

Physical activity and advanced cancer: Evidence of exercise-sensitive genes regulating prostate cancer cell proliferation and apoptosis

Running title: WB-EMS and prostate cancer cell growth

Raphaela Schwappacher<sup>1,2</sup>\*, Kristin Schink<sup>1,2</sup>\*, Svetlana Sologub<sup>1,2</sup>, Walburga Dieterich<sup>1,2</sup>, Dejan Reljic<sup>1,2</sup>, Oliver Friedrich<sup>3</sup>, Hans J. Herrmann<sup>1,2</sup>, Markus F. Neurath<sup>1</sup>, Yurdagül Zopf<sup>1,2</sup>

1) Medical Department 1, Friedrich-Alexander-University Erlangen-Nürnberg, Erlangen, Germany

2) Hector-Center for Nutrition, Exercise and Sports, Medical Department 1, Friedrich-Alexander-University Erlangen-Nürnberg, Erlangen, Germany

3) Institute of Medical Biotechnology, Friedrich-Alexander-University Erlangen-Nürnberg, Erlangen, Germany

\* Authors contributed equally.

Corresponding author: Prof. Yurdagül Zopf, MD, Hector-Center for Nutrition, Exercise and Sports, Ulmenweg 18, 91054 Erlangen, Germany; Phone: 09131/8545218; Fax: 09131/8535228; yurdaguel.zopf@uk-erlangen.de

Keywords: Physical activity; exercise; whole-body electromyostimulation; electric pulse stimulation; prostate cancer; colorectal cancer; cancer cells; proliferation; apoptosis; gene expression

Abbreviations: WB-EMS, whole-body electromyostimulation; EPS, electric pulse stimulation; HSMs, human skeletal muscle myoblasts; CXCL5, C-X-C motif chemokine 5; PPARGC1A, peroxisome proliferator-activated receptor  $\gamma$  coactivator 1 $\alpha$ ; MSTN, myostatin; Bcl2, B-cell lymphoma 2; IL6, interleukin 6; IL5, interleukin 5; IL7, interleukin 7; IL8, interleukin 8; IL15, interleukin 15; BDNF, brain-derived neurotrophic factor; FGF21, fibroblast growth factor 21; IGF1, insulin-like growth factor 1; IGFBP1, IGF binding protein 1; IGFBP3, IGF binding protein 3; CCL2, C-C motif chemokine ligand 2; VEGF, vascular endothelial growth factor; CCNE1, cyclin E1; GHR, growth hormone receptor; CDKN1B, cyclin-dependent kinase inhibitor 1B; CDKN1A, cyclin-dependent kinase inhibitor 1A; CDK, cyclin-dependent kinase; WNT10A, Wnt family member 10A; CASP3, caspase 3; CASP7, caspase 7; DUSP8, dual specificity phosphatase 8; BID, BH3 interacting-domain death agonist; POLE2,

This is an Accepted Article that has been peer-reviewed and approved for publication in The Journal of Physiology, but has yet to undergo copy-editing and proof correction. Please cite this article as an 'Accepted Article'; doi: [10.1113/JP279150](https://doi.org/10.1113/JP279150).

This article is protected by copyright. All rights reserved.

DNA polymerase  $\epsilon$  2, accessory subunit; E2F1, E2F transcription factor 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT1, hypoxanthine phosphoribosyltransferase 1; RPLP0, ribosomal protein lateral stalk subunit P0; PARP, poly (ADP-ribose) polymerase.

### Key points summary

- Physical activity is known to protect against cancer.
- We found that the resistance exercise method whole-body electromyostimulation (WB-EMS) has a significant anti-cancer effect.
- WB-EMS-conditioned serum from advanced prostate cancer patients decreased human prostate carcinoma cell growth and viability *in vitro*.
- Multiplex analysis revealed that genes associated with human prostate cancer cell proliferation and apoptosis are sensitive for exercise.
- Feasible exercise should be part of multimodal anti-cancer therapies, also for physically weakened patients.

## Abstract

Regular physical activity is known to protect against cancer development. In cancer survivors, exercise reduces the risk of cancer recurrence and mortality. However, the link between exercise and decreased cancer risk and improved survival is still not well understood. Serum from exercising healthy individuals inhibits proliferation and activates apoptosis in various cancer cells, suggesting that mechanisms regulating cancer cell growth are affected by exercise. For the first time, we analyzed serum from advanced-stage cancer patients with prostate (exercise group n = 8; control group n = 10) or colorectal (exercise n = 6; control n = 6) cancer, after a 12-week whole-body electromyostimulation (WB-EMS) training (20 min/session, 2x/week; frequency 85 Hz; pulse width 350  $\mu$ s; 6 sec stimulation, 4 sec rest), a tolerable, yet effective resistance exercise for physically weakened patients. We report that serum from these advanced cancer patients inhibits proliferation and enhances apoptosis of human prostate and colon cancer cells *in vitro* using cell growth and death assays (BrdU incorporation, cell counting, DNA fragmentation). Exercise-mimicking electric pulse stimulation (EPS) of human primary myotubes showed that EPS-conditioned myotube medium also impairs human cancer cell viability. Gene expression analysis using a multiplex array of cancer-associated genes and subsequent quantitative RT-PCR, revealed the presence of exercise-sensitive genes in human prostate cancer cells that potentially participate in the exercise-mediated regulation of malignant cell growth and apoptosis. Our data document the strong efficiency of the anti-oncogenic effects of physical activity and will further support the application of regular therapeutic exercise during cancer disease.

## Introduction

A sedentary lifestyle and overweight are significant risk factors for cancer. Regular exercise is known to protect against the development of cancer, and in cancer survivors, exercise reduces the risk of cancer recurrence and mortality (Brown and Gilmore, 2020; Goh et al., 2014; Hojman et al., 2011). Moreover, it is now widely acknowledged that cancer patients can safely participate in adapted exercise programs and that physical activity is an important supportive part of a cancer treatment (Schwartz et al., 2017). Exercise effects in the body are complex and our understanding of how exercise-triggered mechanisms influence cancer development and/or progression is incomplete. Diverse indirect mechanisms are partially responsible for exercise benefits against cancer, e.g. exercise-induced decrease of chronic inflammation (Dieli-Conwright et al., 2018; Goh et al., 2014). In recent years, researchers found significant evidence that skeletal muscle activation directly affects malignant cell growth. Hojman et al. showed, for the first time, that conditioned serum from exercising mice decreases human breast cancer cell growth *in vitro* (Hojman et al., 2011). Further, several *in vivo* and *in vitro* studies suggested that serum from healthy humans or mice after acute or long-term exercise attenuates proliferation and activates apoptosis of human cancer cells (Aoi et al., 2013; Ngo et al., 2002; Rundqvist et al., 2013). Dethlefsen et al. found in a comparative approach that serum from healthy women and patients with stage III breast cancer after acute exercise has similar inhibitory effects on cancer cell culture growth (Dethlefsen et al., 2017). Recently, it was shown that serum from colon cancer survivors after acute high intensity aerobic exercise reduces cancer cell growth *in vitro* (Devin et al., 2019). However, very little is known about the impact of exercise-conditioned serum from advanced-stage cancer patients on malignant cell proliferation and apoptosis. We hypothesized that the anti-cancer effects of exercise also occur in physically weakened, advanced cancer patients, in part cachectic with a deteriorating muscular status. It is recommended that cancer patients should regularly perform resistance training in order to counteract the loss of muscle mass and strength (Schwartz et al., 2017). But traditional resistance training, commonly conducted with weights, can be too strenuous for advanced cancer patients due to poor physical condition or even contra-indicated, e.g. in the presence of bone metastases.

In 2018, we published that whole-body electromyostimulation (WB-EMS) training is

feasible, well tolerated, and effective in improving body composition and function in advanced-stage cancer patients with solid tumors or hematological malignancies under curative and palliative therapy (Schink et al., 2018a; Schink et al., 2018b). WB-EMS is an innovative, time-efficient form of resistance exercise. It is a joint-friendlier, less exhausting exercise training, and thus can be a reasonable alternative for e.g. patients with physical limitations. The electrical muscle stimulation was applied by bipolar impulses at a frequency of 85 Hz and a pulse width of 350  $\mu$ s in bouts of 6 sec stimulation and 4 sec resting intervals (in 20 min sessions, twice a week), which allowed the simultaneous activation of all major skeletal muscle groups (Kemmler et al., 2010; Kemmler and von Stengel, 2013; Schink et al., 2018a). The current intensity during WB-EMS is set to generate a noticeable but comfortable muscle contraction. The current intensity is individually adjusted for each patient, ideally between insufficient and excess training load, in order to achieve the optimal exercise intensity (Filipovic et al., 2012; von Stengel et al., 2012). There is profound evidence that physical training directly affects cancer cells via serum factors; this was demonstrated in cell culture experiments using exercise-conditioned serum from healthy humans and mice (Aoi et al., 2013; Hojman et al., 2011; Rundqvist et al., 2013). Our research objectives for the present *in vitro* study were 1) to learn more about the anti-cancer effects of exercise in advanced cancer patients, and 2) to examine, for the first time, the effectiveness of the exercise method WB-EMS on malignant cell growth and viability. To address these research aims, we used cancer cell cultures in order to analyze WB-EMS serum from stage III/IV cancer patients with prostate carcinomas, a hormone-dependent type of cancer, or with colorectal carcinomas. In contrast to non-exercising cancer patients, our *in vitro* studies indicated that post-WB-EMS serum decreases human cancer cell viability and, as a potential underlying mechanism, suggested that exercise-sensitive genes are involved in the regulation of proliferation and apoptosis of human prostate cancer cells.

## Materials and Methods

To analyze the effect of exercise-conditioned serum on human cancer cell growth *in vitro*, we used serum collected from advanced-stage prostate and colorectal cancer patients, who either underwent exercise via WB-EMS training or acted as non-exercising controls (Schink et al., 2018a).

### Ethical approval

The original study was conducted according to the guidelines of the Helsinki Declaration. The study protocol was approved by the ethics committee of the Friedrich-Alexander-University Erlangen-Nürnberg (Reg.Nr.155\_13B) and is registered at clinicaltrials.gov (NCT02293239). Informed, written consent was obtained from the participants.

### Clinical study design, patient recruitment and WB-EMS exercise intervention

Patient recruitment and design of the original study (conducted from 2014 to 2017) are described in detail in Schink et al. (Schink et al., 2018a). Briefly, patients ( $\geq 18$  yrs old), who were diagnosed with a stage III/IV solid tumor disease and with ongoing anti-cancer therapy, and a Karnofsky performance index between 100% and 60%, were included. After baseline assessment, patients were allocated to an exercise group performing WB-EMS training over a period of 12 wks or to a control group without WB-EMS. Both groups received nutritional counseling during the study to achieve a daily protein intake of  $>1.0$  g/kg bodyweight, and patients with disease-related decreased food intake received medical nutritional therapy. The total energy and nutrient intake of the patients was determined as described in Schink et al. (Schink et al., 2018a), and their individual nutritional intake was monitored throughout the study. The group with WB-EMS performed two training sessions per week for 12 wks. The patients were adapted to WB-EMS by an incremental increase of the training duration up to 20 min/training session. WB-EMS applies low-frequency current impulses ( $< 100$  Hz) with a low current intensity ( $< 100$  mA) via electrodes (Kemmler et al., 2012). The patients wore a vest, a hip belt, and upper-arm and -thigh cuffs with integrated electrodes (miha bodytec GmbH, Gersthofen, Germany). Eight muscle groups were addressed by the WB-EMS application (upper arms, chest, upper back, latissimus, abdomen, lower back, buttocks and thighs (Fig. 1A)). Electric stimulation was mediated by bipolar impulses (frequency 85 Hz, pulse width

350  $\mu$ sec), inducing an intermittent stimulation of a 6 sec impulse phase followed by 4 sec rest. WB-EMS is classified as a resistance exercise. The current intensity was set to trigger a noticeable muscle contraction. Then, the current intensity was raised up to a threshold between being comfortable and inducing discomfort/pain. The current intensity on the miha bodytec device (Fig. 1A) is set via a main regulator (setting for our prostate cancer patients between 70 - 75) and the regulators for each muscle group. During the initial adaption phase, the following settings were applied (data for 6 WB-EMS prostate cancer patients): regulator for the leg electrodes between 18 - 49; buttock 10 - 61; lower back 19 - 39; middle back 16 - 41; upper back 19 - 47; abdomen 16 - 51; breast 18 - 53; arm 14 - 35. After the initial adaption to the training, the following settings were applied (data for 4 WB-EMS prostate cancer patients): leg 41 - 64; buttock 28 - 79; lower back 23 - 47; middle back 24 - 46; upper back 21 - 45; abdomen 28 - 51; breast 33 - 72; arm 23 - 44). The individual and local adjustments are highly relevant due to each subject's current tolerance limits and differences in subcutaneous fat thickness (Kemmler et al., 2012). Our prostate cancer patients in the intervention group performed  $20.75 \pm 2.61$  WB-EMS training sessions out of the scheduled 24 sessions, and our colorectal WB-EMS cancer patients attended  $20.5 \pm 2.43$  trainings. The WB-EMS training was individually supervised by experienced physiotherapists and included light physical exercises (for more details please refer to Schink et al. (Schink et al., 2018a)).

