Changes in plasma concentration of cell-free DNA as a response to physical activity

Keywords
physical activity, mitochondria, nucleus, cell-free DNA

Abstract

Introduction

Plasma concentrations of cell-free DNA (cfDNA) serve as markers of overtraining or muscle injury. We have examined, if nuclear (n) and mitochondrial (mt) cfDNA has the potential as a marker of muscle burden or damage.

Material and methods

Ten healthy, physically active volunteers (6 females, aged 27.1±6.8 years) performed a downhill running test. Samples for cfnDNA and cfmtDNA analysis were collected before, 30 minutes, 1 hour, and 14 days after the downhill run. CfnDNA and cfmtDNA (two markers for both) were analysed using qPCR.

Results

There was an extreme (~ 40-times) increase in cfnDNA at the 30-min time-point against the baseline ($p<0.00001$ for both markers), followed by a quick drop to baseline levels after 1 hour after the end of the downhill run for all subjects. In contrast, plasma levels of cfmtDNA did not increase significantly ($p=0.27$ and 0.12). It reflects the fact, that in 6 subjects, the pattern was similar as for cfnDNA but in 4 subjects rather a decrease of cfmtDNA concentration was observed at the 30-mi time-point. These differences correlate with age, BMI, and sex of the participants. Plasma cfnDNA significantly ($p<0.01$ for all) correlated with concentrations of muscle damage markers such as AST, ALT, and lactate dehydrogenase, and chemokines MIP-1α and IP-10 (positive). No homogenous correlation between cfmtDNA and biomarkers was detected.

Conclusions

Our study confirms the extreme release and clearance of cfnDNA in physically active subjects after strenuous exercise. In contrast, the trajectory of cfmtDNA concentrations seems to have much higher inter-individual variability than cfnDNA concentrations.

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Key words: cell-free DNA; physical activity, nucleus, mitochondria

List of Abbreviations

cfnDNA	– cell-free nuclear DNA
cfmtDNA	– cell-free mitochondrial DNA
ALT	– alanine aminotransferase
AST	– aspartate aminotransferase
BMI	– body mass index
CK	– creatine kinase
CRP	– C-reactive protein
$Fatigue_{end}$	– level of fatigue on a visual analog scale (0-10) after the end of the downhill run
HR_{end} / HR_{max} (%)	– percentage of the maximum heart rate reached at the end of the downhill run
HR_{max}	– maximum heart rate determined at the pre-test
IL	– interleukin
IP-10	– interferon gamma-induced protein 10 (CXCL10)
LD	– lactate dehydrogenase
MCP-1	– monocyte chemoattractant protein 1 (CCL2)
Mgb	– myoglobin
MIP-1α	– macrophage inflammatory protein 1 alpha (CCL3)
MIP-1β	– macrophage inflammatory protein 1 beta (CCL4)
$Pain_{end}$	– level of pain on a visual analog scale (0-10) after the end of the downhill run
PDGF	– platelet-derived growth factor
qPCR	– quantitative polymerase chain reaction

RANTES – regulated on activation/normal T cell expressed and secreted (CCL5)

TNF – tumor necrosis factor

$V_{baseline}$ – speed of running at the beginning of the downhill run

V_{end} – speed of running at the end of the downhill run

V_{max} – maximum speed of running determined at the pre-test

VO_2max – maximal oxygen consumption/uptake/maximal aerobic capacity

VO_{2max} SDS – VO_{2max} standard deviation score

Introduction

Circulating cell-free DNA (cfDNA) represents a new diagnostic marker in many medical fields or biomedical disciplines as in cancer research [1], as a tool for non-invasive prenatal genetic testing [2] or a marker of organ failure [3].

Sports medicine is the field where cfDNA analysis seems to be of extreme interest [4]. Increases in plasma cfDNA after exercise or physical activity have been repeatedly described [5-8] and have been suggested to be a potential hallmark of overtraining [9]. cfDNA concentration increases immediately after exercise and remains elevated for a short time (30-120 minutes), with quick normalisation thereafter [10]. So far, predominantly cfDNA with origin in the nucleus (cfnDNA) has been analysed in sport-related studies.

CfDNA could be detected in all body fluids [11]. Most commonly, cfDNA isolated from plasma is used for the screening. Circulating cfDNA is represented by short (generally 100-180 bp long) double-stranded DNA fragments, mainly derived from cellular apoptosis or necrosis. Active secretion of short DNA fragments has also been described. Low concentrations of cfnDNA (~ 1000-2500 genome equivalent/mL or 0.1-100 ng/mL) in plasma are physiological [12, 13]; high plasma cfDNA concentrations are widely discussed as a biomarker of inflammation, tissue injury or trauma [14].

