead to an overall reduction of metastasis formation.

Materials and methods

Animal studies

Forty-six male C57BL/6NCrl mice, 8–10 weeks of age and weighing 25 ± 2 g, were individually housed due to physical activity tracking in custom-made open cages with contact bedding, tissues, physical activity tracking devices and if applicable, a running wheel. Mice were ad libitum fed with AIN-93 M pellets (Ssniff Spezialdiäten GmbH), had unlimited access to water and were maintained on a 12:12 h light (10 PM)/dark (10 AM) cycle at 20–24°C, 45–60% humidity. Body weight was monitored biweekly. Fat and fat-free mass were monitored weekly using magnetic resonance imaging (EchoMRI-4in1 system, EchoMRI). After 14 weeks, mice were sacrificed under anaesthesia by heart puncture followed by cervical dislocation. All relevant tumour-bearing organs and blood were harvested for analyses.

Ethical approval

This study was conducted in accordance with institutional guidelines for the care and use of laboratory animals, and all animal procedures related to the purpose of the research were approved by the Animal Welfare Body under the Ethical licence of University Utrecht, Medical Center Utrecht, the Netherlands, as filed by the national competent authority, securing full compliance the European Directive 2010/63/EU for the use of animals for scientific purposes.

Exercise interventions and tumour inoculation

Running wheels were offered for 0 h/day (n = 12, TB Control), 3 h/day (n = 12, TB 3 h) or 24 h/day (n = 12, TB Continuous). A non-tumour bearing (TB) control group was also included (Control; n = 10). After a 14 day ‘run in’ period, equal baseline activity in both running groups (TB 3 h and TB Continuous) was confirmed. Prior to surgery, mice were anaesthetised using isoflurane 3–5% for induction and 1.5–2% for maintenance and were administered the analgesic buprenorphine (0.1 mg/kg) subcutaneously. CRC tumour organoids (see below) were disassociated by TrypLE (Gibco), 250 000 single cells were embedded in 10 μL of 75% Rat Tail Type I Collagen (Corning) droplets and 25% neutralization buffer; AlphaMEM powder (Life Technology), 1 M HEPES buffer pH 7.5 (Invitrogen) and NaHCO3 (Sigma). Organoids were dissociated and seeded into collagen droplets at 250 000 cells/
10 μL. After overnight recovery, collagen droplets containing 1-day-old CRC organoids were implanted into the median liver lobe, using microsurgery techniques. Using microsurgical equipment and light microscopy, an incision of 0.5-1 cm was made in the skin, followed by opening the peritoneum wall. A sterile cotton tip was used to position the liver on a sterile gauze outside the abdomen, where an ~2 mm incision was made in the median liver lobe using a scalpel. To prevent excessive bleeding, a cotton tip was placed on the incision until an air-dried collagen droplet was gently pushed in the incision and sealed with Seprafilm (Genzyme), providing a tight seal preventing adhesions of liver tissue to the implantation site. The abdominal wall and skin were sutured, and mice were allowed to recover prior to start of the exercise intervention. In this orthotopic model for metastatic CRC, spontaneous ‘onward’ metastases are formed in non-implanted liver lobes and lungs. To exclude any acute exercise effects, animals were deprived of the running wheel at least 24 h before sacrifice.

Physical activity tracking

Wheel revolutions were monitored continuously by a magnetic sensor using Cage Registration Software (Department of Biomedical Engineering, UMC Utrecht, the Netherlands), and were cumulatively measured every 30 min. The circumference of the running wheel was 40 cm (diameter 12.7 cm). The covered distance was calculated as distance = (revolutions * 40 [cm]) / 105 [km]. Average distance was calculated by averaging the daily covered distance during the dark period on weekdays. Intensity of running (average revolutions/h) was calculated by dividing revolutions during active hours on weekdays by total active hours during that week.

In addition, infrared sensors (dual technology detector DUO 240, Visonic; adapted by R Visser, NIN Amsterdam) were used to cumulatively measure physical activity every 30 min. Data was collected using MED-PC IV software (MED associates). Average physical activity was calculated by averaging the daily physical activity measured during the dark (active) period on weekdays.

