Exercise-induced increases in cell free DNA in human plasma originate predominantly from cells of the haematopoietic lineage

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ABSTRACT

The role of cell free DNA (cfDNA) has been intensively discussed under various pathological conditions and after acute bouts of exercise. To date, there is still no conclusive evidence concerning the cellular origin of cfDNA and the entire mechanism leading to elevated cfDNA concentrations in human plasma and serum. Here, we investigated the cellular origin of cfDNA in sex-mismatched haematopoietic stem cell transplantation (HSCT) and liver transplantation (LT) patients by determining the relative proportion of Y-chromosomal to total nuclear cfDNA. Total nuclear cfDNA and Y-chromosomal cfDNA concentrations were determined in blood plasma before and after an incremental exercise test via quantitative real-time PCR (qPCR). Female HSCT patients showed high proportions of Y-chromosomal cfDNA. Both total nuclear and Y-chromosomal cfDNA increased significantly and in a highly correlated fashion due to exercise. In male HSCT patients with female donors less than 10% of the cfDNA was of Y-chromosomal origin at any point in time and even though the total amount of cfDNA increased during exercise, no increases in Y-chromosomal DNA could be detected. The percentage of Y-chromosomal cfDNA in female LT patients with male donors was very low and levels remained unchanged during exercise. This indicates that cells not derived from the bone marrow, in this case transplanted liver cells, represented only a minor fraction of cfDNA in blood plasma and were not released during acute physical exercise. Even though many physiological conditions may be altered in transplant patients versus healthy people, our results strongly suggest that cells from the haematopoietic lineage are the main source of cfDNA released during acute bouts of exercise.

Keywords: cell free DNA, sex-mismatched transplantation, Y-chromosomal PCR, exercise, graft rejection.

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INTRODUCTION

The potential of cell free DNA (cfDNA) as a biological marker has attracted much interest in various biomedical disciplines. Under physiological conditions, the concentration of cfDNA is low whereas levels increase under chronic and acute pathological conditions like cancer, autoimmune diseases, sepsis and stroke (20, 37, 41, 49). Elevated concentrations of cfDNA have also been reported after acute bouts of exercise (4, 6). For all of these conditions, there is so far no conclusive evidence concerning the cellular origin and the precise mechanisms involved in cfDNA release into serum or plasma. Continuously occurring apoptosis in normal tissues could lead to the presence of cfDNA in healthy subjects (35) whereas elevated levels of cfDNA in cancer patients could result from both apoptosis and necrosis of tumour tissue and surrounding normal cells, autophagy or mitotic catastrophe (34, 40). Apart from cellular damage, strenuous physical exercise can also induce cfDNA levels (2). Since the appearance of DNA fragments in the circulation in response to cell death-stimuli would require several hours (18, 23, 25, 30), these mechanisms seem to be unlikely to account for the immediate increases of circulating cfDNA levels in settings applying short bouts of exercise (10). Several studies have suggested that the spontaneous DNA release from living cells could contribute to the cfDNA pool in blood plasma (39, 42). In a recent study, the rapidly increasing cfDNA concentrations observed during physical exercise were at least partially attributed to the release of neutrophil extracellular DNA traps (7).

In this pilot study we investigated the cellular origin of cfDNA induced by acute bouts of physical exercise. We used a sex-mismatched transplantation model to distinguish donor- from host-derived DNA by quantitative real-time PCR (qPCR), therefore being able to determine whether cfDNA release can be attributed to the transplanted organ. Since it was already shown that baseline cfDNA originates predominantly from the haematopoietic cell lineage (27) we decided to primarily focus on measuring cfDNA release during exercise in sex-mismatched haematopoietic stem cell transplantation (HSCT) patients. Considering the role of the liver in the clearance of DNA from the circulation (16, 44), we also analysed cfDNA release in sex-mismatched liver transplanta-

tion patients (LT). To track the donor-derived DNA in the patients we selectively amplified male DNA by using PCR primers targeting Y-chromosomal sequences. We compared the proportion of male DNA to the amount of total nuclear cfDNA in blood plasma of the patients, quantified by qPCR targeting long terminal repeat (LTR) loci. Whole blood was collected before and after exercise. We show that the majority of cfDNA released during physical exercise is derived from the haematopoietic system. Furthermore, liver cells only contribute to a low extent to the cfDNA pool and it seemed that this cell type does not liberate DNA in response to acute exercise.

MATERIAL AND METHODS

Subjects

We recruited seven HSCT patients at the Department of Internal Medicine III, University Medical Center, Mainz. Five were females with male donors and two male patients received HSCT from a female donor. In addition, five LT patients, who received an orthotropic liver transplant from a deceased male donor, were recruited at the Department of Internal Medicine I, University Medical Center, Mainz. Sex and diagnosis of the patients are shown in Table 1.

We also recruited three healthy male and three healthy female volunteers, serving as controls for the Y-chromosomal qPCR assay, from the Department of Sports Medicine in Mainz.

All experimental procedures were approved by the Human Ethics Committee of Rhineland-Palatine and were in line with the standards of the *Declaration of Helsinki of the World Medical Association.* All subjects were informed orally and in writing about the procedures and the aim of the study and gave written consent to participate.

Exercise protocol

All subjects performed an incremental treadmill test. The exercise protocol started for the patients at a velocity of 2 or 4 km/h and speed was increased 1 km/h every 3 min until volitional exhaustion. To allow for comparability of the protocol duration, the healthy controls started at a speed of 6 km/h with a step-wise increase of 2 km/h every 3 min. Capillary blood samples for lactate measurement were taken from the earlobe before the exercise test, after each incremental step, immediately after and 3, 5 and 10 min post-exercise. Respiratory gas exchange data and heart rate were continuously recorded during the test by spiroergometry and electrocardiogram monitoring, respectively. Borg scale values to monitor the self-reported level of exertion were assessed at the end of each speed step. The subjects were requested to avoid any exercise training for the 24 h before the test.