Two types of internal cfDNA (external bacterial cfDNA could also be detected in plasma [15]) need to be distinguished: cfDNA originating from the nucleus (cfnDNA) and cfDNA with origin from mitochondria (cfmtDNA). As there are commonly several hundreds of mitochondria per one cell (and just one nucleus), the amount of released cfmtDNA in body fluids is expected to be much higher in comparison with cfnDNA. Similarly, also the variability of plasma concentration of cfmtDNA would probably be much higher, as a

consequence of uneven numbers within the cells and the fact, that number of mitochondria could be influenced by the training status, fitness level and type of the exercise [16].

As the so far performed studies focused mostly on cfDNA of nuclear origin, the aim of our study was to analyse the short-time dynamics of plasma cfDNA concentrations, both of nuclear and mitochondrial origin, in a group of physically active healthy subjects after acute strenuous physical activity.

Methods

Participants

The studied cohort comprised 10 healthy, physically active Caucasian volunteers (6 females, 4 males; non-smokers, aged 27.1±6.8 years, BMI 21.9±2.2 kg/m^2) who signed an informed consent and completed a downhill running protocol. Samples of peripheral blood were collected at baseline, 30 min, 1 h, and 14 days after the exercise. Exclusion criteria were diagnosis of any serious disease, history of any musculoskeletal, rheumatic, or autoimmune disease, prescription of any regular medication, pre-/post-test activity limitations as were described in detail previously [17], and finally, non-availability of all four plasma samples in sufficient quality (based on the test for haemolysis).

Ethics

This study was approved by the Ethics Committee of the Institute of Rheumatology in Prague (ID 5689/2015, 1446/2014) and the Department of Rehabilitation and Sports Medicine, Charles University and University Hospital Motol in Prague (ID EK-939/18).

Downhill running protocol

The complete downhill running protocol was described in our previous study [17]. Briefly, all participants underwent two exercise stress tests separated by seven days using a motor-driven treadmill (Valiant Plus (P/N 932902) with Rear Elevation −10% mechanical, Valiant, Lode, Netherlands): (i) a maximal incremental test without inclination (slope 0%) to estimate the aerobic capacity and target exercise intensity for the second test; (ii) the intervention itself, i.e. downhill run at a constant speed corresponding to the intensity of 60% of maximal oxygen consumption (VO_2max), with a negative slope (−10%) for 45 min. Further details were described previously [17].

Peripheral blood samples preparation

Peripheral blood samples were collected into EDTA-treated tubes for plasma preparation and BD Vacutainer SST II Advance tubes for serum collection. Baseline samples and those obtained 14 days post-intervention were centrifuged within 30 minutes of collection. Samples collected at 30 minutes and 1 hour post-intervention were transported on ice (4–8 °C) and were centrifuged within 1 hour. All samples were centrifuged at 3,500 rpm for 10 minutes at 10 °C. Separated serum and plasma samples were aliquoted and stored at –80 °C until further analysis.

Analysis of serum markers of muscle damage and inflammation

Serum levels of traditional markers of inflammation [C-reactive protein (CRP)], and muscle damage [aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK), lactate dehydrogenase (LD), and myoglobin (Mgb)] were analyzed

using Beckman CoulterAU 680 analyzer (Beckman Coulter, USA), as described elsewhere [18].

Serum levels of the following inflammatory cytokines/chemokines were measured by a commercially available Bio-Plex ProTM human Cytokine 27-plex Assay (BIO-RAD, Hercules, CA, USA): interleukin (IL)-1β, lL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17A, eotaxin, fibro-blast growth factor (FGF) basic, granulocyte colony-stimulating factor (G-CSF), granulo-cyte-macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), CXCL10, monocyte chemoattractant protein (MCP-1 alias CCL2), macrophage inflammatory protein (MIP-1α alias CCL3), MIP-1β (also known as CCL4), platelet-derived growth factor (PDGF)-BB, regulated on activation/normal T cell expressed and secreted (RANTES alias CCL5), tumor necrosis factor (TNF), and vascular endothelial growth factor (VEGF). The absorbance of the Bio-Plex ProTM human Cytokine 27-plex Assay was evaluated by Luminex BIO-PLEX 200 System (Bio-Rad, Hercules, CA, USA) [18].

DNA analyses

Cf-DNA was extracted from plasma with Plasma/Serum Cell-Free Circulating DNA Purification Mini Kit (Norgen Biotek, Canada) according to the manufacturer's instructions. The plasma volume of 1000-1500 µL was processed and the elution volume was 45µL.

Two assays specific to human genomic DNA have been used. Amplicon sizes were 129bp (gene ID 79068, referred to as G1; oligo sequences 5'GAGGAGGAGATTGTGTAACTGGA3' and 5'ATCTATTAAAGGAGCTGGACTGTT3') and 136bp (gene ID 3569, referred to as G2; oligo sequences 5'

AGTAAAGGAAGAGTGGTTCTGCT3' and 5'TGGGGCTGATTGGAAACCTTATT3') respectively.

