different extent (average deviations: −3%, −18%, and −33%).

In conclusion, the presented study showed that our measurement procedure, based on ED and trueness-based ID-LC/tandem MS measurement of dialysate, qualifies as a method for standardization of FT_4 measurements. At the same time, our results demonstrate that samples processed according to the C37-A protocol are suitable for use in standardization. These results provide a basis for worldwide standardization of FT_4 measurements under the auspices of the International Federation of Clinical Chemistry and Laboratory Medicine *(23)*. An inaugural meeting of the group is foreseen at the 2006 AACC Annual Meeting *(24)*.

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Cell-Free Plasma DNA as a Novel Marker of Aseptic Inflammation Severity Related to Exercise Overtraining,

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Background: **Circulating free plasma DNA is implicated in conditions associated with tissue injury, including exercise-induced inflammation, and thus is a potential marker for athletic overtraining.**

Methods: **We measured free plasma DNA along with C-reactive protein (CRP), creatine kinase (CK), and uric acid (UA) in 17 recreationally trained men participating**

in a 12-week resistance training regimen (8 resistance multi-joint exercises selected to stress the entire musculature: bench press, squat, leg press, snatch, hang clean, dead lifts, barbell arm curls, and rowing), consisting of 4 training periods (t1, t2, t3, and t4).

Results: **Plasma DNA concentrations increased markedly after t1, t2, and t3 and returned to baseline after t4. There were substantial differences between t2 and t1 and between t3 and t2 plasma DNA concentrations. CRP increased by 300% after t2 and by 400% after t3 (there was no difference between t2 and t3 CRP values) compared with baseline (t0). CK increased only after t3. UA increased after t2 and t3, with a greater increase after t3.**

Conclusions: **This study demonstrates that, after chronic excessive resistance exercise, plasma DNA concentrations increase in proportion to training load, suggesting that plasma DNA may be a sensitive marker for overtraining-induced inflammation.**

Strenuous exercise may lead to transient muscle fiber damage, with indications such as soreness, edema, performance deterioration, and protein release into plasma *(1–6)*. Muscle injury has been attributed to local ATP depletion, calcium homeostasis disturbance, and oxidative stress induced by generation of free radicals *(7, 8)*. These adverse effects are seen in athletes affected by overtraining, which has been defined as an accumulation of exercise training and nontraining stress, leading to long-term decrement in athletic performance (despite continued training) with or without related physiologic and psychological signs. Overtraining can lead to an acute breakdown with subsequent repair of skeletal muscle and is associated with increased susceptibility to infections attributable to changes in the functional status of immune cells *(9)*.

Circulating plasma DNA is altered both quantitatively and qualitatively under a variety of conditions, including tissue injury, pregnancy, cancer, and trauma *(10–13)*. Plasma DNA concentration has been correlated with injury severity and thus is a potential marker for risk stratification *(11)*. We investigated the use of the plasma DNA response as an inflammation marker during a chronic resistance exercise (RE) protocol of progressively increased training volume. We then compared this marker with conventional indices such as C-reactive protein (CRP), creatine kinase activity (CK), and uric acid (UA), which are associated with exercise-induced muscle damage and acute-phase response.

Participants were recruited from a volunteer database, by word of mouth and by fliers posted within the University and the local community. A written informed consent was signed by all participants. Procedures were in accordance with the Helsinki Declaration for the Ethical Treatment of Human Subjects. Ethics approval was given by the institutional review board.

Our study participants were healthy volunteers: age, 21.56 (2.6) years; body weight, 77 (7.1) kg; body height, 1.77 (0.11) m; and body fat, 12.2 (2.1)%. They were 17 recreationally trained men who participated in a 12-week resistance training regimen (8 resistance multi-joint exercises were selected to stress the entire musculature: bench press, squat, leg press, snatch, hang clean, dead lifts, barbell arm curls, and rowing) consisting of four 3-week training periods (t1, t2, t3, and t4). The first and fourth training periods (t1 and t4) included low-volume training: 2 training days per week, 2 sets per exercise, 10–12 reps per set at 70% of their maximal strength (1 repetition maximum, 1RM); t2 included high-volume training, 4 training days per week, 4 sets per exercise, 6–10 reps per set at 75%–85% of 1RM; and t3 included very–high-volume training, 6 training days per week, 6 sets per exercise, and 1–6 reps per set at 85%–100% of 1RM. Consecutive training periods were separated by a 5-day rest period. The mean training volume (tonnage) lifted per training period is presented in Table 1.

