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Long distance running – Can bioprofiling predict success in endurance athletes?

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ABSTRACT

The TransEuropeFootRace (TEFR) was one of the most extreme multistage competitions worldwide. The ultramarathon took the runners over a distance of 4487 km, from Bari, Italy, to the North Cape, Norway, in 64 days. The participating ultra-long-distance runners had to complete almost two marathons per day (~70 km). The race was accompanied by a research team analysing adaptations of different organ systems of the human body that were exposed to a chronic lack of regeneration time. Here, we analyzed runner's urine using mass spectrometric profiling of thousands of low-molecular weight compounds. The results indicated that pre-race molecular factors can predict finishers and separate them from nonfinishers already before the race. These observations were related to the training volume as finishers ran about twice as many kilometers per week before TEFR than nonfinishers, thus apparently achieving a higher performance level and resistance against overuse. While this hypothesis needs to be validated in future long-distance races, the bioprofiling experiments suggest that the competition readiness of the runners is measurable and might be adjustable.

Introduction – The TransEuropeFootRace

Marathon running became very popular among recreational runners since the turn of the millennium. Ultra-marathon (UM) running (shortest distance 50 km) is of more recent interest to runners. It is a testament to human's natural ability to long-distance running using aerobic metabolism [1,2], which also was a significant factor in human evolution [3]. The underlying physiology has drawn increasing scientific interest, however, the available studies comprise only very few investigations into multi-day UM (so-called multistage UM (MSUM) - races of more than 200 km) [2]. To the best of our knowledge, the most extensive investigation (longest MSUM, most subjects) was the mobile magnetic resonance tomography (MRI)-based prospective field-study during the TransEuropeFootRace (TEFR, Fig. 1) [4]. It took the runners over a distance of 4.487 km in 64 days with no day of rest, through six countries from South Italy to the North Cape.

TEFR was an extreme run under the influence of incalculable environmental conditions. It addressed the human capacity for movement in the context of extraordinary distance loading and thus the combined effects of metabolic processes and biomechanical as well as gravitational stress on the human body [4,6]. It was described as "an outstanding model for the study of adaptive responses to extreme load and stress" [7]. TEFR was accompanied by a team of physicians [8] collecting serum and urine of the participants as well as a number of medical parameters including mobile MRI data throughout the race [4,9]. From the generated data, various analyses regarding basic questions on the influence of cross-border (ultra-)endurance running load on different organ systems have been carried out using different medical and scientific methods such as voxel-based morphometry, whole body MRI, cartilage T2*-mapping and MRI-spectroscopy [10-18]. Still, despite more than two decades of ultra-endurance research (for review see ref. [2]), the central question, which structural and metabolic factors or functional circuits determine the limit of physical-aerobic performance in ultra-running, or in other words: What defines a successful UM-runner? remains unsolved.

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TransEuropeFootRace TEFR 2009 [5]

- 11th official transcontinental competition MSUM
- April 19 – June 21, 2009
- From Bari (Italy) to North Cape (Norway)
- 4.487 km in 64 days
- 64 stages: mean distance 70.1 km
- Minimum 44 km, maximum 95.1 km per day
- 44 endurance runners (10 nations, 40 male, 4 female)
- 30 finisher (29 male, 1 female)

Fig. 1. Key data of TEFR and route.

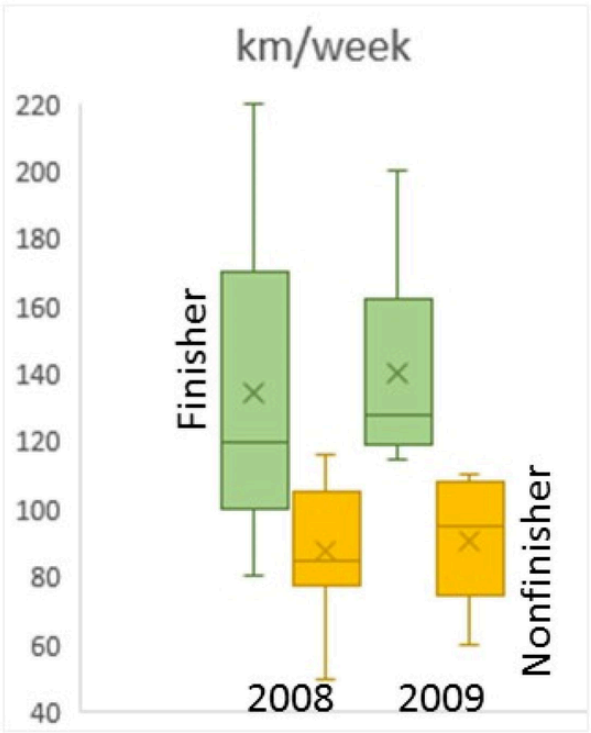

Fig. 2. Mean training volume (km/week) of male runners older than 40 years in the year before and two months before TEFR.

Predictors of performance in MSUM running

Pre-race performance

The most important key factors for a fast UM-race time are extensive previous UM-experience, in particular the number of finished UM-races, fast marathon time, high running speed and, to a lesser extend, a high running volume during training [19-24]. Training for an UM leads to characteristic changes and adaptations in the muscle fiber [25]. For TEFR, we could not confirm that prior running experience had a relevant influence on a successful finishing of the race. The runners had trained for at least 9 years (finishers 9–27 y, nonfinishers 9–26 y) and most participated in multiple marathons and races of different types (6–24 h races, 50–100 km races) on a regular basis. The most striking difference between finishers and nonfinishers was the training volume (finishers: 10–20 h/week, nonfinishers: 8–12 h/week) and intensity (running speed) during the last 14 months before TEFR (Fig. 2), as well as their pre-race records in 50 km- and 24 h-races [26]. It appears that the amount of training was crucial.

Body composition

Endurance exercise leads to a decrease in body mass [27], that is mainly in the most energy-rich substrate - body fat [28-31]. UM races without a break result in a decrease of both body fat and skeletal muscle [13,30,32-35]. The method T1-weighted whole body MRI [36] with added semiautomatic body component analysis [37] used in TEFR (Fig. 3) showed that every subject decreased in body mass BM (index, BMI), total volume (TV), total somatic volume and total visceral volume due to massive loss of total adipose tissue (TAT), respective somatic adipose tissue (SAT) and visceral adipose tissue (VAT) [13].