Two assays specific to human mitochondrial DNA have been used. Amplicon sizes were 93bp (gene ID 4540, referred to as G3; oligo sequences 5' ACCGCTAACAACCTATTCCAAC3' and 5' ATCTGCTCGGGCGTATCATC) and 132bp (gene ID 4514, referred to as G4; oligo sequences 5' CAGGCTTCCACGGACTTCAC3' and 5'CAAAATGCCAGTATCAGGCGG3').

The qPCR was performed in duplicates using the TATAA SYBR® GrandMaster® Mix (TATAA Biocenter, Sweden). The total volume of the reaction was 10µL, the final concentration of the primers was 400nM and the template volume in one reaction was 2µL. The qPCR was performed applying CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA) under the following conditions: pre-denaturation: 95°C for 30 sec; amplification: 45 cycles at 95°C/5 sec followed by 60°C/30 sec and 72°C/10 sec for cfnDNA and 95°C/30 sec; amplification: 35 cycles at 95°C/5 sec followed by 60°C/30 sec and 72°C/10 sec for cfmtDNA.

All assays were validated according to the MIQE guidelines and MIQE assay disclosure. The standard curves and resulting slopes have been used for calculations. The efficiency reached was 100,8% for G1, 100% for G2; 99,8% for G3 and finally 100% for G4.

Statistical analyses

Statistical analysis was performed using STATISTICA 12 (StatSoft, Tulsa, USA). The descriptive statistics are presented as mean ± standard deviation (SD) or median (interquartile range). The graphs were created by GraphPad Prism 5 (version 5.02;

GraphPad Software, La Jolla, CA, USA). Differences between female and male participants were assessed by the Mann–Whitney U test. Repeated measurements were assessed by the Friedman test followed by post-hoc Dunn's test. The relationships between two continuous variables were evaluated by Pearson's correlation coefficient (univariate analysis). P-values less than 0.05 were considered statistically significant.

Given the modest sample size of only 10 participants utilized in the multiple regression models presented in Table 5, there are inherent risks of overfitting. To mitigate these risks, several measures were taken. Firstly, the number of predictors was deliberately limited to essential variables supported by theoretical underpinnings and preliminary analyses. Secondly, we conducted thorough assessments of collinearity among predictors using correlation coefficients to ensure that multicollinearity did not unduly influence the regression estimates.

Results

Intra-correlations between cfDNA markers

There was a strong positive correlation between the baseline levels of both cfnDNA markers ($r=0.980$, $p<0.0001$), as well as between their increase at 30 min after the end of the downhill run compared to baseline ($r=0.998$, $p<0.0001$).

Similarly, there was a strong positive correlation between the baseline concentrations of cfmtDNA G3 and G4 ($r=0.888$, $p<0.001$), and between their change at 30 min after the exercise compared to baseline ($r=0.945$, $p<0.0001$).

Of importance, there was no significant correlation between the baseline levels of cfnDNA markers (G1 and G2) with cfmtDNA markers (G3 and G4) ($p>0.05$ for all four comparisons) suggesting the independence of nuclear and mitochondrial markers.

Changes in cfDNA concentrations

The concentrations of cfnDNA markers uniformly and significantly increased at 30 min after the exercise compared to baseline (43-fold for G1 and 34-fold for G2). At 1h and 14 days after the end of the downhill run, values were comparable to baseline (Figure 1A, 1B).

Surprisingly, the analysis of both cfmtDNA markers (G3 and G4) revealed a numerical increase in 6 and a decrease in 4 participants at 30 min after the end of the downhill run compared to baseline (mean increase 3-fold for both) with a decrease to concentrations comparable to baseline at 1 hour and 14 days after the exercise (Figure 1C, 1D).

Of note, when comparing absolute concentration changes in males (n=4) and females (n=6), males had numerically larger increases at 30 min from baseline for both cfnDNA (1.5- and 1.4-fold higher, respectively), as well as for cfmtDNA markers (3.3- and 5.0-fold higher, respectively).

Correlations of cfDNA with markers of muscle damage

When analysing the dynamics of serum markers of muscle damage, we observed a significant change in LD ($p<0.001$) and myoglobin ($p<0.001$) (Table 1), with a significant increase 30 min and 1 hour after the exercise and a return to baseline levels at 14 days after the downhill run. With one exception (CK at 30 min compared to baseline; $p<0.05$), we did not detect any significant overall changes in the levels of the remaining serum markers of muscle damage (Table 1). Similarly, we did not detect any significant overall changes in serum markers of inflammation such as CRP or any of the inflammatory chemokines/cytokines with measurable levels in at least 7/10 participants (Table 1).