#### Patient serum

Blood samples for cancer cell stimulation were collected at the beginning of the trial and at trial end 1 h after the last training session. Blood samples were centrifugated and the serum was stored at  $-80^{\circ}\text{C}$ . For the afterwards designed *in vitro* study, we used serum from stage III/IV prostate (exercise group  $n = 8$ , control group  $n = 10$ ) and stage III/IV colorectal cancer patients (exercise group  $n = 6$ , control group  $n = 6$ ) under anti-cancer therapy (please see Table 1). Serum from 6 out of 10 control prostate cancer patients was obtained during therapy-relevant attendance at the outpatient uro-oncological unit, Medical Department 5, University Clinic Erlangen. The human serum used in electric pulse stimulation (EPS) assays was a serum pool from 12 healthy individuals (6 male, 6 female), who were not associated with the clinical study; their average age was  $34.2 \pm 10.5$  yrs.

## Cell culture

The human prostate cancer cell line LNCaP was cultured in RPMI without phenol red (Gibco, ThermoFisher Scientific, Waltham, MA, USA) plus 10% FCS Superior (Biochrom, Merck KGaA, Darmstadt, Germany), 100 IU/ml penicillin (Biochrom) and 100 µg/ml streptomycin (Biochrom) at 37°C. The cell line was a kind gift from R. Atreya, Medical Department 1, Friedrich-Alexander-University Erlangen-Nürnberg (RRID:CVCL\_0395; experiments were done in passages 5-25 after purchase). The human prostate cancer cell line DU145 was cultured in RPMI without phenol red, 10% FCS Superior and penicillin/streptomycin at 37°C. The cell line was a kind gift from B. Wullich, Department of Urology, Friedrich-Alexander-University Erlangen-Nürnberg (RRID:CVCL\_0105; cells were kept in culture for no longer than 20 passages). The human prostate cancer cell line PC3 was cultured in DMEM (Gibco), 10% FCS Superior and penicillin/streptomycin at 37°C (RRID:CVCL\_0035; cells were kept in culture for no longer than 30 passages). The human colon cancer cell line HT29 was maintained in DMEM, 10% FCS and penicillin/streptomycin at 37°C. The cell line was a kind gift from R. Lopez-Posados, Medical Department 1, Friedrich-Alexander-University Erlangen-Nürnberg (RRID:CVCL\_0320; experiments were done in passages 15-30 after purchase). The human embryonic kidney cells HEK293T were maintained in DMEM, 10% FCS and 1% penicillin/streptomycin at 37°C. The cell line was a kind gift from A. Kremer, Medical Department 1, Friedrich-Alexander-University Erlangen-Nürnberg (RRID:CVCL\_0045; cells were kept in culture for no longer than 30 passages). Primary human skeletal muscle myoblasts (HSMMs, Lonza, Basel, Switzerland) were cultured according to manufacturer's instructions at 37°C. All cell cultures were mycoplasma-free.

## Human cancer cell growth and apoptosis assays

3 - 5 x 10<sup>3</sup> or 2.5 x 10<sup>4</sup> prostate (LNCaP, DU145, PC3), colon (HT29) cancer cells or non-malignant cells (HEK293T) per well were seeded into 96-well or 48-well plates, respectively, and after overnight incubation at 37°C, cells were serum-starved (0.1% FCS) for 18 - 24 h. Then, cells were treated with medium containing 10% patient serum or EPS-conditioned medium (DMEM/2% FCS/10% human serum from healthy individuals) for 48 h or 96 h at 37°C. 18 h before lysis, cells received 10 µM BrdU, and BrdU incorporation was measured using the Cell Proliferation ELISA Kit (Roche Applied Science, Penzberg, Germany) according to manufacturer's protocol.

For vitality assays, cells were trypsinized and total cell numbers and numbers of apoptotic/necrotic cells were determined using Trypan blue. Treatment-induced DNA fragmentation was measured by the Cell Death Detection ELISA<sup>PLUS</sup> (Roche Applied Science) following manufacturer's instructions.

#### Electric pulse stimulation

$1.5 \times 10^5$ /well primary human skeletal muscle myoblasts (HSMs) were seeded in 6-well plates. After reaching 70 - 80% confluency, HSMs were incubated for 3 - 4 d with DMEM/Ham F12 (Gibco) supplemented with 2% Hyclone<sup>TM</sup> horse serum (GE Healthcare, Chicago, IL, USA) at 37°C for differentiation into myotubes. Effective myogenic differentiation was checked by myogenin expression and the grade of myoblast fusion (data not shown). Medium was changed to 2 ml DMEM with 2% FCS and electric pulse stimulation (EPS; C-Pace EP, Ion Optix, Westwood, MA, US) was performed twice in a period of 24 h in line with the settings of a WB-EMS training unit in patients (20 min; bouts of 6 sec stimulation, 4 sec pause; 85 Hz pulse frequency, 400  $\mu$ sec pulse width, 15 V amplitude). Medium was collected 1 h after the second EPS treatment and stored at -80°C. EPS efficiency was investigated via quantitative RT-PCR (CXCL5, PPARGC1A and MSTN mRNA expression).

#### Multiplex gene expression array

For cancer gene expression analysis,  $1 \times 10^5$ /well low-passage LNCaP cells were seeded into 12-well plates, serum-depleted for 24 h and treated with either 10% of wk 0 and wk 12 serum pools from WB-EMS or control patients, or with a medium pool from EPS-treated human myotubes (from 3 independent treatments) at 37°C. The stimulation period was 24 h and 72 h. Total RNA was isolated with the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. Gene expression analysis was performed using the nCounter<sup>®</sup> PanCancer Pathways Panel (730 genes from cancer-associated pathways; NanoString Technologies, Seattle, WA, USA). 100 ng total RNA from each sample was used to run the analysis with the nCounter<sup>®</sup> FLEX Analysis System (NanoString Technologies). The raw data are available in the GEO database at

<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE135541>, reference number GSE135541. Normalized data from treated LNCaP cells (EPS-conditioned myotube medium / unconditioned myotube medium; WB-EMS group post-intervention serum /

pre-intervention serum, and control group post-intervention serum / pre-intervention serum) were compared and potentially regulated genes were selected for further analysis. The following selection criteria were chosen: a difference in gene expression ratio of > 20% in both, EPS medium-treated vs. untreated samples, and post- vs. pre-intervention samples from the WB-EMS group. Additionally, gene expression in the LNCaP cells stimulated with control cancer patient serum pools should be unaffected or inversely regulated compared to the WB-EMS ratio. Gene expression profiles were examined with the nSolver 4.0 software (NanoString Technologies). Experiments were repeated 3 times for data validation. Total RNA was isolated and reverse-transcribed cDNA was subjected to quantitative RT-PCR. The reference genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase 1 (HPRT1) and/or ribosomal protein lateral stalk subunit P0 (RPLP0) were used to calculate relative mRNA levels with the  $2^{-\Delta\Delta CT}$  method.

#### Quantitative Reverse Transcription PCR (qRT-PCR)

Total RNA was isolated from cells using QIAzol<sup>®</sup> reagent (Qiagen) according to manufacturer's protocol, and 0.5 - 1  $\mu$ g was transcribed into cDNA using iScript<sup>™</sup> synthesis kit (Bio-Rad). qRT-PCR was performed using the CFX Connect Real-Time System and CFX Manager software (Bio-Rad).

#### Primer sequences

Oligonucleotide synthesis was done by Metabion International AG (Planegg, Germany). For gene expression analysis of EPS efficiency and myokines, the following primers were designed/used: Human HPRT1 (forward, f) 5'-CCTGGCGTCGTGATTAGTGAT-3' and (reverse, r) 5'-AGACGTTTCAGTCCTGTCCATAA-3' (PrimerBank ID 164518913c1; PrimerBank Massachusetts General Hospital/The Center for Computational and Integrative Biology/Harvard Medical School); human CXCL5 (f) 5'-CCACTATGAGCCTCCTGTCC-3' and (r) 5'-GATGAACTCCTTGCGTGGTC-3'; human MSTN (f) 5'-GAGACCCGTCGAGACTCCTAC-3' and (r) 5'-AGTGCCTGGGTTTCATGTCAAG-3'; human PPARGC1A (f) 5'-TGAAGACGGATTGCCCTCATT-3' and (r) 5'-GCTGGTGCCAGTAAGAGCTT-3' (PrimerBank ID 116284374c3); human CCL2 (QT00212730, Qiagen); human

IGFBP3 (f) 5'-CCCTGCCGTAGAGAAATGGA-3' and (r) 5'-CCTTCCCCTTGGTGGTGTAG-3'; human IL5 (f) 5'-GGGAATAGGCACACTGGAGA-3' and (r) 5'-TGCAGGTAGTCTAGGAATTGGT-3'; human IL6 (f) 5'-AGCTCTATCTCCCCTCCAGG-3' and (r) 5'-GAGGTGAGTGGCTGTCTGTG-3'; human IL7 (f) 5'-CAACACAGACTCGGCAACTC-3' and (r) 5'-GAACAAGGATCAGGGGAGGA-3'; human IL8 (f) 5'-GCAGAGGGTTGTGGAGAAGT-3' and (r) 5'-ACCCTACAACAGACCCACAC-3'; human IL15 (QT00052066, Qiagen); human VEGFA (f) 5'-ATCCAATCGAGACCCTGGTG-3' and (r) 5'-ATCTCTCCTATGTGCTGGCC-3'; human BDNF (f) 5'-GGCTTGACATCATTGGCTGAC-3' and (r) 5'-CATTGGGCCGAACCTTCTGGT-3'. For gene array validation experiments, the following primers were designed/used: Human RPLP0 (f) 5'-TACACCTTCCCCTTGCTGA-3' and (r) 5'-ATCCCATATCCTCGTCCGAC-3'; human GAPDH (f) 5'-AACGGATTTGGTCGTATTGGG-3' and (r) 5'-TGGAAGATGGTGATGGGATTT-3'; human BID (f) 5'-ACCAGAACCTACGCACCTAC-3' and (r) 5'-CTAGGAACGCTGTTGACATGC-3'; human CASP3 (f) 5'-ACTGGACTGTGGCATTGAGA-3' and (r) 5'-GCACAAAGCGACTGGATGAA-3'; human CASP7 (f) 5'-AGGGACCGAGCTTGATGATG-3' and (r) 5'-GCACAAACCAGGAGCCTCTT-3'; human CCNE1 (f) 5'-CAAACCTCAACGTGCAAGCCT-3' and (r) 5'-GCAGAAGAGGGTGTGCTCA-3'; human CDKN1B (f) 5'-TCTGAGGACACGCATTTGGT-3' and (r) 5'-CAGAACCAGGATTTGGGGAA-3'; human DUSP8 (f) 5'-TACCCATGAGCCTCTCCCA-3' and (r) 5'-CAGGAGTTGCTGGCGTTG-3'; human GHR (f) 5'-CCCAGTTCCAGTTCCAAAGATT-3' and (r) 5'-TCCTCAGTCTTTTCATCTGGCT-3'; human WNT10A (f) 5'-GGCAACCCGTCAGTCTGTCT-3' and (r) 5'-CATTCCCCACCTCCCATCT-3' (Nakamura et al., 2005).

#### Cytokine Array

For simultaneous detection of cytokines/myokines in the medium from untreated or EPS-treated primary human myotubes, a Human Cytokine Antibody Array (120 targets; Abcam, Cambridge, UK) was performed. The assay was done with a pool of myotube medium from 3 independent EPS approaches according to manufacturer's protocol. For chemiluminescence detection the Amersham 600 Imager (General

Electric, Pittsburgh, PA, USA; RRID:SCR\_003070 ) was used. Signals were analyzed using ImageJ software (National Institute of Health, Bethesda, MD, USA). Normalization of the raw densitometry data was performed according to manufacturer's recommendations. After subtracting the mean density of the negative controls (background), the density of each cytokine spot was normalized to the mean positive control signal (for each membrane). The values of the untreated and the EPS-treated medium pools were compared and already established myokines with an upregulation of > 10% in the EPS pool were selected.