Tumour organoid culture

Organoids were derived from spontaneously formed tumours in a transgenic mouse model with conditional activation of the Notch1 receptor and deletion of p53 in the digestive tract. Exome sequencing revealed that all tumours harbour mutations in either the Ctnnb1 or Apc genes, demonstrating classical Wnt pathway activation. CRC organoids were transduced with a lentiviral vector expressing luciferase and dTomato (pULtra-Chilli-Luc, Addgene #48688) and were FACS-purified. CRC organoids were cultured in Advanced DMEM/F12 (Thermo Fisher Scientific), supplemented with 1% penicillin–streptomycin (Gibco), 1% HEPES buffer, 2 mM Glutamax (Invitrogen), 2% B27 supplement (Invitrogen), 100 ng/mL Noggin (produced by lentiviral transfection), 10 nM murine recombinant FGF (PeproTech) and 1 mM N-Acetylcysteine (Sigma-Aldrich). CRC organoids were passaged using TrypLE weekly and medium was refreshed biweekly or at indication. CRC organoid cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Immunohistochemistry and tumour quantification

Lung and liver tissues were fixed in formalin and embedded in paraffin (FFPE); 4 μm FFPE slides were cut from three tissue depths. Antigen retrieval was performed using citrate buffer and endogenous peroxidase was blocked for 30 min. The antibodies used were anti-EpCAM (Sino Biological, 1:500), anti-Vimentin (Cell Signalling, 1:100), anti-αSMA (Abcam, 1:200), BrightVision Poly-HRP-Anti Rabbit Biotin-free (Immunologic, ready-to-use), followed by 3,3′-diaminobenzidine tetrahydrochloride (DAB) incubation and counterstaining with Mayer’s Haematoxylin and Eosin (HE). Collagen type I and III was identified by Picro Sirius Red staining (Sigma Aldrich). Finally, the slides were mounted with coverslips by ClearVue™ Coverslipper (ThermoFisher). Tumours were identified using anti-EpCAM and BrightVision Poly-HRP-Anti Rabbit Biotin-free in liver sections. Lung metastases were identified using HE staining. Tumour lesions were quantified using ImageJ software with the Fiji plugin, through automated selection of total tissue area vs. tumour tissue area and expressed as % area of tissue.

Immunological parameters

Five hundred microlitres of blood plasma was obtained by centrifugation (2000× g, 10 min, RT) of heparin-coated tubes, snap frozen, and stored at −80°C. Systemic inflammation parameters were determined in blood plasma using a multiplex immunoassay (MCYTOMAG-70K, Millipore) based on Luminex xMAP technology (executed by MultiPlex Core Facility, Utrecht, the Netherlands).

Sample size

A sample size of 11 mice was estimated (90% power to detect tumour formation difference of >40%) using a one-way analysis of variance (ANOVA) with a 0.05 two-sided significance level (G*Power 3.1.9.3). An expected drop-out rate of 5% per tumour-bearing group was taken into account, resulting in n = 12 per group. Based on biological variation of physical
activity data obtained from previous studies, non-tumour-bearing control group consisted of 10 mice.

**Statistical analyses**

Seven mice were excluded from analysis, due to decease during tumour implantation (n = 1), decease during in vivo bioluminescent imaging (n = 1) or no detectable primary tumour at the end of the study (n = 5). Physical activity parameters over time were analysed using linear mixed-effects models, using week 2 as baseline activity for individual mice. Normality was tested with Shapiro–Wilk tests, with SPSS (Version 25.0). Groups were compared applying one-way ANOVA (normal data distribution) or Mann–Whitney (non-normal data distribution) tests using GraphPad Prism8. Linear regression curves were plotted and tested with SPSS (Version 25.0). Groups were compared applying Student's t-tests (for normally distributed data) or non-parametric Whitney (non-normal data distribution) tests using GraphPad Prism8. R values <0.05 were considered statistically significant. Figures were created using GraphPad Prism8 and Illustrator. Illustrations were created using BioRender.

**Results**

A novel colorectal cancer organoid-initiated mouse model for studying spontaneous multi-organ metastasis

The liver is the primary site of metastasis formation in CRC, accounting for ~70% of all metastases. In CRC patients, primary tumours are commonly resected immediately after first diagnosis.28 After removal of the primary tumour, liver metastases themselves become the primary source of disseminating tumour cells, causing further ‘onward’ spread and secondary metastasis formation in multiple organs, including the lungs. In the majority of these patients the primary and secondary metastases are inoperable and are usually treated with systemic therapy. To model this clinical situation, we employed an organoid model with multiple features of aggressive CRC, including a high stromal content, a high propensity for epithelial-to-mesenchymal transition, and a high propensity for spontaneous metastasis formation. Moreover, it displays inactivation of both APC and TP53 tumour suppressor proteins, commonly observed in CRC.

