

Physical activity - an important preanalytical variable

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Abstract

The concentration of several biochemical and hematological biomarkers is strongly influenced by a number of preanalytical variables. Several lines of evidence attest that short, middle, and long-term exercise, as well as the relative intensity of physical effort (from mild to strenuous), may influence a broad array of laboratory variables. The amount of extracellular release and clearance from blood of most of these biomarkers is markedly influenced by the biological characteristics of the molecule(s), level of training, type, intensity and duration of exercise, and time of recovery after training. It is hence noteworthy that test results that fall outside the conventional reference ranges in athletes not only may reflect the presence of a given disease, but may frequently mirror an adaptation to regular training or changes that have occurred during and/or following strenuous exercise, and which should be clearly acknowledged to prevent misinterpretation of laboratory data. The aim of this narrative review is to provide an update about the most significant changes of some biochemical and hematological biomarkers in response to physical exercise, for appropriate interpretation of these changes in the context of physically active subjects.

Key words: biomarkers; training; plasma volume; metabolism; cellular damage

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Introduction

The concentration of several biochemical and hematological biomarkers is typically assessed for diagnosis of and screening for a large number of human disorders, since their values may contribute to up to 70% of the clinical decision making (1). Among the most common problems throughout the testing process, biological variability has an important impact on interpretation of test results (1,2). Biological variability basically entails patient-related physical variables such as physical exercise, diet, stress, positional effects, menstruation, circadian rhythm (time-of-day), pregnancy, life style, age, gender, and environmental factors such as climate and altitude (1,3). Accordingly, the values of some biomarkers in physically active subjects should be cautiously interpreted, since their results may fall outside the conventional reference ranges, reflecting an adaptation to regular training or changes that have occurred during and/or fol-

lowing strenuous exercise, rather than the presence of a given disease. Short, middle, and long-term exercise, as well as the relative intensity of the physical effort, may influence a broad array of laboratory variables (4). The biological characteristics of the molecule(s), the level of training, the type, intensity and duration of exercise, and the time of recovery after training are factors that markedly influence the amount of extracellular release and clearance from blood of several biomarkers (2). How frequently physical activity as preanalytical variable affects some clinical chemistry, hematology, and coagulation tests will be discussed in this article. The aim of this narrative review is therefore to describe the implications of physical activity on variability of several biomarkers, and to identify the changes that occur in subjects who undergo regular physical activity or during and/or following strenuous exercise. This review will also

briefly address the different types of exercise and the essential influence of physical activity on laboratory testing. It will then describe the changes induced by physical activity in some biomarkers and hematological parameters conventionally used in clinical practice, to emphasize the concept that, compared with conventional reference ranges, abnormal values in athletes may underlie pathology or injury, but also a simply systemic adaptation to exercise (5).

Types of exercise and its implications

Although it seems obvious that a clear difference exists between the terms "physical activity", "training", and/or "exercise" (6), in this review we will use these concepts interchangeably because the data originate from studies that use these terms as synonymous.

In brief, voluntary muscle exercise can be performed in a constant manner (endurance), in a dynamic (e.g., cycling or running) or static mode, and can also be exhaustive or non-exhaustive, also called strenuous or non-strenuous (5). Exhaustive exercise includes acute, very prolonged exercise, a period of intensified training, non-functional overreaching and overtraining syndrome. It can be performed under aerobic or anaerobic conditions (7). Examples of partially anaerobic exercise include sprint (efforts < 1 min), and exercise under high-force isometric conditions. Only certain types of exercise are completely anaerobic, and these necessarily last for only a few seconds (e.g., power lifting). Contractions during exercise may be isometric (muscle contraction without appreciable muscle shortening) or non-isometric (muscle contraction with muscle shortening), isokinetic (constant velocity throughout movement) or non-isokinetic (changing velocity during contraction), or concentric (muscle shortening) or eccentric (muscle lengthening) (7,8).