#### Immunoblotting

$8 \times 10^4$  LNCaP cells per well were seeded into 12-well plates and after overnight incubation at 37°C, cells were serum-starved (0.1% FCS) for 18 h. Cells were treated with medium containing 10% prostate cancer patient serum or EPS-conditioned myotube medium for 96 h at 37°C. Cells were lysed in cell lysis buffer (0.5% NP-40, 150 mM NaCl, 20 mM Tris pH 7.4, 10 mM phenylmethylsulfonyl fluoride, phosphatase inhibitors (PhosSTOP, Roche Diagnostics, Penzberg, Germany), protease inhibitors (Protease Inhibitor Cocktail Set I, Calbiochem, Merck KGaA). Cell extracts were fractionated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and analyzed by immunoblotting with antibodies specific for poly (ADP-ribose) polymerase (PARP; recognizes full length, 116 kDa, and large fragment, 89 kDa) and cleaved PARP (Asp214; large fragment, 89 kDa; both from Cell Signaling, Danvers, MA, USA; RRID:AB\_2160739, RRID:AB\_10699459). Using horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and enhanced chemiluminescence, the membranes were analyzed with the Amersham 600 Imager and the signals were quantified using ImageJ software.

#### Statistics

Study group differences in Table 1 were analyzed by independent samples t-test or Mann-Whitney test. Graphs depict the mean (SD). The respective number of experiments is stated in the Figure legends. For statistical analysis of BrdU incorporation and DNA fragmentation after stimulation with patient serum, paired (post- vs. pre-intervention data within a patient group) or unpaired two-tailed Student's t-test (post-intervention data between the patient groups) were applied. For

analysis of cell counting and Trypan blue, Wilcoxon signed-rank (post- vs. pre-intervention values within a patient group) and Mann-Whitney test (post-intervention data between the patient groups) were used. For data evaluation of BrdU incorporation, DNA fragmentation and PARP cleavage (Immunoblot) after stimulation with EPS-conditioned myotube medium, unpaired two-tailed Student's t-test was applied, and for cell counting data analysis after EPS-medium treatment Mann-Whitney test was used. For statistics on gene expression data, we applied paired (post- vs. pre-intervention WB-EMS serum) or unpaired (post-intervention serum between the patient groups; EPS-conditioned vs. unconditioned medium) two-tailed Student's t-test. EPS marker and myokine expression in human myotubes after EPS application was examined using unpaired two-tailed Student's t-test. Statistical analysis was performed using GraphPad Prism 8.1.2 (GraphPad Software, San Diego, CA, USA; RRID:SCR\_002798). P values of  $< 0.05$  were considered statistically significant.

## Results and Discussion

In the initial clinical trial advanced-stage cancer patients were examined regarding the effect of WB-EMS (for 12 weeks, 2x/week, 20 min/session; Fig. 1) on bodyweight, skeletal muscle mass and physical function (Schink et al., 2018a). The study flowchart for our *in vitro* investigation focusing on prostate and colorectal cancer patients is shown in Fig. 1B. Table 1 displays the baseline demographic and disease characteristics of the included patients. No significant difference between the study groups within each cancer type were observed regarding performance status (Karnofsky index) and physical functioning (6min-walk test) as well as in body parameters. A relative weight loss of  $> 5\%$  of the bodyweight over 3 - 6 month prior to study entry indicated that some participants may have cancer cachexia (prostate cancer cohort: 20% of the controls, 25% of the WB-EMS patients; colorectal cancer: 66.7% of the controls, 16.7% of the WB-EMS patients). The blood parameter measurement in the prostate cancer cohort showed significant differences between control and WB-EMS patients regarding albumin levels, leucocyte and erythrocyte count.

Our research aim was to use the patient serum collected before the intervention at study entry and post-intervention in order to analyze the anti-cancer effects of WB-EMS exercise on cancer cells *in vitro*. We stimulated the human androgen-sensitive prostate cancer cell line LNCaP with pre- and post-intervention serum from advanced prostate cancer patients with (n = 8) or without WB-EMS training (n = 10) for 96 h. For the first time, we could show that cancer cell growth, determined by proliferation assay and cell counting, was inhibited with WB-EMS exercise serum (Fig. 2A, left). 6 out of 8 patient sera clearly triggered a lower cell proliferation rate (ID 38: P = 0.061; 43: P = 0.723; 68: P = 0.024; 71: P = 0.019; 74: P = 0.080; 79: P = 0.057; 83: P = 0.014; 167: P = 0.009). So far, we have no definite explanation why the serum from 2 WB-EMS patients was without effect. We can speculate that differences in the cancer therapy regimen or individual factors like other medication might be responsible. The BrdU uptake after incubation with the pre-intervention serum from WB-EMS patients did not significantly differ from the values obtained after stimulation with a pool of pre-intervention control patient serum (n = 10; Fig. 2A, left bar). By that, we could exclude that the growth inhibitory effect seen with post- vs. pre-intervention serum in the WB-EMS patients is exclusively caused by an already at baseline existing significant difference in the cell proliferative behavior, compared to control serum. Yet, a subtle higher proliferation rate induced by some pre-intervention WB-EMS patient sera might contribute to the anti-proliferative effects shown in Fig. 2A. Since androgens affect the growth of hormone-sensitive prostate cancer cells, we measured the testosterone amount in each prostate cancer patient serum (Table 2). Of note, 80% of the control patients and all WB-EMS patients were on anti-androgen therapy throughout the trial phase (Table 1). 66.7% of the tested control as well as WB-EMS patient sera showed a decrease in post-intervention testosterone levels. However, the comparison of the testosterone measurements with the BrdU uptake data in Fig. 2A revealed that reduced LNCaP proliferation upon post-WB-EMS serum is not necessarily caused by a lower testosterone amount (e.g. patients 79 and 167). On the other hand, patient 74 had a high pre-intervention testosterone level (3.17 ng/ml), which dropped to 0.24 ng/ml post-intervention; patient 74 serum displayed the most pronounced LNCaP cell growth inhibition (-33.7%; Fig. 2A). In order to investigate if the reduction in serum testosterone is responsible for this, we stimulated the hormone-insensitive prostate

cancer cell lines DU145 and PC3 with pre- and post-intervention serum from patient 74. We also observed a decreased cell proliferation in these hormone-insensitive cells upon post-WB-EMS serum, but not as distinct (data not shown).

A post-intervention serum pool from all WB-EMS patients ( $n = 8$ ) attenuated human prostate cancer cell proliferation after 96 h (Fig. 2B, left; LNCaP, - 10.9%,  $P = 0.014$ ; DU145, - 7.0%,  $P = 0.048$ ; PC3, -10.4%,  $P = 0.011$ ), in contrast to serum taken prior to exercise and from control patients. Remarkably, the growth of human non-malignant HEK293T cells was not inhibited by WB-EMS serum (Fig. 2B, left; for additional statistical analysis, please refer to the legend of Fig. 2). The mean serum testosterone levels shown in Table 2 reflect the testosterone level in the patient serum pools. The testosterone amount in the post-intervention serum from both groups was lower compared to pre-intervention samples (control:  $0.21 \pm 0.22$  ng/ml (pre) to  $0.14 \pm 0.13$  ng/ml (post),  $P = 0.080$ ; WB-EMS:  $0.75 \pm 1.20$  ng/ml (pre) to  $0.24 \pm 0.14$  ng/ml (post),  $P = 0.339$ ; Table 2), indicating again, that changes in serum testosterone do not play a significant role in our cancer cell growth experiments. Of note, we checked whether human serum is an adequate growth environment for the prostate cancer cells. The BrdU incorporation rate after stimulation with 10% baseline serum pools compared to 10% FCS (Fig. 2B, right graph) revealed no significant difference in the proliferation behavior. Patient serum incubation on prostate cancer and non-cancer cells for a shorter period (48 h) showed similar growth-inhibitory effects of the post-WB-EMS serum on the cancer cells (Fig. 2C, left; LNCaP, - 11.6%,  $P = 0.018$ ; DU145, - 9.6%,  $P = 0.051$ ; PC3, - 7.0%,  $P = 0.042$ ). Again, the growth response of the cell lines did not significantly differ under treatment with human compared to bovine serum (Fig. 2C, right). Taken together, these data strongly suggest cancer cell-specific effects of exercise-mediated mechanisms that attenuate cell proliferation.

We also observed that LNCaP viability was impaired by WB-EMS-conditioned patient serum pool (Fig. 2D - F). Total cell count was significantly reduced (- 27.9%,  $P = 0.001$ ; Fig. 2D). Statistical analysis between the groups indicated that the cell numbers after incubation with post-WB-EMS serum is also significantly lower ( $P = 0.022$ ) compared to control serum. Furthermore, apoptotic events, measured by apoptotic/dead cell numbers (from 17.6% to 28.1%,  $P = 0.001$ ; Fig. 2E) and DNA

fragmentation (+ 21.2%,  $P = 0.035$ ; Fig. 2F), were increased upon post-WB-EMS serum. Experimental variation, e.g. incubation time, might be responsible for the difference in apoptotic response upon incubation with post- compared to pre-intervention control serum between the 2 assays (Fig. 2E und F).

Similar to these data in prostate cancer cells, human colon carcinoma cells were also affected after treatment with a serum pool from patients with advanced colorectal cancer after WB-EMS. Upon incubation with post-exercise serum for 96 h or 48 h, proliferation was decreased by 9.1% ( $P = 0.031$ ; Fig. 3A, left) or by 13.2% ( $P = 0.021$ ; Fig. 3B, left), respectively, in the human colon cancer cell line HT29 cells; proliferation of the non-malignant HEK293T cells was uninhibited by post-WB-EMS serum. We also checked HT29 cell proliferation after stimulation with 10% human serum compared to 10% FCS and detected no significant difference in BrdU uptake (Fig. 3A and B, right graphs). As seen in prostate cancer cells, HT29 total cell number was diminished upon exercise serum (- 25.8%,  $P = 0.008$ ; Fig. 3C). Concomitantly, apoptosis/necrosis was increased as detected by Trypan blue inclusion (from 9.4% to 14.4%,  $P = 0.047$ ; Fig. 3D).

Taken together, these results imply that WB-EMS induces defensive humoral factors in cancer patients that target malignant cell growth and viability, independent of their hormonal sensitivity, while non-tumor cell growth is unaffected, a hitherto very important finding. Interestingly, several *in vitro* and *in vivo* studies focusing on prostate cancer described that serum from healthy individuals after acute and long-term exercise contains lower amounts of epidermal growth factor (EGF), fibroblast growth factor 4 (FGF4) and insulin-like growth factor 1 (IGF1) as well as an increased level of insulin-like growth factor-binding protein 1 (IGFBP1) (Ngo et al., 2002; Rundqvist et al., 2013). It is possible that deregulated protein levels might also be responsible, at least in part, for the inhibitory effects of WB-EMS on prostate cancer cell growth seen in our experiments. Barnard and colleagues showed that an exercise-mediated increase in p53 and p21 protein level and a concomitant decrease in the anti-apoptotic protein Bcl2 (B-cell lymphoma 2) can be reasons for reduced prostate carcinoma cell growth (Barnard et al., 2007). Furthermore, serum from early-stage prostate cancer patients that underwent extensive lifestyle changes

including unmonitored regular exercise suppressed prostate cancer cell growth (Ornish et al., 2005).