To model onward metastasis, we developed a micro-surgical liver implantation protocol. Mouse-derived CRC tumour organoids were expanded in BME droplets. Using microsurgical equipment, tumour organoids embedded in collagen droplets were implanted in the liver and the incision was sealed with SepraFilm. A schematic representation of this novel liver implantation procedure is presented in Figure 1A and a complementary video of the microsurgical implantation of tumour organoid procedure is presented in the Supporting Information, Video S1. Over a time frame of 8–12 weeks, mice developed tumours at the implantation site which spontaneously metastasized to the liver and the lungs (Figure 1B, C). The tumour establishment rate in four individual experiments (n = 44) was 86% of all implanted mice of which 74% developed ‘onward’ metastases in non-implanted liver lobes, and 39% developed distant metastases in the lungs. Immunohistochemistry for EpCAM could be used for quantification of primary tumour size and liver metastases. However, lung metastases were readily detected by HE staining (Figure 1D). Primary tumours and metastases were characterized by extensive collagen deposition (Sirius Red staining) and the formation of reactive tumour stroma, revealed by staining for the cancer associated fibroblast markers α-smooth muscle actin (α-SMA) and vimentin (Figure 1E).

Two voluntary exercise interventions that differ in running distance and intensity

To assess the effects of voluntary exercise on metastasis formation, two exercise regimens were investigated (Figure 2). Mice that were offered the running wheel for 3 h or continuously ran average distances of 2.3 ± 0.3 km/night or 6.6 ± 3.0 km/night, respectively (Figure 3A). Over the course of 14 weeks, the mice that had access to the running wheel for 3 h per day ran total distances of 223 ± 29 km. Mice that had access to the running wheel continuously ran 603 ± 290 km during the exercise intervention period (Figure 3B). While the total running distance was significantly higher in the continuous exercise group, the intensity of running (i.e. wheel revolutions/h) was significantly lower compared with the 3 h group (Figure 3C). The average running distance of mice with continuous access to the running wheel significantly decreased from week 8 onwards (Figure 3D). Total physical activity significantly increased with increased access to the running wheel (Figure 3E). In the absence of running wheels, tumour-bearing control mice showed significantly lower physical activity compared with tumour-bearing mice in both exercising groups (Figure 3E). This voluntary exercise model therefore allows us to compare two intervention modalities that significantly differ in both running distance and exercise intensity.

Exercise alters body weight and composition in tumour-bearing mice

Exercise led to a decrease in body weight in both groups (3 h, continuous access) compared with sedentary mice with or without tumours (Figure 4A), regardless of the body...
Figure 1. A novel colorectal cancer organoid-initiated mouse model for studying spontaneous multi-organ metastasis. (A) Schematic overview of novel organoid-initiated mouse model for studying spontaneous multi-organ metastasis from liver metastases of CRC. (B) Tumour organoids culture. Left to right; BME droplet containing mature tumour organoids prior to single cell making; mature tumour organoids prior to single cell making; 250,000 1 day old organoids in collagen droplet before implantation in the liver. (C) Macroscopic images, left to right; primary tumour in liver; liver tissue harbouring a single liver metastasis; lung tissue. (D) Immunohistochemical EpCAM staining in liver, indicating primary tumour and metastatic lesions; HE staining in lung tissue, indicating multiple lung metastatic lesions. (E) Immunohistochemical staining of primary tumours. Left to right; Sirius red staining indicating collagen bands; αSMA staining indicating stromal bands; vimentin staining indicating stromal bands. Scale bars = 1000 μm.
weight gain that was caused by tumour progression. Moreover, both exercise interventions affected body composition as fat mass was significantly reduced over time and at sacrifice in both groups (Figure 4B, C), while food intake over time remained comparable between all groups (Figure 4D). Tumour formation per se had a minor effect on the formation of fat mass (Figure 4E). Neither tumour progression nor exercise had an impact on the formation of fat-free mass over time (Figure 4F). Longer distance-lower intensity exercise significantly reduced the ratio of fat-over-fat-free mass at sacrifice (Figure 4G). Thus, regardless of intensity or duration, running exercise significantly affected body weight and composition.

