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Laboratory medicine: health evaluation in elite athletes

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Abstract: The need to evaluate the health status of an athlete represents a crucial aim in preventive and protective sports science in order to identify the best diagnostic strategy to improve performance and reduce risks related to physical exercise. In the present review we aim to define the main biochemical and haematological markers that vary significantly during and after sports training to identify risk factors, at competitive and professional levels and to highlight the set up of a specific parameter's panel for elite athletes. Moreover, we also intend to consider additional biomarkers, still under investigation, which could further contribute to laboratory sports medicine and provide reliable data that can be used by athlete's competent staff in order to establish personal attitudes and prevent sports injuries.

Keywords: athlete's health; biomarkers; sports medicine.

Introduction

Laboratory medicine in sport can be considered a preventive and protective science that has, as a fundamental aim, the evaluation of the condition of an athlete. As exercise is the most important modulator of metabolism, laboratory medicine has the task of monitoring the athlete's health and identifying the best strategy to improve performance and reduce risks related to the strenuous physical exercise [1, 2].

In the modern conception of competitive sports, a complex psycho-balance between workloads and recovery provide for an adequately monitoring through specific laboratory tests to achieve top performance and success

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in competitions [3–7]. It is also important to consider how exercise affects analytical processes taking into account that it could influence the pre-analytical phase in a direct and also in an indirect manner, consequently altering the response of the metabolism [8, 9].

The use of biochemical and haematological screening tests to evaluate risk factors in people who practice sports is of relevance and increasing interest at the amateur, competitive and elite level [1].

It is well known that specific conditions, in particular those related to some forms of inherited cardiovascular diseases (CVDs) or metabolic defects, may not be evident especially in the absence of family history and specific symptoms, which may lead to severe accidents, and may be even fatal [10, 11]. It is therefore essential to identify all determinants that can define the phenotype of athletes, to determine the metabolic characteristics and the adaptation and response mechanisms of the individual athlete to specific environmental stresses.

Several authors are currently debating the need to set up a specific panel of parameters for elite athletes, to preserve health and highlight any potential overtraining that could cause injuries. This review has the aim to identify and describe a specific subset of parameters aimed at evaluating an athlete's health (Figure 1) and thus to set up adequate therapeutic actions directed to optimise sport performances without increasing the risk of injuries. In particular, we focussed the interest on the main parameters that are modified during and after physical exercise,

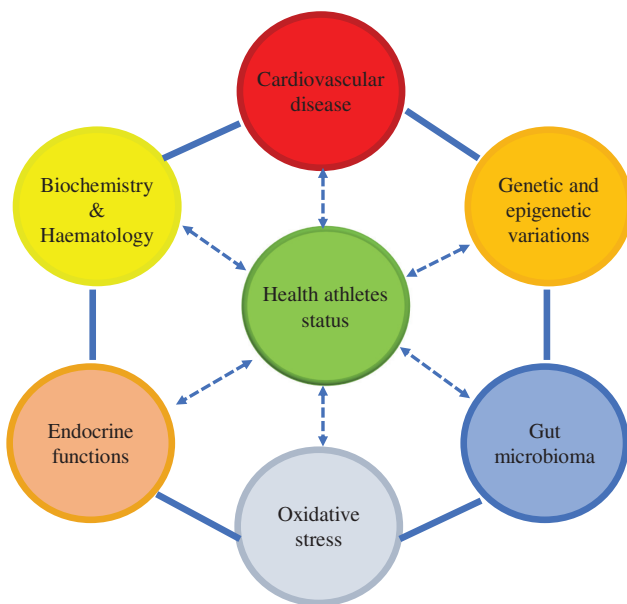


Figure 1: Scheme of diagnostic tool aimed at evaluating athlete's health.

together with others still under investigation, which could give an additional future contribution to sports medicine. Among these, we focussed primary attention on the haemostasis balance for its relevance to cardiovascular risk, the endocrine system that can affect adaptive hypothalamic-pituitary secretory responses to physical stress, the biochemical and epigenetic parameters, the cardiovascular risk along with the lipoproteins metabolism and the antioxidant supplementations currently used by athletes. Besides, we also describe the influence of some genetic variations on the health status of athletes. The evaluation and the knowledge of these parameters might represent a diagnostic tool in the medical laboratory for elite athletes.

