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Assessing the Exercise-Related Kinetics of cfDNA, ctDNA, DNase I Activity and Cytokines in Patients with Solid Tumors: A Pilot Study

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Abstract

Background: Circulating cell-free DNA (cfDNA), circulating tumor DNA (ctDNA), and inflammatory cytokines have prognostic and predictive value in oncology. Here, we aim to explore the kinetics of cfDNA, ctDNA, and cytokines upon physical exercise in a pilot cohort of cancer patients compared to healthy controls.

Methods: Patients with solid tumors (n=12) and age-matched controls (n=6) were recruited to perform an all-out cardiopulmonary bicycle test. Blood samples were collected before (Pre), directly after (Post), and 90 min after the test (+90), and cfDNA, ctDNA (KRAS mutation), DNase I activity, and cytokine levels were measured.

Results: Cardiopulmonary exercise testing was well feasible in cancer patients, and data from eight patients and five controls were included for explorative statistical evaluation. cfDNA levels increased from Pre to Post and decreased to baseline +90 min in all subjects. cfDNA concentrations and DNase I activity clearly correlated in the control but not in the cancer group. Neutrophil-associated MPO, MRP8/14, and NGAL showed strong responses to exercise. The percentage of ctDNA, detected only in one cancer patient, decreased after acute exercise.

Conclusion: In our study we could safely perform cardiopulmonary exercise testing with patients with different cancer entities and subsequently run biomarker analysis. Our preliminary results hint on an exercise-triggered release of cfDNA and neutrophil-derived cytokines in cancer patients.

Keywords: cfDNA, Cytokines, Cancer, Exercise, ctDNA, Solid Tumor

Introduction

Circulating cell-free DNA (cfDNA), including the tumor-derived DNA fraction (circulating tumor DNA = ctDNA), has become an important marker implemented in the field of liquid biopsy for cancer management [1]. During the last three decades, numerous studies have indicated that cancer patients show higher levels of cfDNA than the healthy population [2]. As reviewed by Bronkhorst *et al.*, cfDNA concentrations correlate with tumor size, disease stage, and metastatic burden [3]. The clearance mechanisms are not clarified in detail; however, at least three mechanisms are described, including direct degradation by nucleases, including DNase I and DNase1L3, active uptake by the reticuloendothelial system in the liver and spleen, and passive filtration by the renal system (reviewed in [4]).

Multiple screening approaches have been developed to detect tumor-specific genomic and epigenetic alterations. In addition to PCR, next-generation sequencing (including whole-exome or whole-genome sequencing), cancer personalized profiling by deep sequencing (CAPP-Seq [5]), tagged-amplicon deep sequencing (TAm-Seq [6]), and targeted or whole-genome methylation sequencing[7] were developed to detect mutations, copy number aberrations, cfDNA fragmentation profiles [8], or cancer-specific epigenetic signatures [7]. Detection of *KRAS* point mutations is specifically important since anti-EGFR therapy is ineffective in the presence of *KRAS* mutations [9]. We developed a highly specific nested qPCR targeting *KRAS* point mutations to detect and quantify minute amounts of circulating ctDNA, which was utilized to monitor the release of ctDNA during surgery [10].

While the applications of cfDNA in cancer liquid biopsy are increasing, the significance of preanalytics and standardization has become increasingly visible. ctDNA is very low concentrated in relation to background cfDNA, making it hard to detect. The choice of anticoagulant, blood processing, and cfDNA isolation method strongly determine the composition of cfDNA populations in a sample [11]. Especially for, but not limited to, the early-stage diagnosis of cancers, where ctDNA concentrations are very low, it is mandatory not to dilute ctDNA by increasing background cfDNA [12]. In this context, it is important to understand to what extent environmental factors, such as physical exercise, circadian rhythms, or nutrition, may influence the outcome of cancer liquid biopsies and therefore should be considered for the standardization of sampling for liquid biopsies [11-13].

Physical exercise has shifted from a perceived threat to patients to a widely accepted supportive treatment in cancer therapy and rehabilitation in only a few decades. Physical exercise not only reduces the cancer incidence but also reduces cancer recurrences for some types of cancer and inhibits tumor growth [14]. It is assumed that the positive effects of exercise are partly related to the dynamic interaction between the tumor microenvironment and host immune reactivity, mediated by cytokines and other signaling molecules [15]. The feasibility and safety of exercise interventions in cancer patient cohorts of nearly all exercise intensities have been demonstrated in various patient studies (e.g., [15-17]). Even "exotic" and very strenuous disciplines such as high-intensity interval training [18] or high-intensity strength training [19] find use. Intriguingly, acute exercise bouts lead to an immediate rise in cfDNA levels under healthy conditions and in several pathologies [20-22]. The kinetics of cfDNA in response to incremental exercise test in patients with solid tumor is unknown.

Here, we elucidate the feasibility of assessing the impact of cardiopulmonary exercise testing on circulating DNA species in a small cohort of patients with heterogenous cancer entities. Via minimally invasive direct measurement of cfDNA we examined the effect of an acute physical

exercise bout on cfDNA levels in cancer patients. Moreover, using highly specific nested qPCR targeting *KRAS* point mutations (ctDNA) we detected one case of *KRAS* point mutation (ctDNA) in this exercise setting. Additionally, we analyzed the kinetics of inflammatory cytokines in a subset of randomly selected samples.

Materials & Methods

Ethical Approval and Consent to Participate

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Commission of the state of Rhineland-Palatinate, Mainz, Germany (837.033.11 (7575)). Written informed consent was obtained from all study participants.