**Prolonged exercise with lower intensity stimulates lung metastasis formation**

Primary tumour formation in the liver and metastatic progression to non-implanted liver lobes was quantified using IHC EpCAM staining (Figure 5A). Lung metastasis formation could not be quantified by EpCAM (due to high background staining of the lung tissue) and was quantified using HE staining (Figure 5B). Primary tumour growth and metastasis formation in the liver and the lungs were not significantly different between sedentary mice and mice who had 3 h access to the running wheel (Figure 5C). By contrast, lung metastasis formation was significantly increased in mice who had continuous access to the running wheel (Figure 5D), while liver metastasis formation was reduced (Figure 5E). When assessing the spatial distribution of tumour formation, mice with continuous access to the wheel showed the largest tumour load with more lung metastases, whereas mice with 3 h access showed increased liver metastases. Both exercise groups showed a trend towards increased total tumour area compared with sedentary mice (Figure 5F).

**Systemic inflammation is induced in mice performing prolonged exercise with lower intensity**

We reasoned that prolonged exercise could influence whole body physiology and that this could be reflected by changes in systemically released inflammatory mediators. Both exercise regimens induced changes in inflammatory mediators in the blood plasma of all groups of tumour-bearing mice at sacrifice (Figure 6A, Table S1). Mice with continuous access to the running wheel displayed higher blood levels of interleukin 1-beta (IL-1β), IL-10, IL-12 (p70), Leukaemia Inhibitory Factor (LIF), Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted (RANTES) and Tumour Necrosis Factor alpha (TNFα), when compared with sedentary mice, or mice with 3 h access to the running wheel (Figure 6B). Higher levels of these inflammatory cytokines showed a significant positive correlation with the formation of lung metastases (Figure 6C), but not liver metastases (Figure 6D). These data suggest that prolonged low-intensity exercise induces systemic inflammation, which could in turn lead to changes in metastatic organotropism.

**Discussion**

The lack of a general consensus regarding the effects of exercise on metastasis formation in preclinical models calls for the generation of standardized disease-specific models.19 In the present report we have used colorectal cancer organoids to...
develop a novel orthotopic mouse model to study spontaneous 'onward' metastasis to multiple organs. In order to metastasize, the tumour cells in this model must complete the entire metastatic cascade, including dissemination from the tumour bulk, survival in the circulation, extravasation, and colonization of distant organ sites, including contralateral liver lobes and the lungs. Strikingly, we found that prolonged exercise with lower intensity resulted in a shift from liver to lung metastasis formation, while total tumour and metastasis load was not affected. This shift in metastatic organotropism was...
Figure 4 Exercise modalities significantly impact body weight and composition over time in tumour-bearing mice. (A) Body weight (g), (B) fat mass (g), and (C) fat free mass (g) measured over time. (D) Food intake (g) per day measured over time. (E) Fat mass (g) measured at sacrifice. (F) Fat free mass (g) measured at sacrifice. (G) Fat over fat free mass ratio measured at sacrifice. Data are presented as mean ± SEM. Control n = 9; TB control n = 9, TB 3 h n = 10, TB continuous n = 10. Statistical differences were tested using a one-way ANOVA.
Exercise may affect tumour establishment and progression in a positive or negative manner through several mechanisms. First, exercise has direct effects on the tumour microenvironment by inducing angiogenesis, reducing hypoxia and enhancing intra-tumoural drug exposure. However, angiogenesis also promotes intravasation of metastatic cells into the circulation. By contrast, enhanced blood flow induces shear stress, causing less favourable conditions for disseminating tumour cells. Second, exercise can enhance platelet formation, enabling metastatic cells to migrate in clusters, decreasing their vulnerability to shear stress in circulation. Third, the tumourigenic potential of breast cancer cells is reduced upon exposure to conditioned serum by high-intensity exercise due to activation of the Hippo/YAP pathway by catecholamines.

Fourth, Schwapperacher et al. discovered exercise-sensitive oncogenes in prostate cancer, providing a link between exercise and genomic stability as well as exercise-related epigenetic modifications.

Whether (any of) the above mechanisms play a role in the observed shift in metastatic organotropism in our study is unclear. Metastatic organotropism is regulated by many factors including intrinsic properties of cancer cells, characteristics of organ microenvironments, and cancer cell-organ interactions. Exercise can have direct effects on the tumour microenvironment by influencing the ‘seed and soil’ mechanisms of metastasis. In addition, systemic factors, such as inflammatory cytokines, also play a major role.