Lean (muscle) tissue

UM-runners in very long races face an ongoing negative energy balance with a decrease in solid mass [38,39]. As the race progresses this leads to the utilization of muscle tissue for energy provision. For longer UMs, the proportion of fat and protein to the energy contribution seems to increase [40,41]. In TEFR, not every runner lost total lean tissue (TLT) or somatic lean tissue (SLT); some of them showed increases, others decreases [13].

Visceral adipose tissue

The finisher group of TEFR had significantly less adipose tissue volume percentage than the nonfinishers at the beginning of the race; nonfinisher had 71.5% more VAT, 28.0% more SAT and in total 26.6% more TAT than the finishers [13]. Conversely, the lean tissue difference of percentage volume was significantly smaller in nonfinishers compared to finishers for TLT (−6.9%) and SLT (−8.1%). Low body fat [23,42], respective the percentage of body fat and BMI [43], were specified as some of the most important anthropometric predictors for a fast UM-race [44]. In TEFR, the independent variable VAT showed the fastest and most significant decrease compared to SAT and lean tissue compartments, respectively. VAT was detected as the most sensitive morphometric parameter regarding the risk of not finishing the TEFR and it showed a direct relationship to pre-race records in 50 km- and 24 h-races as well as training volume (km) and intensity (mean speed) one year before TEFR [13].

Intramyocellular lipid

In the pathogenesis of insulin resistance and maximum oxygen capacity (VO_2max: endurance capacity), not only the fat content, but also its distribution plays a central role. Mobile proton-MR-spectroscopy

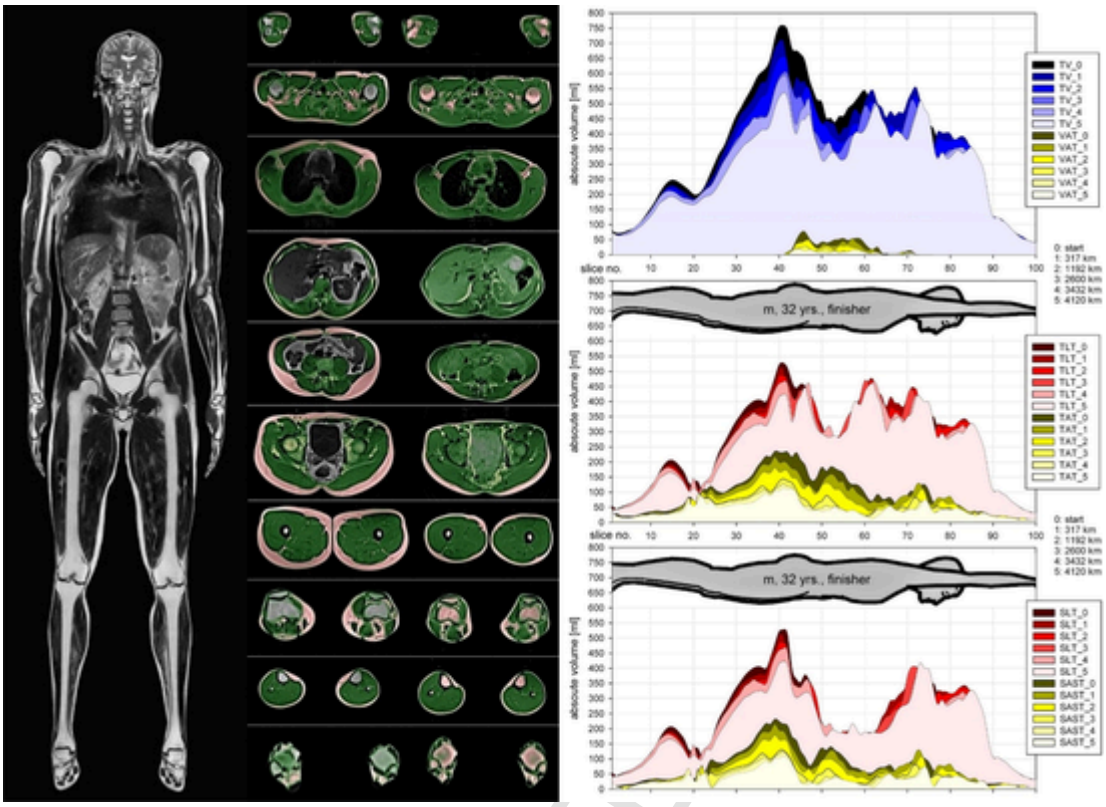

Fig. 3. Whole body MRI with semiautomatic segmentation and differentiation von fat and lean body compartiments. Decrease of tissue compartiments in the course of TEFR in a 32 year old male finisher. (Whole body MRI-sequence, T1w TSE 2D transversal: flip angle 180°, echo time 12 ms, repetition time 490 ms, slice thickness 10 mm, spacing between slices 20 mm, field of view 1991 cm^2, matrix size 256*196 px, pixel size 1.9922 mm iso, pixel bandwith 120, image number 90–120 var, image acquisition time appr. 20 min).

(^{1}H-MRS) measurements [45,46] regarding intramyocellular lipid (IMCL) content showed a comparatively high level of IMCL-accumulation in the calf muscle (tibialis anterior muscle) in our subjects before the start of TEFR and in some cases a significant increase during TEFR (Fig. 4). IMCL, spherical fat droplets in the cytoplasm of skeletal muscle cells, are correlated with insulin sensitivity and VO_2max. In ^{1}H-MRS, the IMCL-peak has to be distinguished from the extramyocellular lipids (inhomogeneous septa attached between the muscle fiber bundles; Fig. 4), as both fat clusters contribute to the spectrum [47,48]. Using ^{1}H-MRS, elevated IMCL in untrained individuals with high body fat (TAT above average) and insulin resistance as well as in trained athletes with comparatively low body fat (SAT / VAT reduced) and high insulin sensitivity was detected [49,50]. In lean subjects, VO_2max correlated positively with IMCL, which is consistent with our TEFR ^{1}H-MRS data and a recent 7 T ^{1}H-MRS comparative analysis with triathletes [51]. In untrained subjects, however, IMCLs correlated negatively with insulin sensitivity [49]. The explanation lies in the U-shaped curve of the IMCL concentration in relation to the percentage of body fat and VO_2max (Fig. 5) [49]. The two subject groups with increased IMCL, which are clearly opposite in terms of aerobic fitness level, can be differentiated on the basis of the fat distribution in the body (VAT, SAT, intrahepatic lipids) [50]. Therefore, measurements of aerobic fitness and body fat are essential for the interpretation of IMCL and its relationship to insulin sensitivity.