The magnitude of physiological stress and the resultant responses are a combination of intensity of physical activity and physiological demands related to the fitness of the exerciser. For example, acute high-intensity exercise such as cycling 30 km in 1 hour would constitute a relatively light bout of

exercise for a fit endurance cyclist, representing a relative low physiological stress. However, for a less fit or sedentary subject, attempting the same amount of exercise may be very close to maximum capacity and would thereby represent a strenuous effort, associated also with a high physiological stress. The clinical implications of this aspect are that the same bout of exercise may produce very different responses and changes in a wide range of biomarkers and hematological parameters depending on level of fitness (9). Thus, for an unfit subject, changes in some biomarkers may be evident, whereas for a fit practitioner, the modification of circulating biomarkers may only be evident after more strenuous bouts of exercise such as marathons or ultramarathons (10-13), iron-man triathlon or ultra-distance races (e.g., Tour de France) (8).

Therefore, although it is frequently challenging to operate a clear separation, we can basically discriminate the effects of exercise on laboratory tests in two major clusters:

- i) Short-term acute adaptations to high-intensity/strenuous exercise.
- ii) Long-term chronic adaptations to regular exercise.

Influence of physical activity on laboratory test results

Plasma volume changes (PV)

PV change is an important factor that contributes to the variability of several parameters in blood, and assumes special significance in athletes, who may be subjected to intense dehydration especially when performing strenuous exercise under extreme environmental conditions (14-20). Increases or decreases in PV related to the type of training exercise have been described:

Hemoconcentration: transient exercise-induced dehydration or hypohydration can produce a reduction in PV, and consequently hemoconcentration. It is usually associated with acute exercise and in function of warm environment, humidity, reduced fluid intake, exercise intensity, etc... (21).

Plasma volume expansion or hemodilution: blood volume is higher in trained individuals compared with untrained individuals. The larger blood volume after long-term endurance training is mostly due to an increase of plasma volume (plasma expansion) and erythrocyte volume (14,22).

Increased basal metabolism

It is well-known that exercise increases basal energy demands and, therefore, basal metabolism (1,18,19). These adaptations to exercise produce many fluctuations in several analytes.

Increases in cellular damage

Exhaustive or acute physical exercise causes an increased generation of reactive oxygen species (ROS), and magnifies oxidative stress in muscle and other organs, resulting in cell damage (23,24). Therefore, oxidative stress is clearly involved in cellular damage induced by exercise.

The main laboratory parameters affected by physical activity

Skeletal muscle, liver and cardiac damage biomarkers

Middle and long-term endurance and/or strenuous exercise triggers transient elevations of muscular and cardiac biomarkers such as cardiac troponins (Tns), natriuretic peptides, neutrophil gelatinase associated lipocalin (NGAL) alanine aminotransferase (ALT), creatine kinase (CK), aspartate aminotransferase (AST), lactate dehydrogenase (LD) among others (2,25-28).

Strenuous exercise such as marathon and ultramarathon may induce transitory elevation of Tns concentrations (29). Normal physical activity can also induce Tns rise in healthy individuals. In a small study on adolescents, more than half of the participants developed Tn increase after basketball training (30). The same has been reported after a treadmill test (31). In a study of cardiac healthy middle-aged subjects who were subjected to a maximal bicycle stress test, almost half of them had a Tn increase after exercise (32). In these stud-

ies the Tn levels exceeded the 99th percentile of the upper reference range (URL) in several subjects. These elevated values can formally fulfil the criteria for acute myocardial infarction. For instance, in 105 endurance athletes, both cardiac Tns (cTnI and cTnT) concentrations showed exercise-induced increases in (74% and 47%, respectively) (33). Likewise, increased cTnT are frequently found in recreational runners after a marathon race (34). In addition, increases in CK and CK-MB activity were also observed. These increments were neither related to athlete's age, nor to the presence of cardiovascular risk factors. In long-distance runners, CK, CK-MB, AST, and ALT were above the corresponding clinical cut-off values at rest (35). Furthermore, significant elevations above rest were observed for all biomarkers except ALT after a 21 km run, with cTnI, CK, CK-MB, LD and AST remaining increased over baseline up to 24 h post-workout (35).

Kratz and co-workers (36) showed that several samples from trained marathoners obtained before the race had laboratory results exceeding the conventional reference range. For instance, increases were noted in CK-MB (11%) and ALT (5%) (36). Lippi *et al.* also observed that the number of half-marathon runners which exceeded the conventional reference ranges did not change during the race for AST and LD, whereas both CK and CK-MB increased significantly (11), concluding that results obtained in physically active subjects should be cautiously interpreted since abnormal values may reflect an adaptation to regular training rather than an underlying disease.