Possible mechanisms are exercise-induced changes in lymphatic angiogenesis and epithelial-mesenchymal plasticity regulating cancer stemness, influencing which tumour cells

Figure 5 Lung metastasis formation is stimulated with prolonged exercise with lower intensity. Immunohistochemical quantification on FFPE slides of primary tumours, liver metastases and lung metastases. (A) Transplanted tumour in the liver (blue arrow) and distant liver metastases (yellow circles). Quantification using EpCAM staining (% area tumour). (B) Distant lung metastasis (yellow circles). Quantification using HE staining (% area tumour). (C) Primary tumour in liver (% area tumour). TB control n = 9, TB 3 h n = 7, TB continuous n = 8. (D) Lung metastasis (% area tumour). TB control n = 9, TB 3 h n = 10, TB continuous n = 9. (E) Liver metastasis (% area tumour). TB control n = 8, TB 3 h n = 9, TB continuous n = 8. (F) Spatial distribution of total tumour, as presented by the sum of primary tumour, liver and lung metastases (% area tumour). Data are presented as mean ± SEM. Statistical differences were tested using a one-way ANOVA. Scale bars = 1 cm.
Figure 6  Systemic inflammation is induced in mice performing prolonged exercise with lower intensity. (A) Heatmap of average expression levels of cytokines and chemokines relative to TB control, data obtained from multiplex immunoassay of blood plasma based on Luminex xMAP technology. (B) Cytokine expression levels of IL-1β, IL-10, IL-12 (p70), LIF, RANTES, and TNFα (pg/μL). (C) Pearson correlations of lung metastases (% area) with cytokine expression levels of IL-1β, IL-10, IL-12 (p70), LIF, RANTES, and TNFα (pg/μL). (D) Pearson correlations of liver metastases (% area) with cytokine expression levels of IL-1β, IL-10, IL-12 (p70), LIF, RANTES, and TNFα (pg/μL). TB control $n = 4$, TB 3 h $n = 7$, TB continuous $n = 7$. Data are presented as mean plasma concentration (pg/μL) ± SEM.
are more prominent to lodge to and colonize distant organs. Moreover, exercise-induced changes in inflammatory cytokines could lead to the recruitment of macrophages to the pre-metastatic niche in lung, leading to stromal remodelling resulting in a pro-metastatic microenvironment.33 Moreover, high TGF-beta signalling can cause tumour cells to form lung metastasis.35 Future research is needed to investigate whether exercise influences metastatic organotropism through any of the above and/or additional mechanisms.

A direct comparison of exercise modalities in preclinical models with patient exercise programmes is complex. Nevertheless, time-restricted exercise interventions (3 h/day in our model) are likely to bear more resemblance to the exercise programmes that are currently advised for cancer patients, than continuous exercise interventions. Indeed, mice with unrestricted access to the wheels ran extremely long distances, approaching 10 km per day, every day, and some animals ran more than 1000 km during 14 weeks. In human athletes, ultra-endurance exercise leads to extensive systemic inflammation,36 a condition that was also observed in mice with unrestricted access to the running wheels. We hypothesize that increased inflammation observed in the continuous exercise group is, at least partially, mediated via an increased stress response as a result of extreme exercise for an extended period of time. Potential mechanisms behind this phenomena are associated with the activation of the sympathetic system, release of stress hormones, such as catecholamines and prostaglandins, and activation of the systemic inflammatory response.36 In addition, tumour progression per se could contribute to systemic inflammation.

In previously published animal models, not all exercise interventions led to significant changes in body mass, and if reported, decreases in body mass were modest.37,38 The magnitude of body weight and composition alterations observed in our study were remarkable. Longer duration with low intensity exercise resulted in significantly reduced fat mass and total body mass. The effects of shorter duration exercise with higher intensity were less pronounced. As expected, we did not find significant effects on lean mass, as lean mass is predominantly increasing after resistance exercise, and not with aerobic exercise.39

During the final weeks of exercise intervention, running distances per day were decreased in the prolonged low-intensity exercise group. Although all mice were still exercising during the last weeks of the intervention, they did take more breaks between consecutive running periods, resulting in shorter overall covered distances. This decrease may be caused by advancing physical discomfort due to tumour progression over time. In addition, the use of the running wheel could be gradually decreasing over time due to habituation to its presence.