Haemostasis

Haemostasis is a balanced process responsible for maintaining the blood flow, with important diagnostic relevance to predicting and following-up CVD [12]. It has been well demonstrated that strenuous exercise, although it is beneficial for health, may be considered as a risk factor of a thrombotic event. Activation of haemostasis depends on the level and strength of the exercise [13, 14]. Exercise induces a significant increase in factor VIII activity, and this occurs with a significant shortening of activated partial thromboplastin time [15]. A concomitant enhancement of tissue plasminogen activity results in significant increases in tissue plasminogen activity antigen (tPA) and total fibrin/fibrinogen degradation products, and a significant decrease in tissue plasminogen activator inhibitor-1 activity (PAI-1). Increases in coagulation and fibrinolytic activity changes in parallel during exercise. It can be assessed that, whereas the enhanced fibrinolytic activity during exercise appears to counterbalance the increase in blood coagulability, this haemostatic balance is not maintained during recovery. This perturbed blood haemostasis might represent an enhanced risk for coronary artery thrombosis and may contribute to exercise-related cardiovascular events [16]. Several findings indicate that exposure to hot and cold ambient temperatures during exercise may enhance thrombotic potential, thus increasing the possibility of cardiovascular events. Platelet activation has been described in response to cold exposure together with an increase in plasma levels of some coagulation factors [17]. On the other hand, other authors described the effect of heat exposure on blood composition after a 164-km road cycling event in a hot environment. Heat stress leads to an increase of concentrations of platelets, platelet factor 4 (PF4), β -TH tromboglobulin (β -TG) activity and thrombin-antithrombin complexes

(TAT) thus indicating an alteration of blood homeostasis. The activation of the fibrinolytic system with the increase of D-Dimer is a strategy to protect blood homeostasis [18, 19]. Also, the exercise intensity influences the coagulation and fibrinolytic responses. The coagulation unbalances observed after strenuous exercise could increase the thrombophilic risk in unknown carriers of the protein C system defects [20]. Moreover, it has been demonstrated that concentrations of coagulation factors TAT, tPA and PAI-1 are modified in subjects exerting greater effort [21, 22]. An appropriate volume and intensity of exercise is necessary to prevent CVD. To date, several studies have indicated that high volumes of extreme aerobic exercise can have an adverse effect on CVD similarly to physical inactivity [23]. Some authors described in a study, that American runners (51 min/week) have lower CVD mortality risk compared to non-runners [24]. Similarly, Taiwanese people performing moderate-intensity exercise (92 min/week), showed a reduction in CVD mortality compared to their inactive counterparts [25]. Moreover, Arem et al., in a meta-analysis study including 661,137 American and European men and women, demonstrated that the physical exercise reduced CVD mortality in 20% compared to sedentary control subjects [26]. The haemostasis is unbalanced in elite athletes:

- it can be used to evaluate the risk for coronary artery thrombosis
- exposure to hot and cold ambient temperatures during exercise may enhance thrombotic potential, leading to cardiovascular events.

Hormones

It is well accepted that healthy athletes present hormonal conditioning adaptations [27, 28]. The impact of exercise training on the endocrine system in young athletes is very complex. Several factors such as training intensity and duration, nutrition and energy balance, gender, age, sex and sexual maturation status, affect adaptive hypothalamic-pituitary secretory responses to physical stress (Figure 2).

The thyroid is a gland secreting metabolically active hormones responsible for growth, maturation, energy expenditure and body substrate turnover [29]. Thyroid hormones are great regulators of energy metabolism and may influence energy processes during physical exercise. Hypothyroidism is associated with decreased caloric expenditure, while hyperthyroidism is associated with increased metabolism. Both have systemic effects.

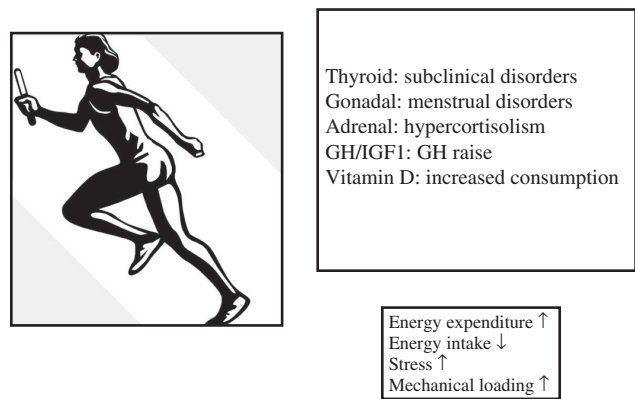


Figure 2: Intense training triggers circulating hormone levels variations because of the increase in energy expenditure, stress and mechanical loading and decrease of energy intake.