Brain volume

The massive negative energy burden during TEFR was also indicated by the significant loss of grey matter (GM, ~6%) in the brain (normal, physiological reduction of brain volume < 0.2% / year) [10,11]. This global, after 7 months reversible, GM volume loss had a specific spatial distribution [11], but substantial structural brain lesions did not occur during TEFR [10]. The accentuated GM volume reduction may be associated with decreased demands during daily running of very long distances in areas responsible for higher brain functions. Furthermore, the need for energy conservation may drive the reduction of high energy consuming default mode network circuits.

Soft tissue overuse

Approximately 50–60% of UM-participants experience musculoskeletal pain [52], in particular muscular pain in the lower limb mainly due to skeletal muscle injury [2,53] and muscle soreness [54]. Various studies using MRI have shown fluid retention around tendons, tissue edema, and damage to cartilage [55]; such overuse injuries are the most common reason why UM runners have to interrupt training [56]. The main reason (71.4%) for not finishing TEFR (45.5% of the subjects) was intolerable pain in the legs due to overuse syndrome of the soft tissues [4], resulting in (peri-)myotendinal inflammations and fasciitis in the upper and lower legs (Fig. 6). Nearly every TEFR participant, however, showed, more or less severe, overuse soft tissue reactions in inflammation of the myotendinous fascial structures of the legs. The immense amount of mechanical stress on the musculoskeletal system when running nearly two marathons daily over a period of nine weeks can lead to these overuse syndromes without the obligatory necessity of prevalent (intrinsic) factors like less specific pre-race performance and too much VAT and SAT [13].

Metabolic adaption and running economy

Rates of metabolic processes are drastically changed by intensive exercise [57]. Oxygen uptake rises ~20-fold and the energy turnover

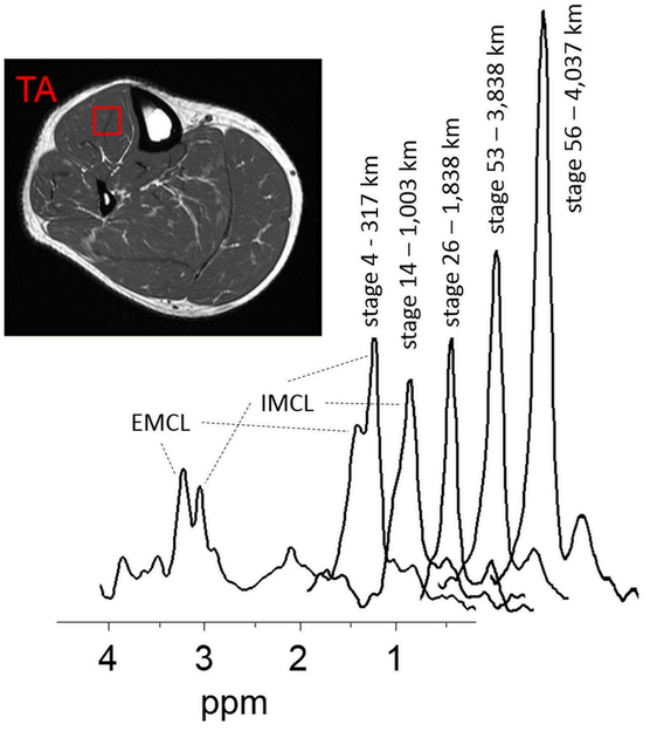

Fig. 4. MRS-measurement data of a 43 year old female nonfinisher showing increasing IMCL-peaks in the course of TEFR (TA: region of interest in tibialis anterior muscle, IMCL: intramyocellular lipids, EMCL: extramyocellular lipids). (MR-spectroscopy (MRS) sequence, single-voxel stimulated-echo acquisition mode (STEAM): echo time 20 mm, repetition time 2000 ms, voxel of interest 11 × 11 × 20 mm^3, 40 acq., image acquisition time appr. 10 min).

can equate 6 g of glucose per minute, more than is typically freely available in the human body (~4 g) [58]. In the working muscles, the rate of adenosin triphosphate (ATP) hydrolysis can increase 100-fold [59] so that ATP must be synthesized within fractions of a second [60]. Hormones like catecholamines are also affected with respect to the regulation of heart rate and blood flow [61,62]. Both protein synthesis and breakdown are elevated [63]. Prolonged exercise also promotes the generation of reactive oxygen species and induces oxidative stress, transient renal impairment and inflammation [64]. Energy metabolism, anabolism and catabolism are thus profoundly changed in response to exercise and have been associated with an increase in the concentrations of lactate, pyruvate, fatty acids, acylcarnitines, ketone bodies and nucleotides and a decrease in the concentrations of bile acids [65]. Commonly studied metabolites in exercise science act within cellular pathways for ATP production such as glycolysis (e.g., pyruvate and lactate), β-oxidation of free fatty acids (FFA, e.g., palmitate) and ketone bodies (e.g., β-hydroxybutyrate) [66].

Like in TEFR, other reports on multistage endurance events also showed catabolism [67,68]. In general, UM runners are unable to meet their energy demands during a race by nutrition [69,70] and in some cases a considerable energy deficit arises [70-72]. Reasons are insufficient energy intake, suppression of appetite and digestive problems during the race [70]. After the first thousand kilometers the mean loss of TV per km, mainly caused by SAT and VAT decrease, declined constantly to more than half until the end of TEFR [13]. Even seasoned UM-athletes experienced further adaptation of their economization mechanisms during TEFR. UM-finishers were more likely to consume and deliver the required energy during performance than non-finishers [73]. If running economy could not be sacrificed in an UM [74,75] and the amount of change in running mechanics depended on the duration of running and distance towards a fatigue state, respectively [75,76], the necessity of the organism to optimize the running economy / running style during progression of the race to a high-end level (as low an energy consumption as possible, but maintaining of redox balance) is mandatory in transcontinental MSUM.