In the present study we have modelled late-stage CRC in immunocompetent animals, in particular the clinically relevant situation of ‘onward metastasis’. One other preclinical study examined the effect of exercise on CRC lung metastasis formation in mice, using tumour inoculation via tail vein injection.40 This study showed that forced treadmill running was associated with a survival benefit but had no effect on lung metastasis formation. However, voluntary wheel running in a murine mammary adenocarcinoma model,40 or forced swimming in a liver cancer model, caused increased lung metastasis formation.17,18 In the latter study, lung metastasis formation was increased by extreme, but not moderate, swimming exercise.18 This is in line with our finding that extreme but not moderate running exercise increases lung metastasis formation. The concordance between the two completely distinct model systems further suggests that the effects of extreme exercise on lung metastasis formation may be a generic phenomenon. Duration and intensity may therefore be important variables in determining the effects of exercise on metastasis formation.

In the present study, we did not observe beneficial effects of exercise on metastasis progression. Our study is distinct from other exercise studies in that it uses a combination of organoid and microsurgery technologies to create a model for spontaneous multi-organ metastasis in immunocompetent mice. Conventional models often use highly aggressive and in vitro selected 2D tumour cell lines that have often been in culture for decades, and are mostly performed in immunodeficient mice. Genetically engineered models usually have a relatively low penetrance of metastasis formation (1–50%), and a long and heterogeneous latency period,41,42 which complicates their application in intervention studies. In our model of orthotopic transplantation of syngeneic tumour organoids we observe a robust incidence of spontaneous metastasis formation (84%) when compared with the genetic models. This increase in robustness, and the relatively long disease progression time of 14 weeks, uniquely allowed us to model exercise interventions. This might explain the different outcomes found in our model compared with other model systems. To further validate the observed effects in our study will require testing of additional CRC models of spontaneous metastasis. These may be syngeneic mouse models, or models using patient-derived tumour organoids in ‘humanized’ mice. In addition, the effects of voluntary exercise versus forced treadmill exercise on metastatic organotropism should be investigated, also with varying exercise doses. Hence, more research on the dose-dependent effects of exercise is necessary to understand how exercise influences metastasis formation and their response to systemic therapies, as synergistic effects of exercise and therapy have been reported.43

In conclusion, our results suggest that exercise has the potential to influence the patterns and extent of metastasis in CRC, and that the degree and intensity of exercise are likely
to be important variables. A major question remains whether exercise-induced changes in metastatic organotropism have the potential to impact patient survival and whether such effects are dependent on the stage of the disease. In addition, the influence of exercise on chemotherapy efficacy and on toleration of its toxic side effects need to be evaluated. Models of late-stage metastatic CRC in immunocompetent animals, such as the one presented in this report, are ideally suited to start addressing these questions. Ultimately, this knowledge will be crucial for rational future development of physical activity recommendations for cancer patients.

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Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Median, minimum and maximum values (pg/μL) of cytokines per group, data derived from multiplex immunoassay of blood plasma based on Luminex xMAP technology

Movie S1. Supporting information

Conflict of interest

Miriam van Dijk is employed by Danone Nutricia Research. Liza A. Wijler, Daniëlle A. E. Raats, Andre Verheem, Anna M. den Otter, Helene Rundqvist, Anne M. May, and Onno Kranenburg do not declare conflicts of interest.