Table 1. Sex and diagnosis of the transplantation patients.

Patient	Sex of donor	Sex of recipient	Age (years)	BMI	Diagnosis	Months after transplantation	Infection	hsCRP (mg/l)	GVHD
HSCT1	M	F	23	25.8	sAA	13	No	53.8	No
HSCT2	M	F	41	29.1	AML	5	Sinusitis *	1.7	No
HSCT3	M	F	48	20.8	c-B-ALL	3	No	45.0	No
HSCT4	M	F	53	26.9	MM	13	parvovirus B19	2.4	Yes
HSCT5	M	F	58	24.3	Pre-B-ALL	5	No	0.4	Yes
HSCT6	F	M	24	21.5	Pro-T-ALL	11	No	8.9	No
HSCT7	F	M	59	22.6	MDS	28	Chronic respiratory infection	9.4	Yes
LT1	M	F	56	22.8	Hepatitis B/D liver cirrhosis	79	No	1.1	No
LT2	M	F	43	26.4	Acute drug induced liver failure (non-paracetamol)	96	No	1.9	No
LT3	M	F	57	22.1	Hepatitis C Liver cirrhosis	98	No	0.2	No
LT4	M	F	63	22.1	Hepatocellular carcinoma Alcoholic liver cirrhosis	29	No	1.1	No
LT5	M	F	56	20.9	Primary sclerosing cholangitis	19	No	0.2	No

HSCT: Haematopoietic stem cell transplantation; LT: Liver transplantation; M: Male; F: Female; BMI: Body Mass Index; ALL: Acute lymphoblastic leukaemia; pre-B-ALL: pre-B cell acute lymphoblastic leukaemia; c-B-ALL: common B cell acute lymphoblastic leukaemia; Pro-T-ALL: pro-T cell acute lymphoblastic leukaemia; AML: Acute myeloid leukaemia; MDS: Myelodysplastic syndrome; MM: Multiple myeloma; sAA: Severe aplastic anaemia; BM: Bone marrow; PB: Peripheral blood; hsCRP: high-sensitivity C-reactive protein; GVHD: graft-versus-host disease * Partial lung resection with pneumonia before transplantation.

Blood sampling and processing

10 ml of EDTA-anticoagulated blood were taken from the antecubital vein before, immediately after and 90 min after the treadmill exercise. The blood samples were centrifuged at 4°C, 1,600**g* for 10 min. In a second high-speed step the plasma was centrifuged at 4°C, 16,000**g* for 5 min to remove cellular debris. The plasma samples were stored at -20°C and cfDNA concentrations were measured within a maximum of 4 weeks. 2.5 ml of venous blood were sent to an external laboratory for the analysis of complete blood cell counts and other blood parameters. Capillary blood samples were measured with the lactate analyser Biosen 5130 (EKF Diagnostics, Magdeburg, Germany).

DNA extraction

Since column-based DNA extraction kits may not be as efficient as traditional methods for isolating low quantities of DNA from bodily fluids (48), we established a manual, non-column based phenol-chloroform method. 50 µl of plasma were diluted with 250 µl phosphate buffered saline (PBS, Life Technologies, Darmstadt, Germany) to a total volume of 300 µl. 1/100 Vol of Triton X-100 (Carl Roth, Karlsruhe, Germany) were added, samples were incubated at 98°C for 5 min and then cooled on ice for 5 min. Samples were mixed with 1 Vol Phenol:Chloroform:Isoamyl Alcohol, pH 8.0 (Sigma-Aldrich, Taufkirchen, Germany), vortexed for 30 s and centrifuged at 20°C, 16,000**g* for 10 min. The upper aqueous phase was pipetted off and DNA was precipitated with 2.5 Vol of 100% ethanol, 1/10 Vol 3 M sodium acetate, pH 5.2 and 20 µg Glycogen (Life Technologies, Darmstadt, Germany) overnight at -20°C. The next day, the precipitate mixture was centrifuged at 4°C, 16,000**g* for 30 min. DNA pellets were washed two times with 70% ethanol and a third time with 100% ethanol. After each washing step the samples were centrifuged at 4°C, 16,000**g* for 5 min. Pellets were dried for about 20 min at 55°C and eluted with 50 µl TE buffer, pH 8.0 (Life Technologies, Darmstadt, Germany). Samples were further incubated at 37°C for 30 min to completely dissolve the DNA.

Quantitative real-time PCR

Quantification of total nuclear cfDNA was based on the amplification of long terminal repeats (LTRs) of the human endogenous retrovirus K family (HERV-K LTR5 Hs). Since fragmented cfDNA is characterized by less intact target amplicons (21), we chose these multi-locus primers to enable a more precise and sensitive quantification of cfDNA. Sequence analysis revealed that LTR elements are represented in the cfDNA pool in an equal proportion as in genomic DNA (43), which minimizes a target-specific bias in quantification of cfDNA. Sequence information for primer design was obtained from the UCSC Genome Browser on the February 2009 human reference sequence (GRCh37) (33). The online software Primer3 was used to design multi-locus primers 5'-ACC GAG ACA TTC CAT TGC C -3' and 5'- GCC TCT TGC AGT TGA GAC AAG -3' targeting a 70 bp fragment of a LTR5 sequence with 195 matches in the human haploid genome. The binding frequency was assessed by aligning the primers using the *in-silico* PCR tool of the UCSC Genome Browser. For the quantification of Y-chromosomal cfDNA we used a set of primers targeting DYZ1 sequences (8): sense (5´-GTCCATTACACTACATTCCC -3´) and antisense (5´-AATGCAAGCGAAAGGAAAGG -3´) to amplify a 77 bp sequence.