Also, thyroid hormones are the primary regulators of cardiac function and myocardial arteriolar density. In athletes, hypothyroidism causes a decrease in exercise capacity and athletic performance. Subclinical hypothyroidism has been associated with hypercoagulability, impaired vascular function, atherosclerotic CVD and reduced submaximal exercise capacity. Symptoms of subclinical hypothyroidism include physical fatigue, dry skin, constipation, muscle cramps and decreased athletic performance or exercise capacity in athletes. In the case of hyperthyroidism, athletes may present unwanted weight loss, despite adequate nutrition, tachycardia and a decrease in the level of performance. Subclinical hyperthyroidism shows cardiovascular effects (tachycardia, increased risk of arrhythmias, ventricular hypertrophy and reduced systolic performance) on effort and decreased exercise tolerance [29]. An adequate analysis of thyroid function in athletes would allow to set-up an appropriate therapy aimed to resume sport without risks.

Moreover, gonadal and adrenal steroids are generally determined as biomarkers of athletes overtraining. In particular, testosterone and cortisol are used as markers of anabolism and catabolism, respectively [30]. Testosterone plays a role on the strength and ability of a muscle to exercise adaptations, whereas cortisol can inhibit the neuromuscular system [31]. Cortisol increase and lose circadian rhythm as a result of psychophysical stress in chronic exercise, potentially leading to several diseases such as cancer and obesity [32].

Some authors [33, 34] recently demonstrated that gonadal steroids circulating levels of elite athletes significantly differ based on gender and sporting categories. They observed lower testosterone values in men than in women. Healy et al. [33] suggested that the decrease of testosterone levels is most probably a form of “central”

hypothalamic hypogonadism as seen in weight-loss and exercise-related amenorrhoea [35–40]. Female athletes often present low oestrogen levels and menstrual disorders as a consequence of overtraining and/or inadequate dietary intake [41]. It has been recently demonstrated that athletes may revert menstrual disorders maintaining the same exercise intensity, but with an increase of the dietary energy intake [42]. Dehydroepiandrosterone (DHEA) levels were significantly associated with muscular enzymes activity, suggesting that this hormone may be involved in muscular traction during exercise [43]. Several studies reported that female athletes performing long-distance running showed testosterone and DHEAS circulating levels moderately lower compared to athletes playing sports requiring strength, power and speed [44, 45]. However, some factors affect androgen concentration such as oral contraceptive, which cause higher sex hormone binding globulin (SHBG) concentration [46], congenital adrenal hyperplasia associated with high testosterone levels and oligo- and amenorrhoea, usually linked with low androgen levels [30]. Several studies [35, 37, 47] demonstrated that circulating or salivary cortisol increase during high-intensity exercise in several sports. In this framework, little is known about DHEA variations [48, 49]. Sartorio et al. showed that some athletes, mostly females, present higher growth hormone (GH) levels, but standard insulin-like growth factor I (IGF-1), probably due to occasional GH pulses [50]. Other authors reported that GH levels in elite athletes present differences between sports, probably associated with differences in ages. GH levels were higher in elite athletes than in non-elite athletes and sedentary subjects without any modification of IGF-1 levels; this is probably due to a strong positive correlation between GH levels and intensity of training [51]. Moreover, GH levels during acute exercise are associated with pubertal status: higher GH peaks were observed in children with more advanced pubertal stages.

The principal GH isoform present in the circulation is the 22-kDa isoform [52]. It has been demonstrated that physical exercise increased the release of non-22 kDa and dimeric forms [53].

The pharmaceutical, recombinant GH preparation is represented only by the 22-kDa one, so the ratio between the 22 kDa and the other isoforms can be used as an indicator of hGH abuse [54]. Physical exercise leads to an increase of IGF-1, causing an improvement in inflammatory signalling and sarcopenia [55]. Moreover, an IGF-1 rise may foster the onset of insulin-resistance [56].