Electric pulse stimulation (EPS) of cultured skeletal muscle cells at the myotube stage can be used as an *in vitro* model of exercise (Nikolic et al., 2012; Nikolic et al., 2017; Scheler et al., 2013). We applied EPS to human myotubes with parameters similar to the WB-EMS training (85 Hz, 15 V, 400  $\mu$ s band width, 6 s stimulation, 4 s rest; 2 x 20 min on two consecutive days), and could show that known EPS-responsive genes like C-X-C motif chemokine 5 (CXCL5;  $P = 0.0004$ ) and peroxisome proliferator-activated receptor  $\gamma$  coactivator 1 $\alpha$  (PPARGC1A;  $P = 0.003$ ) are increased, and Myostatin (MSTN) is downregulated ( $P = 0.072$ ; (Fig. 4A) (Nedachi et al., 2008; Nikolic et al., 2012; Tarum et al., 2017). In accordance with the observed WB-EMS serum effects, we found that EPS-conditioned medium decreased proliferation of LNCaP cells by 7.7% ( $P = 0.007$ ), of PC3 cells by 7.4% ( $P = 0.0003$ ) and with lower efficacy in DU145 cells (- 3.5%,  $P = 0.003$ ; Fig. 4B). In line with that, total LNCaP cell count was significantly reduced after EPS medium incubation (- 18.8%,  $P = 0.005$ , left bars), while cell death, quantified by apoptotic/dead cell numbers (from 14.3% to 19.8%,  $P = 0.0007$ , right bars; Fig. 4C) and DNA fragmentation (+ 83%,  $P = 0.017$ ; Fig. 4D), was increased. We obtained similar results in colon cancer cells. EPS-conditioned medium reduced proliferation of HT29 cells by 10.6% ( $P = 0.0007$ ; Fig. 4B). Total HT29 cell count was also significantly decreased (- 15.4 %,  $P = 0.025$ , left bars), while concomitantly, the apoptotic/dead cell number (from 13.6% to 19.0%,  $P = 0.014$ , right bars; Fig. 4E) was enhanced. In contrast to that, non-malignant HEK293T cell growth was not significantly affected by the EPS medium ( $P = 0.307$ ; Fig. 4B). These data indicate that mechanisms induced by muscle cell stimulation, either *in vivo* (WB-EMS) or *in vitro* (EPS), are responsible for cancer cell growth inhibition. Moreover, the direct comparison of both approaches with yet very similar effects allow to eliminate all non-muscle origins of respective soluble factors and track those down to muscle-secreted modulators, for which myokines represent prime candidates (Hoffmann and Weigert, 2017; Karstoft and Pedersen, 2016; Piccirillo, 2019).

Stimulated skeletal muscle releases specific cytokines and growth factors which facilitate communication with various body tissues. Hundreds of secretory proteins

are considered as myokines, including interleukins like IL6 and IL15, and growth factors like fibroblast growth factor 21 (FGF21) and brain-derived neurotrophic factor (BDNF) (Aoi et al., 2013; Karstoft and Pedersen, 2016; Schnyder and Handschin, 2015a; Whitham and Febbraio, 2016). We were curious about the effectiveness of our EPS approach to induce myokine expression and/or release. We analyzed EPS-conditioned medium and the medium from untreated myotubes regarding their cytokine/myokine composition. A protein array revealed that several established myokine are induced and/or secreted by our EPS protocol (Table 3)(Raschke et al., 2013; Scheler et al., 2013). Together with corresponding gene expression experiments we could showed that the following myokines are upregulated by EPS (Table 3): BDNF (fold change protein 2.58; fold change mRNA  $1.41 \pm 0.49$  ( $P = 0.0332$ )), CCL2 (1.12;  $1.35 \pm 0.51$  ( $P = 0.057$ )), IGFBP3 (1.13;  $2.31 \pm 1.01$  ( $P = 0.001$ )), IL5 (6.40;  $3.18 \pm 3.58$  ( $P = 0.107$ )), IL6 (1.49;  $3.91 \pm 1.96$  ( $P = 0.0002$ )), IL7 (protein not detectable;  $3.34 \pm 1.07$  ( $P = 0.0001$ )), IL8 (1.42;  $1.73 \pm 0.82$  ( $P = 0.019$ )), and vascular endothelial growth factor (VEGF/VEGFA; 1.63;  $2.12 \pm 0.76$  ( $P = 0.0004$ )). No increase in IL15 protein secretion could be determined (0.78), but IL15 mRNA level was elevated ( $2.06 \pm 0.50$ ,  $P = 0.0001$ ). Other established myokines like FGF21, leukemia inhibitory factor (LIF) and myonectin were not included in the cytokine array (Karstoft and Pedersen, 2016; Schnyder and Handschin, 2015b; Whitham and Febbraio, 2016).

As mentioned before, Hojman et al. showed for the first time, that conditioned serum from exercising mice decreases human cancer cell viability via exercise-induced factors. They attributed part of the observed pro-apoptotic effects of exercise serum to the muscle-derived factor Oncostatin M (Hojman et al., 2011). Gannon and co-workers found that Irisin, a pivotal protein mediating several beneficial effects of exercise on the body, affects breast cancer cell viability *in vitro* (Gannon et al., 2015). Also, exercise-induced SPARC (secreted protein acidic and rich in cysteine) was shown to suppress colon tumorigenesis *in vivo*, and decreases proliferation and concomitantly increases apoptosis of colon carcinoma cells *in vitro* (Aoi et al., 2013). It will be very interesting to examine if myokines play a role in WB-EMS-mediated interference with cancer cell growth.

In our study here, we were curious how cancer cell gene expression is affected by exercise, a research area involving epigenetic changes as well as modulation of upstream signal transduction networks. We performed a gene expression screen with 730 cancer-associated genes on LNCaP cells either stimulated with WB-EMS or control patient serum, or with EPS-conditioned or unconditioned medium from human myotubes. After incubation for 24 h and 72 h, the expression of 11 (4 up, 7 down) and 15 genes (6 up, 9 down), respectively, was equally regulated by both treatments (Fig. 5A). From these hits, we chose to examine the expression of genes (potentially) involved in cancer cell proliferation and apoptosis (Table 4). In validation experiments, we observed in the 24h-response gene group (Fig. 5B) that mRNA expression of cyclin E1 (CCNE1) is downregulated by treatment with post-WB-EMS serum ( $P = 0.004$ ) as well as with EPS-conditioned medium ( $p = 0.1379$ ). Furthermore, post-intervention CCNE1 levels significantly differed between the groups ( $P = 0.014$ ). Cyclins are key players during cell cycle and regulate cyclin-dependent kinases. The complex of cyclin-dependent kinase 2 (CDK2) and cyclin E is essential to drive G1/S transition (Malumbres and Barbacid, 2009). CCNE1 overexpression seems to be associated with poor survival of cancer patients (Zhao et al., 2018). A study in prostate cancer cells suggested that downregulation of CCNE1 suppresses proliferation and induces G1/S arrest (Zhang et al., 2019), which is in line with our results. We also found significantly lower levels of growth hormone receptor (GHR) mRNA in cells treated with post-WB-EMS serum ( $P = 0.041$ ) as well as EPS medium ( $P = 0.018$ ). We also detected significantly different GHR levels when comparing mRNA expression upon post-intervention serum between the groups ( $P = 0.0001$ ). The observed downregulation might be in part responsible for the anti-proliferative effects of exercise via WB-EMS. It was shown, e.g., that GHR mRNA expression is 80% higher in prostate carcinomas than in benign prostate hyperplasia, and GH/GHR-mediated production of IGF1 seemed to play an important role in tumor progression (Dehkhoda et al., 2018; Weiss-Messer et al., 2004). Moreover, we discovered that treatment with EPS-conditioned medium clearly decreased WNT10A (Wnt family member 10A) expression levels ( $P = 0.057$ ) in LNCaP cells. We also found a strong trend towards a downregulation with WB-EMS serum, and a significant difference in WNT10A mRNA levels between the groups upon post-intervention serum ( $P = 0.027$ ). Wnt signaling was discovered in cancer

models, and mutations in Wnt pathways are frequently observed in carcinomas. In accordance with our data, knockdown of WNT10A suppressed proliferation of, e.g., renal and ovarian cancer cells (Hsu et al., 2012; Li et al., 2017). Additionally, we detected that the expression of the cell cycle-dependent kinase inhibitor 1B (CDKN1B) is increased upon the EPS medium stimulus ( $P = 0.007$ ) and the post-WB-EMS serum ( $P = 0.057$ ). CDKN1B controls the cell cycle, i.e. G0 to S phase transition, by regulating the activity of the cyclin E/CDK2 complex (Chu et al., 2008). Thus, CDKN1B upregulation might be partly responsible for the observed suppression of LNCaP cell growth. CDKN1B ablation/downregulation enhanced the aggressive prostate carcinoma phenotype, and in general, decreased CDKN1B levels can be detected in various cancers with poor outcome (Borriello et al., 2011; Roy et al., 2008). As mentioned above, Barnard et al. could show that another regulator of cell cycle progression, CDKN1A (p21), is also downregulated in prostate cancer cells by exercise serum from healthy subjects (Barnard et al., 2007). It will be interesting to unravel the relationship between CDKN1B and the CCNE1/CDK2 complex in cancer cells under the influence of exercise, since we could show that CDKN1B mRNA levels are up- and CCNE1 levels are downregulated by an exercise stimulus. Unfortunately, the exercise-mediated downregulation of E2F1 (transcriptional activator) and POLE2 (DNA polymerase  $\epsilon$  subunit 2) seen in the gene expression screen could not be confirmed (data not shown). Data validation in the 72h-response group (Fig. 5C) revealed that WNT10A mRNA is still decreased, by both post-WB-EMS patient serum ( $P = 0.117$ ) and EPS-conditioned medium ( $P = 0.033$ ), hinting towards a more prominent role of WNT10A in the exercise-mediated control of human prostate cancer cell growth. We also found that expression of dual-specificity phosphatase 8, a tyrosine phosphatase targeting several mitogen-activated protein kinases (MAPKs), is enhanced (post-WB-EMS serum  $P = 0.097$ ; post-intervention serum WB-EMS vs. control group  $P = 0.012$ ; EPS  $P = 0.0076$ ). Abnormalities in MAPK signaling have been implicated in multiple human malignancies. Little is known about the physiological function of DUSP8, with contradicting function(s) in cancer cells (Nunes-Xavier et al., 2011). Further analysis should specify if DUSP8 upregulation in “exercise-exposed” LNCaP cells is involved in their proliferation control. We also detected that the expression level of the BH3 interacting-domain death agonist BID is elevated by treatment with post-WB-EMS

serum for 72h, with a significant difference between BID mRNA levels upon stimulation with post-intervention serum from the WB-EMS or control group ( $P = 0.039$ ). In EPS medium-stimulated cells we detected a trend towards an upregulation of BID. Among others, BID is essential for initiation of apoptosis signaling; it engages all Bcl-2 pro-survival proteins and is therefore a potent cell killer (Willis and Adams, 2005). However, the significance of BID expression in malignancies is unclear (Song et al., 2010). BID upregulation upon exposure to exercise might have pro-apoptotic effects specifically on LNCaP cells, but further experiments are needed for clarification. Additionally, we identified that caspase 7 (CASP7) expression is significantly increased under the described conditions (post- vs. pre-WB-EMS serum  $P = 0.033$ , post-intervention serum WB-EMS vs. control group  $P = 0.054$ ; EPS  $P = 0.0164$ ). Caspases, sequentially activated, play pivotal roles in apoptotic events. Downregulation of, e.g., CASP7 was described in colon cancer samples compared with normal mucosa, and resistance to apoptosis in renal cancer cells correlated with loss/downregulation of, e.g., CASP7 and 3 levels (Kolenko et al., 1999; Palmerini et al., 2001). In our gene expression screen, caspase 3 (CASP3) mRNA was borderline increased upon both stimulation with post-WB-EMS serum or EPS-conditioned medium for 72 h (Table 4). Validation experiments corroborated this result (post- vs. pre-WB-EMS serum  $P = 0.173$ , post-intervention serum WB-EMS vs. control group  $P = 0.051$ ; EPS  $P = 0.044$ ; Fig. 5C), suggesting that CASP3 upregulation might also contribute to exercise-induced apoptosis in prostate cancer cells. According to the literature, prostatic as well as breast and ovarian cancer cells show significant loss of CASP3 expression (Devarajan et al., 2002; Winter et al., 2001). Confirmatory gene expression experiments shown in Fig. 5B revealed, that CASP3 and CASP7 mRNA are also upregulated after shorter stimulation (24h) with post-WB-EMS serum or EPS-conditioned medium (CASP3: post- vs. pre-WB-EMS serum  $P = 0.099$ ; EPS  $P = 0.019$ , and CASP7: post-intervention serum WB-EMS vs. control group  $P = 0.016$ ; EPS  $P = 0.0004$ ), pointing towards a more dominant function of caspase 3 and 7 in exercise-mediated apoptosis/death of prostate cancer cells. Cleavage of poly(ADP ribose) polymerase 1 (PARP) is a hallmark of apoptosis. This cell death substrate has a canonical caspase 3/7 cleavage site (Boucher et al., 2012; Oliver et al., 1998). In order to corroborate our gene expression results regarding caspase 3/7, we performed cleaved PARP immunoblot analysis on LNCaP cell lysates after

stimulation with control and WB-EMS prostate cancer serum as well as with medium from untreated and EPS-treated myotubes (96 h; Fig. 5D and E). After densitometry analysis, we found only a minor trend towards an increased PARP cleavage after incubation with post-WB-EMS serum (Fig. 5D). However, stimulation of LNCaP cells with EPS myotube medium led to a significant, almost 2-fold enhancement in PARP cleavage (Fig. 5E, densitometry graph,  $P = 0.011$ ). We could speculate that the EPS-conditioned medium can exert “cleaner”, more specific effects compared to the medium plus serum from the patients, which contains potentially interfering compounds and factors from the anti-cancer therapy and/or other medication. This discrepancy in the cell death analysis became already apparent in the DNA fragmentation experiments, where fragmentation was more pronounced upon EPS medium (Fig. 4D) than with medium plus post-WB-EMS serum (Fig. 2F). The WB-EMS/EPS-mediated upregulation of CASP3 and 7 gene expression seems to correlate with the EPS-induced PARP cleavage. We propose a mechanistic link between muscle stimulation and cancer cell death, initiated by an exercise-induced muscle-derived player that can activate the caspase pathway via the regulation of CASP3/7 mRNA expression. This, in turn, leads to an increased cleavage of the caspase 3/7 substrate PARP and ultimately to enhanced DNA fragmentation and cell death.