Oxidative stress and redox balance

It is evident that a marathon places immense strain on the energy-producing pathways of the athlete, leading to extensive protein degra-

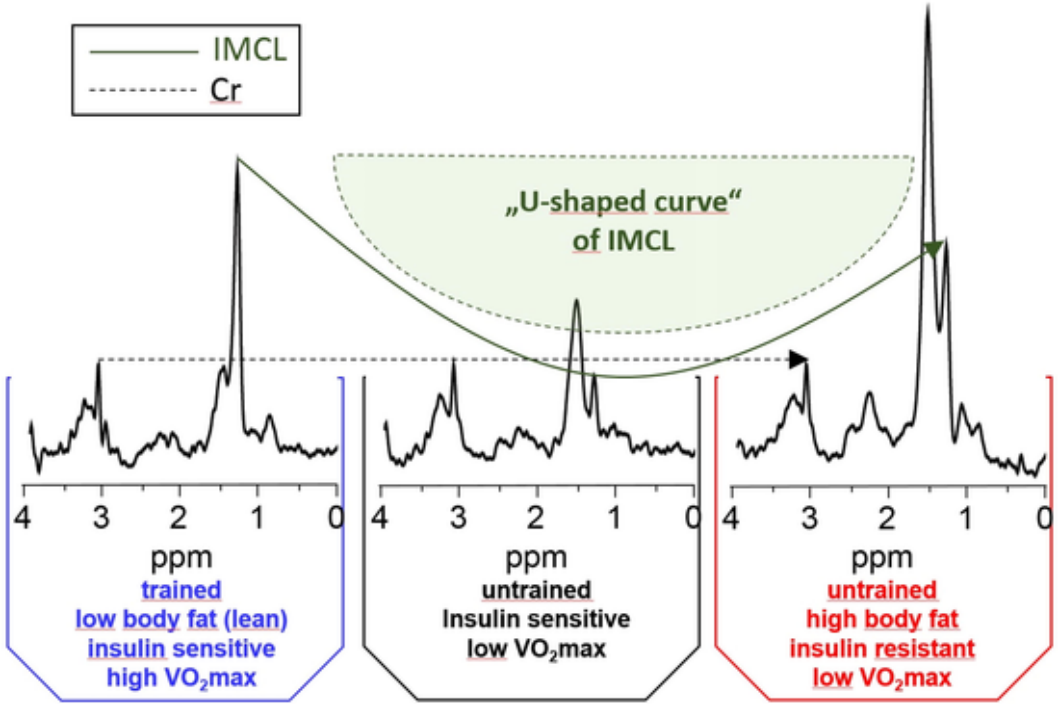

Fig. 5. Relationsip between VO_2max, percentage of body fat and IMCL (tibialis anterior muscle). Modified from [49].

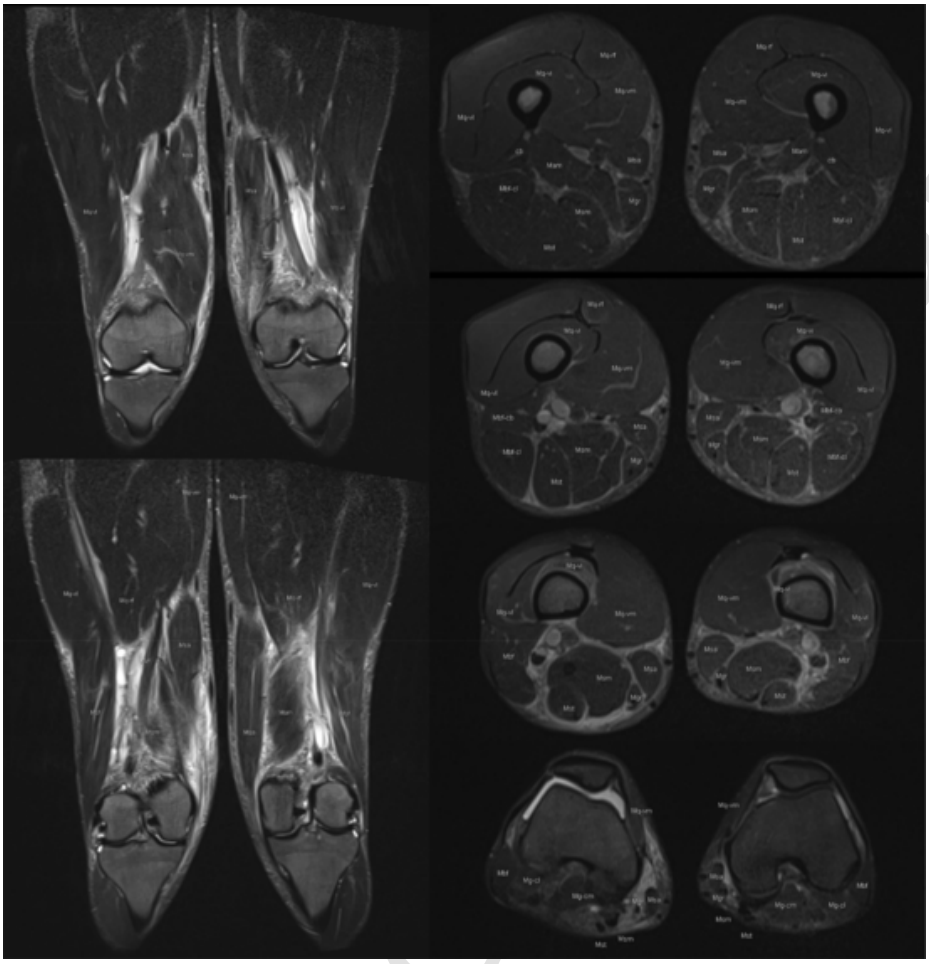

Fig. 6. Example of intra- and perimuscular inflammation (* T2 signal increased, "bright") of the bilateral thigh soft tissues of a 47-year-old nonfinisher (stage 58 – 4,037 km). Mq: quadriceps, vl: vastus lateralis, vi: vastus intermedius, vm: vastus medialis, rf: recuts femoris, Mbf: femoral biceps; cb: caput brevis, cl: caput longus, Mst: semitendinosus, Msm: semimebranosus; Mgr: gracilis; Msa: sartorius. (MRI sequences upper leg, TIRM 2D transversal: flip angle 140°, echo time 62 ms; repetition time 12530 ms, inversion time 130 ms, slice thickness 3 mm, spacing between slices 3.9 mm, field of view 512 cm^2, matrix size 384*192 px, pixel size 0.833 mm iso, pixel bandwith 180, image acquisition time 2:08 min; PDw TSE fs 2D coronar: flip angle 150°, echo time 39 ms, repetition time 6730 ms, slice thickness 3 mm, S spacing between slices 3.9 mm, field of view 512 cm^2, matrix size 320*160 px, pixel size 1.0 mm iso, pixel bandwith 150, image acquisition time 2:14 min).