A prevalent insufficiency of vitamin D among athletes has been reported, although they practice outdoor or indoor sports [57]. Some authors suggested that this

insufficiency is due to the expression of vitamin D receptor in skeletal muscles [58, 59]. Therefore, it is conceivable that in athletes the intense muscle activity leads to a high level of vitamin D consumption [60].

Blood concentrations of vitamin D can be easily measured by current laboratory methods and should be considered a relevant biomarker in athletes as this hormone is crucial to support the sustained skeletal and myocardial muscle contractility.

Significant circannual rhythms for vitamin D has been demonstrated [60]. The medical staff of elite athletes have to take into account this finding in order to prevent vitamin D deficiencies or insufficiencies, which can affect muscle activity and bone homeostasis.

Based on the existing literature data, the following recommendations for monitoring hormonal changes in elite athletes are suggested:

- careful evaluation of hormonal status together with an assessment of their performance goals is required before an athletic performance begins
- regular hormonal status follow-up needs to be planned to avoid interference with an athlete's health status and sports specific goals.

Complete blood counts

Complete blood count (CBC) is the most critical laboratory exam used to define the physiological or pathological status of a patient. The blood count consists of a numerical quantification of white blood cells and their percent division, red blood cells and platelets with all their indices (i.e.: hemoglobin concentration [cHb], hematocrit [HCT], red cell distribution width [RDW], mean cell volume [MCV], mean cell hemoglobin [MCH] for red blood cells and mean platelet volume [MPV] and platelet distribution width [PDW] for platelets). In sports medicine, the blood count along with the biochemical exam and other specific laboratory analyses contribute to define the health status of athletes and shed light on any potential use of doping.

In many elite athletes, CBC can reveal a low concentration of haemoglobin that is often associated with a reduction in serum iron and ferritin. The compromise of iron storages is considered the leading cause of the “athlete's anaemia”. However, anaemia in athletes is not always associated with a reduction of iron storage, and other mechanisms can be involved. As a matter of fact, in elite athletes the increase in total blood volume, physiologically derived from a rapid and early adjustment of the cardio-circulatory system to exercise, generally exceeds the production of red blood cells, whose change is slow

due to loitering rate of erythropoiesis, thus producing an apparent anaemia known as “dilution anaemia”.

During exercise, the cardiovascular system has the fundamental role to respond to an increase in oxygen demand from the working muscle and to transport metabolic CO₂ back to the lungs for expiration. Other than being involved in O₂ and CO₂ transport mediated by haemoglobin, red blood cells are also responsible for a variety of other functions strictly related to exercise performance. Red blood cells release adenosine triphosphate (ATP) and nitric oxide (NO) that trigger vasodilatation and improve blood flow to the working muscle [61]. Besides, they influence blood buffering capacity by transporting CO₂ and binding H⁺ to haemoglobin. As a consequence, an adequate amount of red blood cells is required in circulation during physical exercise, but they are not the unique determinants to take into account.

Several strategies concur in the body in facilitating the increased demand for oxygen during exercise, which includes not only an increased muscle blood flow [62] but also a decrease in Hb-O₂ affinity that favours O₂ unloading from haemoglobin. Hb-O₂ affinity physiologically changes throughout the body; during exercise differences in factors, such as temperature, pH and CO₂ further modify the binding between oxygen and haemoglobin to optimise the affinity in the lung and its decrease in capillaries in working muscles. Interestingly, during exercise, no significant changes in the level of the allosteric effector 2,3-diphosphoglycerate have been described [63].

An additional significant aspect in this framework concerns the O₂ transport capacity, which should be high in elite athletes and is dependent on factors such as cHb, HCT, the total Hb mass and the total red blood cell volume in circulation. Several studies showed that HCT is lower in athletes than in sedentary individuals [63–65]. In particular, Sharpe et al., showed that a short-term modification of HCT could be foreseen as a consequence of a decrease in plasma volume when a fluid replacement was insufficient during exercise. Indeed, total haemoglobin and/or red blood cell volume has to be measured in addition to cHb and HCT to evaluate oxygen transport capacity correctly.