Univariate analysis of potential relationships between the change (30 min–baseline) in cfnDNA concentrations and biomarkers of muscle damage and inflammation, and selected anthropometric and functional parameters revealed significant positive correlation of both cfnDNA markers with AST, ALT, LD (Table 2), MIP-1α, IP-10 (Table 3) and a negative correlation with the difference between the speed of running at the start and end of the downhill run ($V_{baseline} – V_{end}$) (Table 4). Univariate analysis of potential relationships between the change (30 min – baseline) in levels of cfmtDNAs demonstrated a significant positive correlation with MCP-1 and PDGF (Table 3) as well as with BMI (Table 4).

Lastly, parameters from univariate analyses with $p<0.25$ were taken into a multivariate logistic regression analysis which provided the final four models of the significant predictors of plasma concentrations development of the cfDNA at 30 min after the end of the downhill run compared to baseline. This analysis revealed that the strongest predictors of the concentration increase in cfnDNA G1 and G2 were the increase in the speed of running ($V_{baseline} – V_{end}$), male sex and the increase in LD and CRP levels. A larger increase in CRP levels and higher BMI were the strongest predictors of the concentration increase in both cfmtDNA markers (G3 and G4). In addition, larger increases in LD levels and higher age were also significantly associated with the increase of the cfmtDNA G3 concentration (Table 5).

Discussion

We have highlited differences between the development of plasma concentrations of nuclear and mitochondrial cfDNA concentrations in young physically active Caucasian subjects. Concentrations of cfnDNA increased dramatically after intensive physical activity

and the development profile of concentrations was extremely uniform among individuals. In contrast, fluctuation of cfmtDNA was far less dramatic and was observed in only about 60% of subjects, other subjects did not increase the concentrations of cfmtDNA significantly. As expected the amount of detected cfmtDNA was 100-1000-fold higher than concentrations of cfnDNA.

In general, massively increased plasma levels of cfDNA have been described after both intensive or endurance activity and independent to wether aerobic or anaerobic activity has been applied [19-21]. The increase is very quick, as well as the decline to the baseline after the end of the exercise. It is worth mentioning that in professional sportsmen, levels of plasma cfDNA remain slightly increased over baseline longer time after the activity [6, 7]. In professional football players, cfnDNA increased dramatically after the season game (approximately 23-fold) [6] and remained significantly (albeit only ~1.5-fold) increased one day after the game [7]. CfnDNA also correlates with training. In identical subjects, sprint training sessions pronounced higher cfDNA concentrations in the case of long (5 min) in comparison with short (1 min) pauses. Out of all biomarkers analysed (e.g., IL-6, CRP, or CK) only concentrations of cfnDNA correlate with the distance covered during the game.

Interestingly, it cannot be excluded, that the massive peak of the cfnDNA after exercise is possibly detectable in trained subjects only. For example, untrained but physically active male controls reveal a gradual decrease in plasma cfnDNA (the highest concentration has been detected before the exercise) [22] after maximal aerobic and anaerobic tests in contrast to professional athletes. On the other hand, it has been described that cfnDNA increase has an inverse relationship to individual training levels [23].

It is possible, that trained subjects developed some kind of adaptation, which allows the organism to more efficient cfnDNA excretion, whose role in details remains unknown.

Sugasawa *et al.* [8] performed a next-generation sequencing of plasma cfnDNA in marathon runners and reported that not all DNA fragments increase the concentrations uniformly. The extreme marker originating from chromosome 1 occurs in 16,000-fold abundance compared to markers from other genomic regions. The cfDNA fragments identified seem to be highly sensitive markers of extreme physical stress.

It has also been reported (for review see 4), that cfnDNA correlates with other metabolic markers of muscle damage. Our findings further support this, as we have detected a strong correlation of both cfnDNA markers with lactate dehydrogenase but not with creatine kinase, which has been correlated with cfnDNA in some [24] but not all [5,25] previous studies. Although no significant change was detected in AST or ALT on the group level, slight changes on the individual level were associated with robust uniform changes in cfnDNA markers. Additionally, two serum biomarkers of inflammation (MIP-1α and IP-10) have been also consistently associated with plasma cfnDNA markers in our study.

A different pattern has been observed in the case of cfmtDNA markers. There was no association with any markers of muscle damage. A slight correlation has been, however, found between cfmtDNA concentrations and CRP, MCP-1, and PDGF. It is important to note, that these markers don't overlap with inflammatory markers associated with cfnDNA.

Nonetheless, it can be concluded, that post-exercise development of plasma concentrations of cfnDNA is generally uniform, with a high increase after exercise and a quick decrease afterward, albeit some exceptions exist and some unclarities remain to be

elucidated. The observed robust increase in cfnDNA markers might lack specificity due to several potential cellular sources of release upon various forms of stress or pathological conditions, but might provide a much higher sensitivity to even milder affection of skeletal muscles as evidenced by a 40-fold increase in cfnDNA markers compared to 35% increase in CK, 32% in LD and 220% in myoglobin levels in our study.