The amplification of total nuclear cfDNA and Y-chromosomal cfDNA was performed on a CFX384 Touch™ Real-Time PCR detection system (Bio-Rad, München, Germany) under the following conditions for the LTR assay: initial denaturation at 95°C for 5 min and 40 cycles of denaturation at 94°C for 15 s, annealing at 61°C for 30 s, and extension at 80°C for 30 s and for the DYZ1 assay: initial denaturation at 95°C for 5 min and 40 cycles of denaturation at 94°C for 15 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s. The experiments were performed in triplicates with a final volume of 5 µl per single reaction containing 1.6 µl of template DNA, 2.6 µl master mix containing 0.1 U/µl HotStar*Taq* Plus Polymerase (Qiagen, Hilden, Germany), 2 x PCR Buffer (Qiagen, Hilden, Germany), 1 µM $MgCl_2$ (Qiagen, Hilden, Germany), 0.4 mMdNTPs (Carl Roth, Karlsruhe, Germany), 0.28 x SYBR green (Sigma-Aldrich, Taufkirchen, Germany), 5 nM FITC (Sigma-Aldrich, Taufkirchen, Germany), and 0.8 µl primer-mix at a final concentration of 312 nM. Non-template controls (NTC) and positive controls for inter-plate calibration were also analysed in triplicate within each PCR run. Formation of the expected PCR product was confirmed by melting curve analysis.

The LTR assay was established and optimised with a genomic reference standard including the target sequences for the LTR5 primer set. The standard was generated from human genomic DNA (Novagen, Merck, Darmstadt, Germany) by PCR using primers 5´ TTC TCA AAG AGG GGG ATG TG 3´ and 5´ GTG GGA AGG GAA AGA CCT GA 3´ to amplify a 400 bp-fragment of a LTR-sequence. Sequence information was obtained from the UCSC Genome Browser on the February 2009 human reference sequence (GRCh37) (33). The amplification was performed on a Mini Thermal Cycler (Bio-Rad, München, Germany) with the following conditions: initial denaturation at 95°C for 5 min and 40 cycles of denaturation at 94°C for 15 s, annealing at 59°C for 30 s, and extension at 72°C for 30 s. The PCR product was TA-cloned in a pCR®2.1 vector (Invitrogen, Carlsbad, CA, USA) and the sequence was confirmed by Sanger sequencing (StarSEQ®, Mainz, Germany). For standard preparation the PCR product was cut out of the vector and the stock solution of genomic reference standard was determined fluorophotometrically (NanoDrop 3300, Thermo Fisher Scientific Inc., Waltham, MA). The DYZ1 assay was established with male genomic DNA (gDNA). Prior to amplification the high-molecular gDNA was treated for 1 h at 37°C with the restriction enzymes *DdeI* and *RsaI* (NEB, Frankfurt/M., Germany), followed by a heat-inactivation-step at 65°C for 20 min. We selected the restriction enzymes *DdeI* and *RsaI* for the fragmentation of high-molecular genomic DNA to mimic fragmented cfDNA. The coordinates of the restriction sites in the human genome were taken from an online database: http://tools.neb.com/~posfai/TheoFrag/grch38_site_counts.tools.html. The enzymes digest DNA into fragments of 200-600 bp (also confirmed by agarose gel electrophoresis) which corresponds to the typical cfDNA size profile of 180-200 bp and multiples of this (17, 22, 32). Furthermore, *DdeI* and *RsaI* were selected

since both enzymes can be combined in one reaction without activity loss.

DNA concentrations were converted into copy numbers by using the online program Finnzymes - DNA copy number calculation. Standards were prepared in defined copy numbers, ranging from 2 x 10^5 to 50 copies/µl for the LTR assay, and from 32 to 0.0125 copies/µl for the DYZ1 assay. The differences in the absolute copy numbers between both assays arise from the type of the PCR standards (specific sequence versus total genomic DNA) and, consequently, a different calculation basis for copy numbers. One copy of specific sequence is equivalent to one molecule of double-stranded DNA. One copy of genomic DNA corresponds to one human genome copy with a molecular weight of 3.3 pg. In subsequent qPCR analysis, these calibrators were used to construct standard curves by plotting the quantification cycle (Cq) value against the logarithm of calibrator copy number in each dilution. We also assessed the lower limit of quantification (LOQ), defined as the minimal concentration that could be quantified with 80% accuracy. The LOQ was determined with each reference standard measured in seven replicates. The LOQ of the LTR assay was set to 50 copies/µl, corresponding to a DNA concentration of 0.78 pg/µltemplate. The LOQ of the DYZ1 assay was set to 0.0125 copies/µl of genomic DNA which is equivalent to a DNA concentration of 0.09 pg/µl template.

Statistical analysis

The qPCR data were captured with the MyIQ5 Optical System Software, Version 2.4 (Bio-Rad, München, Germany). Microsoft® Excel 2007 was used for data analyses. We considered p values of p values less than 0.05 to be statistically significant ($p<0.001$***, $p<0.01$**, $p<0.05$*) and performed statistical analysis with JMP 11 (SAS Institute Inc., Cary, NC, USA). All data are presented as mean (±SD). Changes in cfDNA concentrations at the various points in time were compared by a nonparametric Wilcoxon-Test. Since the overall data were not normally distributed, a Spearman's rho test was calculated for nonparametric correlations.

RESULTS

Demographic data and exercise parameters

Seven HSCT patients (mean (±SD) age 44 (14) y, BMI 24.6 (2.8) kg/m^2) and five LT patients (mean (±SD) age 55 (7) y, BMI 22.9 (1.9) kg/m^2) performed an incremental treadmill test until volitional exhaustion. To evaluate the specificity of the Y-chromosomal qPCR assay, we monitored six healthy subjects (mean (±SD) age 28 (2) y, BMI (21.3 (1.6) kg/m^2, serving as positive (men) and negative (women) controls. To get an idea about the individual level of exhaustion, the values of peak oxygen consumption (VO_{2peak}), Borg scale rating of perceived exertion (RPE) and blood lactate concentration were assessed at maximal intensity (Table 2). The patients had RPE values of mean (±SD) 16.8 (1.7), VO_{2peak} of 22.4 (6.4) ml/kg/min) and lactate levels of 5.1 (2.7) mmol/l and the healthy controls had RPE values of 19.3 (0.5), VO_{2peak} of 44.4 (8.4) ml/kg/min) and lactate levels of 9.3 (1.5) mmol/l. Exercise parameters and physiological measures at the endpoint of the incremental treadmill test are shown in Table 2.