dation, oxidative stress, and autophagy [77]. The human organism depends on stable blood glucose levels in order to maintain its metabolism. Glucose maintenance at physiological levels is a fundamental mechanism of counteracting excessive oxidation due to its involvement in ATP and nicotinamide adenine dinucleotide phosphate (NADPH) production [78]. Intracellular glycolysis is postulated as the central mechanism to produce nicotinamide adenine dinucleotide (NADH) and ATP [79]. However, under conditions of limited glucose supply and/or excessive metabolic demand, there are alternative ways of producing NADH and ATP such as the oxidation of fatty and amino acids or usage of ketone bodies. Complex multilevel regulatory interactions provide a sufficient flow of glucose to tissues in order to maintain a redox balance. During times of hunger or glucose deficit, activating catabolic programs can maintain energy production in most organs [80]. The pentose phosphate pathway (PPP) is long known as major pathway of glucose metabolism. But in contrast to glycolysis, glucose is essential for the functioning of the PPP and cannot be replaced by other metabolites. The PPP creates and maintains NADPH levels, which are essential for numerous metabolic processes: reduction of oxidized glutathione and protein thiols, synthesis of lipids and DNA [81], detoxification of xenobiotics [82], the fight against infections and regulatory redox signaling. Current evidence provides new insights into the positive metabolic effects of exercise through modulation of redox homeostasis [78]. Cells prioritize the metabolism of glucose through the PPP over standard reactions of upper glycolysis in order to maintain a suffcient NADPH/NADP + ratio needed for the counteraction of acute oxidative challenge biosynthesis and/or generation of superoxide during immune responses or physiological redox signaling [78,83].

Hypothesis and method – TEFR urine profiling

We have employed metabolic profiling of the urine of TEFR participants using high-definition mass spectrometry (MS) to tackle the problem of metabolic fitness in UM. Both targeted and untargeted MS experiments have become of interest for metabolomics studies in recent years, because they allow the analysis of hundreds to thousands of small molecules in single experiments (for review see [65] and Table 1 for latest research). Our experiments indicate that prospective finishers can be distinguished from nonfinishers already before the race based on their metabolic profile. Therefore, we hypothesize that bioprofiles reflect the fitness level of the body and can be used to predict the outcome of a long-distance run.

The aim of our experiments was to clarify whether or not substances could be identified which provide any indication for the runner's ability to finish a transcontinental MSUM. We did not only perform traditional specific analyses on the basis of the study protocol [6],

Table 1
Recent metabolomics research in sports adding to the 2020 review of the field [65].

Exercise	Title	Ref.
Marathon / UM	Factors influencing post-exercise proteinuria after marathon and ultramarathon races	[84]
Marathon	The unaided recovery of marathon-induced serum metabolome alterations	[85]
	Runners' metabolomic changes following marathon	[86]
	Differential time responses in inflammatory and oxidative stress markers after a marathon: An observational study	[87]
	Association between hematological parameters and iron metabolism response after marathon race and ACTN3 genotype	[88]
	The altered human serum metabolome induced by a marathon	[77]
	Fluid Metabolism in Athletes Running Seven Marathons in Seven Consecutive Days	[89]
UM	Changes in urine components and characteristics during a 415-km mountain ultra-marathon	[90]
Soccer	Integration of metabolomics and proteomics to reveal the metabolic characteristics of high-intensity interval training	[91]
Football	Urine metabolomic analysis for monitoring internal load in professional football players	[92]
	Changes of differential urinary metabolites after high-intensive training in teenage football players	[93]
Athlets	Distinct microbiome composition and metabolome exists across subgroups of elite Irish athletes	[94]
Cycling	Adaptation of exercise-induced stress in well-trained healthy young men	[95]
Standardized exercise test	An NMR-based approach to identify urinary metabolites associated with acute physical exercise and cardiorespiratory fitness in healthy humans-results of the Karlsruhe Metabolomics and Nutrition study	[96]
Bench step exercise	Metabolic profiling of eccentric exercise-induced muscle damage in human urine	[97]

but we also applied an undirected methodology and examined urine with an unbiased, hypothesis-generating approach. We hypothesized that high-resolution MS profiling of the low mass components in the urine of ultra-endurance athletes would generate insights with regard to the probability of success in TEFR. The determination of the structure is based on the measured molecular weight and gas phase fragments of the biomolecule. The profiles of hundreds of small molecules (filter cut-off 10 kDa) were measured in an untargeted manner using data-independent high-definition MS and investigated with respect to the parameters endurance performance and success. MS bioprofiling [65,98] has evolved in recent years following the increasing importance of MS in the life sciences and the parallel improvements in the corresponding bioinformatics and computer infrastructure. These developments enable the comparison of huge sets of molecular information (big data). Such profiles represent molecular systems as a whole and, thus, reflect bodily changes in a more comprehensive manner. This is an advantage, because disease or strain never affect a singular substance alone even though a single compound may be prominently involved. Differences in bioprofiles may highlight groups of molecular variables and target them for subsequent more specific research. The

method relies on well-matched biological replicates and delivers statistically relevant data. Samples from 24 participants (among them 4 females, Table 2) taken on different days during the race were measured.

A total of 8741 compounds was determined and principal component analysis (PCA) was used to screen the results for molecular factors. PCA is a procedure of multivariate statistics used to simplify and visualize complex datasets by converting a set of correlated measurements into a set of linearly uncorrelated variables (PC) where the first PC represents the largest possible variance [99]. The method allows to elucidate whether or not sample sets carry features which distinguish them with a certain probability. Gender clearly had a great impact on the extraction of differentiating compounds due to the influence of abundant hormones and their metabolites as discussed before [100]. Conclusively, sample cohorts had to be selected in a gender-specific manner. In this study, only four females participated (with one finisher), which was not sufficient for valid statistical analysis, so that only male runners were considered further. Moreover, special medication taken by some probands such as cough syrup affected the urine profile and created outliers. Age is also an important factor in medical studies as the visible body changes reflect on the molecular level and we noted considerably different profiles for the two men which were much younger (26 and 27 years of age) than the bulk of the male runners (41–65 years). Sample sets also differed between the measurements on day 1 and the following days as the use of sports gels, diclofenac and pain medication increased with the strain on the body (Fig. 7A). In such a set-up, artificial contributions to the urine profile were hard to distinguish from those resulting from metabolic changes so that we focused on the analysis of the samples from day 1 of the race and male runners older than 40 years.

Table 2
Urine sample collection. Samples discussed in the text are marked in grey. G – gender, A – age, F/nF – finisher/nonfinisher.