Erythropoietin (EPO) might modify HCT values. Physiologically produced EPO binds to EPO receptors in bone marrow on progenitor cells in erythroblastic islands [66], where it stimulates the proliferation of red blood cells and prevents apoptotic destruction of newly formed cells. HCT not only affects the oxygen transport capacity but has a strong influence on the rheological properties of blood, which are affected among others by the concentration of plasma proteins, the physicochemical properties of the red blood cell plasma membrane (deformability), the

cellular haemoglobin concentration (cytosolic viscosity), the flow velocity (aggregation), and the temperature [67]. It is well-documented that the increase in whole blood viscosity during exercise, reverses rapidly, which is a consequence of haemoconcentration and dehydration.

The reticulocyte count (RT) represents a fundamental parameter in sports medicine and anti-doping testing, but significant discrepancies can be found between measurements obtained with different analytical systems (analytical variability). In the reticulocyte count, cellular stability can be ensured if blood samples are kept at constantly cold temperatures (277.15 K). Marked intra-individual variability represents the main discovery to be evaluated when changes induced by the exercise are observed or an illegal procedure is suspected. Reticulocyte variability is influenced by seasonal factors related to training and competition programmes and by the type of sporting discipline [68, 69].

A collection of data from published studies indicates that there are some highly stable parameters, such as haemoglobin and erythrocytes, while others (e.g. reticulocytes, mean volume of red blood cells and haematocrit) appear to be less stable. Regardless of the analytical methodology, the stability of the haematological parameters could be improved by sample refrigeration [70, 71].

The blood count analyses are determinant to:

- protect the athletes from anaemia, one of the most common pathological conditions present in athletes
- identify any potential use of doping.

Biochemistry

Monitoring the biochemical parameters of elite athletes is useful to evaluate their health status [72, 73] and to shed light on a metabolic deficiency that might be asymptomatic. There are several biomarkers which vary during and after sporting performance. Sports performance induces cellular changes within the body, increasing cytokine levels and bringing about changes in liver, kidney, muscle, heart, energy and bone metabolism. The choice of the parameters described depends on the number of studies in the literature applied to the various sports disciplines.

Liver metabolism

Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and γ -glutamyltransferase (GGT) are enzymes

measured in serum or plasma to investigate liver disease. In athletes, the concentration of serum aminotransferases should consider the release of AST from muscle and of ALT and GGT mainly from the liver, when bilirubin can be elevated because of continuous haemolysis, which is typical of exercise [3]. Increases in AST and ALT, after long-distance exercise such as an ultramarathon, induces chronic liver injury. In particular, ALT and GGT serve as specific markers for liver injury, and their levels are increased after long-distance running, and the degree of liver injury is linked to the intensity and duration of the performance. A study was performed on 15 endurance athletes with similar physical and demographic characteristics who participated in a marathon, a 100 km or a 308 km ultramarathon. AST and ALT increased significantly after completion of all courses, compared with the baseline taken before the race, and were significantly higher after the 308 km race than the marathon or the 100 km race. The decline in hepatic function caused by running the ultramarathon is related to changes in the liver cell membrane by lipid peroxidation because of impaired blood flow and the release of free radicals. This suggests that the liver undergoes a temporary decline in function during long-distance rather than short-distance running [74]. Bilirubin derives from the breakdown of red blood cells in the body. In athletes, the principal cause of increased bilirubin is haemolysis and consequent catabolism of haemoglobin. This phenomenon is due, during physical exercise, to mechanical factors that cause an accelerated breakdown of red blood cells [75]. Total bilirubin concentration increases with the increase of intensity of hard physical work. This is true in different sports, for example, in rugby and triathlons [76].

Muscle metabolism

Parameters of muscle metabolism consist of the kinase (CK) and lactate dehydrogenase (LDH) and are typically increased after exercise. Creatine kinase (CK) is an enzyme present in three plasma isoforms: creatine kinase isoenzyme-3 (CK-MM) (predominantly muscular), CK-MB (prevalently cardiac) and CK-BB (cerebral). Serum CK concentration proportionally increases with physical exercise and tissue damage. The damage reflects sports performance and is evident at the biochemical level with the presence of increased muscle protein levels such as CK, where the most represented enzyme is CK-MM, LDH and myoglobin, together with weakness, loss of strength, muscle cramps at the physical level [77–79]. Failure to return to baseline values (incomplete recovery) underlines the presence of trauma or overtraining. It is necessary to