Participants and Sample Collection

A total of 12 tumor patients and 6 age matched healthy subjects were recruited at the University Medical Center Mainz, Mainz, Germany, and the St. Hildegardis Hospital Mainz, Mainz, Germany, and the department of Sports Medicine of the Johannes Gutenberg-University of Mainz, Mainz, Germany. Inclusion criteria for the tumor group were a located solid tumor, in the age between 40-70 years. Exclusion criteria were chemotherapy within the last 48 h before the examination, radiation within the last 7 days before the examination, enterostomy, and untreated anemia. Indications of diseases of the lungs, cardiovascular system, liver, kidney, or seizure disorders that rule out acute exercise. Furthermore, known addictions (alcohol, drugs, tablets) and the use of anticoagulant medication impeded inclusion in the study. All procedures were carried out in accordance with the Declaration of Helsinki. Before the exercise test, the sports capability of all the participants was confirmed by a physician at the Department of Sports Medicine.

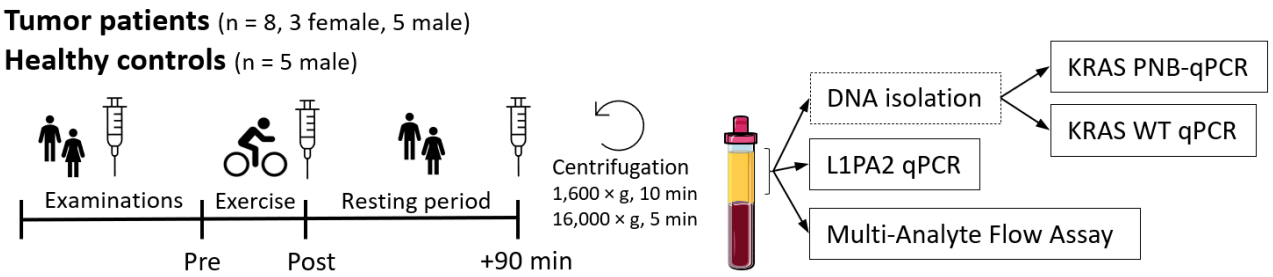

Figure 1: Study design. K3-EDTA plasma was collected before, directly after, and 90 min after all-out incremental cycling exercise and centrifuged two-times before cell-free DNA (cfDNA), KRAS circulating tumor DNA (ctDNA) and circulatory cytokine analysis

Cardiopulmonary Exercise Test

To determine the VO_{2peak}, a supervised cardiopulmonary exercise test was performed by an experienced exercise physiologist. The stepwise incremental cycling exercise test until exhaustion was performed on an ergoselect 200 Cycling ergometer (ergoline GmbH, Bitz, Germany). Respiratory gas exchange data were recorded breath by breath by a metabolic unit (Ergostik, Geratherm Respiratory GmbH, Bad Kissingen, Germany), and electrocardiogram was recorded continuously using Cardiopart 12 Blue ECG Pro Amedtec (AMEDTEC Medizintechnik Aue GmbH, Aue, Germany). The individualized workload protocol contained steps of 3 min duration with different initial loads and increasing resistance. An experienced physician individualized the protocol based on the treatment history, comorbidities, and physical condition. Blood pressure was monitored throughout rest and exercise as recommended [23]. At the end of each workload, the subjects were asked for their rating of perceived exhaustion (RPE) [24]. Lactate concentrations were determined in capillary blood taken from the earlobe after each step of the incremental test with an automatic lactate analyzer Biosen 5130 (EKF Diagnostics, Magdeburg, Germany). The exercise test was terminated when subjective exhaustion was reached or if any of the general indications for exercise termination were observed[23]. Notably, the cardiopulmonary capacity was determined at peak exercise (VO_{2peak}), as recommended for clinical populations, and not at maximum VO_{2max}[23].

Blood Handling, Processing, and DNA Isolation

Venous blood samples were taken before the test, directly after and +90 min after the end of the test using K3-EDTA Monovettes (Sarstedt, Nümbrecht, Germany). All blood samples were centrifuged for 10 minutes at 1,600 × g within 10 minutes after withdrawal, and the resulting plasma was centrifuged again in a new tube for another five minutes at 16,000 × g, both at 4 °C. Plasma samples were stored at -20 °C. cfDNA was isolated from fractions of the plasma samples using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions with two changes. First, no carrier RNA was used in the protocol, and second, the DNA was eluted in water instead of the included buffer.

Quantification of cfDNA and ctDNA

The concentrations of cfDNA and ctDNA were measured using three different qPCR approaches. First, total cfDNA concentrations were measured using an ultrasensitive qPCR approach amplifying DNA in diluted plasma, without the need for DNA isolation. Second, isolated DNA was used to quantify the seven most common KRAS-specific mutations, and third, mutation-independent KRAS qPCR was used to determine the percentage of mutated fragments.

Total cfDNA was quantified from diluted plasma amplifying an L1PA2 retrotransposable element with approximately 3340 hits in the human genome, as described previously [25]. In short, the plasma was diluted 1:40 with PCR grade water and then mixed with Tego Buffer, Velocity Polymerase, $MgCl_2$ (all Bioline, Luckenwalde, Germany), SYBR Green, FITC (both Sigma-Aldrich Co., Taufkirchen, Germany), and 0.34 mM of a primer pair specific for a sequence of 90 bp in the aforementioned LINE element (5'-TGCCGCAATAAACATACGTG-3' and 5'-GACCCAGCCATCCCATTAC-3'). A CFX384 Bio-Rad (Bio-Rad, Munich, Germany) cycler was used with the following protocol: 35 cycles of 94 °C for 10 s, 64 °C for 40 s and 75 °C for 10 s, followed by a melting curve. All measurements were run in triplicate.