Taken together, we could show that exercise regulates growth of human cancer cells, regardless of their hormone dependency. For the first time, we demonstrated that *in vivo* exercise in form of WB-EMS during advanced cancer disease can inhibit cancer cell proliferation and concomitantly activate apoptotic events *in vitro*, and provide evidence that genes associated with cancer cell growth and viability are regulated by exercise. Our comparative approach using both patient sera and supernatant from myotube cultures following exercise, revealed similar effects on cancer cell proliferation and apoptosis. Therefore, we can target the origin of the observed anti-cancer effect directly to muscle cells, suggesting that one or several myokines might be released during exercise (please refer to Table 3), (Karstoft and Pedersen, 2016)). Future studies should aim for identification of (potentially) involved myokines. We are convinced that our data further support the application of adjuvant exercise as part of multimodal therapeutic concepts for cancer patients, also for physically weakened patients. Regular exercise not only stabilizes the physical and

mental status of cancer patients, but also seems to actively battle cancer development and progression.

### **Additional Information**

#### Data availability statement

The raw data are available in the GEO database at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE135541>, reference number GSE135541.

#### Competing interests

None.

#### Author contribution

R.S., K.S. and Y.Z. were involved in all aspects of the study: (1) conception or design of the work, (2) acquisition, analysis or interpretation of data for the work and (3) drafting the work or revising it critically for important intellectual content. S.S., W.D., D.R., O.F., H.J.H. and M.F.N were involved in (2) and (3). All authors have approved the final version of the manuscript.

#### Funding

This research work was supported by the H.W. & J. Hector Foundation, Weinheim, Germany.

## References

- Aoi, W., Naito, Y., Takagi, T., Tanimura, Y., Takanami, Y., Kawai, Y., Sakuma, K., Hang, L. P., Mizushima, K., Hirai, Y., *et al.* (2013). A novel myokine, secreted protein acidic and rich in cysteine (SPARC), suppresses colon tumorigenesis via regular exercise. *Gut* *62*, 882-889.
- Barnard, R. J., Leung, P. S., Aronson, W. J., Cohen, P., and Golding, L. A. (2007). A mechanism to explain how regular exercise might reduce the risk for clinical prostate cancer. *European journal of cancer prevention : the official journal of the European Cancer Prevention Organisation (ECP)* *16*, 415-421.
- Borriello, A., Bencivenga, D., Criscuolo, M., Caldarelli, I., Cucciolla, V., Tramontano, A., Borgia, A., Spina, A., Oliva, A., Naviglio, S., and Della Ragione, F. (2011). Targeting p27Kip1 protein: its relevance in the therapy of human cancer. *Expert Opin Ther Targets* *15*, 677-693.
- Boucher, D., Blais, V., and Denault, J. B. (2012). Caspase-7 uses an exosite to promote poly(ADP ribose) polymerase 1 proteolysis. *Proceedings of the National Academy of Sciences of the United States of America* *109*, 5669-5674.
- Brown, J. C., and Gilmore, L. A. (2020). Physical Activity Reduces the Risk of Recurrence and Mortality in Cancer Patients. *Exerc Sport Sci Rev* *48*, 67-73.
- Chu, I. M., Hengst, L., and Slingerland, J. M. (2008). The Cdk inhibitor p27 in human cancer: prognostic potential and relevance to anticancer therapy. *Nature reviews Cancer* *8*, 253-267.
- Dehkhoda, F., Lee, C. M. M., Medina, J., and Brooks, A. J. (2018). The Growth Hormone Receptor: Mechanism of Receptor Activation, Cell Signaling, and Physiological Aspects. *Front Endocrinol (Lausanne)* *9*, 35.
- Dethlefsen, C., Hansen, L. S., Lillelund, C., Andersen, C., Gehl, J., Christensen, J. F., Pedersen, B. K., and Hojman, P. (2017). Exercise-Induced Catecholamines Activate the Hippo Tumor Suppressor Pathway to Reduce Risks of Breast Cancer Development. *Cancer research* *77*, 4894-4904.
- Devarajan, E., Sahin, A. A., Chen, J. S., Krishnamurthy, R. R., Aggarwal, N., Brun, A. M., Sapino, A., Zhang, F., Sharma, D., Yang, X. H., *et al.* (2002). Down-regulation of caspase 3 in breast cancer: a possible mechanism for chemoresistance. *Oncogene* *21*, 8843-8851.
- Devin, J. L., Hill, M. M., Mourtzakis, M., Quadriatero, J., Jenkins, D. G., and Skinner, T. L. (2019). Acute high intensity interval exercise reduces colon cancer cell growth. *The Journal of physiology* *597*, 2177-2184.
- Dieli-Conwright, C. M., Parmentier, J. H., Sami, N., Lee, K., Spicer, D., Mack, W. J., Sattler, F., and Mittelman, S. D. (2018). Adipose tissue inflammation in breast cancer survivors: effects of a 16-week combined aerobic and resistance exercise training intervention. *Breast cancer research and treatment* *168*, 147-157.
- Filipovic, A., Kleinoder, H., Dormann, U., and Mester, J. (2012). Electromyostimulation--a systematic review of the effects of different electromyostimulation methods on selected strength parameters in trained and elite athletes. *Journal of strength and conditioning research / National Strength & Conditioning Association* *26*, 2600-2614.
- Gannon, N. P., Vaughan, R. A., Garcia-Smith, R., Bisoffi, M., and Trujillo, K. A. (2015). Effects of the exercise-inducible myokine irisin on malignant and non-malignant breast epithelial cell behavior in vitro. *International journal of cancer Journal international du cancer* *136*, E197-202.
- Goh, J., Niksirat, N., and Campbell, K. L. (2014). Exercise training and immune crosstalk in breast cancer microenvironment: exploring the paradigms of exercise-induced immune modulation and exercise-induced myokines. *Am J Transl Res* *6*, 422-438.
- Hoffmann, C., and Weigert, C. (2017). Skeletal Muscle as an Endocrine Organ: The Role of Myokines in Exercise Adaptations. *Cold Spring Harb Perspect Med* *7*.
- Hojman, P., Dethlefsen, C., Brandt, C., Hansen, J., Pedersen, L., and Pedersen, B. K. (2011). Exercise-induced muscle-derived cytokines inhibit mammary cancer cell growth. *American journal of physiology Endocrinology and metabolism* *301*, E504-510.

- Hsu, R. J., Ho, J. Y., Cha, T. L., Yu, D. S., Wu, C. L., Huang, W. P., Chu, P., Chen, Y. H., Chen, J. T., and Yu, C. P. (2012). WNT10A plays an oncogenic role in renal cell carcinoma by activating WNT/beta-catenin pathway. *PloS one* 7, e47649.
- Karstoft, K., and Pedersen, B. K. (2016). Skeletal muscle as a gene regulatory endocrine organ. *Current opinion in clinical nutrition and metabolic care*.
- Kemmler, W., Engelke, K., and von Stengel, S. (2012). Effects of Whole-Body-Electromyostimulation on Sarcopenia in Lean, Elderly Sedentary Women. The TEST-III Study. *Deut Z Sportmed* 63, 343-+.
- Kemmler, W., Schliffka, R., Mayhew, J. L., and von Stengel, S. (2010). Effects of whole-body electromyostimulation on resting metabolic rate, body composition, and maximum strength in postmenopausal women: the Training and ElectroStimulation Trial. *Journal of strength and conditioning research / National Strength & Conditioning Association* 24, 1880-1887.
- Kemmler, W., and von Stengel, S. (2013). Whole-body electromyostimulation as a means to impact muscle mass and abdominal body fat in lean, sedentary, older female adults: subanalysis of the TEST-III trial. *Clinical interventions in aging* 8, 1353-1364.
- Kolenko, V., Uzzo, R. G., Bukowski, R., Bander, N. H., Novick, A. C., Hsi, E. D., and Finke, J. H. (1999). Dead or dying: necrosis versus apoptosis in caspase-deficient human renal cell carcinoma. *Cancer research* 59, 2838-2842.
- Li, P., Liu, W., Xu, Q., and Wang, C. (2017). Clinical significance and biological role of Wnt10a in ovarian cancer. *Oncol Lett* 14, 6611-6617.
- Malumbres, M., and Barbacid, M. (2009). Cell cycle, CDKs and cancer: a changing paradigm. *Nature reviews Cancer* 9, 153-166.
- Nakamura, Y., Nawata, M., and Wakitani, S. (2005). Expression profiles and functional analyses of Wnt-related genes in human joint disorders. *The American journal of pathology* 167, 97-105.
- Nedachi, T., Fujita, H., and Kanzaki, M. (2008). Contractile C2C12 myotube model for studying exercise-inducible responses in skeletal muscle. *American journal of physiology Endocrinology and metabolism* 295, E1191-1204.
- Ngo, T. H., Barnard, R. J., Tymchuk, C. N., Cohen, P., and Aronson, W. J. (2002). Effect of diet and exercise on serum insulin, IGF-I, and IGFBP-1 levels and growth of LNCaP cells in vitro (United States). *Cancer causes & control : CCC* 13, 929-935.
- Nikolic, N., Bakke, S. S., Kase, E. T., Rudberg, I., Flo Halle, I., Rustan, A. C., Thoresen, G. H., and Aas, V. (2012). Electrical pulse stimulation of cultured human skeletal muscle cells as an in vitro model of exercise. *PloS one* 7, e33203.
- Nikolic, N., Gorgens, S. W., Thoresen, G. H., Aas, V., Eckel, J., and Eckardt, K. (2017). Electrical pulse stimulation of cultured skeletal muscle cells as a model for in vitro exercise - possibilities and limitations. *Acta physiologica (Oxford, England)* 220, 310-331.
- Nunes-Xavier, C., Roma-Mateo, C., Rios, P., Tarrega, C., Cejudo-Marin, R., Tabernero, L., and Pulido, R. (2011). Dual-specificity MAP kinase phosphatases as targets of cancer treatment. *Anti-cancer agents in medicinal chemistry* 11, 109-132.
- Oliver, F. J., de la Rubia, G., Rolli, V., Ruiz-Ruiz, M. C., de Murcia, G., and Murcia, J. M. (1998). Importance of poly(ADP-ribose) polymerase and its cleavage in apoptosis. Lesson from an uncleavable mutant. *The Journal of biological chemistry* 273, 33533-33539.
- Ornish, D., Weidner, G., Fair, W. R., Marlin, R., Pettengill, E. B., Raisin, C. J., Dunn-Emke, S., Crutchfield, L., Jacobs, F. N., Barnard, R. J., *et al.* (2005). Intensive lifestyle changes may affect the progression of prostate cancer. *The Journal of urology* 174, 1065-1069; discussion 1069-1070.
- Palmerini, F., Devilard, E., Jarry, A., Birg, F., and Xerri, L. (2001). Caspase 7 downregulation as an immunohistochemical marker of colonic carcinoma. *Hum Pathol* 32, 461-467.
- Piccirillo, R. (2019). Exercise-Induced Myokines With Therapeutic Potential for Muscle Wasting. *Frontiers in physiology* 10, 287.
- Raschke, S., Eckardt, K., Bjorklund Holven, K., Jensen, J., and Eckel, J. (2013). Identification and validation of novel contraction-regulated myokines released from primary human skeletal muscle cells. *PloS one* 8, e62008.