Table 2. Exercise parameters and physiological measures at the endpoint of the incremental treadmill test.

Subjects	Sex	Velocity (km/h)	Lactate (mmol/l)	VO_{2peak} (ml/kg/min)	Borg RPE value	Heart rate (beats/min)
HSCT	male	8.0 (1.6)	6.0 (2.3)	30.6 (6.4)	18.5 (1.5)	156.5 (31.5)
HSCT	female	6.3 (1.2)	6.4 (2.4)	21.3 (5.1)	16.4 (1.6)	164.6 (13.1)
LT	female	6.6 (0.6)	3.4 (1.9)	20.6 (4.1)	16.6 (1.5)	138.4 (21.7)
HC	male	16 (0.6)	10.3 (1.4)	51.3 (2.0)	19.7 (0.5)	194.7 (2.5)
HC	female	13.4 (2.5)	7.7 (0.7)	37.3 (6.6)	18.7 (0.5)	199 (6.5)

Values are given as mean (±SD); VO_{2peak}, peak oxygen consumption; RPE, rating of perceived exertion
HSCT: Haematopoietic stem cell transplantation; LT: Liver transplantation; HC: Healthy controls.

Quantification of total nuclear cfDNA and Y-chromosomal cfDNA

To evaluate qPCR performance in terms of sex specificity of the Y-chromosomal assay and for the agreement of DNA quantification by both methods we measured total nuclear (LTR sequences, amplified by the LTR 5 assay) and Y-chromosomal cfDNA (Y chromosomal DYZ sequences, amplified by the DYZ1 assay) concentrations in healthy males and females. The proportion of Y-chromosomal cfDNA amongst total nuclear cfDNA in male healthy controls was mean (±SD) 95.1 (25.2) % (Figure 1A). The amount of Y-chromosomal DNA at every point in time in female plasma was below the LOQ of the qPCR assay and therefore not quantifiable (Figure 1 B). One exception, however, was a post-exercise sample which gave weak, but positive signals when amplified with the Y-qPCR assay. In this case the relative proportion of Y-chromosomal cfDNA to total nuclear cfDNA was <0.1%. This discrepancy could be due to sequence homologies between the sex chromosomes, which can drive low level of unspecific amplification (19). Deviations from the theoretically expected proportion of 100% in healthy males could be explained by using two qPCR assays to quantify cfDNA which differ in terms of their target amplicons, PCR protocols, PCR calibrators and reaction efficiency. However, the fold-increase of cfDNA post-exercise compared to baseline levels is comparable between both assays (mean (±SD) 7.2 (0.3) for LTR 5 and 6.0 (1.56) for DYZ1, respectively) in the healthy male controls. There was a significant correlation between total nuclear cfDNA and Y-chromosomal cfDNA in healthy male controls ($r= 0.95$, $p= 0.001$) and no correlation between both variables in the healthy female controls ($r= 0.29$, $p= 0.81$).

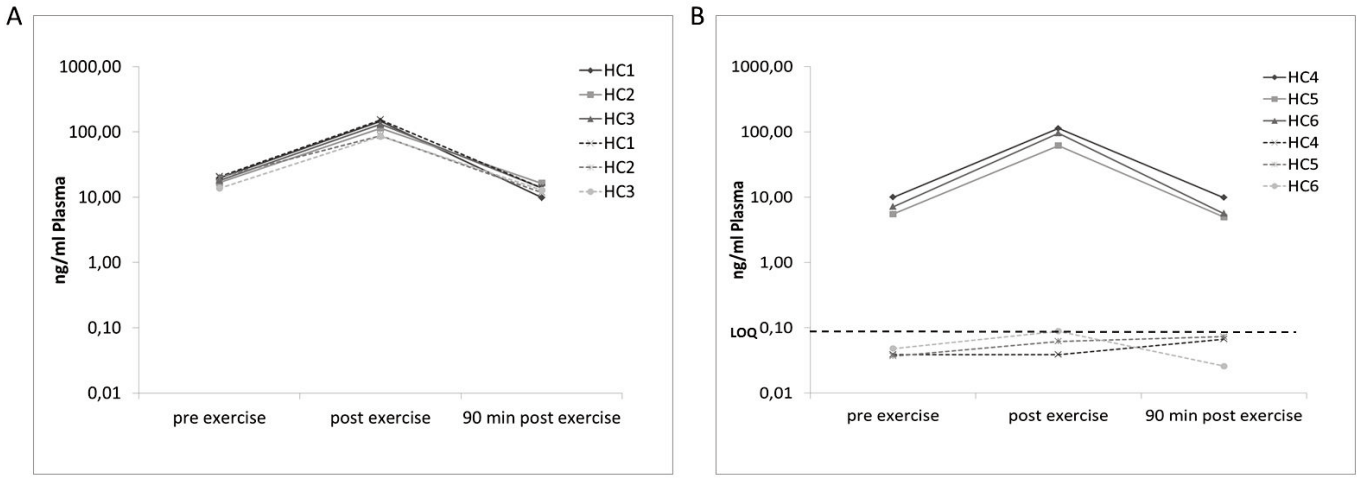

Figure 1: Mean total cfDNA and Y-chromosomal cfDNA concentrations in healthy male (A) and healthy female controls (B) (solid lines: total nuclear cfDNA, dashed lines: Y-chromosomal cfDNA). The LOQ indicates the lower limit for the quantification of Y-chromosomal DNA concentrations.