As regards the variations observed after exercise, published data are incongruent for many analytes depending on type, duration and intensity of exercise. For instance, Takahashi *et al.* (37) reported that only LD increased after a rugby match lasting 10 min, while CK, AST, and ALT remained basically unchanged. On the other hand, other studies showed that maximal physical exercise (e.g., running) induces transient elevation of muscle and hepatic enzymes (38). When exercise loading exceeds the limit of muscle ability, CK and LD leak into the interstitial fluid and are released into circulation (25). Moreover, in well-trained athletes a

positive correlation has been found between mean sprint velocity and activities of LD isoenzymes 4 and 5 (39). AST is contained in striated muscle and increases with ALT and CK after exercise (40). A study in 37 marathon runners evaluated biological parameters before a marathon, and 4 and 24 hours afterward (36). An increase in ALT and AST was observed in samples collected 4 hours after the marathon, and remained elevated 24 hours afterward. An acute bulk of aerobic physical exercise also produced significant changes in the activity of traditional biomarkers of liver injury such as gamma-glutamyl transpeptidase (GGT), AST, LD, CK (10). ALT, AST, LD and CK also increased after a Wingate test (30 seconds of maximal sprint against a constant resistance related to body mass – 0.087 kg / kg body mass –) (3). Furthermore, Lippi *et al.* observed in 10 healthy trained Caucasian males who performed 21-km run significant elevations in glycogen phosphorylase isoenzyme BB (GPBB), carbonic anhydrase III (CAIII), heart-type fatty acid-binding protein (H-FABP), CK-MB, and myoglobin immediately after the race (2). The concentrations of GPBB and H-FABP returned to baseline after 6 and 3h, respectively, while those of CAIII, CK-MB and myoglobin remained elevated up to 24 h after the run (2). More recently, Salvagno *et al.* studied the pre- and post-competition concentrations of high-sensitivity cTnI, N-terminal-pro-B-type natriuretic peptide (NT-proBNP) and galectin-3 in 18 trained athletes who performed a 60-km ultramarathon run (41). The concentration of all these biomarkers measured in the post-race samples was remarkably increased when compared with the values obtained on baseline values. In particular, the median increase when compared with baseline values was 3.3-fold for cTnI, 3.5-fold for NT-proBNP, and 2.4-fold for galectin-3, respectively (41).

A study performed in healthy endurance athletes reported that increases in NT-proBNP can be found in a major part of obviously healthy athletes after prolonged strenuous exercise and are correlated with exercise time (33). The concentration of NT-proBNP also increased in seven well-trained endurance runners (five male and two female) who participated in the Two Oceans 56 km ultramarathon

trial (42). In a study performed in professional cyclists, NT-proBNP values were found to be lower than those of sedentary controls (19). The natriuretic peptides are neurohormones that typically reflect a condition of increased cardiac stress and exhibit a ductile response to physical exercise. Therefore, the reduced levels of NT-proBNP in trained subjects can be interpreted as a physiological and potentially beneficial adaptation of heart structure and function to regular physical exercise. The acute increase of NT-proBNP values after exercise is also predictable, and has been previously described in endurance athletes and in marathoners (34,43), as an acute and benign consequence of increase cardiac output (34). Exercise performed at 50% of the maximal heart rate increases NT-proBNP concentrations over pre-exercise levels in patients with heart failure, whereas no similar increase was observed in reference individuals for the same or even higher exercise intensity (44). Thus, physical exercise before sampling may have an effect on NT-proBNP concentrations.