Blood samples were drawn at baseline (participants abstained from resistance training for at least 8 weeks before the study) and at 96 h after the last training session of each period.

We isolated plasma DNA from 400 μL plasma after 10 min centrifugation at 800*g*, followed by 10 min centrifugation at 16 000*g*. DNA was extracted with the QIAamp DNA Blood MiniKit (Qiagen) and eluted into 50 μL of RNase/DNase-free H_2O. We measured free-DNA by quantitative real-time PCR with the LightCycler (Roche Diagnostics) with the following primers: forward, 5′-AGG TGA ACG TGG ATG AAG TT-3′ and reverse, 5′-AGG GTA GAC CAC CAG CAG CC-3′, for the amplification of a 189-bp fragment of the β-globin gene. PCR amplification reactions were performed with the LightCycler Fast Start DNA Master SYBR Green I (Roche Diagnostics). The PCR conditions included a first denaturation step of 95 °C (10 min), followed by 40 cycles of 95 °C (10 s), 95 °C (5 s), and 95 °C (20 s) with a temperature ramp of 95 °C/s *(14)*. All samples were analyzed in duplicate. Each reaction included a calibration curve of 6.6 ng to 6.6 pg genomic DNA made from 4 serial dilutions of purchased human genomic DNA (0.2 g/L in 10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0), (Roche Diagnostics GmbH, Cat. No. 11691112001) plus 2 known dilutions (50 and 500 pg DNA) used as controls in all experiments. DNA values were expressed as genome equivalents per milliliter (GenEq/mL), corresponding to 6.6 pg DNA.

We measured CK, UA, and CRP with the COBAS INTEGRA 800 Clinical Chemistry System (Roche Diagnostics).

We analyzed data with SPSS-PC and calculated means (SE). We evaluated time differences with ANOVA repeated measures and used a Bonfferoni test for post hoc comparisons. Data normality was tested with the 1-sample Kolmogorov-Smirnoff test; therefore a nonparametric test was not necessary. Statistical significance was set at $P < 0.05$.

Plasma DNA increased significantly after t1, t2, and t3, and returned to baseline concentrations after t4 (Table 1, Fig. 1). Plasma DNA concentrations differed significantly

for t2 vs t1 and for t3 vs t2. CRP increased compared with baseline by 300% after t2, and by 400% after t3 (there was no difference between t2 and t3 values). CK increased only after t3 (Table 1). UA (Table 1) increased after t2 and t3, with a greater increase after t3. Performance increased after t2 and declined thereafter (Table 1).

An overtraining response to the exercise protocol was demonstrated by a decreased performance level, despite increased training and subsequent lack of recovery within 3 weeks, and despite decreased training volume in t4 *(9)*. Tissue damage and inflammation were confirmed by increased plasma DNA and CRP concentrations. After each training period, Pearson correlation coefficients were calculated between plasma DNA, CRP, CK, UA, and training load. The results showed substantial correlations between plasma DNA and CK at t2 ($r = 0.793$, $P < 0.01$) and at t3 ($r = 0.744$, $P < 0.01$). We observed no statistically significant correlations between plasma DNA, CRP, CK, and UA. Plasma DNA concentrations were significantly related to training load (average tons lifted in all exercises per week in each training period) only in t2 ($r = 0.755$, $P < 0.01$) and t3 ($r = 0.786$, $P < 0.01$).