References

1. Brown JC, Gilmore LA. Physical activity reduces the risk of recurrence and mortality in cancer patients. Exerc Sport Sci Rev 2020;48:67–73.
2. Hojman P, Gehl J, Christensen JF, Pedersen BK. Molecular mechanisms linking exercise to cancer prevention and treatment. Cell Metab 2018;27:10–21.
3. Gerritsen JKW, Vincent AJPE. Exercise improves quality of life in patients with cancer: a systematic review and meta-analysis of randomised controlled trials. Br J Sports Med 2016;50:796–803.
4. Mustian KM, Alfano CM, Heckler C, Kleckner AS, Kleckner IR, Leach CR, et al. Comparison of pharmaceutical, psychological, and exercise treatments for cancer-related fatigue. JAMA Oncol 2017;3:961–968.
5. Cormie P, Zopf EM, Zhang X, Schmitz KH. The impact of exercise on cancer mortality, recurrence, and treatment-related adverse effects. Epidemiol Rev 2017;39:71–92.
6. Chen YJ, Li XX, Ma HK, Zhang X, Wang BW, Guo TT, et al. Exercise training for improving patient-reported outcomes in patients with advanced-stage cancer: a systematic review and meta-analysis. J Pain Symptom Manage 2020;59:734–749.e10.
7. Campbell KL, Winter-Stone KM, Wiskemann J, May AM, Schwartz AL, Courteney KS, et al. Exercise guidelines for cancer survivors: consensus statement from international multidisciplinary roundtable. Med Sci Sports Exerc 2019;51:2375–2390.
8. Patel AV, Friedenreich CM, Moore SC, Hayes SC, Silver JK, Campbell KL, et al. American College of Sports Medicine roundtable report on physical activity, sedentary behavior, and cancer prevention and control. Med Sci Sports Exerc 2019;51:2391–2402.
9. Ashcraft KA, Peace RM, Betof AS, Dewhirst MW, Jones LW. Efficacy and mechanisms of aerobic exercise on cancer initiation, progression, and metastasis: a critical systematic review of in vivo preclinical data. Cancer Res 2016;76:4032–4050.
10. Ruiz-Casado A, Martin-Ruiz A, Pérez LM, Provencio M, Fiuza-Luces C, Lucia A. Exercise and the hallmarks of cancer. Trends Cancer 2017;3:423–441.
11. Pedersen L, Christensen JF, Hojman P. Effects of exercise on tumor physiology and metabolism. Cancer J 2015;21:111–116.
12. Hoffmann-Goetz L, MacNeil B, Arumugam Y. Tissue distribution of radiolabelled tumour cells in wheel exercised and sedentary mice. Int J Sports Med 1994;15:249–253.
13. Pedersen L, Idron M, Olofsson GH, Lauenborg B, Nookaew I, Hansen RH, et al. Voluntary running suppresses tumor growth through epinephrine- and IL-6-dependent NK cell mobilization and redistribution. Cell Metab 2016;23:554–562.
14. Alvarado A, Gil da Costa RM, Faustino-Rocha AI, Ferreira R, Lopes C, Oliveira PA, et al. Effects of exercise training on breast cancer metastasis in a rat model. Int J Exp Pathol 2017;98:40–46.
15. Goh J, Ladiges W. Voluntary wheel running in mice. Curr Protoc Mol Biol 2015;8:283–290.
16. Tsai MS, Kuo ML, Chang CC, Wu YT. The effects of exercise training on levels of vascular endothelial growth factor in tumor-bearing mice. Cancer Biomark 2013;13:307–313.
17. Smeda M, Przyborowski K, Proniewski B, Zakrzewska A, Kaczor D, Stojak M, et al. Breast cancer pulmonary metastasis is increased in mice undertaking spontaneous physical training in the running wheel; a call for revising beneficial effects of exercise on cancer progression. Am J Cancer Res 2017;7:1926–1936.
18. Zhang Q, Zhang B, Zhang K, Meng X, Jia Q, Zhang Q, et al. Moderate swimming suppressed the growth and metastasis of the transplanted liver cancer in mice model: with reference to nervous system. Oncogene 2015;35:4122–4131.
19. Rincon-Castanedo C, Morales JS, Martin-Ruiz A, Valenzuela PL, Ramirez M, Santos-Lozano A, et al. Physical exercise effects on metastasis: a systematic review and meta-analysis in animal cancer models. Cancer Metastasis Rev 2020;39:91–114.
20. Lambert AW, Pattabiraman DR, Weinberg RA. Emerging biological principles of metastasis. Cell 2017;168:670–691.
21. Roberts CJ, Stuhrl KL, Hillard CJ. Swim stress differentially affects limbic contents of 2-arachidonoylglycerol and 2-oleoylglycerol. Neuroscience 2012;204:74–82.
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22. Yanagita S, Aramiya S, Suzuki S, Kita I. Effects of spontaneous and forced running on activation of hypothalamic corticotropin-releasing hormone neurons in rats. *Life Sci* 2007;80:356–363.

23. Moraska A, Deak T, Spencer RL, Roth D, Fleschner M. Treadmill running produces both positive and negative physiological adaptations in Sprague-Dawley rats. *Am J Physiol Regul Integr Comp Physiol* 2000;279:1321–1329.

24. Svensson M, Rosvall P, Boza-Serrano A, Andersson E, Lexell J, Deierborg T. Forced treadmill exercise can induce stress and increase neuronal damage in a mouse model of global cerebral ischemia. *Neurobiol Stress* 2016;5:8–18.

25. Obradović MMS, Hamelin B, Manevski N, Couto JP, Sethi A, Coissieux M-M, et al. Glucocorticoids promote breast cancer metastasis. *Nature* 2019;567:540–544.