monitor CK concentrations to define the athlete's condition with muscle damage and consequently to decide the potential return to physical exercise. The LDH, as the CK enzyme, is present in the plasma in the form of five different LDH1-5 isoenzymes that are expressed in several tissues in varying percentages. In particular, isoenzyme 5 (LDH5) is muscle specific and increases in muscle trauma. Altered LDH levels are physiological in athletes and in subjects that practice physical exercise with constancy. Myoglobin constitutes 2% of the pro-total muscle cells and is rapidly released following cell injury. It is mostly an early index of acute heart failure myocardium [80]. Moreover, urea and CK can be used as indicators of muscle recovery, in fact, high values of urea and CK are related to a lesser state of muscle recovery [81]. The urea is a product of protein metabolism and indirectly signals muscle metabolism, and high concentrations indicate accelerated catabolism of muscle proteins and a gluconeogenesis rate in response to physical training affecting the performance of athletes [82]. A study on seven moderately-trained White male triathlon athletes showed a moderate increase of plasma urea level after running and cycling events as compared to swimming and resting condition [83]. The acid uric is a final waste product of protein, amino acids and DNA and changes in levels of uric acid are related to the production of energy and muscle damage during the exercise [84]. A study showed that usually, an increase in the concentration of acid uric occurs after 20–30 min of intense physical training. Furthermore, as high levels of plasma uric acid are associated with increased incidences of hypertension in adults, it would be interesting to monitor this parameter in athletes at different times after exercise [85].

Renal metabolism

In athletes, renal metabolism is followed by measuring serum creatinine concentration. The interpretation of this parameter should be considered by the body mass index (BMI) of the athletes and phase of the competitive season. The evaluation of serum creatinine values represents the most widely used measure of renal function in clinical medicine and athletes should represent an important feature to identify modifications of renal blood flow during training and competitions. In different studies it has been demonstrated that in athletes the creatinine concentrations are higher than those found in sedentary people (the standard reference range in the general population is 0.7–1.3 mg/dL for adult males). Banfi and Del Fabbro, showed that the distribution of the serum creatinine concentrations in professional athletes from eight

different sports, was not homogeneous; the athletes differed in aerobic/anaerobic metabolism, training loads, length of competitions and periods of training and competitions [86]. In endurance athletes, the serum creatinine concentrations observed was lower than those of sedentary controls [87] and also in cyclists, the serum creatinine concentrations found were lower than those observed in controls [88]. The different types of sport and the different anthropometrical characteristics of athletes should influence different values of creatinine concentrations.

Glucose metabolism

Blood glucose concentration is regulated by complex neuro-hormonal and metabolic mechanisms that maintain values within the limits of normality. Glucose is one of the products deriving from the digestion and absorption of carbohydrates and represents a primary energy source for cells and the central nervous system. Elevated postprandial blood glucose is an independent predictor for developing metabolic complications such as CVD, type II diabetes mellitus and obesity [89]. During exercise and training, there are adaptations in glucose metabolism which improve glucose utilisation in athletes and are beneficial for reducing insulin insensitivity in non-athletes. Glucose metabolism differs slightly for different sports disciplines, as revealed in laboratory levels. Regular exercise is a standard recommendation as a means to manage blood glucose including post-prandial blood glucose responses [90]. Activity increases uptake of glucose by up to 50-fold through the simultaneous stimulation of three key steps: delivery, transport across the muscle membrane and intracellular flux through metabolic processes (glycolysis and glucose oxidation) [91]. Duration and intensity of exercise play a pivotal role in glucose uptake by skeletal muscle. Glucose uptake into the skeletal muscle can be stimulated through single, acute bouts of exercise via translocation and activation of glucose transporter type 4 (GLUT4) to the muscle membrane. Beneficial effects of only bout exercise on postprandial glucose responses extend to low effort modalities such as light to moderate intensity walking and standing. However, due to the level of intensity, these forms of exercise require substantial time commitments of at least 20–30 min or repeated bouts [92].