Cell-free DNA bearing cancer-specific *KRAS* mutations (ctDNA) was amplified with a highly sensitive nested qPCR approach called PNB-qPCR (Pooled, Nested WT-Blocking qPCR) [10]. With this technique, we were able to quantify down to 0.01% and to detect down to 0.003% of mutant DNA of the seven most common *KRAS* mutations, including G12D, G12V, G12A, G12S, G12C, G12R, and G13D [10]. The nested qPCR protocol comprised a first- and a second-round PCR. In the first round of PCR, a 110 bp PCR product was amplified, including a WT-specific 3'-C6 amine blocking primer for mutation enrichment (for primer sequences, see supplementary material in [10]). The PCR protocol consisted of 20 cycles of 10 s at 98 °C and 30 s at 69 °C, followed by 5 min of final elongation at 72 °C, including 0.002 U/µl Phusion® Hot Start Flex DNA Polymerase with the accompanying 1× HF buffer, 0.5 mM $MgCl_2$ (NEB, Ipswich, MA), 200 µM dNTPs, 400 µM outer primer mix, 1 µM blocking primer, and 14 µl of template and H_2O in a final volume of 50 µl. In the second round of PCR, the preamplified sample (1:50 dilution) was amplified with seven mutation-specific ARMS primers, including an LNA probe for maximum specificity. The reaction mix included 1× SsoAdvanced™ Universal Probes Supermix (Bio-Rad, Munich, Germany), 400 nM forward ARMS and reverse primer, 200 nM LNA probe, and 3.2 µl of template for a total of 8 µl per well. There were three different qPCR protocols, depending on the primer pair used.

For the mutation-independent quantification of *KRAS,* exon 2 was targeted outside of the mutation hotspots (5′-GAATATAAACTTGTGGTAGTTGGAGC-3′ and 5′-CTGAATTAGCTGTATCGTCAAGG-3′) using the *KRAS* WT LNA probe ([6FAM]CTC[+T][+T]GC[+C][+T]ACGC[+C]A[BHQ1]). The reaction mix of this qPCR was identical to the second round of the PNB-qPCR. The qPCR conditions were 2 min at 95 °C followed by 50 cycles of 5 s at 95 °C and 30 s at 67 °C and a final elongation step of 5 min at 72 °C.

DNase Activity ELISA

An ELISA to measure DNase I activity was performed with 9 µl of plasma for all plasma samples according to the manufacturer's instructions (ORGENTEC, Mainz, Germany).

Multi-Analyte Flow Assay

Cytokines were quantified using a human inflammation panel (13-plex) and a human vascular inflammation panel (12-plex) (LEGENDplex™, Biolegend) according to the manufacturer's instructions. Diluted plasma samples were mixed with bead-coated capture antibodies specific for IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, IL-33 (inflammation panel) or Myoglobin, Calprotectin (MRP8/14), Lipocalin A (N-GAL), MMP-2, Osteopontin (OPN), Myeloperoxidase (M-PO), Serum Amyloid A (SAA), IGFBP-4, ICAM-1 (CD54), VCAM-1 (CD106), MMP-9, Cystatin C (vascular inflammation). After an incubation time of 1 h, the beads were washed twice before adding the biotin-labeled detection antibodies for 1 h. Following a final incubation with streptavidin-PE, the beads were analyzed using a FACSCanto II cytometer (BD). Analysis was performed using the LEGENDplex™ analysis software v8.0. The software distinguishes between 12 or 13 different analytes on the basis of bead size and internal dye.

Statistical Analysis

All statistical tests were performed using R (version 4.1.0) and rstatix package (version 0.7.2). The figures were created using ggplot2 (version 3.1.4). Normal distribution of the data was tested with the Shapiro–Wilk test. The homogeneity of variances was assessed with Levene's test. The cfDNA and cytokine data were log normalized to achieve a normal distribution. For the graphical presentation of the data, the back-transformed mean of the log values is displayed. In the text, the mean and standard deviation (SD) of the untransformed data are presented. If not otherwise stated, parametric mixed ANOVA tests with Bonferroni-Holm adjusted post hoc tests were computed to identify statistically significant differences. T tests or Wilcoxon tests were used for normal or nonnormal distributed data, respectively. Correlations were computed using Pearson correlation coefficient. A P value ≤.05 was considered significant.

Results

Study Cohort

Twelve tumor patients and six healthy control subjects were recruited for inclusion in the study. One patient was excluded prior to the exercise test because of high blood pressure on two separate occasions. One test could not be performed due to technical difficulties, and the patient abandoned the study afterwards. Two other patients completed the test, but no blood could be taken directly after the exercise test; therefore, no data were available for analysis. Additionally, the 90-minute resting sample could not be taken from one patient because of insufficient blood flow. This patient was not excluded from the study. For one control patient, VO_{2peak} and RER could not be determined due to unreliable data. Therefore, eight tumor patients (five male, three female) and five age-matched healthy male control subjects were included for explorative statistical evaluation. In the patient cohort, four subjects had colon cancer, two of whom had metastases. Two patients had esophageal carcinoma, and one patient had gastric cancer. One had mammary and thyroid carcinoma with distant metastases.

Bicycle Ergometry and Physiological Parameters

The starting resistance for the stepwise bicycle ergometry test ranged from 20 to 75 watts with a mean of 26.3 ± 5.8 for the tumor group and 58 ± 16.0 for the control group. The starting watts were chosen with respect to the estimated fitness of the subjects. The number of completed steps was not significantly different between the tumor group (4.2 ± 1.5) and the control group (6.3 ± 2.1), P = 0.107 (Table 1). However, the control group reached significantly higher maximal watts (P = 0.007). Thus, the control group performed higher loads for a longer time. In line with this, the control subjects showed a significantly higher lactate increase at exhaustion with a mean of 9.9 ± 3.4 mmol/l compared to the control subjects (5.7 ± 1.8) and a significantly higher mean VO_{2peak} (35.3 ± 6.3 ml/min/kg) compared to the tumor group (22.5 ± 9.3 ml/min/kg). However, the RPE and RER were similar between the groups (Table 1), indicating that all subjects reached the level of maximum exhaustion (P> 0.05).