Circulating DNA has been shown to increase 9- to 17.5-fold after long-distance running (21–246 km) *(7, 14)*, but changes in concentration in response to chronic overload training have not been investigated. We measured plasma DNA 96 h after the last training session to look for changes reflecting chronic exercise-induced inflammation. Indeed, plasma DNA remained increased for 96 h postexercise after the t1, t2, and t3 periods. Increased plasma DNA concentrations have persisted up to 3 h after major injury in trauma patients *(15)*, 2 or more days (as in this study) after exhaustive exercise *(14)*, and several days in patients with multiple organ dysfunction syndrome *(10)*.

Increased plasma DNA concentrations correlated with injury severity in trauma and stroke patients *(11, 16)* indicate that circulating DNA concentration is a potential risk stratification marker. We observed a significant correlation of plasma DNA with CK (in t2 and t3) but not with CRP. Demonstration of a similar response of plasma DNA to progressive training that does not lead to overtraining would indicate that plasma DNA can be used as an overtraining marker.

Muscular overtraining impairs athletic performance, increases susceptibility to infections, and induces muscle tissue injury as well as psychological, immunological, and biochemical dysfunctions *(9)*. Although there is a strong demand for early diagnosis of overtraining, specific sensitive markers have not been identified. CRP is a sensitive marker for inflammation regardless of etiology *(17)* and increases in response to intense exercise *(14, 18, 19)*. Our results provide evidence of a delayed (4 days) CRP response to RE, which was also seen (3–6 days) after intense running *(20, 21)*. In our study, basal CRP concentrations were within the reference interval (0–10 mg/L), but after t3, mean CRP (4.8 mg/L) approached the reference interval for acute infections (5 mg/L for adults) *(22)*. Unlike plasma DNA, however, CRP concentrations did not reflect the difference in magnitude of exercise overload between t3 and t4. Similarly, CK increased only after t3. The response of plasma DNA, but not of CRP or CK, seemed to be proportional to the exercise training overload.

The mechanisms underlying the presence of circulating DNA are generally unknown. Apoptosis, necrosis, active release, and impaired clearance have been implicated *(23–25)*, but information regarding their role during exercise is scarce. RE has been reported to generate reactive oxygen species (ROS) and oxidative stress *(26)*. ROS have been associated with postexercise inflammation and muscle damage propagation *(27)*. An inflammatory response during the repair of overtraining-induced muscle damage *(28)* promotes neutrophil and macrophage infiltration of

Table 1. Training volume and performance as well as CK, CRP, and UA concentration changes at rest (Baseline), and after low- (t1), high- (t2), very high- (t3), and low-volume (t4) resistance training

	Baseline	t1	t2	t3	t4
Mean training volume, tonnage/wk (SE)[a]	N/A	2.2 (0.2)	7.8 (0.6)[d]	14.8 (1.4)[d,c]	1.7 (0.3)[f]
Performance (maximal strength), kg (SE)[b]	96.6 (17.1)	99.2 (11.7)	109.6 (14.8)[c,d]	104.5 (14.9)[c]	107.2 (12.5)[c,d]
Cell-free plasma DNA, 8GenEq/mL (SE)	31.4 (13.8)	143.5 (22.9)[c]	289.9 (41.1)[c,d,e]	605.7 (116.4)[c–f]	74.8 (29.6)[c,d,f]
	(14.0–65.9)	(76.6–180.6)	(196.9–383.0)	(251.8–959.5)	(27.7–141.8)
CK activity, units/L (SE)	102.6 (19.1)	134.5 (20.1)	296.6 (49.6)	368.1 (44.0)[c,d]	161.7 (23.8)[f]
	(59.4–149.8)	(86.7–82.2)	(185.5–407.6)	(264.4–513.9)	(105.7–217.7)
CRP, mg/L (SE)	0.84 (0.17)	0.86 (0.19)	3.364 (1.07)[c,d]	4.8 (1.04)[c,d]	1.42 (0.30)[f]
	(0.45–1.24)	(0.42–1.31)	(1.00–5.77)	(1.89–6.64)	(0.73–2.11)
UA, mg/L (SE)	39.1 (4.0)	42.4 (4.0)	51.2 (3.7)[c]	66.6 (5.8)[c,d]	43.8 (4.3)[f]
	(30–48.2)	(33.3–51.5)	(42.8–59.6)	(53.4–79.5)	(34.1–53.5)