G	A	Nationality	F/nF	Day of sampling 2009					
				April	May			June	
m	**41**	Japan	nF	19.					
m	**43**	France	nF	19.					
m	**46**	Germany	nF	19.					
m	**49**	France	nF	19.	03.	18.		01.	
m	**51**	Germany	nF	19.	03.	16.			
m	**52**	Netherlands	nF	19.					
m	**58**	Japan	nF	19.					
m	**60**	Netherlands	nF	19.	04.	17.	26.		
m	**62**	Japan	nF	19.		18.	26.		
m	**64**	Japan	nF	19.					
m	**44**	Germany	**F**	19.	03.	17.		02.	15.
m	**47**	Netherlands	**F**	19.	04.			01.	15.
m	**50**	Switzerland	**F**	19.	03.	17.		01.	15.
m	**53**	Taiwan	**F**	19.	04.	17.		02.	15.
m	**59**	Germany	**F**	19.	04.	17.		01.	15.
m	**63**	Japan	**F**	19.	03.	18.		01.	18.
m	**65**	Japan	**F**	19.	04.	18.		02.	17.
m	**65**	France	**F**	19.	03.			01.	17.
m	26	Japan	**F**	19.	04.	18.		01.	17.
m	27	Germany	**F**	19.	03.	18.		03.	16.
f	45	Germany	**F**	19.	03.	18.		01.	15.
f	46	Netherlands	nF	19.	04.	17.		01.	06.
f	46	Japan	nF	19.	03.	18.		02.	15.
f	68	Germany	nF	19.					

Pilot data discussion

As is evident in Fig. 7B, in this setting, finishers and nonfinishers were well separated based on 223 mass spectrometric signals meeting the filter parameters (ANOVA $p \leq 0.05$, fold value ≥ 2). This was a surprising observation as it implicated the presence of molecular information defining the post-training / pre-run condition of the sportsmen and their capability to finish the marathon. Some of the detected mass signals could be assigned to structures using the available metabolite databases. Among them were tri- and tetrapeptides, which were measured with up to 8-fold higher intensity in nonfinishers. Conspicious were also fatty acids and lipids (sphingolipids, glycerophospholipids) and oligosaccharide-containing compounds, which were, due to the high structural variance at equal mass, like the peptides, assigned only tentatively (*m/z* 265.214, 10E-heptadecene-8-ynoic acid, 10-fold higher in nonfinisher; *m/z* 761.618, PE-Cer(d16:1(4E)/24:0(2OH)), 4-fold higher in nonfinisher; *m/z* 450.305, PI(O-20:0/20:5(5Z,8Z,11Z,14Z,17Z)), 6-fold higher in finisher; *m/z* 353.074, 4-(4-deoxy-α-D-gluc-4-enuronosyl)-D-galacturonate, 2-fold higher in finisher, *m/z* 870.326, 1,3-alpha-D-mannosyl-(1,2-N-acetyl-alpha-D-glucosaminyl)-1,2-alpha-D-mannosyl-1,2-alpha-D-mannosyl-D-mannose, 4-fold higher in nonfinisher). Given the high structural variability in some substance classes such as FFA and lipids (e.g. same mass - different structure) ambiguities may still remain. Therefore, we refrain from pinpointing individual compounds from our bioprofiles until proper validation has been performed.

Thiaminetriphosphate (TTP, *m/z* 505.033, 8-fold higher in finisher) was also matched. TTP is the triphosphate ester of the water-soluble essential vitamin thiamine (vitamin B_1), which has antioxidant, erythropoietic, mood modulating, and glucose-regulating functions. It is involved in cell energy metabolism [101,102]. Exercise has been suggested to affect thiamin status by increasing thiamin-dependent mitochondrial enzymes and needs through tissue repair and maintainance [103,104]. Like glucose, the FFA concentration is significantly higher at the end of endurance exercises compared to the resting value [105]; the use of fat as energy substrate was measured as the increase in FFA during an UM [106,107]. Endurance training improves exercise performance and insulin sensitivity, and these effects may be in part mediated by enhanced fat oxidation. The use of FFA in the serum (ß-oxidation) [108] as a relevant source of energy [109,110] in connection with the mobilization/catabolism of tissue fat has been widely documented in the endurance field [111,112]. In the anaerobic area (metabolism), this can lead to metabolic acidosis (ketoacidosis) [113,114]. Several recent reports indicate that the balance of skeletal muscle phosphatidylcholine (PC) and phosphatidylethanolamine (PE) is a key determinant of muscle contractile function and metabolism. Muscle PC and PE are altered by exercise and total muscle PC and PE are positively related to insulin sensitivity while PC:PE ratio is inversely related to insulin sensitivity in humans [115,116]. Phosphatidylserine is externalized during cell apoptosis as an “eat me” signal [117] and therefore a marker of catabolic processes occurring in consumptive and wasting life situations such as cancer or TEFR [10,13].

Recent ultra-high field MRS-based intramyocellular (IM) investigations concluded that single proton-based (^{1}H-)MRS cannot provide an exclusive IM-biomarker of (aerobic) muscular fitness or training status [51]. A combination of different, cross-sectionally related IM-parameters (e.g., phosphodiester PDE), assessable during a multi-nuclear MRS-session (e.g., phosphorus (P-) and ^{1}H-MRS), provides much more information about the complex intracellular energy metabolism [51]. The ATP-based phosphocreatinine (PCr) synthesis (P-MRS) and also the IMCL (^{1}H-MRS) is increased in triathletes [51] and probably also in ultra-runners [49]; their recovery after aerobic energetic exhaustion is improved. The VO_2max-capacity is in addition to the IMCL also posi-