To determine whether exercise-induced cfDNA levels originate from cells of the haematopoietic lineage, we measured the total nuclear cfDNA and the Y-chromosomal cfDNA concentrations in HSCT patients before and after exercise (Figures 2 and 3).

The percentage of donor DNA in female blood plasma was mean (±SD) 46.6 (12.2) % across all time points (Figure 2 A). Accordingly, the concentrations of total nuclear cfDNA (mean (±SD) before: 77.6 (97.9) ng/ml, after: 140.0 (136.4) ng/ml, 90 min after exercise: 75.7 (101.8) ng/ml) and Y-chromosomal cfDNA (before: 30.8 (39.8) ng/ml, after: 70.8 (81.5) ng/ml, 90 min after exercise: 33.5 (50.7) ng/ml) in plasma showed similar kinetics during and after the exercise test (Figure 2 B). Total nuclear cfDNA increased mean (±SD) 2.97 (1.99)-fold ($p = 0.17$) and Y-chromosomal cfDNA increased 3.23 (1.98)-fold ($p = 0.12$) compared to baseline (Figure 2 C).

We observed a significant correlation between total nuclear cfDNA and Y-chromosomal cfDNA ($r= 0.98$, $p < 0.0001$) across all points in time. One female HSCT patient, who suffered from c-B-ALL, had a relapse (HSCT3) and showed increased total nuclear cfDNA levels at baseline with a lower proportion of Y-chromosomal cfDNA (32.2%) in comparison to the other relapse-free HSCT patients. This phenomenon could be expected since the host tumour cells are devoid of Y-chromosomal DNA.

The results obtained from the female patients were counterchecked with two male HSCT patients with a female donor. The percentage of Y-chromosomal cfDNA, representing host-derived DNA in this transplantation setting, in blood plasma across all time points was mean (±SD) 9.0 (2.8) % (Figure 3 A). Total nuclear cfDNA concentrations (mean (±SD) before: 76.0 (62.7) ng/ml, after: 179.7 (24.4) ng/ml, 90 min after exer-

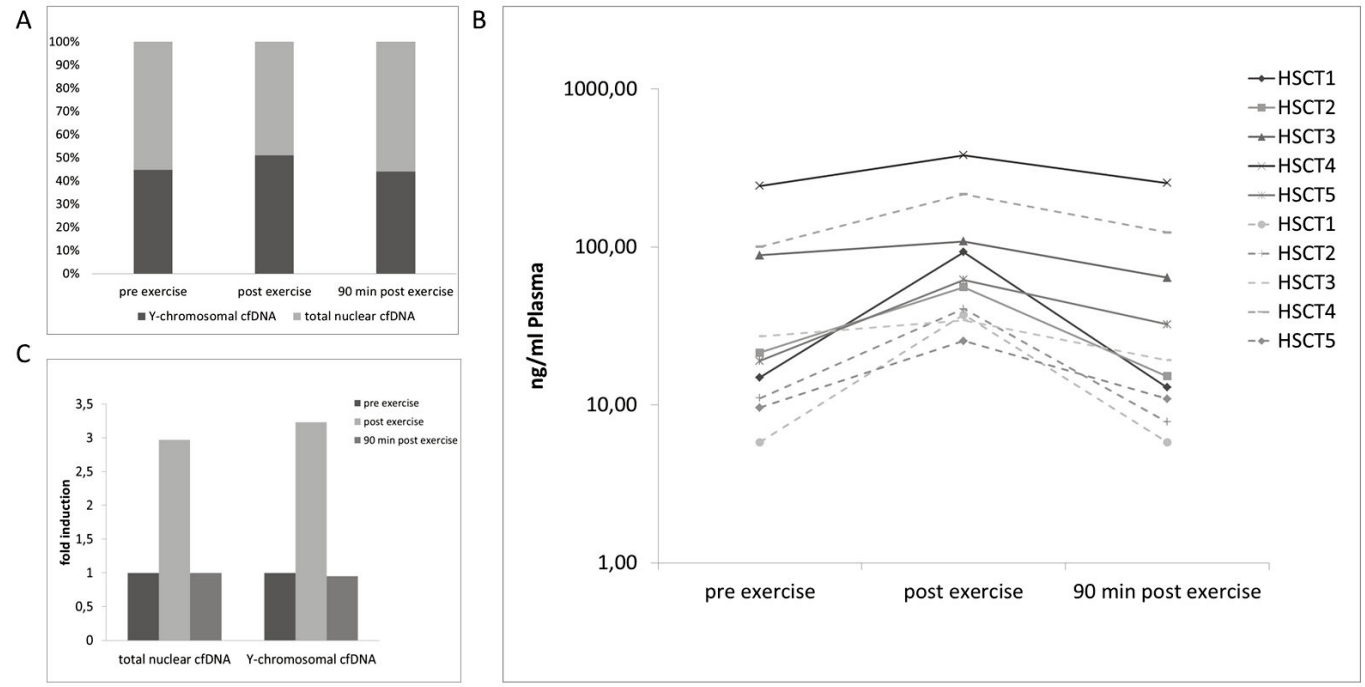

Figure 2: Percentage of Y-chromosomal cfDNA (A), absolute values (B), and fold-changes (C) of Y-chromosomal and total nuclear cfDNA in blood plasma of female HSCT patients with male donors (solid lines: total nuclear cfDNA, dashed lines: Y-chromosomal cfDNA).