- Roy, S., Singh, R. P., Agarwal, C., Siriwardana, S., Sclafani, R., and Agarwal, R. (2008). Downregulation of both p21/Cip1 and p27/Kip1 produces a more aggressive prostate cancer phenotype. *Cell Cycle* 7, 1828-1835.
- Rundqvist, H., Augsten, M., Stromberg, A., Rullman, E., Mijwel, S., Kharaziha, P., Panaretakis, T., Gustafsson, T., and Ostman, A. (2013). Effect of acute exercise on prostate cancer cell growth. *PloS one* 8, e67579.
- Scheler, M., Irmeler, M., Lehr, S., Hartwig, S., Staiger, H., Al-Hasani, H., Beckers, J., de Angelis, M. H., Haring, H. U., and Weigert, C. (2013). Cytokine response of primary human myotubes in an in vitro exercise model. *American journal of physiology Cell physiology* 305, C877-886.
- Schink, K., Herrmann, H. J., Schwappacher, R., Meyer, J., Orlemann, T., Waldmann, E., Wullich, B., Kahlmeyer, A., Fietkau, R., Lubgan, D., *et al.* (2018a). Effects of whole-body electromyostimulation combined with individualized nutritional support on body composition in patients with advanced cancer: a controlled pilot trial. *BMC cancer* 18, 886.
- Schink, K., Reljic, D., Herrmann, H. J., Meyer, J., Mackensen, A., Neurath, M. F., and Zopf, Y. (2018b). Whole-Body Electromyostimulation Combined With Individualized Nutritional Support Improves Body Composition in Patients With Hematological Malignancies - A Pilot Study. *Frontiers in physiology* 9, 1808.
- Schnyder, S., and Handschin, C. (2015a). Skeletal muscle as an endocrine organ: PGC-1alpha, myokines and exercise. *Bone* 80, 115-125.
- Schnyder, S., and Handschin, C. (2015b). Skeletal muscle as an endocrine organ: PGC-1alpha, myokines and exercise. *Bone* 80, 115-125.
- Schwartz, A. L., de Heer, H. D., and Bea, J. W. (2017). Initiating Exercise Interventions to Promote Wellness in Cancer Patients and Survivors. *Oncology (Williston Park)* 31, 711-717.
- Song, G., Chen, G. G., Hu, T., and Lai, P. B. (2010). Bid stands at the crossroad of stress-response pathways. *Current cancer drug targets* 10, 584-592.
- Tarum, J., Folkesson, M., Atherton, P. J., and Kadi, F. (2017). Electrical pulse stimulation: an in vitro exercise model for the induction of human skeletal muscle cell hypertrophy. A proof-of-concept study. *Experimental physiology* 102, 1405-1413.
- von Stengel, S., Kemmler, W., Engelke, K., and Kalender, W. A. (2012). Effect of whole-body vibration on neuromuscular performance and body composition for females 65 years and older: a randomized-controlled trial. *Scand J Med Sci Sports* 22, 119-127.
- Weiss-Messer, E., Merom, O., Adi, A., Karry, R., Bidosee, M., Ber, R., Kaploun, A., Stein, A., and Barkey, R. J. (2004). Growth hormone (GH) receptors in prostate cancer: gene expression in human tissues and cell lines and characterization, GH signaling and androgen receptor regulation in LNCaP cells. *Molecular and cellular endocrinology* 220, 109-123.
- Whitham, M., and Febbraio, M. A. (2016). The ever-expanding myokinome: discovery challenges and therapeutic implications. *Nat Rev Drug Discov* 15, 719-729.
- Willis, S. N., and Adams, J. M. (2005). Life in the balance: how BH3-only proteins induce apoptosis. *Curr Opin Cell Biol* 17, 617-625.
- Winter, R. N., Kramer, A., Borkowski, A., and Kyprianou, N. (2001). Loss of caspase-1 and caspase-3 protein expression in human prostate cancer. *Cancer research* 61, 1227-1232.
- Zhang, C., Zhu, Q., Gu, J., Chen, S., Li, Q., and Ying, L. (2019). Down-regulation of CCNE1 expression suppresses cell proliferation and sensitizes gastric carcinoma cells to Cisplatin. *Biosci Rep* 39.
- Zhao, H., Wang, J., Zhang, Y., Yuan, M., Yang, S., Li, L., and Yang, H. (2018). Prognostic Values of CCNE1 Amplification and Overexpression in Cancer Patients: A Systematic Review and Meta-analysis. *J Cancer* 9, 2397-2407.

## Figures, Tables and Legends

### Figure 1

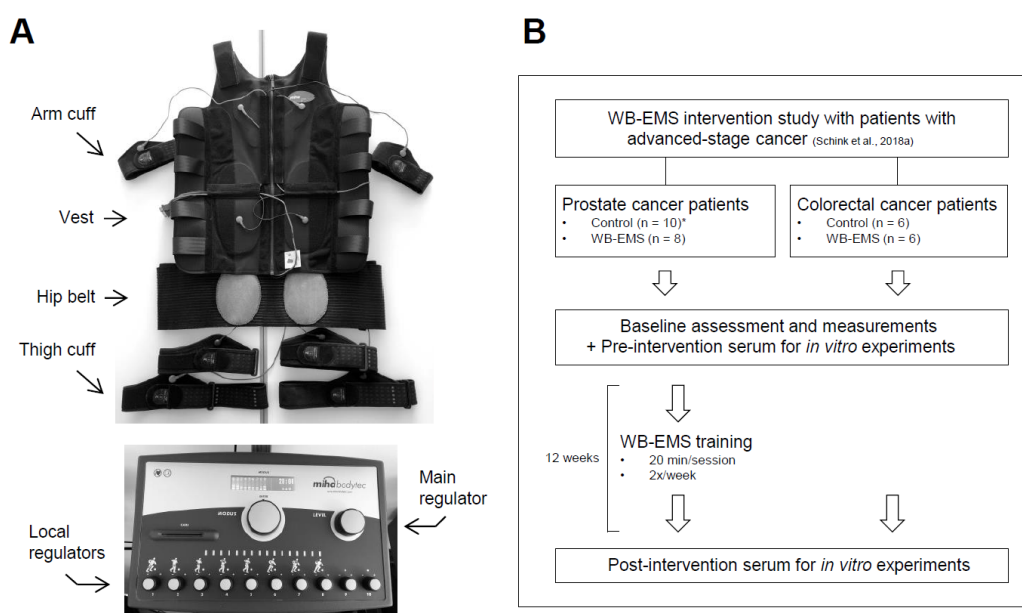


Figure 1: Whole-body electromyostimulation (WB-EMS) equipment and study flowchart. (A) The upper picture displays the vest, hip belt, and upper-arm and -thigh cuffs with integrated electrodes. Electric stimulation was mediated by bipolar impulses (frequency 85 Hz, pulse width 350  $\mu$ sec; 6 sec impulse phase followed by 4 sec resting phase; 20 min per training session). Eight muscle groups are simultaneously addressed by the WB-EMS application (upper arms, chest, upper back, latissimus, abdomen, lower back, buttocks and thighs). The lower picture shows the operating unit of the miha bodytec WB-EMS training device, with the main regulator and the local regulators for each muscle group. (B) Study overview. The patients participated in our previous controlled pilot trial “Effects of whole-bodyelectromyostimulation combined with individualized nutritional support onbody composition in patients with advanced cancer: a controlled pilot trial” (Schink et al., 2018a). \* 6 out of 10 control prostate cancer patients were recruited after the clinical trial from the outpatient uro-oncological unit, Medical Department 5, University Clinic Erlangen.

Figure 2

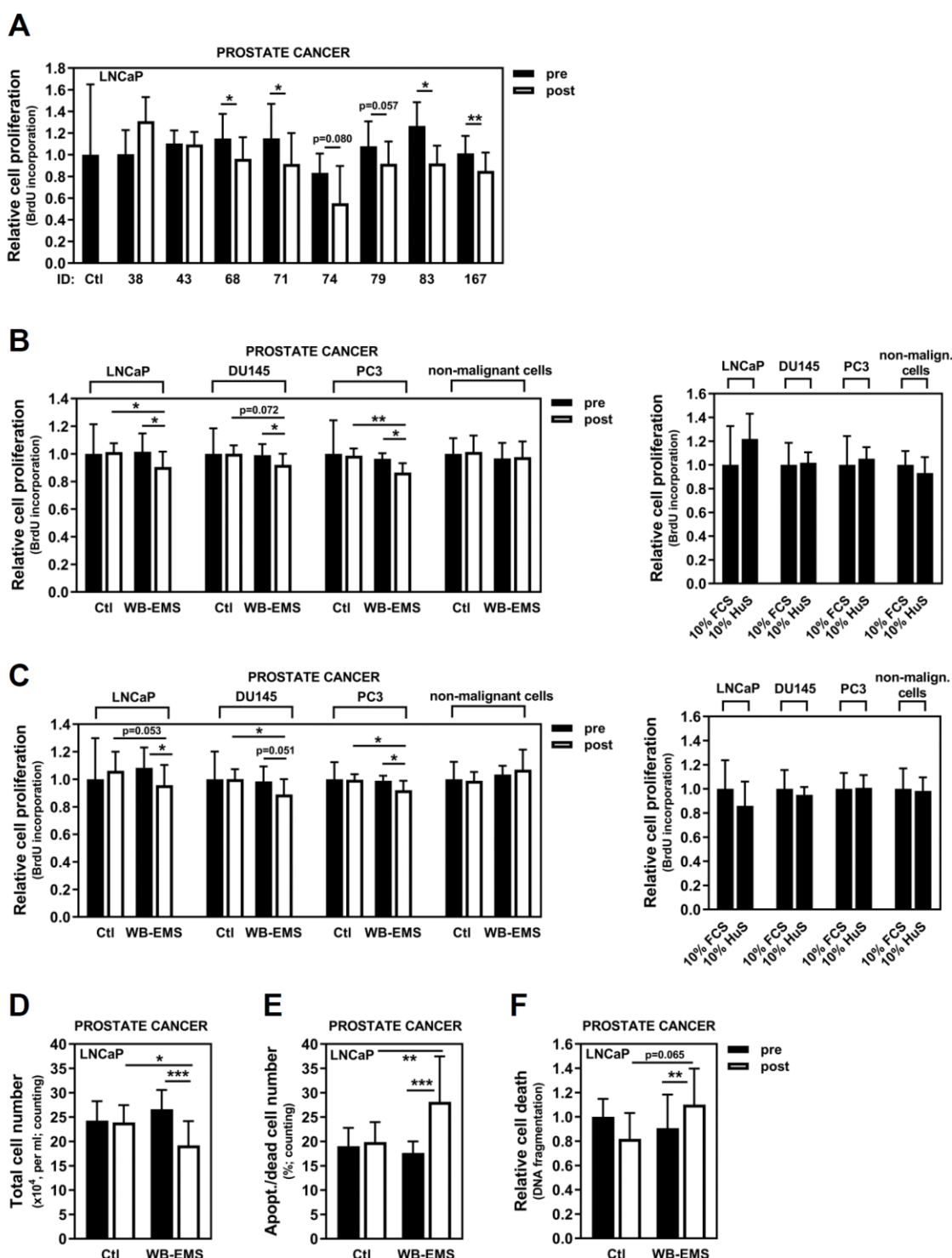


Figure 2: Prostate cancer patient serum after WB-EMS inhibits prostate carcinoma cell growth. (A) The prostate cancer cell line LNCaP was serum-starved in RPMI medium containing 0.1% FCS for 18 - 24 h at 37°C, and stimulated for 96 h with 10% serum from stage III/IV prostate cancer patients taken at study entry (pre) or after a

12 week (post) WB-EMS exercise regimen. Cell proliferation was measured via BrdU incorporation into DNA and is shown relative to the values after stimulation with a pre-intervention serum pool from control patients (n = 10). Mean (SD) of at least 6 independent experiments per patient is shown. (B) Prostate cancer cells (LNCaP, DU145, PC3) and the control non-malignant cells HEK293T were serum-starved (RPMI/0.1% FCS or DMEM/0.1% FCS, respectively) and incubated for 96 h at 37°C with serum pools from WB-EMS (n = 8) or control patients (n = 10) with advanced prostate tumors, and BrdU uptake was measured (left). Statistical analysis on the proliferation data with post-intervention serum between the two patient groups revealed a distinct difference in the growth response upon the post-WB-EMS serum pool compared to the control pool (LNCaP, P = 0.035; DU145, P = 0.073; PC3, P = 0.002). Mean (SD) of at least 7 independent experiments per cell line is shown. The right graph depicts the growth response of the cells upon 10% FCS compared to 10% human serum (pre-intervention control pool from prostate cancer patients; mean (SD), n = 5 - 8). (C) Similar to Fig. 2B, cells were serum-starved but incubated for a shorter period (48 h) with control and WB-EMS serum pools at 37°C, and BrdU incorporation was measured (left). Statistical analysis of the proliferation upon incubation with post-intervention serum between the groups showed also a significant difference (LNCaP P = 0.053; DU145 P = 0.023; PC3 = 0.044). Mean (SD) of at least 5 independent experiments per cell line is shown. The right graph depicts the cell proliferation upon 10% human compared to 10% bovine serum (mean (SD), n = 5 - 7). (D and E) LNCaP were serum-starved and treated with serum pools from WB-EMS or control patients at 37°C. After 96 h, cell viability was examined by determination of total cell numbers (D; numbers per ml x 10<sup>4</sup>) and the number of apoptotic/dead cells via Trypan blue inclusion (E; % of total cell number; statistical analysis between the groups after post-intervention serum incubation, P = 0.003). Mean (SD) of 11 independent experiments. (F) Apoptotic DNA fragmentation was measured after 48 h of WB-EMS serum stimulation (shown relative to pre-intervention control serum values; statistical analysis between the groups after post-intervention serum incubation, P = 0.065). Mean (SD) of 8 independent experiments. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001; paired and unpaired two-tailed Student's t-test in A, B and C; Wilcoxon signed-rank and Mann-Whitney test in D, E and F.