Post-exercise development of plasma cfmtDNA seems to be an independent model. In a majority of our subjects, cfmtDNA increases 30 minutes after the downhill run, but not to such an extent as cfnDNA. It is a rather surprising finding, as the number of mitochondria per cell is usually several hundreds, and the potential for release of the mtDNA seems to be much higher than in the case of nDNA. One of the possible explanations could be the fact, that nDNA release (as a response to the physical performance) is an active and highly regulated process, but the mtDNA release is based rather on catabolic processes and circulating cfmtDNA reflects a passive leakage from broken mitochondria [26]. Moreover, post-hoc power analysis confirmed that our study was underpowered to detect statistically significant changes in G3 and G4.

In an animal model [27], it has been shown that mitochondrial membrane permeability significantly increases after downhill running, reaching the peak 12 hours after exercise. The simultaneous relatively slow increase of the DNA oxidative damage marker observed in this study supports the idea, that also skeletal muscles are negatively affected.

Not only that number of mitochondria is not identical in different types of cells [28]. Their number is very variable also over time and some variability has been described even in the cells from identical tissues [29, 30]. Further, the number of mitochondria increases as a response to the training [16, 31], or with the age of the subject [32]. This variability

might be reflected in the variability of the plasma concentration of the cfmtDNA, and might support our findings of age and BMI being the important predictors of variability in cfmtDNA.

To the best of our knowledge, there are only a few studies focused on post-exercise cfnDNA as well as on cfmtDNA simultaneously with conflicting results. Similarly to our study, males after short-term treadmill exercise did not show increased cfmtDNA immediately after the excercise [33]. Healthy volunteers reveal a slight steady increase of plasma cfmtDNA until 90 minutes after the controlled ergo-spirometry cycle test [34] as well as after incremental treadmill exercise [35]. A slight increase of cfmtDNA has been observed in moderately trained young men immediately after treadmill exercise [36].

Our study has several limitations. One limitation of the study is the small sample size of only 10 participants. This sample size increases the risk of not detecting a true effect when it does exist, known as a false negative. Additionally, the limited number of participants may compromise the generalizability of the findings to a broader population, as it may not accurately represent the characteristics or behaviours of a larger group. Secondly, future studies should include also other forms of exercise, a control physically inactive group, and additional sampling time points to better elucidate the relationship between cfDNA and inflammatory markers, thereby supporting the generalizability of our findings for clinical practice. Also the sex needs to be taken into account in future studies, as we have potentially observed differences (especially in cfmtDNA concentrations) between males and females.

Our downhill run protocol was designed to mimic real-life muscle strain/damage induced by acute submaximal excentric exercise in physically active healthy volunteers, thus our findings cannot be generalizable to other physical activities (as for example

resistance training or interval sprints). Our original cohort of 18 volunteers [17] was significantly reduced to 10 due to the non-availability of all four plasma samples in sufficient quality. Nevertheless, this limited number of participants provided homogeneous results with respect to an increase in concentrations of cfDNA markers 30 min after exercise and a quick decrease to normal levels afterwards, with the exception of cfmtDNA in 4 volunteers. The PCR detection error and the methodology of preselection of several multicollinear variables for our regression analysis models might have led to slightly different outcomes for the most significant predictors of variability of cfnDNA and cfmtDNA, respectively.

Despite the interest and high potential in defining and planning training loads, analysis of cfDNA is not widely used as it has its pitfalls for several reasons. As cfDNA degrades rapidly, standardised pre-analytical and analytical requirements will need to be defined in the future. At the same time, however, it must be taken into account that the collection and subsequent processing of samples from athletes "in the field" is much more difficult than in clinical studies. Albeit the “normal” values are published and generally accepted, they shall be considered rather as estimates, as no epidemiological study of plasma cfDNA concentrations, neither nuclear nor mitochondrial, has been reported. Sport-related studies have been based on longitudinal data, where data are collected at a time scale at several points. It makes it possible to partially ignore the fact that comparison of cfDNA between subjects is challenging mostly due to the high interindividual variability.

It is important to note, that studies published to date are difficult to compare. Not only technical differences during sample treatment and DNA isolation and methods used

for screening but also different types and durations of physical activity should not be overlooked. Thus, a large uniform future studies are warranted.

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Table 1. Systemic levels of markers of muscle damage, CRP, and selected cytokines/chemokines before and after the downhill run.