Table 1: Baseline and exercise performance characteristics of study participants allocated by group (Tumor = T, Control = C), and time point (Pre, Post exercise)

	Timepoint Pre	***P* between condition at timepoint Pre**	**Timepoint Post**	***P* within condition (Pre to Post)**	***P* between condition at timepoint Post**
N (male/female)	T: 8 (5/3) C: 5 (5/0)				
Age	T: 53.5 ± 3.2 C: 54.8 ± 6.6	P_T = 0.864			
BMI (kg/m$^{2)}$	T: 25.6 ± 3.2 C: 24.6 ± 3.4	P_T = 0.607			
Heart rate (1/min)	T: 92.1 ± 12.2 C: 72.6 ± 9.1	**P_T = 0.011**	T: 151.7 ± 24.6 C: 177 ± 15.6	**T: P_T< 0.001 C: P_T< 0.001**	$P_{T=}$ 0.067
Systolic BP	T: 115.3 ± 14.0 C: 129.5±14.8	P_T = 0.147	T: 180.4 ± 50.2 † C: 222.4 ± 29.7	**T: P_T= 0.006 C: P_T< 0.001**	P_T = 0.128
Diastolic BP	T: 77.3 ± 9.2 C: 83.0 ± 15.4	P_W = 0.152	T: 86.8 ± 20.4 C: 93.6 ± 10.3	T: P_W = 0.280 C: P_T≤ 0.537	P_T = 0.516
Lactate (mmol/L)	T: 1.1 ± 0.28 C: 0.9 ± 0.15	P_T = 0.089	T: 5.7 ± 1.8 C: 9.9 ± 3.4	**T: P_T< 0.001 C: P_T< 0.001**	**P_T = 0.017**
VO_2 (ml/min/kg)	T: 5.0 ± 1.1 C: 4.5 ± 0.2	P_T = 0.301	T: 22.5 ± 9.3 C: 35,3 ± 6.4	**T: P_W< 0.001 C: P_T< 0.001**	**P_W = 0.030**
RER			T: 1.13 ± 0.09 C: 1.1 ± 0.06		P_T = 0.52
RPE			T: 18.4 ± 1.5 C: 18.6 ± 1.1		P_T = 0.781
Steps until exhaustion			T: 4.2 ± 1.5 C: 6.3 ± 2.1		P_W = 0.107
Max. watt			T: 122.5 ± 54.4 C: 227.0 ± 57.2		**P_T = 0.007**

Data are presented as mean ± standard deviation (SD). The *P* values for between-group differences were calculated using unpaired t tests (P_T) or Wilcoxon tests (P_W). The Pre to Post exercise within-group differences were calculated using paired t tests (P_T) or Wilcoxon tests (P_W), depending on normal distribution. The tumor group included patients with colon cancer (n = 2), metastatic colon cancer (n = 2), esophageal cancer (n=2), gastric cancer (n = 1) and mammary and thyroid carcinoma with distant metastases (n = 1). BMI: body mass index, BP: blood pressure, RER: respiratory exchange ratio, RPE: rating of perceived exertion.

cfDNA and DNase I Activity Analysis

First, we wanted to examine the release and clearance kinetics of total cfDNA in response to an all-out physical exercise test in the tumor group and the control group. A mixed ANOVA on the cfDNA concentrations revealed a statistically significant interaction between the time points Pre, Post, +90 min and the condition cancer vs. healthy ($F(2, 20) = 15.348$, $P< 0.001$, partial $\eta2 = 0.605$), indicating

that the cfDNA concentrations of the two groups differed over the time course of the bicycle test. As shown in Figure 2A, cfDNA increased significantly 1.75-fold in the cancer group from 42.0 ng/ml to 73.6 ng/ml and decreased to 44.9 ng/ml +90 min. In the control subjects, cfDNA concentration increased 3.90-fold from 18.5 ng/ml to 72.3 ng/ml and decreased to 12.6 ng/ml. The fold-change increases (Post/Pre) and decreases (Post/+90 min) were significantly higher in the control group than in the cancer group (increase 3.90 vs. 1.75, P = 0.008 and decrease 6.0 vs. 1.89, P = 0.002, respectively). As shown in Figure 2B, the groups did not differ at the time points Pre 41.99 ± 35.97 ng/ml vs. 18.5 ± 3.59 ng/ml (P = 0. 127) and Post 73.62 ± 77.71 vs. 72.32 ± 27.61 ng/ml (P = 0.492) but differed significantly at the timepoint +90 min (P = 0.006) with 44.90 ± 38.19 ng/ml in the cancer group, compared to 12.63 ± 3.97 ng/ml in the control group.