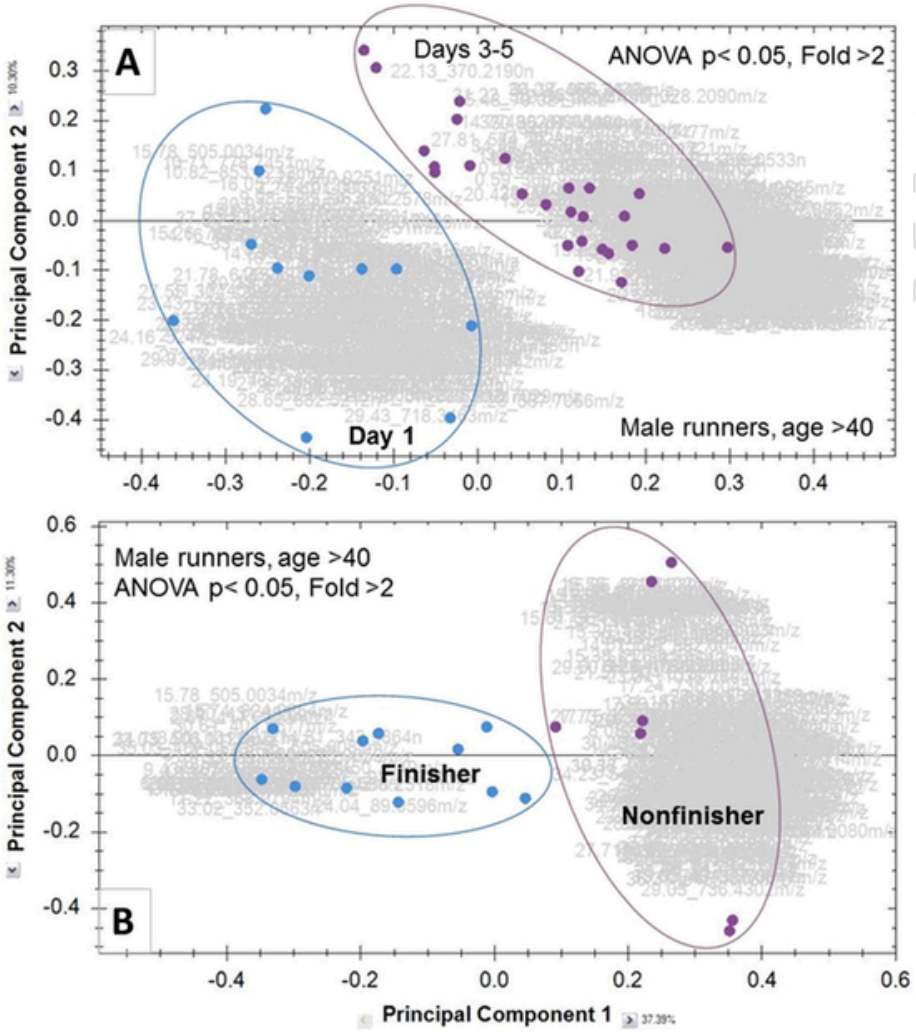

Fig. 7. PCA for urine profiles of samples of male runners older than 40 years (ANOVA $p < 0.05$, fold > 2). A) Samples from day 1 and days 3–5. The values for day 2 fell between the two data clouds and were omitted for better illustration. B) Day 1. PCA demonstrates the variance between the molecular profiles as sample sets separate into groups.

tively correlated with the PDE concentration in skeletal muscle [51] (measured by P-MRS: increased PCr-to-ATP flux [118]).

The homeostatic control of the energy balance, e.g., the optimization of the relationship between energy intake and consumption, is a major concern in UM due to its high energy demands [2]. Processes in energy metabolism and the management of focal tissue inflammatory and systemic oxidative stress, possibly also involving the central nervous system, are affected. Targeted metabolomics studies have indicated elevated serum concentrations of various carbohydrate/glycolysis intermediates during short strenuous physical activity; the utilization of free glucose is suggested as the preferred primary energy source [108-110]. Alterations to the tricarboxylic acid (TCA) cycle intermediates were induced by longer duration and lower intensity exercise such as a marathon [121]. Free glucose and carbohydrate stores can be depleted within 1–2 h after the start, which leads to the utilization of alternative fuel substrates (lipids and amino acids) for energy production [110]. Increased lipolysis activity results in elevated serum glycerol and FFA [109,110]. FFA produce acetyl-CoA via β-oxidation and subsequent energy via the TCA cycle and imbalanced NADH:NAD + ratio (additional strain placed on the electron transport chain) [108]. The increased synthesis of acetyl-CoA could also ascribe for the elevated ketone concentrations [110], as it is a key component of ketogenesis. If the strenuous physical activity continues beyond the athlete's lipid store capacity, or if the regular lipid oxidation pathways become saturated [122], the metabolism shifts towards protein catabolism [109]. Amino acids are primarily oxidized to pyruvic acid and acetyl-CoA, both of which can serve as TCA cycle influx substrates for energy production [108]. Protein degradation alters purine catabolism, resulting in elevated adenosine monophosphate, inosine monophosphate, hypoxanthine, xanthine, uric acid and allantoin (uric acid derivative) [121]. The latter is a surrogate index of oxidative stress [109]. The measurement of these molecules has largely been restricted to targeted methodologies. Only little is known about the holistic metabolic changes in athletes [77] and nearly nothing for UMs.

The measured compounds indicate, not surprisingly, differences in lipid and sugar, respective energy, metabolism, between the runner groups and may explain their different performance and power of endurance; they will have to be validated and evaluated separately in dedicated studies to clarify their impact (e.g. lipids [119]). This is an important step, because untargeted MS does not, despite the use of high-resolution technology (assignment from metabolite databases with an error of less than 10 ppm (< 0.001%)), always deliver unambigouous identification of all biomolecules [120].

Conclusion

There may be a training volume and intensity where the body switches to a higher performance level. This is reflected in the relationship between pre-race performance and body composition in the com-

parison of TEFR finishers and nonfinishers, and also in the urine profiles. This hypothesis will have to be tested and validated in future races (Fig. 8). It is however fascinating to envision that a molecular pattern can be used to define a training status of long-distance runners and/or indicate limited functioning of the organ systems leading to early drop-out of the race – sufficiently large proband cohorts for comparison provided. It can lead to the ability to assess the performance of the entire organism in advance with regard to specific requirements, e.g. in certain competitive sports such as running, and can later may be used for exercise programmes for athletic competitions.

However, bioprofiling only delivers reliably information when the groups are well matched and experiments are standardized. Major differences such as gender or age may cause failure of the analysis, in particular for comparatively small cohorts. Our study was a retrospective investigation which prompts us to look closer at the prominent molecular features in the respective bioprofiles. It is worth studying different substance classes such as FFA and lipids in more detail, because they