Parameter	Baseline	30 min	1 hour	14 days	p^{12} ;p^{13}; p^{14}; p^{24}; p^{34}; p*
AST, µkat/L	0.38 (0.31-0.41)	0.39 (0.34-0.45)	0.38 (0.32-0.45)	0.36 (0.33-0.37)	all n.s.
ALT, µkat/L	0.35 (0.29-0.50)	0.34 (0.25-0.52)	0.38 (0.20-0.49)	0.34 (0.23-0.44)	all n.s.
CK, µkat/L	1.93 (1.36-3.44)	2.60 (1.49-3.28)	2.52 (1.72-4.05)	2.20 (1.60-2.74)	0.37; **0.02**; 0.86; 0.79; 0.29; 0.25
LD, µkat/L	2.76 (2.33-3.22)	3.64 (2.83-4.25)	3.44 (2.71-3.93)	2.65 (2.50-3.01)	**0.003**; **0.007**; 0.56; **0.002**; **0.020**; **0.001**
Myoglobin, µg/L	50.5 (41.7-58.2)	162 (136-202)	260 (195-367)	48.2 (42.0-57.5)	**0.001**; **0.001**; 0.52; **0.001**; **0.001**; **0.001**
CRP, mg/L	0.44 (0.23-1.32)	0.45 (0.18-0.98)	0.47 (0.17-0.81)	0.47 (0.24-0.70)	all n.s.
IL-9, pg/mL	78.6 (52.1-103.0)	84.0 (73.4-102.7)	81.5 (64.6-99.3)	91.3 (66.2-109.8)	all n.s.
MIP-1α, pg/mL†	1.02 (1.02-1.79)	1.49 (1.02-1.89)	1.02 (0.36-1.65)	1.02 (1.02-1.37)	all n.s.
MIP-1β, pg/mL	163 (108-210)	175 (137-213)	173 (110-207)	183 (126-227)	all n.s.
MCP-1, pg/mL†	8.92 (4.39-15.75)	11.00 (9.55-17.30)	10.59 (8.71-14.72)	9.34 (5.28-11.95)	all n.s.
RANTES, ng/mL	3.79 (2.07-8.00)	4.36 (2.97-5.90)	4.25 (2.96-5.27)	4.56 (3.32-8.90)	all n.s.
TNF, pg/mL†	26.3 (21.2-30.1)	33.9 (26.3-37.5)	26.3 (23.8-35.1)	36.3 (27.6-42.4)	all n.s.
Eotaxin, pg/mL	39.9 (25.7-53.2)	44.1 (29.9-49.4)	35.3 (28.5-43.2)	36.1 (25.7-49.0)	all n.s.
IP-10, pg/mL	331 (244-384)	250 (192-391)	252 (172-378)	331 (236-412)	all n.s.
PDGF, pg/mL†	189 (85-316)	1,563 (878-2,111)	362 (143-740)	223 (177-327)	0.97; 0.41; 0.37; **0.04**; 0.72; 0.41

Data are presented as median (interquartile range). Statistically significant differences ($p<0.05$) are marked in bold: p^{12}, baseline vs 30 min; p^{13}, baseline vs 1 hour; p^{14}, baseline vs 14 days; p^{24}, 30 min vs 14 days; p^{34}, 1 hour vs 14 days; p*, Friedman test; n.s., not significant;

†, data available only for 9 (MCP-1, TNF), 8 (MIP-1 α) and 7 (PDGF) participants

AST- asphartataminotransfrase; ALT – alaninaminotransferase; CK – creatine kinase; LD – lactatedehydrogenase; CRP – C reactive protein; IL – interleukine; MIP – macrophage inflammatory protein; MCP – monocyte chemoattractant protein; RANTES - Regulated on Activation, Normal T-cell Expressed and Secreted protein; TNF – tumor necrosis factor; IP-10 – interferon gamma-induced protein 10; PDGF – platelet derived growth factor)

Table 2. Univariate relationships of the change in cfDNA concentrations with the change in serum biomarkers of muscle damage (30 minutes after the end of the downhill run vs. baseline).

		AST	ALT	CK	LD	Mgb
cfnDNA G1	r	**0.672**	**0.707**	0.504	**0.810**	0.198
	n	**10**	**10**	10	**10**	10
	p	**0.03**	**0.02**	0.14	**0.004**	0.58
cfnDNA G2	r	**0.702**	**0.717**	0.520	**0.843**	0.204
	n	**10**	**10**	10	**10**	10
	p	**0.02**	**0.02**	0.12	**0.002**	0.57
cfmtDNA G3	r	0.318	0.537	0.049	0.533	0.315
	n	10	10	10	10	10
	p	0.37	0.11	0.89	0.11	0.38
cfmtDNA G4	r	0.168	0.438	-0.126	0.360	0.369
	n	10	10	10	10	10
	p	0.64	0.21	0.73	0.31	0.29

Statistically significant (Pearson's correlation coefficient) differences ($p<0.05$) are highlighted in bold.

Effect size for Pearson's correlation coefficient (r): absolute value of r (effect size) 0.0-0.1 (negligible), 0.1-0.3 (small), 0.3-0.5 (moderate), 0.5-1.0 (strong).