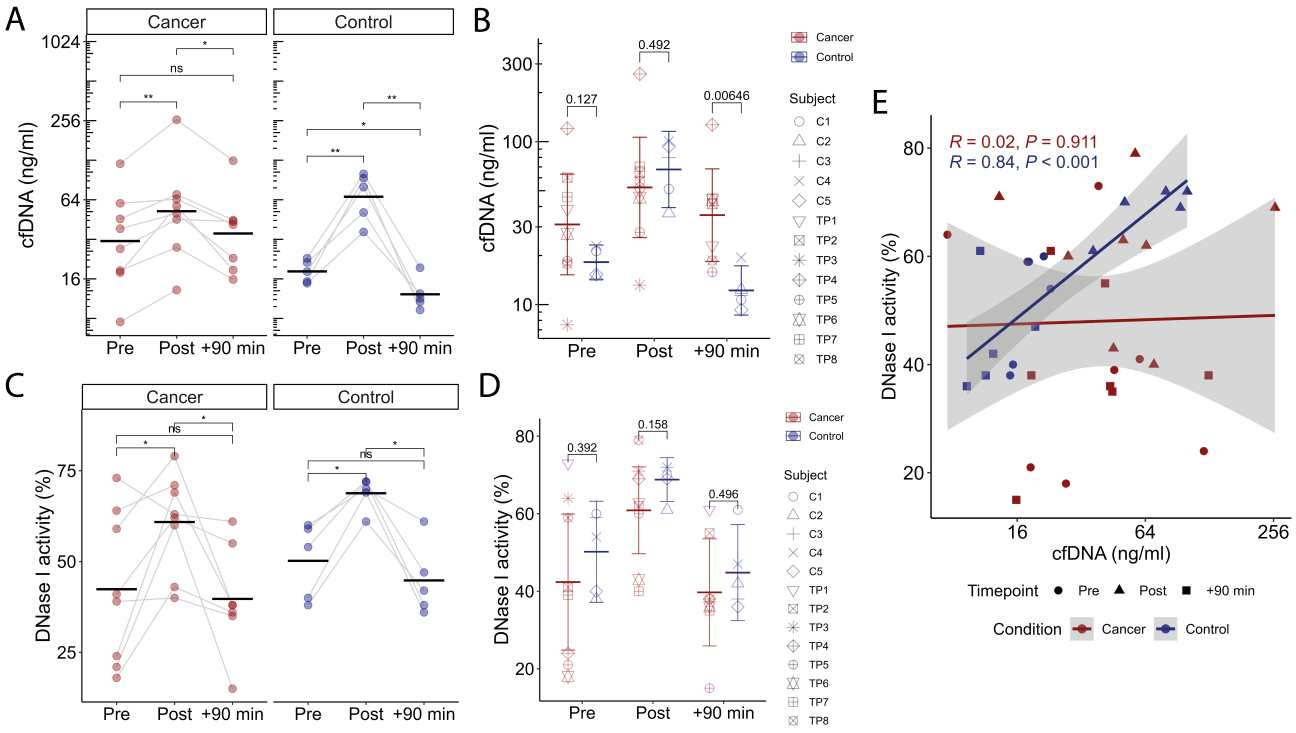

Figure 2: A: Individual development of cfDNA concentrations in cancer and control group over test time. B: Comparison of cfDNA concentrations in tumor and control group at different time points. Mean and 95% CI values are shown. c: DNase I activity (%) in tumor and control group over test time, and D: between groups. E: Correlation between cfDNA concentration and DNase I activity (%). * = $P < 0.05$, ** = $P < 0.01$, ns: not significant

The kinetics of DNase I activity were more consistent in the control group than in the tumor group, and all subjects showed increases in response to acute exercise. DNase I activity (%) increased significantly from Pre 50.2 ± 10.5 to Post 68.8 ± 4.5, $P = 0.01$, and decreased to 44.8 ± 9.9 + 90 min (Figure 2D). In the cancer group, DNase I activity increased from 42.4 ± 21.0 to 60.9 ± 13.4 ($P = 0.046$) and decreased to 39.7 ± 14.9. In both groups, no difference was detected from Pre to +90 min (Figure 2C). Additionally, no significant differences were found between the groups at the different time points (Figure 2D).

Importantly, there was a clear correlation between cfDNA concentration and DNase I activity in the control group (Figure 2E), with a Pearson correlation coefficient of R=0.84, P < 0.01, whereas the parameters did not correlate in the cancer group.

Circulating Tumor DNA (ctDNA)

Next, we screened all cancer patients' plasma samples taken during the physical exercise test for circulating DNA containing KRAS mutations (circulating tumor DNA, ctDNA) by PNB-qPCR. One patient was KRAS mutation positive, and mutated fragments were quantified in all three

samples taken. ctDNA concentrations were 0.106 ng/ml before the test (Pre), 0.14 ng/ml directly after the test (Post) and 0.097 ng/ml +90 min. In percentage of total cfDNA, this equals 2.16, 0.396, and 1.51% of total cfDNA, respectively. The ctDNA percentages were calculated as a quotient of the concentration of the mutated KRAS sequence and total KRAS DNA concentration in the isolated cfDNA quantified by qPCR (Figure 3).