Figure 3

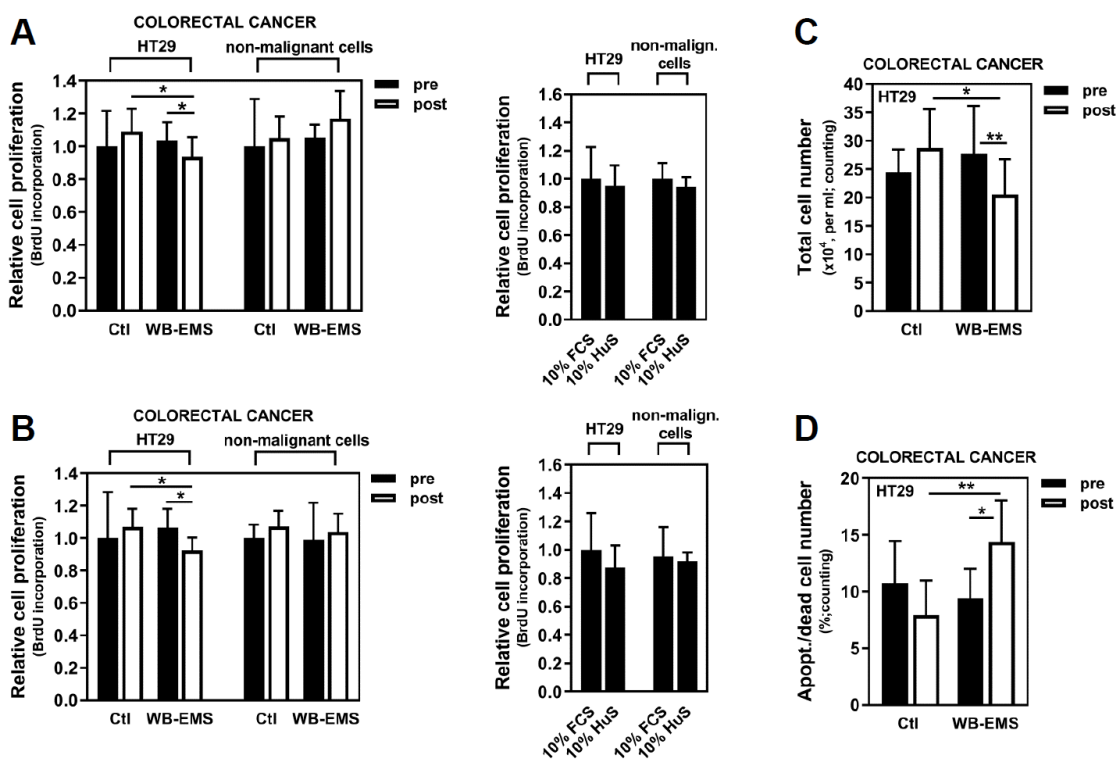


Figure 3: WB-EMS-conditioned serum from colorectal cancer patients attenuates colon carcinoma cell viability. (A) HT29 colon cancer and non-malignant HEK293T cells were serum-starved (DMEM/0.1% FCS) and incubated for 96 h at 37°C with serum pools from WB-EMS (n = 6) or control patients (n = 6) with advanced colorectal tumors. BrdU incorporation into DNA was measured via ELISA. Additional significant difference in the proliferation response was found upon incubation with post-intervention serum pools from both study groups (P = 0.046). Mean (SD) of at least 4 independent experiments is shown. The right graph depicts cell proliferation under incubation with 10% FCS vs. 10% human serum (pre-intervention, colorectal cancer patients; mean (SD), n = 3). (B) Similar to Fig. 3A, cells were serum-starved, but incubated for a shorter period (48 h) at 37°C with control and WB-EMS serum pools, and BrdU uptake was determined. Additional significant difference in the growth response was detected by comparing the data from both study groups after treatment with post-intervention serum pools (P = 0.013). Mean (SD) of at least 3 independent experiments is shown. The right graph depicts BrdU uptake upon incubation with 10% FCS vs. 10% human serum (pre-intervention, colorectal cancer patients; mean (SD), n = 3). (C and D) Serum-depleted HT29 cells were treated with

WB-EMS and control serum pools. 96 h later, cell viability was determined by cell counting (C; numbers per ml,  $\times 10^4$ ; statistical analysis between the groups after post-intervention serum incubation,  $P = 0.039$ ). The number of apoptotic/dead cells was measured via Trypan blue inclusion (D; % of total cell number; statistical analysis between the groups after post-intervention serum incubation,  $P = 0.001$ ). Mean (SD) of at least 7 independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ ; paired and unpaired two-tailed Student's t-test in A and B; Wilcoxon signed-rank and Mann-Whitney test in C and D.

Figure 4

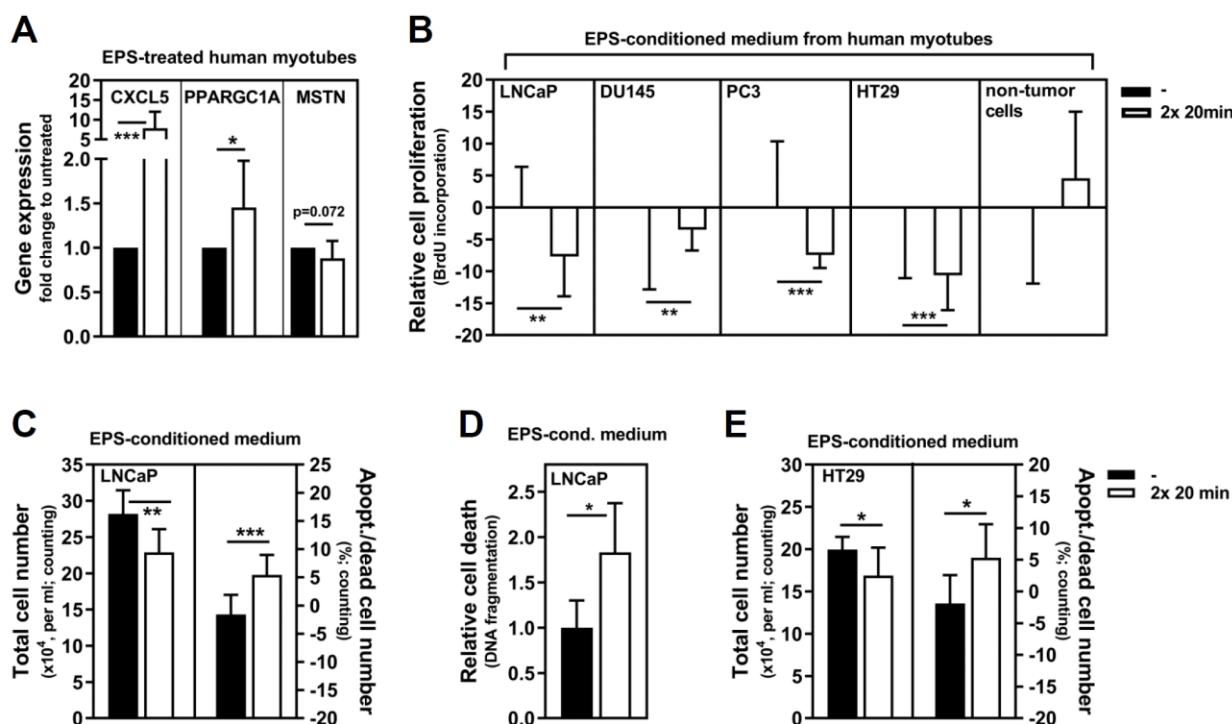


Figure 4: Prostate and colon cancer cell proliferation and apoptosis are affected by EPS-conditioned medium from human myotubes. (A) Human myotubes were treated with EPS (6 sec stimulation followed by a 4 sec pause, 85 Hz, pulse width of 400  $\mu$ s, 15 V) for 20 min on 2 consecutive days. After another 1 h, total RNA was isolated and analyzed for EPS marker expression (CXCL5, PPARGC1A and MSTN). The graph depicts the mean (SD) from at least 8 representative experiments. (B) Prostate cancer (LNCaP, DU145, PC3), colon cancer (HT29) and non-cancer (HEK293T) cells were serum-starved and then treated with EPS-conditioned medium from *in vitro*-differentiated human myotubes for 48 h at 37°C. The conditioned medium contained 2% FCS and 10% human serum from 12 randomly selected healthy individuals, who were not associated with the clinical study. Cell proliferation was measured by BrdU incorporation. The graph shows the relative proliferation of untreated cells as 0%, and the percentage difference in proliferation (after treatment with EPS-conditioned compared to unconditioned medium). Mean (SD) of at least 4 independent experiments. (C and D) The effect of EPS-conditioned myotube medium on total (per ml  $\times 10^4$ ) and apoptotic/dead LNCaP cell numbers was measured by cell counting and Trypan blue inclusion (C) and by DNA fragmentation (D). Mean (SD) of 9 - 10 (C) and 5 (D) independent experiments is shown. (E) Total and apoptotic/dead HT29 cell numbers upon EPS myotube medium were analyzed as in Fig. 4C. Mean (SD) of 9 independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ; unpaired two-tailed Student's t-test in A, B and D; Wilcoxon signed-rank test in C and E.

Figure 5

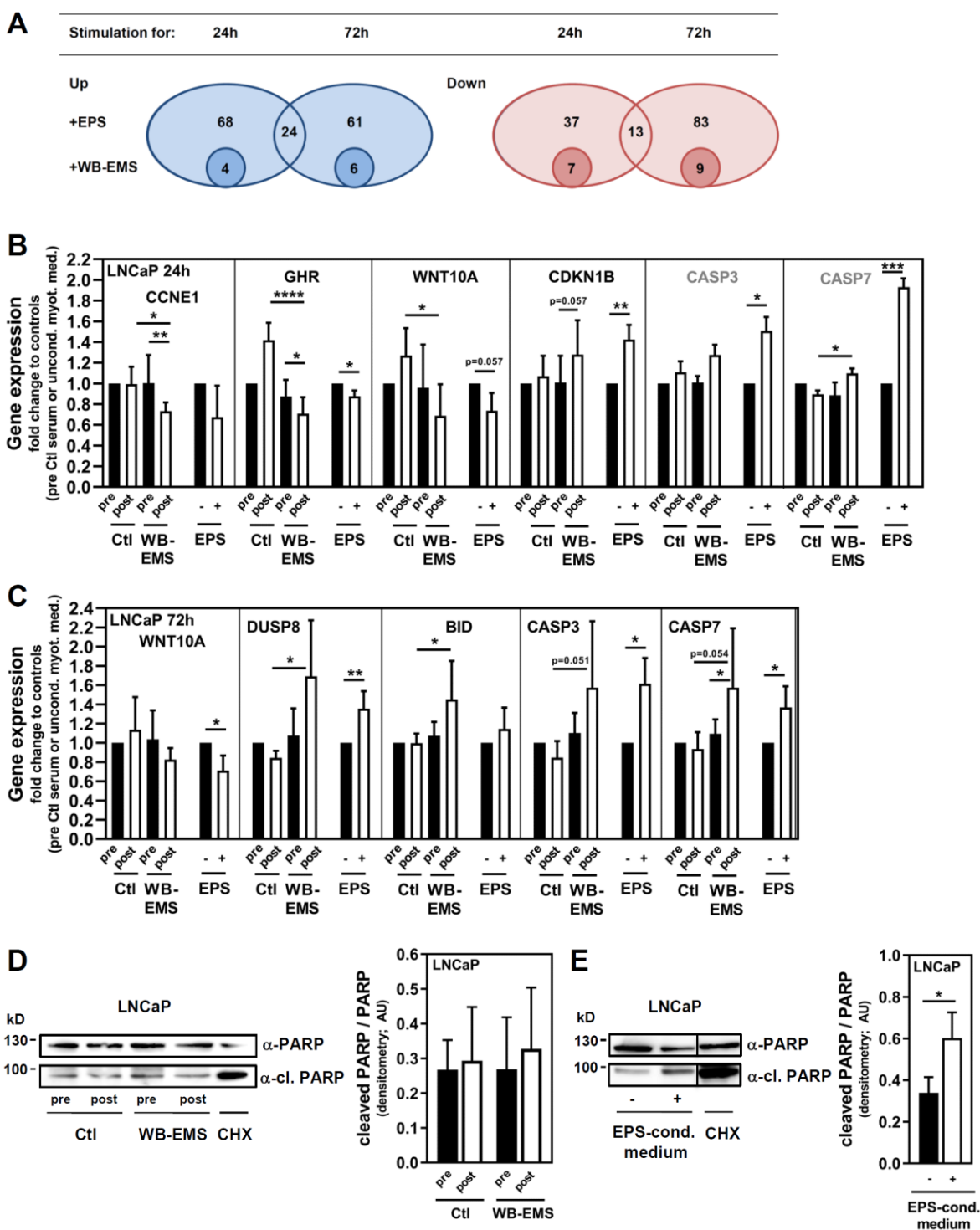


Figure 5: Specific genes associated with prostate cancer cell proliferation and apoptosis are regulated by exercise. (A) The scheme shows the result from a cancer-associated gene expression screen (730 genes; GEO database GSE135541) in “exercise-exposed” LNCaP cells based on NanoString technology (treatment either with post- vs. pre-intervention serum pools from WB-EMS (n = 8) or control prostate cancer patients (n = 10), or with EPS-conditioned human myotube medium