AST- asphartataminotransfrase; ALT – alaninaminotransferase; CK – creatine kinase; LD – lactatedehydrogenase; Mgb – myoglobin

Table 3. Univariate relationships of the change in cfDNA concentrations with the change in selected serum biomarkers of inflammation (30 minutes after the end of the downhill run vs. baseline; evaluated by Pearson's correlation coefficient).

		CRP	IL-9	MIP-1α	MIP-1β	MCP-1	RANTES	TNF	Eotaxin	IP-10	PDGF
cfnDNA G1	r	0.501	0.118	**0.878**	0.016	0.203	0.260	0.432	-0.213	**0.653**	0.272
	n	10	10	**8**	10	9	10	10	10	**10**	7
	p	0.14	0.74	**0.004**	0.96	0.60	0.47	0.21	0.55	**0.04**	0.55
cfnDNA G2	r	0.484	0.113	**0.869**	0.003	0.217	0.262	0.439	-0.194	**0.655**	0.288
	n	10	10	**8**	10	9	10	10	10	**10**	7
	p	0.156	0.756	**0.005**	0.99	0.575	0.46	0.20	0.59	**0.04**	0.53
cfmtDNA G3	r	**0.705**	0.194	0.503	0.233	**0.662**	0.520	0.528	-0.126	0.393	**0.782**
	n	**10**	10	8	10	**9**	10	10	10	10	**7**
	p	**0.02**	0.59	0.20	0.51	**0.05**	0.12	0.12	0.73	0.26	**0.04**
cfmtDNA G4	r	0.595	0.234	0.256	0.302	**0.671**	0.515	0.441	-0.125	0.155	**0.752**
	n	10	10	8	10	**9**	10	10	10	10	**7**
	p	0.07	0.52	0.54	0.40	**0.05**	0.12	0.20	0.73	0.67	**0.05**

Statistically significant differences ($p<0.05$) are highlighted in bold.

Effect size for Pearson's correlation coefficient (r): absolute value of r (effect size) 0.0-0.1 (negligible), 0.1-0.3 (small), 0.3-0.5 (moderate), 0.5-1.0 (strong).

CRP – C reactive protein; IL – interleukine; MIP – macrophage inflammatory protein; MCP – monocyte chemoattractant protein; RANTES - Regulated on Activation, Normal T-cell Expressed and Secreted protein; TNF – tumor necrosis factor; IP-10 – interferon gamma-induced protein 10; PDGF – platelet derived growth factor)

Table 4. Univariate relationships of cfDNA concentrations with selected anthropometric and functional parameters evaluated by Pearson's correlation coefficient

		Age	BMI	V_{max}	$V_{baseline}$	V_{end}	$V_{baseline} - V_{end}$	HR_{max}	HR_{end} / HR_{max} (%)	VO_{2max}	VO_{2max} SDS	Pain $_{end}$	Fatigue $_{end}$
cfnDNA G1	r	-0.247	0.320	-0.272	-0.078	-0.505	**-0.831**	0.342	0.529	0.123	-0.433	0.126	0.195
30 min-	n	10	10	10	10	10	**10**	10	10	10	10	10	10
baseline	p	0.49	0.37	0.45	0.83	0.14	**0.003**	0.33	0.12	0.73	0.21	0.73	0.59
cfnDNA G2	r	-0.243	0.333	-0.295	-0.094	-0.512	**-0.824**	0.331	0.538	0.108	-0.450	0.108	0.217
30 min-	n	10	10	10	10	10	**10**	10	10	10	10	10	10
baseline	p	0.50	0.35	0.41	0.80	0.13	**0.003**	0.35	0.11	0.77	0.19	0.77	0.55
cfmtDNA G3	r	-0.435	**0.757**	0.062	0.265	0.041	-0.267	0.572	0.462	0.329	-0.559	-0.464	-0.055
30 min-	n	10	**10**	10	10	10	10	10	10	10	10	10	10
baseline	p	0.21	**0.011**	0.86	0.46	0.91	0.46	0.08	0.18	0.35	0.09	0.18	0.88
cfmtDNA G4	r	-0.483	**0.822**	0.223	0.417	0.306	0.023	0.596	0.282	0.425	-0.464	-0.567	-0.207
30 min-	n	10	**10**	10	10	10	10	10	10	10	10	10	10
baseline	p	0.16	**0.004**	0.56	0.23	0.39	0.95	0.07	0.43	0.22	0.18	0.09	0.57

Statistically significant differences ($p<0.05$) are highlighted in bold.

Effect size for Pearson's correlation coefficient (r): absolute value of r (effect size) 0.0-0.1 (negligible), 0.1-0.3 (small), 0.3-0.5 (moderate), 0.5-1.0 (strong).