[a] Mean number of tons lifted per week for all resistance exercises employed in the protocol. NA, not applicable.
[b] Mean maximal strength in 1 representative exercise (squat).
[c] Significant difference with baseline (T0).
[d] Significant difference with t1.
[e] Difference between t3 and t4.
[f] Significant difference between t4 and t3.
[g] Significant difference with t4. Minimum and maximum values are shown in parentheses.

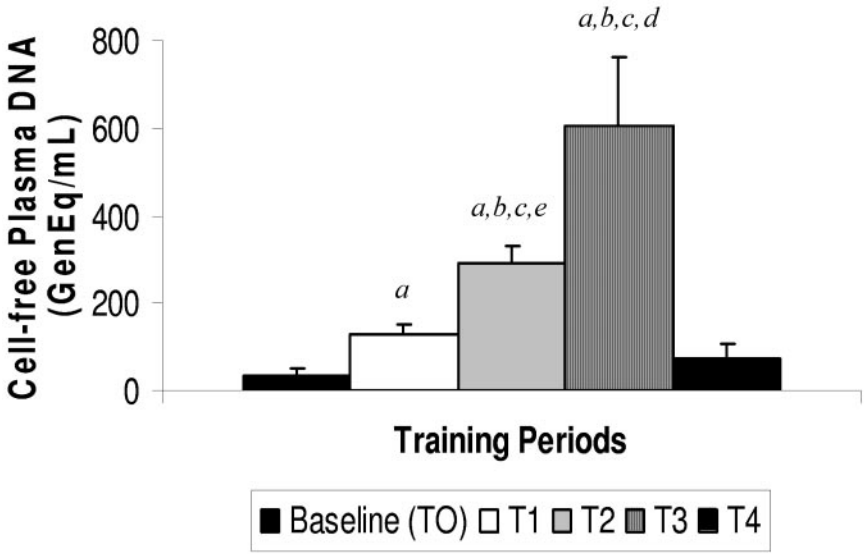

Fig. 1. Cell-free plasma DNA concentration in 17 study participants measured sequentially at rest (t0) and after low- (t1), high- (t2), very high- (t3), and low-volume (t4) resistance training.

[a] Significant difference with baseline (t0)
[b] Significant difference with t1.
[c] Significant difference between t3 and t2.
[d] Significant difference between t4 and t3.
[e] Significant difference with t4.

muscles *(2)*, most likely initiated by ROS *(29)*. Thus, peroxidative muscle damage is exacerbated during post-exercise inflammation. Neutrophils and macrophages generate superoxide *(30)*, which may be converted to hydrogen peroxide, which then reacts with superoxide in the presence of a transition metal to form hydroxyl radical *(31)*. After exercise, neutrophil counts in muscle are increased for 5 days, and peak macrophage counts occur at 2 to 7 days *(1–2)*. UA, which we used as an oxidative stress marker because of its free-radical scavenging properties, increased after t2 and t3, indicating that chronic RE induced oxidative stress. ROS generation leads to DNA damage in the form of DNA cross-links, oxidized nucleosides, and strand breaks *(32)*. More work is needed to elucidate the origin of plasma DNA during exercise.

This study demonstrates that plasma DNA concentrations increase in proportion to training load after chronic excessive RE, suggesting that plasma DNA may be a sensitive marker for monitoring and quantification of overtraining in athletes. In particular, our finding that plasma DNA was substantially correlated with mean training load during periods of high-volume and very–high-volume training (t2 and t3) supports this theory. Plasma DNA measurement is noninvasive and requires limited time and personnel. Further investigation of the origin of plasma DNA in the overtrained state is warranted.

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