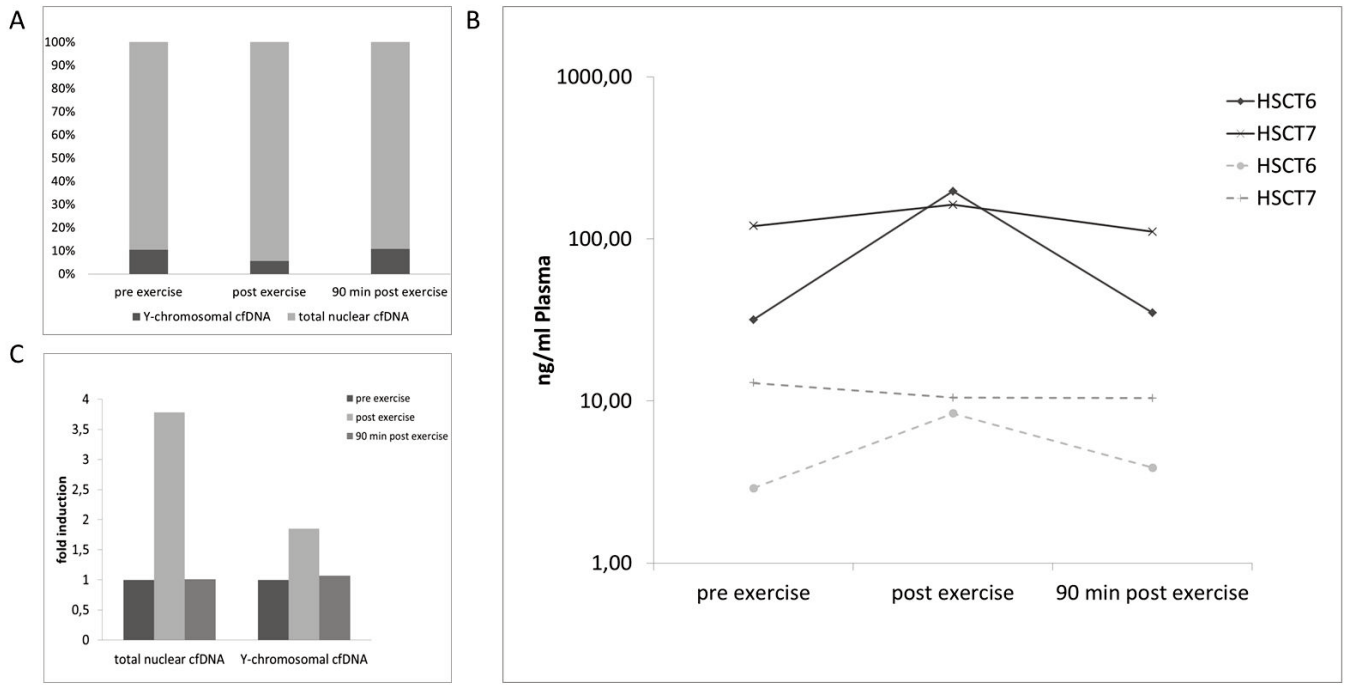

Figure 3: Percentage of Y-chromosomal cfDNA (A), absolute values (B), and fold-changes (C) of Y-chromosomal and total nuclear cfDNA in blood plasma of male HSCT patients with female donors (solid lines: total nuclear cfDNA, dashed lines: Y-chromosomal cfDNA).

cise: 73.0 (53.7) ng/ml) increased 3.78 (3.44)-fold (p = 0.12) in response to acute exercise (Figure 3B/C). Y-chromosomal cfDNA concentrations (mean (±SD) before: 7.9 (7.1) ng/ml, after: 9.4 (1.5) ng/ml, 90 min after exercise: 7.2 (4.6) ng/ml) was 1.85 (1.47)–fold (p =1.0) elevated compared to baseline levels (Figure 3B/C).

In order to investigate whether there is a contribution of non-bone marrow-derived cells to exercise-induced cfDNA levels, we analysed total nuclear cfDNA and the Y-chromosomal cfDNA concentrations in five female sex-mismatched LT patients. The percentage of liver-derived cfDNA in blood plasma across all time points was mean (±SD) 2.1 (1.4) % (Figure 4 A). Total nuclear cfDNA concentrations (mean (±SD) before: 59.3 (69.2) ng/ml, after: 160.6 (193.8) ng/ml, 90 min after exercise: 55.8 (87.4) ng/ml) increased 2.57 (0.42)-fold (p = 0.17) in response to acute exercise. In contrast, Y-chromosomal DNA concentrations (mean (±SD) before: 0.73 (0.50) ng/ml, after: 0.74 (0.57) ng/ml, 90 min after exercise: 0.53 (0.40) ng/ml) remained unchanged (0.99 (0.15)-fold) during and after the test (p = 0.60) (Figure 4 B/C). In one case (LT1) concentrations of Y-chromosomal cfDNA