Hematological and related parameters

Red blood cells (RBC), hemoglobin (Hb), hematocrit (Hct) and reticulocytes (Ret %)

Increases in exercise training might justify a significant drop in RBC and Hb concentration (45). Schumacher *et al.* reported that exercise training alone did not change Hb concentrations in athletes when compared with untrained controls (46). However, Bojadjev and Taralov showed that chronic (i.e., more than one year) high-intensity training decreases Hb concentration in pubescent boys and girls: 7.21% and 5.61% lower, respectively (47). Banfi and Del Fabbro (48), reported that Ret% and Hb decreased in rugby players throughout one competitive season. Similarly, Hb values also decreased in cyclists during a competitive season when compared with values measured before the start of training and/or competition (48). Again, Banfi and collaborators observed that Hb, RBC and Hct decreased in professional cyclists during the *Giro d'Italia*, stabilizing in the second part, but at the end of the race all values were lower as compared with the baseline (6.7%, 6.8%, and 4.9%, re-

spectively), while Ret% remained stable (49). Wirnitzer and Faulhaber also reported decreases in both Hb and Hct after an eight-day mountain bike race (13.4% and 16%, respectively) (50), whereas Hu and co-workers observed lower mean cell hemoglobin concentration (MCHC) in physically inactive men after 20-week of resistance training (control 0.40% vs. training - 0.74%) (51). Likewise, in both cross-country and alpine-skiers, decreased Hb concentrations have been also found (52,53). Recently, Diaz *et al.* (54) reported that Hb concentrations remained stable in elite triathletes along four consecutive seasons, albeit fluctuations related to training volumes and stimuli could also be observed. Interestingly, Lombardi *et al.* reported that swimming in cold water induced significant increases in RBC (55), probably due to significant hemoconcentration and were then attributed to sympathetic system activation and subsequent vasoconstriction. Moreover, Hct and RBC increased after a Wingate test (3). It is also noteworthy that lower Hb concentration for most of the year were found in ironman triathletes as compared with untrained subjects (56). The effects of chronic exercise training on Hb and RBC values have a physiological explanation, in that the large expansion in PV that occurs with chronic endurance training is accompanied by a decrease in blood Hb and RBC concentration (14,22).

White blood cells (WBC)

Strenuous exercise typically triggers leukocytosis (57,58). Kratz *et al.* (36) described the mean WBC in a group of 37 male and female athletes, which rose from 5.6 to 17.0 ($\times 10^9 / L$) 4 hours after running a marathon. Twenty-four hours after the race, this change was persistent, although not significant. As was the case in other studies, post-race WBC differential demonstrated that elevation in WBC was primarily due to an increase in neutrophil value, although a mild monocytosis was also noted. Thus, Lombardi *et al.* observed that swimming in cold water increases WBC values in fifteen healthy subjects (55). The relative number of leukocytes did not change significantly, apart from a strong decrease of the eosinophils population, a strong increase in the total number of neutrophil granu-

locytes, lymphocytes and monocytes was noted (55). The same pattern was observed after a Wingate test (3). The best explanations of exercise-induced leukocytosis include demargination of WBCs secondary to augmented blood flow during exercise, an acute inflammatory response due to tissue injury, and exercise-induced increases in epinephrine and cortisol levels (36,59). Exercise induced leukocytosis should not be confused with another underlying infectious or inflammatory process, which would be characterized by other suggestive clinical findings.

Hemostasis

Excess physical activity in patients immediately prior to collection can lead significant effects on hemostasis (18). The best-known acute effects are related to acute phase reactants, which may rise due to physical activity, and include fibrinogen, von Willebrand factor (VWF), and Factor VIII (60). Exercise activates coagulation (61), and physiological stress is associated with changes in both the coagulation and fibrinolytic systems (62). Thus, exercise may impact on the assessment of coagulation and fibrinolysis in an exercise intensity-dependent manner (63). Platelet aggregation is enhanced during, and within 1 h after, moderate exercise (64). Platelets count increased significantly in fifteen healthy subjects after swimming in cold water (55).

Renal function markers

The increased blood flow through the muscles during exercise causes a dramatic decrease of circulation in the renal district, which seems directly proportional to extent and duration of exercise (65). Several mechanisms have been suggested to explain the perturbation of renal hemodynamics induced by exercise, including an increased sympathetic nervous system outflow as well as a heightened activity of two important vasoconstrictors such as angiotensin II and vasopressin (66). These changes are counterbalanced by a local mechanism of autoregulation, which is aimed to preserve the glomerular filtration rate (GFR). The resulting increased filtration fraction, which can be

twice as high as in resting condition, partially limits the transfer of metabolites or substances through the glomeruli and reduces the extent of exercise proteinuria (67). A post-exercise, transient proteinuria (i.e., with half-time of approximately 1 hour) is commonplace in athletes and appears to be directly related to the intensity (and duration) of exercise (67). It is mainly due to a combined mechanism of increased clearance of plasma proteins due to increased glomerular permeability, and a partial inhibition of tubular reabsorption of macromolecules (66).