Bone metabolism

Bone metabolism is profoundly affected in sports medicine both directly, by an intensity of load and

indirectly through the activation of many endocrine axes. In response to exercise, myokines and adipokines are involved in the fine regulation of bone metabolism in response to energy availability. Furthermore, bone regulates energy metabolism by communicating its energetic needs thanks to osteocalcin which acts on pancreatic β -cells and adipocytes [93]. Miyamoto et al. [94] conducted a study on 56 female athletes aged 18–22 years, performing different sports like basketball, and who participated in running, fencing, yachting, canoeing, gymnastics or swimming and divided the participants into stress fracture and non-fracture subjects. The subjects of the fracture group showed higher CK and LDH levels and lowered osteocalcin and undercarboxylated osteocalcin compared to the non-fracture subjects. The monitoring of these biomarkers could be an approach to predict stress fractures in young female athletes combined to an evaluation of related symptoms. Recently studies have emphasised the co-regulation of bone and energy metabolism and the central role of the equilibrium between carboxylated and undercarboxylated forms of osteocalcin. Cycling is known to induce bone resorption affecting the energy homeostasis. Lombardi et al. [93] aimed to understand the acute physiological responses to a cycling stage race concerning bone turnover and energy metabolism and the probable co-regulative mechanisms underlying their relationship. For this aim, nine professional cyclists performing the Giro d'Italy stage race in 2011, were tested for bone and energy metabolism markers (bone alkaline phosphatase, tartrate resistant acid phosphatase 5b, total and undercarboxylated osteocalcin, leptin and adiponectin) and related hormones (cortisol and testosterone) at days -1 (pre-race), 12 and 22 during the race. During the run, the enhanced resorption occurred along with a relative increase in the concentration of the undercarboxylated form of osteocalcin, indirectly related to adipokines modifications, leptin decrease and adiponectin increase. Also a reduction of cortisol was observed, while testosterone levels were unmodified. These results support the evidence of a stringent involvement of bone in the regulation of energy metabolism probably related to absolute and relative concentrations of undercarboxylated OC, adipokines concentrations, BMI, fat mass (%), and the energy expenditure.

Alkaline phosphatase (ALP) is an enzyme expressed in many tissues throughout the body and is especially abundant in hepatic, skeletal and renal tissue. Liver disease or bone disorders most commonly cause elevated levels of ALP in the blood. Variances in ALP values have been described in different sports and performances to verify if physical exercise can modulate the expression of the ALP enzyme. In ultramarathons participating in a 161 km race

at altitude, the ALP levels evaluated were significantly increased; consequently, this biomarker can be considered as an indicator of renal disease and bone disorders in athletes [95]. ALP levels were evaluated in healthy women who performed brisk walking on a treadmill for 30 min with or without 5 kg of weight in a backpack. Variances in ALP values within subjects after exercise were statistically significant and these results reveal a stimulating effect on bone turnover after 30 min of brisk walking [96].

Blood lipid profile

Blood 'lipid profile' is the varying levels of lipids in the blood and is commonly formed by low-density lipoprotein-cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C) and triglycerides (TG). Total cholesterol is a measure that includes LDL-C and HDL-C. More sensitive tests report the total: HDL-C ratio, or non-HDL-C levels that are positively associated with CVD [97]. TGs in plasma derived from fats eaten in foods or from other energy sources and their excess of in plasma are associated with CVD [98]. A meta-analysis study reported improvements in the lipid profile following sports activities, describing shreds of evidence that exercise can create a positive effect on the pathogenesis, symptomatology and physical fitness of individuals with dyslipidaemia [99]. The differences between athletes and sedentary individuals are mainly due to the concentration of HDL-C in physically active individuals, although some discrepancies between sports disciplines exist [3]. Also, a dose-response relationship between increases in physical exercise and improvements in TGs and HDL-C was reported in previously sedentary subjects [100]. Sports activities seem to enhance the ability of skeletal muscles to utilise lipids as opposed to glycogen, thus reducing plasma lipid levels [101]. All these biochemical parameters, which vary proportionally to the increase of physical exercise, must be continuously evaluated to:

- ensure the athlete's health
- cure and prevent injuries
- decide the potential return to physical exercise

Urine test

In sports medicine, debates involving several authors underline the need to adopt a non-invasive sample collection approach with a minor risk of contamination that allows obtaining timing samples without stressing

athletes. Indeed, the use of non-invasive methods protects athletes from fluctuations in concentrations of metabolites due to stress or pain [102]. A urine test is the first non-invasive investigation required for the assessment of renal function.