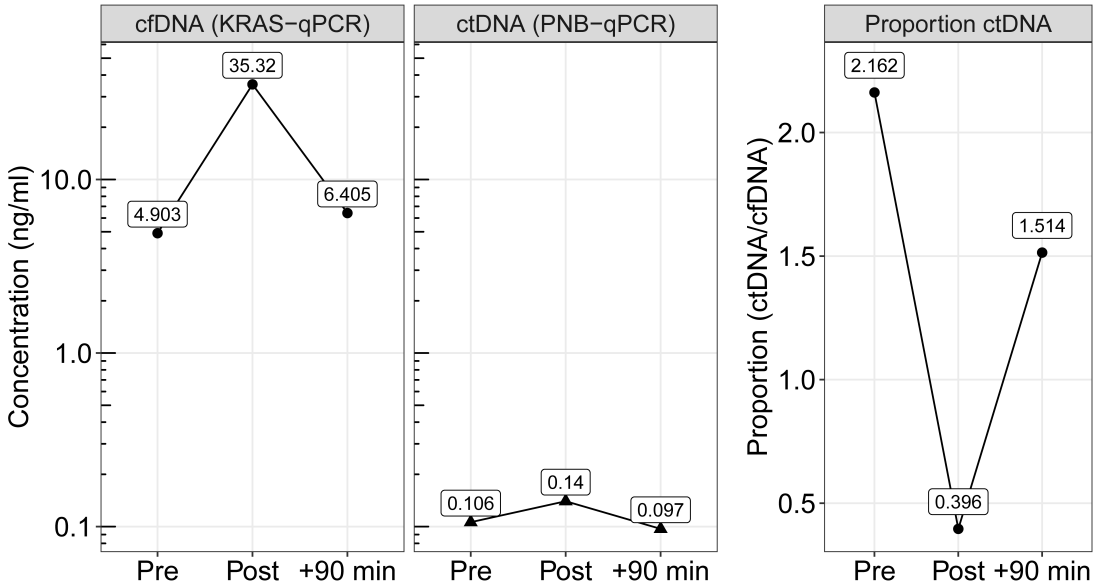

Figure 3: Kinetics of cfDNA, ctDNA and the proportion of ctDNA (cfDNA/cfDNA) in the single subject who was tested positive for circulating *KRAS* mutation.

Multi-Analyte Flow Assay

In a subset of randomly selected samples including six cancer patients and three healthy controls, we studied the kinetics of 25 inflammatory proteins included in the LEGENDplex™ inflammation panel (13-plex) and a human vascular inflammation panel (12-plex). We could show that meaningful measurements are possible for cancer patients in the selected exercise setting: As shown in Figure 4A, the concentration of circulatory proteins increased or decreased for a number of targets in response to acute exercise in the control group as well as in cancer patients. MPO, MRP8/14, NGAL, and SSA showed the strongest log2 mean differences (Post – Pre exercise). IGFBP-4, OPN, and VCAM-1 were the strongest downregulated proteins. The picture was similar in the cancer patients and control subjects. A combination of the data (patients and controls) showed that MRP8/14, MPO, NGAL and SAA were significantly upregulated ($P < 0.05$) (Figure 4A). None of the cytokines were significantly regulated from Pre exercise to +90 min. Figure 4B illustrates the kinetics of the four significantly upregulated cytokines.

A cluster analysis for the z transformed protein concentration values shows a differentiation into the control and cancer groups (Figure 4C). Unpaired *t* test comparisons of the normally distributed data with homogenous variances indicated that IL-10 ($P = 0.001$), IL-6 ($P = 0.011$), IL-33 ($P = 0.018$) and IL-23 ($P = 0.048$) differed significantly at the time point Pre between the cancer and control groups (Figure 4D). However, due to the small sample size, the accuracy of the statistical tests is limited.