Creatinine

Banfi and Del Fabbro found that professional athletes at rest such as professional cyclists, triathletes, basketball players, racing motorcyclists, soccer players, alpine skiers, and rugby players have significantly lower serum creatinine concentration than healthy sedentary individuals (68). In addition, significant differences between each group of athletes of different sports modalities were also observed, and this has been attributed to the well established relationship existing between creatinine and BMI (e.g., rugby players have creatinine concentrations higher than those recorded in the general population, while cyclists have values lower than those of the general population). Scharhag *et al.* reported that mean creatinine concentration significantly increased after exercise, irrespective of the baseline muscle mass and of the body mass index (33). Otherwise, professional cyclists and cross country skiers displayed lower serum creatinine concentration than healthy sedentary individuals (15,69), although these values were measured during periods of competition (summer), and after a 24 h period of rest from the last training session. Again Lippi *et al.* showed that the mean serum creatinine level was significantly higher in sedentary subjects than in amateur and professional cyclists, being also higher in amateur than in professional cyclists (70). In addition, the mean estimated GFR was significantly lower in the sedentary population than in the subgroups of amateur and professional cyclists, concluding that the average intensity of daily physical exercise was inversely associated with serum creatinine and posi-

tively associated with the estimated GFR (70). After the race, creatinine concentrations also increased significantly in marathon and half-marathon runners (71). Creatinine concentrations also increased after a Wingate test (3). Serum and urinary creatinine increased significantly in 16 trained male athletes who ran a 60 km ultramarathon by 38% and 78%, respectively. The eGFR contextually decreased by 31%. In 6 out of 16 athletes (38%), the acute post-exercise increase of serum creatinine met the criteria of acute kidney injury (AKI). A significant correlation was found between pre- and post-exercise changes of serum creatinine (28). Accordingly, the use of reference intervals based on general populations is not recommended in sports medicine, to avoid misinterpretation of data. It is also noteworthy that recent evidence attests that the use of cystatin C for monitoring renal function in athletes may be more appropriate than using creatinine-based equation, since this biomarker is indeed less biased by muscle mass and body mass index (72).

NGAL (neutrophil gelatinase associated lipocalin)

NGAL is a 25-kDa acute phase protein produced by the kidney tubule studied as a potential biomarker of AKI and also up-regulated in other pathological conditions such as atherosclerosis and myocardial infarction (73,74). This biomarker has been studied in 16 trained male athletes who ran a 60 km ultramarathon (28), and increased serum and urinary NGAL values (by 1.6-fold and 7.7-fold, respectively) were found (28).

Iron metabolism

Iron and ferritin

Several studies showed a high incidence of iron deficiency in athletes, particularly in runners and cyclists. Decreased serum ferritin values have been found in male runners compared with the control group (75). In addition, it was also observed that female marathon runners had low ferritin levels (76). Interestingly, women may have an increased prevalence of exercise-related alterations in body iron because of a net negative iron balance (77). In middle- and long-distance runners, serum ferritin

was borderline when compared with a group of non-runners (78). After a 6-week strength training program in 12 male, mean ferritin values dropped from 75 to 49 $\mu\text{g/L}$, whereas no changes were appreciated in serum iron or transferrin levels (79). Iron deficiency was also detected in 17% of males and 40% to 47% of females high school athletes (80). During an 8-month training season significantly decreased serum ferritin values were noted in swimmers (81). In professional cyclists who participated in the *Giro d'Italia*, a significant decrease in serum iron concentration was noted after the race (49).

Hepcidin

Hepcidin is a liver-produced peptide which acts as the major regulator of iron metabolism in the body (82). Hepcidin is expressed in response to a wide range of physiological changes such as inflammation, hypoxia and elevated iron levels (83). In addition, the main mediators of hepcidin activity are the inflammatory cytokine Interleukin-6 (IL-6) and oxidative stress (84). In several studies by Peeling *et al.*, the authors extensively observed that hepcidin significantly increase after exercise in both moderately and highly trained subjects (82,85,86). Roekner *et al.* also noted significant increase in hepcidin 24 h post-run in moderately trained females (87).