BMI – body mass index; V – speed of running; HR – heart rate; VO_2 – oxygen consumption/uptake/maximal aerobic capacity; SDS – standard deviation score

Table 5. Multivariate regression analysis models - Significant associations of cfDNA concentrations and selected biochemical, anthropometric and functional parameters

DNA concentration	Laboratory, anthropometric and functional parameters	b	p-value	Adjusted R^2
cfnDNA G1 30 min-baseline	$V_{baseline} - V_{end}$	-12,226	<0.001	0.831
	Female sex	-4,822	0.01	
	CRP $_{30\ min - baseline}$	14,454	0.02	
	LD $_{30\ min - baseline}$	9480	0.04	
cfnDNA G2 30 min-baseline	$V_{baseline} - V_{end}$	-7,859	<0.001	0.814
	Female sex	-3,028	0.02	
	LD $_{30\ min - baseline}$	9,281	0.03	
	CRP $_{30\ min - baseline}$	10,557	0.04	
cfmtDNA G3 30 min-baseline	CRP $_{30\ min - baseline}$	804,947	<0.001	0.977
	BMI	114,347	<0.001	
	LD $_{30\ min - baseline}$	119,340	0.03	
	Age	9,438	0.04	
cfmtDNA G4 30 min-baseline	BMI	126,657	<0.001	0.887
	CRP $_{30\ min - baseline}$	579,582	0.003	

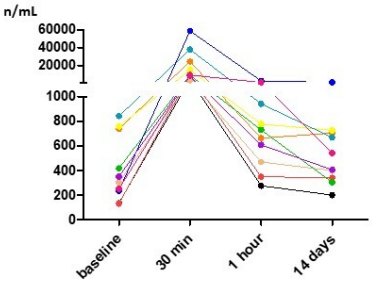
Note: b, regression beta coefficient; adjusted R^2, adjusted coefficient of determination

V – speed of running; BMI – body mass index; CRP – C reactive protein; LD – lactatedehydrogenase

physically active volunteers
6 females, 4 males
aged 27.1±6.8 years
BMI 21.9±2.2 kg/m^2

45 min downhill run
-10% slope
constant speed corr. to 60% of VO_2max

Plasma cf-nuclear DNA

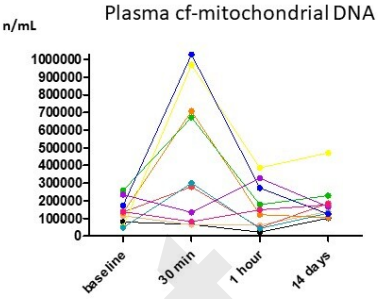


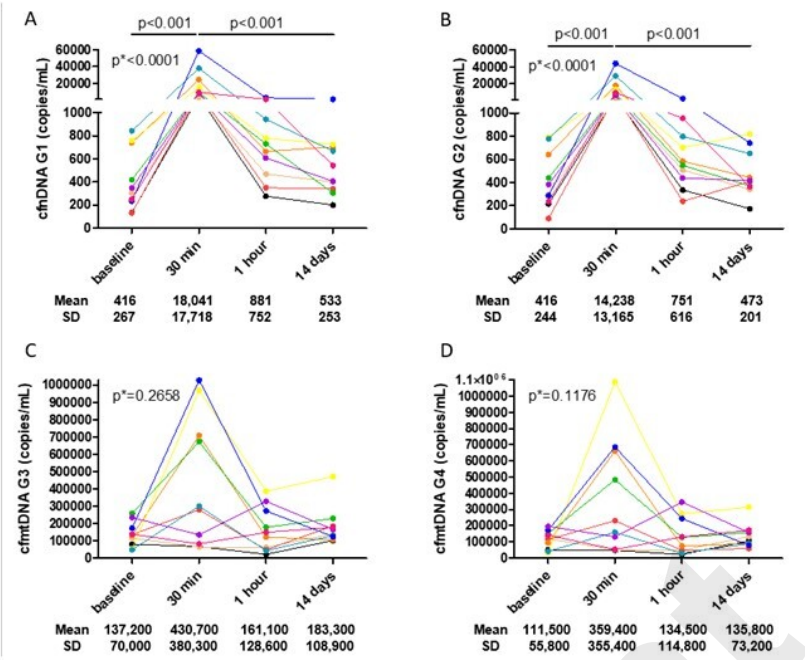
cf nuclear DNA - extreme increase 30-min after the activity
- quick drop to baseline levels after 1 hour
- correlation with muscle damage markers

cf mitochondrial DNA - no homogenous concentration changes
- no correlation with muscle damage markers

Males had numerically larger increases of both cfnDNA as well as cfmtDNA markers than females

Plasma cf-mitochondrial DNA





p*, overall change assessed by the Friedman test; p, statistically significant change between selected time-points assessed by the post hoc Dunn's test (time points on axis x are not in a scale); mean and standard deviation (SD) of concentrations for G3 and G4 are rounded to hundreds

Gene expression levels of cfnDNA G1 (A), cfnDNA G2 (B), cfmtDNA G3 (C), and cfmtDNA G4 (D) before and after the downhill run