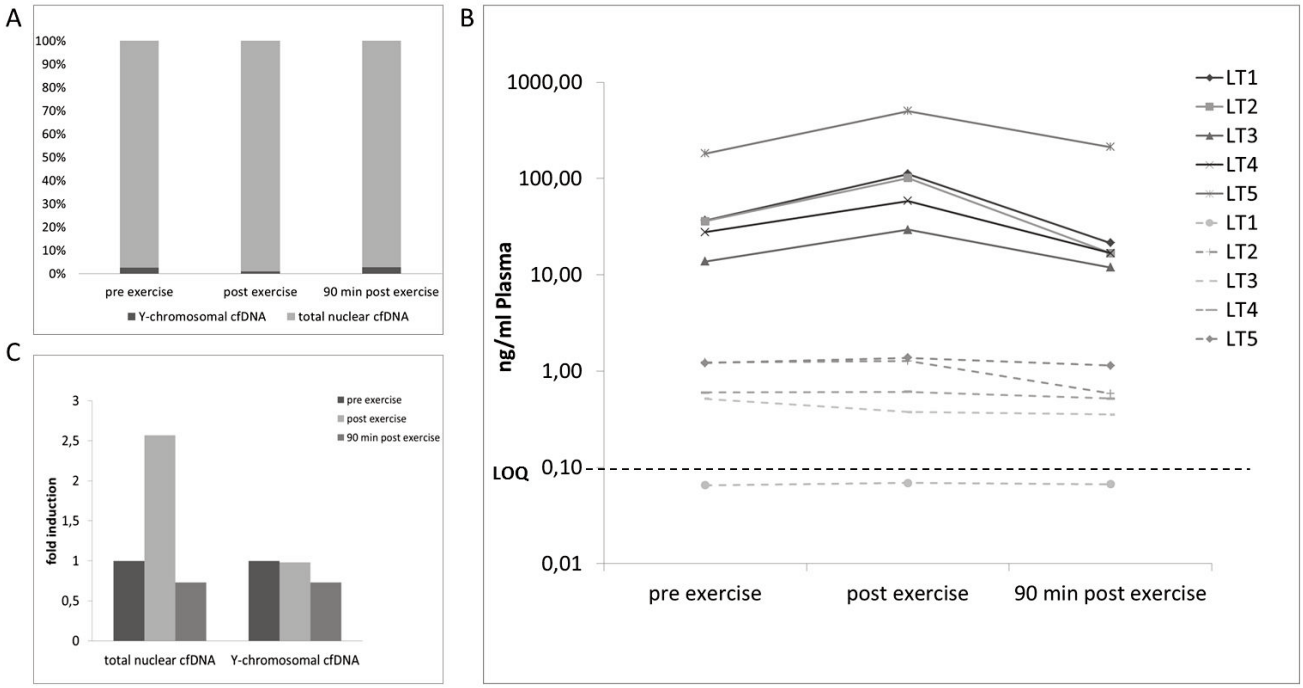

Figure 4: Percentage of Y-chromosomal cfDNA (A), absolute values (B), and fold-changes (C) of Y-chromosomal and total nuclear cfDNA in blood plasma of female HSCT patients with male donors (solid lines: total nuclear cfDNA, dashed lines: Y-chromosomal cfDNA).The LOQ indicates the lower limit for the quantification of Y-chromosomal DNA concentrations.

were below the LOQ of the qPCR assay. There was no significant correlation between total nuclear cfDNA and Y-chromosomal cfDNA (r=0.29, p = 0.31).

In summary, our results indicated that in response to exercise the majority of cfDNA is released by cells of the haematopoietic system. Liver cells only contribute to a low extent to the baseline cfDNA pool and this cell type would not release significant amounts of DNA in response to exercise. Table 3 summarises the individual results of the patients.

Table 3. Diagnosis and chimerism results of transplantation patients, increase of total and Y-chromosomal cfDNA levels after exercise and the percentage of Y-chromosomal cfDNA.

Patient	Sex of donor	Sex of recipient	Chimerism % (PB)	Chimerism % (BM)	Increase total nuclear cfDNA (pre-post) (fold change)	Increase Y-chromosomal cfDNA (pre-post) (fold-change)	% Y of total nuclear cfDNA (mean pre/post/90+)	Correlation total nuclear and Y-chromosomalcfDNA
HSCT1	M	F	87	---	6.22	6.41	43.5	0.98***
HSCT2	M	F	---	100	2.59	3.68	61.7	
HSCT3	M	F	---	73	1.22	1.26	32.4	
HSCT4	M	F	100	---	1.56	2.15	51.4	
HSCT5	M	F	---	100	3.26	2.65	44.0	
HSCT6	F	M	100	100	6.21	2.89	8.6	n.a
HSCT7	F	M	100	---	1.35	0.81	9.3	
LT1	M	F	---	---	3.02	1.05	0.19	0.29
LT2	M	F	---	---	2.81	1.03	3.6	
LT3	M	F	---	---	2.13	0.73	3.93	
LT4	M	F	---	---	2.12	1.02	2.21	
LT5	M	F	---	---	2.76	1.13	0.7	

p<0.001***, p<0.01**, p<0.05*
HSCT: Hematopoietic stem cell transplantation; LT: Liver transplantation; BM: Bone marrow; PB: peripheral blood. n.a: no analysis, no statistical analysis possible with 2 patients.

simple qPCR application, as previously described by others (15, 27, 28). The benefits offered by this qPCR-based approach compared to other methods like SNP-based sequence analysis (5, 38, 47) are time and cost efficiency. Furthermore, there is no need to analyse genomic DNA from the donor, which should also not be underestimated in terms of donor-anonymity. Finally, our approach allows a higher level of inter-individual comparability of the outcome, since we were able to measure all individuals with the same PCR assays.

DISCUSSION

In this pilot study we studied the cellular origin of cfDNA in human plasma released during exercise. In line with the findings of other studies (6, 7, 9), we showed that exhaustive short-term treadmill exercise led to increases of cfDNA concentrations. Increases of cfDNA in response to exercise have already been described for various exercise settings, e.g. endurance treadmill exercise (13), half- and ultra-marathon (2, 4), high-intensity cycling exercise (7), rowing (44) and weightlifting (3). However, the physiological or pathophysiological relevance of cfDNA increases in sports remains elusive (9), since essential questions regarding the cellular source and release mechanisms of cfDNA have not been answered yet. Here, we addressed the question of the cellular origin of cfDNA by exercising sex-mismatched HSCT and LT patients. This enabled us to study the relative contribution of bone marrow-and non-bone marrow-derived cells to the cfDNA pool in blood plasma. We distinguished donor-specific from recipient-specific DNA by targeting Y-chromosomal sequences in a