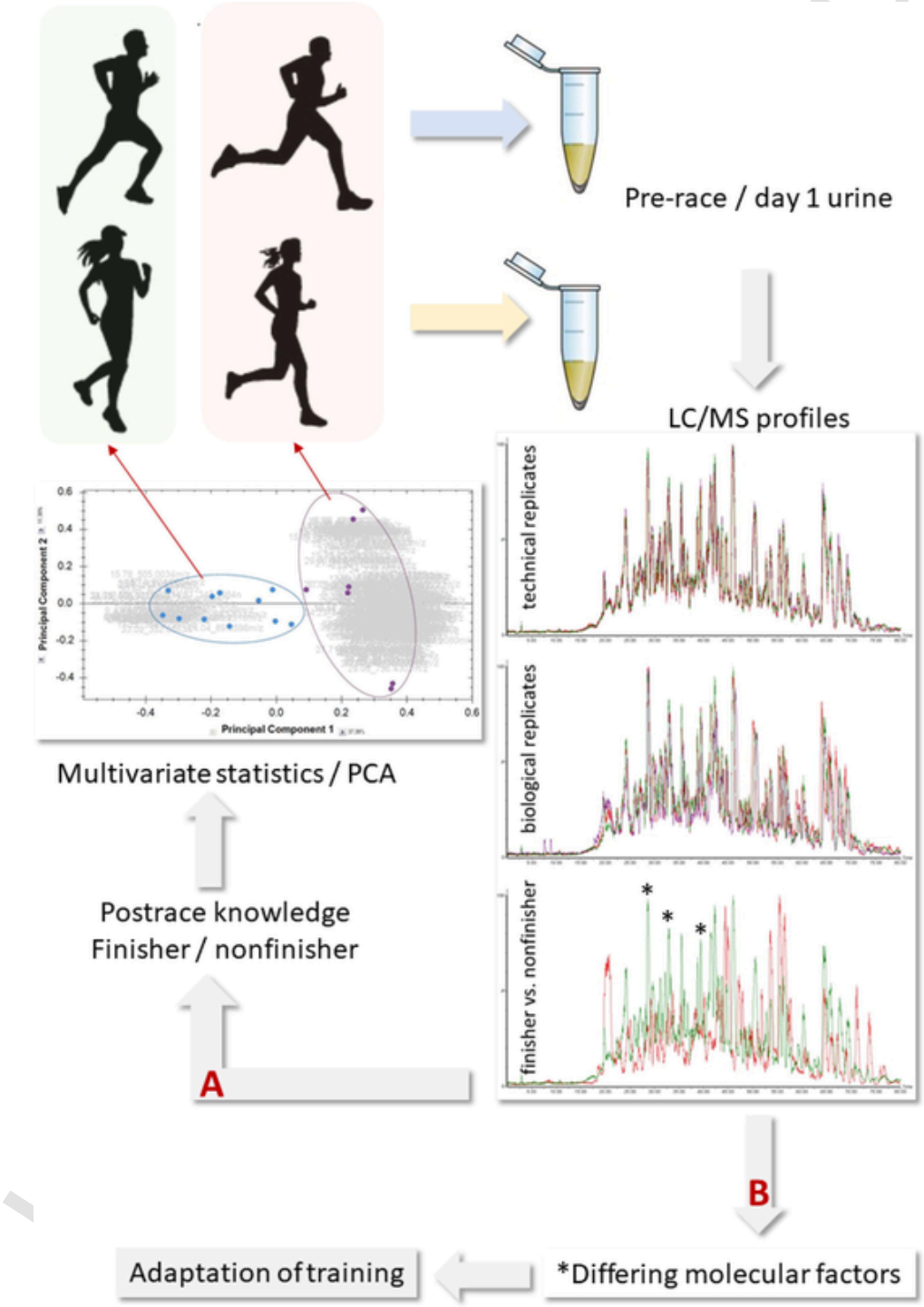

Fig. 8. Hypothesis testing in future races. Runners urine is collected on day 1 of the race and subjected to metabolic profiling and statistical analysis (A). Profiles of finishers and nonfinishers differ and responsible molecules can be identified (B).

are promising targets for controlling the physiological or pathophysiological energy balance processes (insulin sensitivity, intramyocellular glycerol-content, etc.) being affected by endurance exercise and obesity [115,116,123,124]. This will take considerable time. But is it true that we can already test runners and predict (MS)UM outcome? In principle, the answer is yes with respect to TEFR, but we do not have data from other UMs for validation yet. More long-distance runs should be investigated in the same manner (Fig. 8). As a common feature, all the compounds profiled indicate processes in energy metabolism, reparative- and/or detoxifying and (anti-) inflammatory processes between the runner groups and may explain their different performance, power of endurance and potential to "overrun" burden-related focal inflammation and systemic oxidative stress.

The best option for performance and fast recovery in an (MS)UM is specific, appropriate training with high training volume [125] and long race experience [126]. The specificity of the training has a direct influence on the UM-performance, which, apart from the individual will, can be explained by a specific adaptation of the organism and optimization of systemic, organic and intracellular energy supply metabolisms, respectively. The overall premise is that small molecules measured in a biological medium report the physiological state in that organism. To what extent specific training and regenerative behavior before an ultra-run show relationships (e.g., overtraining, functional insufficiency, optimized energy delivery metabolism) will be left to future studies.

The "optimization" of intra- and extracellular regeneration, repair and energy metabolisms plays a central role in the ultrathlete. The better these are, the better the performance ("survival of the fitest"). The urine bioprofiles distinguished finishers from nonfinishers at day 1 of TEFR (males older than 40 years) and indicated differing molecular factors. Based on the data available (urine bioprofiling and MRS), we postulate that TEFR athletes with higher training volume and better pre-race performance had a better adapted energy metabolism before the race than "less well" trained TEFR participants. They were better prepared for the demands of prolonged ultra-endurance burden or negative energy balance (fat loss, proteinolysis, catabolism, high oxidative stress, high inflammatory load, etc.); happening both on the systemic-organic extracellular as well as on the intracellular level (IMCL, TTP, thiamine). This leads to the hypothesis, that urine (and serum) molecular profiling predicts performance in endurance sports and allows to control the benefit of intervention (specific training, nutrition, recovery behavior, physical therapy, food supplementation, drugs) on the basis of epidemiological, therapeutic, pathophysiological or other scientific evidence.

Experimental

Urine was collected from 24 runners on up to 5 days during the run (Table 2). It was aliquoted and stored at −20 °C until further use. For profiling, 250 µl of urine was filtered using a molecular cut-off of 10 kDa (Amicon 0.5 ml units) and diluted 1:10 with 0.1% formic acid (solvent A for liquid chromatography (LC)). Experiments were performed using reversed-phase-LC coupled to mass spectrometry with Synapt G2 Si ion mobility mass spectrometer / M−Class UPLC (Waters Corp.; 30 min gradient; solvent system 100% water versus 100% acetonitrile, both containing 0.1% formic acid; trap column V/M Symmetry C18 100 Å 5 µm, 180 µm × 20 mm; reversed phase column HSS T3 1.8 µm 75 µm × 200 mm; 0.5 µl injection volume; MSe mode, m/z 50–2000). Progenesis (nonlinear diagnostics/Waters Corp.) was used for statistical analysis. Compound assignment was performed using ChemSpider, LipidBlast, Progenesis Metascope and the NIST library within Progenesis as well as Massbank [127].

Uncited reference

[5].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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