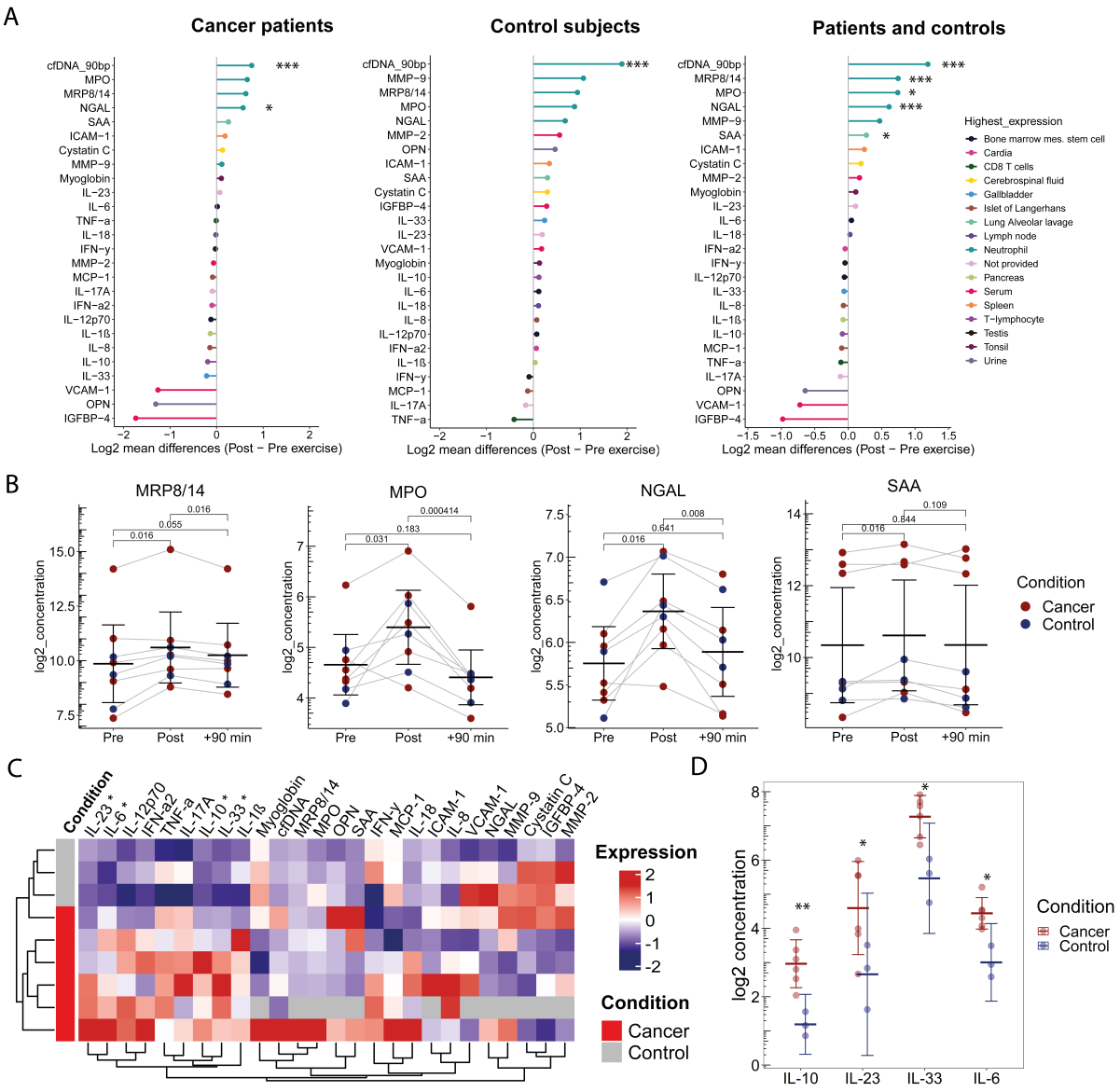

Figure 4: Circulatory cytokine expression changes in response to exercise. A: Log2 mean differences from Pre to Post exercise in cancer patients, control subjects, and pooled samples (cancer and control subjects) indicate that neutrophils mainly contribute to expression changes. The highest expression for the proteins were taken from Gene–cards - Human Genes | Gene Database | Gene Search providing the highest protein expression in normal tissues and cell lines. B: Kinetics of the differentially expressed plasma proteins. C: Heatmap for z normalized expression at baseline levels in cancer patients and healthy controls. Grey fields display values below limit of detection. D: Explorative description of significantly elevated cytokines in cancer patients compared to healthy controls at baseline. Paired or unpaired t tests or Wilcoxon tests were conducted to compare the groups, without p-value correction. (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$). ns: not significant.

Discussion

The purpose of the study was to obtain first evidence on the effects of acute exercise on the kinetics of cfDNA, ctDNA and DNase I activity in a small pilot cohort of patients with solid cancers in comparison with age-matched control subjects in order to be able to plan fully-powered follow-up studies. Cardiopulmonary exercise testing was well feasible in the tumor group as well as the healthy control group, and the dropout rate due to medical, personal, or technical reasons was comparable to other studies. In our pilot analysis of blood markers, we found significant increases in cfDNA, DNase I activity, and inflammatory cytokines in response to exercise in both groups. The cfDNA kinetics differed significantly between the groups. A clear correlation between cfDNA and DNase I activity could be detected in healthy subjects but not in cancer patients, indicating disordered DNase I and cfDNA homeostasis. Neutrophil-associ-

ated inflammatory cytokines (MPO, MRP8/14 and NGAL) showed high increases in response to exercise, suggesting a large contribution of neutrophils to the exercise-mediated effect. In our cancer cohort, we identified one subject who was positive for KRAS ctDNA. In this case, the kinetics of cfDNA and ctDNA indicate that exercise does not increase the absolute ctDNA concentration but decreases the percentage of ctDNA compared to the total amount of cfDNA, indicating that exercise can be a relevant preanalytical factor impairing the results of liquid biopsies.

cfDNA is known to circulate in the blood of healthy individuals at low concentrations. Various pathophysiological conditions, including hematopoietic and solid cancers, are associated with increased cfDNA levels. Emerging evidence suggests that cfDNA, in the form of nucleosomes, is not only a waste product of cell death but also has pathogenic or even functional roles [4], including acting as a proinflammatory stimulant and promotor of tumor growth (reviewed in [4,26]). Therefore, low levels of cfDNA at rest and efficient cfDNA clearing during exercise are likely to have beneficial effects. In our age-matched cohorts, the cancer patients showed ~ 2-fold higher cfDNA values at rest, although the difference was not statistically significant ($P > 0.05$). In line with former studies, we found that cfDNA levels increase in response to acute exercise, with more pronounced increases in healthy cohorts than in diseased subjects [21,22]. The cfDNA levels increased 1.75- or 3.90--fold in patients and controls, respectively. Importantly, this indicates that cancer patients do not have an overshooting cfDNA increase in response to acute exercise.

The clearance of cfDNA is related to the activity of different nucleases [4]. As shown by Velders et al., exercise is a potent stimulus to enhance the activity of circulating DNase I in healthy subjects in response to a stepwise incremental exercise test on a rowing ergometer [27]. We showed that acute exercise triggers DNase I activity in healthy subjects and cancer patients. In both cohorts, the activity increased significantly, reducing to baseline levels +90 min (Figure 2C). We did not find significant differences in DNase I activity between the groups at any time point analyzed (Figure 2D); however, a clear correlation between cfDNA and DNase I activity was detected for healthy controls but not for the cancer group (Figure 2E). This finding might indicate a disordered balance between cfDNA and DNase I activity in tumor patients. However, since the tumor patients had a more or less efficient clearance of the cfDNA, it can be expected that other nucleases contribute to cfDNA homeostasis and DNase I activity is not the main factor regulating cfDNA levels. Future studies should consider analyzing the activity of other relevant nucleases, including DNase1L3. Since DNase I activity has been described to be up- or downregulated in different kinds of cancer (reviewed in [4]), larger studies with more homogenous cohorts could help to better identify how and which nuclease counterbalances the increased levels of cfDNA. Notably, Ondracek et al. studied the effect of 8 months of consequent exercise on DNase I activity in patients with a cardiovascular risk factor. The authors showed that in the group of subjects who achieved performance gains, the cfDNA levels decreased, whereas DNase activity increased [28]. Moreover, the results from animal studies indicate that DNase I injection led to increased survival time, a reduced number of cancer cells, and inhibition of metastasis (reviewed in [29]). Thus, long-term exercise interventions that affect DNase activity could have beneficial effects in cancer patients.