Our results indicate that cells of the haematopoietic lineage are the main source of DNA released by a short incremental exercise until volitional exhaustion. The most intriguing evidence comes from the data revealed from the two male sex-mismatched HSCT patients. Both showed considerable increases in total nuclear cfDNA of 42 ng/ml and 166 ng/ml post-exercise compared to pre-exercise.The respective increases of the Y-chromosomal cfDNA that reflects the DNA released from all other cells of the body except the HSCT cells from the female donor were 5.5 ng/ml increase and 2.6 ng/ml decrease, respectively. Accordingly, there does not seem to be any significant release of cfDNA due to exercise from other sources than the cells of the haematopoietic lineage. In line with this, sex-mismatched female HSCT patients showed a high (around 50%) and constant proportion of Y-chromosomal cfDNA relative to total nuclear cfDNA over the course of the experiment and the correlation of the respective absolute values was very high (r = 0.98; Table 3). In contrast to this, female sex-mismatched LT patients had very low proportions of Y-chromosomal cfDNA throughout all points in time with no correlation between Y-chromosomal and total nuclear cfDNA (r = 0.29;

Table 3) indicating that neither liver cells in general nor transplanted cells in particular contribute to cfDNA concentrations before exercise. Physical exercise exerts numerous effects on haematopoietic cells, as reflected by transient lymphocytosis, neutrophilia, monocytosis and activation of leukocytes and platelets (12, 45). Acute exercise and mitogen- and antigen-stimulation could activate T-cells (45). It has been reported that lymphocytes secrete DNA *in vitro* in response to mitogen-stimulation or in the presence of antigens (14). Interestingly, T-cell derived but no endothelial-cell specific DNA could be detected in the plasma of cancer patients (22). However, enucleation of erythroblasts during erythropoiesis could also contribute to the cfDNA pool in blood plasma (31). Concerning the release mechanisms, Breitbach *et al.* discussed that composite effects of different physiological stress parameters under exercise conditions could be responsible for increasing cfDNA levels (10). The authors suggest that, due to acute stress, cfDNA concentrations increase rapidly by a spontaneous unknown active or passive release mechanism (10). A further explanation could be the active release of cfDNA via neutrophil extracellular traps (NETs) (11). A current study has shown that post-exercise blood contains NET-like structures (7). They observed morphologic signs of NETosis in blood smear samples and found a striking correlation of cfDNA levels with the granule-derived enzyme myeloperoxidase in human plasma (7). Our results indicate that cells from the haematopoietic lineage respond to physical exercise by rapidly releasing DNA in the circulation, although the contribution of different cell subsets is currently unknown.

The results also permit some conclusions concerning the clinical use of cfDNA. Total baseline cfDNA concentrations are higher in the transplantation patients compared to healthy individuals. Increased levels of cfDNA concentrations in other pathological conditions were already shown in several studies (1, 20, 36, 37). Higher levels of cfDNA in the transplant could be due to infections or unspecific activation of the innate immune system, but may also occur due to acute transplant rejection or graft damage. Three of our patients (HSCT4, HSCT5 and HSCT7) suffered from graft-versus-host disease (GVHD), three from infections (HSCT2, HSCT4 and HSCT7), one from a relapse (HSCT3) and one HSCT patient (HSCT1) who had neither an infection, nor a GVHD, had very high plasma hsCRP values (> 40ng/ml). Unfortunately, given this situation it was not surprising that neither high total nuclear nor high Y-chromosomal cfDNA values could be clearly attributed to one of the three factors. HSCT3, who suffered a relapse, showed higher total cfDNA levels and lower proportions of donor-derived DNA than the relapse-free patients. This could be due to the fact that more host-derived blood cells were released from the bone marrow in the circulation with a concomitant reduction of donor-specific cells. The question whether elevated host- or donor-derived cfDNA concentrations in HSCT patients could serve as a biomarker was beyond the scope of this pilot study and should be analysed in larger patient populations. Higher levels of donor-specific DNA in three LT patients (LT2-4) could be a result of tissue damage in the transplanted organ and, possibly, of emerging graft rejection at a low level. However, none of the LT patients had clinical signs of acute or chronic rejection at the time of examination. Long-term monitoring would be necessary to elucidate if the concentration of liver-specific DNA in blood plasma rises prior to the first clinical signs of transplant rejection. However, given the high sensitivity and rapid dynamics, graft-derived cfDNA could indicate graft injury earlier compared to conventional markers (24). In a kinetic study using a renal allograft model in the rat, donor-derived DNA concentrations peaked shortly before acute rejection (29). The measurement of donor-specific cfDNA could therefore be used as a clinical marker for the detection and monitoring of rejection and the evaluation of relapse after transplantation (5, 26, 27, 38, 46).

Our study has several limitations. We only detected a mean of 46.6% of donor DNA in the female HSCT plasma instead of values close to 90% (assuming that 10% of cfDNA in plasma is derived from other tissues or organs). This could be due to technical reasons, such as the underestimation of DNA concentrations determined with the Y-qPCR assay. However, the percentage of donor-derived cfDNA could reflect the success of grafting. Therefore, the percentage of donor-derived cfDNA in blood plasma should be compared to the chimerism found in different compartments (e.g. bone marrow, full blood, different blood cell types). Unfortunately, the chimerism results presented here (see Table 3) are incomplete and retrospective. Since the percentage of donor-derived cells changes continuously, chimerism has to be assessed at the day of the exercise test, ideally at every point in time. Furthermore, the analysis of chimerism in specific subsets of blood cells, such as granulocytes, lymphocytes, monocytes or reticulocytes could decipher the cellular origin of cfDNA more precisely. A further limitation of our study is the fact that only sex-mismatched transplantation patients can be analysed with our qPCR system. When studying non sex-mismatched patients different methods, based on distinct individual sequence differences, must be implemented.

Taken together, our results suggest that cfDNA released during acute bouts of exercise mainly originated from cells of the haematopoietic linage. In future, questions like the use of host- or donor-derived cfDNA concentrations as a biomarker in transplant patients should be analysed in larger patient populations. In addition, various transplant patients could be analysed to determine a possible involvement of other organs to the cfDNA pool after physical exercise. Elucidating these mechanisms is important in terms of the physiological role and, consequently, evaluating the validity of cfDNA as a biomarker for exercise and clinical diagnostics.

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