vs. unconditioned medium (medium pool from 3 independent EPS treatments)). Normalized data (WB-EMS group post-intervention / pre-intervention serum and control group post-intervention / pre-intervention serum; EPS-conditioned / unconditioned medium) were compared and potentially regulated genes were selected for further analysis (please refer to Table 4). A difference in gene expression ratio of > 20% between EPS medium-treated and -untreated LNCaP cells, and between post- and pre-WB-EMS serum-treated cancer cells was selected as measure for significance. Additionally, gene expression in LNCaP cells stimulated with a post-intervention serum pool from control prostate cancer patients had to be unaffected or inversely regulated compared to the WB-EMS ratio. After a 24 h treatment with EPS-conditioned medium from human myotubes, 68 genes were up- and 37 genes were downregulated. Within these groups of genes, the expression of 4 genes was also increased by post-WB-EMS patient serum and the expression of 7 genes was decreased by WB-EMS. The stimulation with EPS medium for 72 h yielded 61 upregulated and 83 downregulated genes. Among these, the expression of 6 genes was also higher and the expression of 9 genes was lower after a post-WB-EMS patient serum incubation run in parallel. Genes of these intersections (potentially) controlling cancer cell growth and apoptosis were chosen for further analysis. (B) Expression of genes involved in cancer cell proliferation and cell death was measured in serum-starved LNCaP cells after stimulation for 24 h at 37°C with either WB-EMS exercise or control serum from advanced prostate cancer patients, or EPS-conditioned medium pool from human myotubes. mRNA expression levels were detected using qRT-PCR and are depicted as fold change (relative to the expression in cells treated with post-intervention control serum pool). CASP3 and CASP7 are grayed out since they did not fit the initial selection criteria after the gene expression array, but significant changes in mRNA expression could be detected in the validation analysis using qRT-PCR. (C) As described in Fig. 5C, LNCaP gene expression was quantified after 72 h treatment with post-WB-EMS serum or EPS-conditioned medium. Graphs depict the mean (SD) of 5 - 6 independent experiments for patient serum incubation and 3 independent experiments for EPS medium treatment. (D) Immunoblot analysis of PARP cleavage after WB-EMS serum stimulation. LNCaP cells were serum-deprived for 18 h and treated with control and WB-EMS serum for 96 h. As a control for cell death-associated PARP cleavage, cells were treated with 10 µg/ml cycloheximide (CHX) for 24 h. Cell lysates were analyzed using  $\alpha$ -cleaved (cl.) PARP and PARP immunoblotting. Signal intensities from 4 independent experiments were quantified (including background and protein loading; mean (SD), graph). (E) LNCaP cells were serum-starved, incubated with EPS-conditioned or unconditioned myotube medium for 96 h, and analyzed as described in (D). The graph depicts densitometry results from 4 independent experiments (mean (SD)). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ; paired (for comparison of the serum effect post- vs. pre-WB-EMS (B, C and D)) and unpaired (for comparison of the post-intervention serum effect of WB-EMS vs. control group, as well as for the EPS-conditioned medium effect (B, C and E) two-tailed Student's t-test.

**Table 1**

	Prostate Cancer			Colorectal Cancer		
	Control (n=10)	WB-EMS (n=8)	P	Control (n=6)	WB-EMS (n=6)	P
<b>Sex</b>			-			-
Male	10 (100%)	8 (100%)		2 (33.3%)	3 (50%)	
Female				4 (66.7%)	3 (50%)	
<b>Age [y]</b>	68.2 ± 6.4	69.9 ± 13.3	0.729*	53.8 ± 15.6	60.0 ± 14.6	0.496*
<b>Tumor Stage (UICC)</b>			-			-
III	-	1 (12.5%)		-	1 (16.7%)	
IV	10 (100%)	7 (87.5%)		6 (100%)	5 (83.3%)	
<b>Oncological Therapy</b>			-			-
Chemotherapy (CT)	2 (20%)	-		4 (66.7%)	4 (66.7%)	
Hormone Therapy (HT)	-	2 (25%)		-	-	
CT + HT	8 (80%)	2 (25%)		-	-	
Other Combined Therapies	-	4 (50%)		2 (33.3%)	2 (33.3%)	
<b>Karnofsky Index [%]</b>	80 ± 7.1 (n=4)	76.3 ± 13.0	0.388**	75.0 ± 5.5	73.3 ± 8.2	1.0**
<b>6min-Walking Distance [m]</b>	486.3±64.2 (n=4)	494.4 ± 106.2	0.966**	521.2 ± 112.3	520.8 ± 126.3	1.0**
<b>Body Parameters</b>						
Body Weight [kg]	86.3 ± 9.4	86.9 ± 12.2	0.913*	78.7 ± 15.9	73.6 ± 21.2	0.653*
Weight Loss in the last 3-6 month [%]	4.2 ± 5.6 (n=4)	3.7 ± 5.3	0.909**	8.9 ± 8.6	1.8 ± 2.8	0.058**
Body Mass Index [kg/m <sup>2</sup> ]	28.5 ± 2.1	27.5 ± 3.2	0.442*	28.5 ± 6.2	25.1 ± 5.3	0.334*
Skeletal Muscle Mass [kg]	26.3 ± 4.4 (n=4)	28.4 ± 5.4 (n=7)	0.523*	23.1 ± 5.4	22.3 ± 7.1	0.829*
<b>Blood Parameters</b>						
Albumin [g/l]	38.3 ± 3.8 (n=9)	42.2 ± 2.6	0.034*	40.3 ± 1.6	40.6 ± 4.0	0.867*
C-reactive Protein [mg/l]	20.7±23.6 (n=9)	16.0 ± 35.9	0.863*	17.9 ± 31.0	14.0 ± 28.9	0.826*
Creatinine [mg/dl]	1.0 ± 0.3 (n=9)	0.9 ± 0.2	0.678*	0.74 ± 0.1	0.8 ± 0.2	0.374*
Hematocrit [%]	34.3 ± 4.4	38.2 ± 6.5	0.152*	34.1 ± 3.0	38.1 ± 3.8	0.074*
Hemoglobin [g/dl]	11.4 ± 1.5	12.7 ± 2.1	0.133*	11.7 ± 1.2	12.7 ± 1.4	0.218*
Leucocytes [x10 <sup>3</sup> /μl]	7.6 ± 2.9	4.6 ± 2.1	0.034**	5.9 ± 1.1	5.5 ± 0.8	0.853**
Erythrocytes [x10 <sup>6</sup> /μl]	3.7 ± 0.4	4.2 ± 0.7	0.034**	4.4 ± 0.3	4.2 ± 0.4	0.818**
Thrombocytes [x10 <sup>3</sup> /μl]	248 ± 84.9	193.3 ± 56.2	0.274**	276.2 ± 106.5	242.3 ± 53.3	0.513**

Table 1: Pre-intervention characteristics of the prostate and colorectal cancer patient cohorts. Values are given as mean (SD). Where appropriate, statistical analysis was done using independent samples t-test\* or Mann-Whitney test\*\*.

Table 2: Serum testosterone measurements of the prostate cancer cohort. Values are given as mean from duplicate measurements. Pre- and post-intervention serum from 6 patients per group could be examined. No significant difference in testosterone levels (ng/ml) between pre- and post-intervention serum within each group was determined (mean (SD); two-tailed paired Student's t-test).

**Table 2**

Serum Testosterone [ng/ml]					
Control (n = 6)			WB-EMS (n = 6)		
ID	pre	post	ID	pre	post
K01	0.17	0.12	68	0.50	0.47
K02	0.12	0.33	71	0.38	0.12
K04	0.08	0.06	74	3.17	0.24
K05	0.12	0.03	79	0.08	0.08
K07	0.24	0.27	83	0.23	0.20
K08	0.52	0.32	167	0.15	0.32
Mean ± SD	0.21 ± 0.22	0.14 ± 0.13	Mean ± SD	0.75 ± 1.20	0.24 ± 0.14
P	0.080		P	0.339	

Table 3: Established myokines are regulated by electric pulse stimulation based on WB-EMS training parameters. Primary human myotubes were treated with EPS (85 Hz, 400  $\mu$ sec pulse width, 6 sec stimulation followed by 4 sec rest, 15 Volt, 20 min) on two consecutive days. A serum pool from 3 independent EPS treatments was used for a cytokine array in order to identify EPS-regulated protein factors. The fold change in myokine protein expression in myotube EPS-conditioned vs. unconditioned medium is shown. The same set of myokines was analyzed regarding their mRNA expression in EPS-treated human myotubes. \*VEGF protein was not specified on the Cytokine Array, but VEGFA-specific primers were used for gene expression analysis (fold change to untreated; mean (SD) from 7 - 9 independent experiments; two-tailed unpaired Student's t-test).

**Table 3**

	Cytokine Array (Medium)	Gene Expression (Myotubes)	
	Fold change EPS-cond. / uncond.	Fold change EPS-treated / untreated Mean $\pm$ SD	P P
<b>BDNF</b>	2.58	1.41 $\pm$ 0.49	0.033
<b>CCL2</b>	1.12	1.35 $\pm$ 0.51	0.057
<b>IGFBP3</b>	1.13	2.31 $\pm$ 1.01	0.001
<b>IL5</b>	6.40	3.18 $\pm$ 3.58	0.107
<b>IL6</b>	1.49	3.91 $\pm$ 1.96	0.0002
<b>IL7</b>	not detectable	3.34 $\pm$ 1.07	0.0001
<b>IL8</b>	1.42	1.73 $\pm$ 0.82	0.019
<b>IL15</b>	0.78	2.06 $\pm$ 0.50	0.0001
<b>VEGFA*</b>	1.63	2.12 $\pm$ 0.76	0.0004

Table 4: Potential exercise-sensitive genes regulating prostate cancer cell proliferation and apoptosis after multiplex analysis. Fold change in gene expression after incubation with pre- and post-intervention patient serum or EPS-conditioned medium for 24 h or 72 h are listed. CASP3 is grayed out since it did not fit our initial selection criteria (i.e. the ratio of EPS vs. untreated ratio > 20%), but could potentially be important.

	Gene name	Fold change WB-EMS serum post / pre	Fold change Control serum post / pre	Fold change Myotube medium EPS-cond. / uncond.
24 h	CCNE1	-1.28	1.10	-1.23
	CDKN1B	1.49	-1.28	1.27
	E2F1	-1.31	1.02	-1.33
	GHR	-1.23	1.26	-1.20
	POLE2	-1.31	1.11	-1.42
	WNT10A	-1.26	1.00	-1.26
72 h	BID	1.39	-1.14	1.27
	CASP3	1.24	-1.01	1.10
	CASP7	1.33	-1.24	1.50
	DUSP8	1.43	1.02	1.87
	WNT10A	-1.43	1.00	-1.22