Cytokines have a critical role in cancer development and serve as the means of communication between the immune system and healthy and malignant cells. Here, we ran pilot tests on the cytokine response after an acute bout of cycling exercise in a subgroup of our cancer patients. We found the highest increases in inflammatory cytokines that are most highly expressed in neutrophils, indicating that neutrophils largely contribute to the exercise-mediated effect. Acute physical exercise leads to profound and transient changes in blood cell count and functional capacity of the cells [30]. This includes the release of cytokines that interact in a coordinated system activating immunospecific pathways and interact with the (host)-tissue-tumor microenvironment [31]. In our study, four cytokines were significantly upregulated in response to acute exercise (Figure 4A, B). None of the cytokines showed significant differences from Pre to +90 min. MPO, MRP8/14 and NGAL were most strongly increased in response to exercise. All of them are highly expressed in neutrophils [32]. In response to their activation, the cells can extrude neutrophil extracellular traps (NETs). NETs are net-like structures composed of

DNA-histone complexes and proteins, including MPO, NGAL, MRP8/14, and others [32]. The process of NET release typically takes several hours; however, rapid release mechanisms, called vital netosis, have been described [33,34]. Using targeted bisulfite sequencing, we have recently shown that in healthy persons, neutrophils are the major source of cfDNA released in response to exercise [35]. More recently, Mattox et al. identified that neutrophils are the major source of cfDNA in patients with pancreatic, colorectal, lung, or ovarian cancer, who show elevated cfDNA levels [36]. Neutrophils have attracted attention because of their role in cancer promotion [26,37]. The regular activation and renewal of the pool of circulating neutrophils by exercise might have beneficial effects in cancer patients as well as in the healthy population. Neutrophil released cytokines, including neutrophil elastase, which are released in response to exercise, could have beneficial effects on the development of cancer and tumor growth. Cui et al. showed that neutrophils release catalytical active neutrophil elastase which kills cancer cells and attenuates tumorigenesis [38]. Next to the effect of acute exercise, we compared the cytokine levels under resting conditions. The plasma levels of IL-23, IL-33, IL-6, and IL-10 were higher in cancer patients than in healthy controls, which has been described in different cancer entities, including colorectal cancer [39].

We analyzed all patient samples for the occurrence of KRAS mutations using mutant-specific PCR to evaluate a possible stimulating effect of exercise on ctDNA concentrations. In our study, one patient was positive for the G12R KRAS mutation and had colon cancer with residual liver metastasis. The ctDNA concentration was only slightly elevated after the cycling test (0.106 vs 0.14 ng/ml), but the relative proportion of ctDNA in the total pool of cfDNA decreased considerably directly after exercise. This led to a reduction in the mutated fraction in the circulation. The ctDNA and cfDNA concentrations at +90 min were similar to the Pre values. This finding highlights the importance of considering physical exercise as a preanalytical factor for cancer-liquid biopsies. Kuligina et al. studied the effect of physical activity in a relevant real-world scenario. In addition to the effects of circadian rhythm and food intake, the authors measured the concentration of ctDNA in 18 subjects 15 min before and 15 min after walking upstairs for two stairwells [13]. The authors did not detect changes in tumor-derived ctDNA copies [13]. However, studies with varying relevant physical activity modes, times and loads should be performed to comprehensively elucidate the impact of exercise on ctDNA detection in different cancer types. Exercise could increase or decrease the levels of ctDNA in plasma samples, having advantages or disadvantages for ctDNA detection in subjects with undiagnosed disease [40].

The study includes several limitations, which should be addressed in follow-up studies with higher sample sizes and more homogenous patient collectives. First, due to the small sample size of the study, type II statistical errors are possible. Moreover, although we analyzed the seven most common KRAS mutations, ctDNA was detected only in a single patient. Larger and well-characterized cohorts need to be studied to identify the acute and chronic effects of exercise on ctDNA levels. Notably, during exercise, blood flow decreases in the abdominal organs, whereas the thoracic blood volume within the lung largely increases [41]. It cannot be excluded that ctDNA increases during exercise in patients with lung cancer, since blood flow increases in this organ. In this case, physical activation could have beneficial effects on ctDNA detection. Additionally, we did not account for plasma volume changes, which can occur after short duration, high intensity exercise. In elderly circulatory healthy subjects, the plasma volume decreased by 14% after four progressive 5-min intervals on a cycling ergometer. In our case, the plasma volume changes could be responsible for the slight increase in ctDNA after exercise [42]. The consideration of this bias is especially important for biomarkers that are only slightly regulated in response to exercise. Finally, although our control subjects were age matched and the number of steps during the test was similar, the healthy subjects performed higher maximal power output. It cannot be excluded that the higher load led to higher cfDNA increases, and future studies might include control subjects with similar cardiorespiratory fitness.

Conclusions

Here, we could show that cardiopulmonary exercise testing followed by cfDNA, and cytokine analysis is well feasible in cancer patients. The results of our pilot analysis hint at a different reaction to acute physical exercise of can-

cer patients than healthy controls with respect to cfDNA clearance by DNase I. In both cohorts, the cfDNA levels increase immediately after the exercise bout and reduce at least to Pre values +90 min, whereas cfDNA and DNase I only correlated in control subjects. The latter finding might indicate a disordered homeostasis in the cancer group potentially involving other nucleases and mechanisms for cfDNA clearance than in healthy controls, which should be addressed in more detail in follow-up studies. Exercise mainly leads to the release of neutrophil-related cytokines and further research is required to study to what extent the beneficial health effects of exercise are related to neutrophil activation. Moreover, our study highlights the need to include physical activity in preanalytical considerations in cancer liquid biopsy.

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Ethics Approval and Consent to Participate

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Commission of the state of Rhineland-Palatinate, Mainz, Germany (837.033.11 (7575)). Written informed consent was obtained from all study participants.

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