26. Arranz A, Venihaki M, Mol B, Androulidaki A, Dermitzaki E, Rassouli O, et al. The impact of stress on tumor growth: peripheral CRF mediates tumor-promoting effects of stress. *Mol Cancer* 2010;9:261.

27. Charron M, Kuperstein I, Barriere C, El Marjou F, Cohen D, Vignjevic D, et al. Concomitant Notch activation and p53 deletion trigger epithelial-to-mesenchymal transition and metastasis in mouse gut. *Nat Commun* 2014;5:5005.

28. Brenner H, Kloor M, Pox CP. Colorectal cancer. *Lancet* 2014;383:1490–1502.

29. Brown JC, Rhim AD, Manning SL, Brennan L, Mansour AI, Rustgi AK, et al. Effects of exercise on circulating tumor cells among patients with resected stage I-III colon cancer. *PloS ONE* 2018;13:e0204875.

30. Wang J-S, Chang C-Y, Chow S-E, Chen Y-W, Yang C-M. Exercise modulates platelet-nasopharyngeal carcinoma cell aggregation and subsequent tissue factor and matrix metalloproteinase activities. *J Appl Physiol* 2007;103:763–770.

31. Dethlefsen C, Hansen LS, Lillelund C, Andersen C, Gehl J, Christensen JF, et al. Exercise-induced catecholamines activate the hippocampus tumor suppressor pathway to reduce risks of breast cancer development. *Cancer Res* 2017;77:4894–4904.

32. Schwappacher R, Schink K, Sologub S, Dietrich W, Relic J, Friedrich O, et al. Physical activity and advanced cancer: evidence of exercise-sensitive genes regulating prostate cancer cell proliferation and apoptosis. *J Physiol* 2020;598:3871–3889.

33. Nan X, Wang J, Liu HN, Wong STC, Zhao H. Epithelial-mesenchymal plasticity in organotropism metastasis and tumour immune escape. *J Clin Med* 2019;8:747.

34. van Doorslaer de Ten Ryen S, Deldicque L. The regulation of the metastatic cascade by physical activity: a narrative review. *Cancer* 2020;12:153.

35. Padua D, Zhang XH-F, Wang Q, Nadal C, Gerald WL, Gomis RR, et al. TGFβ primes breast tumors for lung metastasis seeding through angiopoietin-like 4. *Cell* 2008;133:66–77.

36. Stelzer I, Kröpf JM, Fuchs R, Pekovits K, Mangge H, Raggam RB, et al. Ultra-endurance exercise induces stress and inflammation and affects circulating hematopoietic progenitor cell function. *Scand J Med Sci Sports* 2015;25:e442–e450.

37. Manzanares G, Brito-da-Silva G, Gandra PG. Voluntary wheel running: patterns and physiological effects in mice. *Braz J Med Biol Res* 2019;52:e7830.

38. Khamoui AV, Park B-S, Kim D-H, Yeh M-C, Oh S-L, Elam ML, et al. Aerobic and resistance training dependent skeletal muscle plasticity in the colon-26 murine model of cancer cachexia. *Metabolism* 2016;65:685–698.

39. Brown JC, Winters-Stone K, Lee A, Schmitz KH. Cancer, physical activity, and exercise. *Compr Physiol* 2012;2:2775–2809.

40. Jee H, Chang JE, Yang EJ. Positive prehabilitative effect of intense treadmill exercise for ameliorating cancer cachexia symptoms in a mouse model. *J Cancer* 2016;7:2378–2387.

41. Gomez-Cuadrado L, Tracey N, Ma R, Qian B, Brunton VG. Mouse models of metastasis: progress and prospects. *Dis Model Mech* 2017;10:1061–1074.

42. Xu Y, Zhang L, Wang Q, Zheng M. Comparison of different colorectal cancer with liver metastases models using six colorectal cancer cell lines. *Pathol Oncol Res* 2020;26:2177–2183.

43. Schadler KL, Thomas NJ, Galie PA, Bhang DH, Roby KC, Addai P, et al. Tumor vessel normalization after aerobic exercise enhances chemotherapeutic efficacy. *Oncotarget* 2016;7:65429–65440.

44. von Haeling S, Morley JE, Coats AJS, Anker SD. Ethical guidelines for publishing in the *Journal of Cachexia, Sarcopenia and Muscle*: update 2017. *J Cachexia Sarcopenia Muscle* 2017;8:1081–1083.