Inflammation and infection biomarkers

C-reactive protein (CRP)

Strenuous exercise increases the concentration of CRP, and this variation is potentially dichotomous, being dependent upon the physical exercise *per se* or attributable to muscle damage following strenuous muscular efforts, trauma or even sports injuries (88). For instance, CRP concentrations, as measured with a conventional assay, increased between day 1 ($0.36 \pm 0.25 \text{ mg/L}$) and day 22 ($2.51 \pm 2.60 \text{ mg/L}$) in professional cyclists during the *Giro d'Italia* (49). In marathon runners, CRP levels measured with a conventional assay increased significantly from pre-race to post-race, increasing further 24h after the race (89). In healthy adults a higher frequency of physical activity was associat-

ed with significantly lower CRP concentrations measured with a standard technique (limit of analytical sensitivity of 0.22 mg/dL) (90). Margeli *et al.* found that CRP levels (analytical sensitivity of the method was not specified) markedly increased by 152-fold over the baseline at the end of an ultra-distance foot race of continuous, moderate intensity exercise of 246 km distance, remaining elevated for more than 48 hours (91). In another study also performed in fifteen healthy endurance-trained runners who participated in the 2006 Spartathlon ultra distance running race (246 km), the concentration of CRP (analytical sensitivity of the method was not specified) was dramatically increased after the end of the race (116-fold increase) (92).

Interleukin 6 (IL-6)

IL-6 is a key player in the inflammatory response. Changes in IL-6 concentrations $> 60\%$ (2 times the biological variation) are likely to reflect changes in disease activity and not only preanalytical or normal biological variability. Exercise (cycling) increases serum IL-6 in healthy subjects (93). Markedly increased (by 8000-fold over the baseline) IL-6 levels were found at the end of an ultradistance foot race of continuous, moderate intensity exercise of 246 km distance, with return to normal within 48h (91). IL-6 concentrations were also increased in seven well-trained endurance runners who participated in the Two Oceans 56 km ultramarathon trial (42), as well as in fifteen healthy endurance-trained runners who participated in the 2006 Spartathlon ultra distance running race (246 kilometres) (92).

suPAR

The soluble urokinase plasminogen activating receptor (suPAR) is a circulating protein, with a molecular weight comprised between 20 to 50 kDa (94). Circulating suPAR predicts cancer, cardiovascular disease, diabetes and mortality in the general population (95), whereas systemic concentrations of suPAR correlate positively with markers of organ dysfunction and severity-of-disease classification system scores (96). Serum suPAR concentration did not significantly increase in soccer players after a match (97). Therefore, at variance with oth-

er consolidated inflammatory biomarkers, suPAR seems to be less influenced by physical exercise.

Hormones

Several hormones undergo acute changes as a consequence of physical activity, as recently reviewed by Viru (98). In brief, cortisol and β -endorphin increases during or after high intensity short-duration exercise, and during exercise of lower intensity when exceeding 2-3h. It has also been reported that testosterone increases with high-intensity exercise, but may decrease if the exercise is very prolonged. Physical activity could positively influence the age-related decline of male testosterone concentrations (99). Insulin decreases during exercise but increases post-exercise to facilitate muscle glycogen replenishment and training adaptations (98). The serum concentrations of testosterone, cortisol, and growth hormone (GH) were measured during and after combined strength (S) and endurance (E) loading sessions, reversing the order of exercises (ES vs. SE) in recreational endurance trained subjects (100). After SE, augmented cortisol concentrations were observed.

Thereafter, testosterone decreased at 24 and 48 h, being significantly lower than those observed after ES sessions. In addition, a significant difference in testosterone levels was observed between ES and SE loadings in women. GH response in men also differed significantly after combined loading sessions (100).

Concluding remarks

Healthy individuals are susceptible to variations of several biological markers after physical exercise. In some cases these levels may exceed the decision thresholds used to diagnose pathological conditions. This has consequences for assessment of health status. Patients with certain elevated or decreased analytes and uncertain findings should therefore be asked whether they had been physically active around the time when the test was taken or maybe regularly physical active. Alternatively, the abstention from physical exercise 48 h before blood sampling should be considered.

Potential conflict of interest

None declared.

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