The test is useful to screen a disease and/or infection of the urinary tract and also to check the treatment procedure of such conditions [103]. The urine test is prescribed in sports medicine (release of the fitness assessment during the competitive sports medical examination) for a necessary assessment of the health state associated with a check for sports fitness. Moreover, the urine sample collection represents the gold standard, among laboratory tests, to detect the use of illegal substances [104].

Several studies show how physical strength exercise can cause both proteinuria and haematuria in elite athletes. Proteinuria can occur when a reduction in blood supply to the renal district causes a consequent accumulation of proteins in the urine, whereas haematuria can be caused by small vascular traumas due to prolonged physical stress [105].

It is well known that hydration levels influence the concentrations of urinary biomarkers. In particular, hyper-hydration results in a dilution effect, whereas when hypo-hydration occurs, it is possible to observe a concentration consequence. The variations of biomarkers due to modifications in hydration status could suggest corrective indications for exercise performance and, therefore, the challenges associated with urinary biomarker correction and hydration status.

In both cases, the conditions are not necessarily pathological and revert to the end of the competitive performance [106, 107].

Creatinine is now accepted as a first urinary biomarker of hydration status for its constant secretion rate [108, 109], although the simple quantification is necessary to take into account several limitations associated with urinary creatinine correction. It has been demonstrated that the lean body mass acts as a significant factor in its excretion. Urinary creatinine levels are higher in men or lean individuals than women and obese individuals, respectively [110, 111].

Physical exercise also influences the creatinine volume excretion. An increase of 50%–100% following a strenuous physical exercise performed by 6-mile runners, 100 km marathoners and 70–90 km skiers has been demonstrated. Further studies showed that also in rugby players a substantial increase in creatinine is due to an aerobic and anaerobic component of the game.

Another study on athletes lifting weights investigated the excretion of the muscle damage marker 3-methyl

histidine after resistance exercises. This marker decreases during the 48 h following the training [102].

Another biomarker used for early diagnosis of acute kidney injury (AKI) and renal damage is neutrophil gelatinase-associated lipocalin (NGAL). In a study on athletes, who practice endurance cycling, an increase of 2.73-fold urinary NGAL 48 h after training, compared to a non-cyclist control group without clinical signals or symptoms, or even any alterations in other biomarkers of kidney disease was demonstrated. These findings suggest a metabolic response induced by exercise that does not mean disease but indicates the kidney as a target organ in endurance cycling exercise [112].

All these biomarkers mentioned can be easily tested and monitored through a non-invasive investigation, allowing:

- a rapid diagnosis of the urinary tract and renal disease
- the evaluation of modifications in hydration status suggesting corrective indications to ensure and enhance performances

Epigenetic

Some studies show that exercise can modulate the epigenetic mechanisms associated with a variety of human diseases; in fact, exercise can be a powerful environmental stimulus by inducing an epigenetic regulation and consequently changes in gene expression [113, 114]. Epigenetics is defined as variations of gene expression independent of those mitotically stable and in some cases inheritable, through mechanisms such as acetylation of histone proteins, DNA methylation and hydroxymethylation, and miRNAs regulation [115]. Regarding acetylation, the function of histone deacetylases (HDAC) is to remove the acetyl groups from histones in order to increase the chromatin condensation, which in turn leads to decreased transcriptional activity. Modulation of histone deacetylases is observed during physical exercise in skeletal muscle. Some studies show the involvement of many HDAC and Sirtuin in the regulation of cellular tropism, and many of them are related to the muscle atrophy process. An increase in mRNA levels of E1A binding protein p300, CREB binding protein, histone acetyltransferase (Pcaf), HDAC2, HDAC4, HDAC4, HDAC6 and Sirt1, but a decrease in the mRNA level of HDAC7 proteins was observed in the muscle atrophy process. The over-expression of HDAC4 proteins is correlated to atrophy of muscle fibres, and moreover, the knockout of HDAC4 is capable of attenuating muscular atrophy promoted by denervation [116]. Another

study identified imprinted genes in skeletal muscle gene networks and observed exercise-associated DNA methylation alterations. In particular, some genes significant for muscle gene networks: *RBI* (retinoblastoma 1), *MEG3* (maternally expressed 3), *UBE3A* (ubiquitin protein ligase E3A), *PLAGL1* (pleomorphic adenoma gene-like 1), *SGCE* (sarcoglycan epsilon), *INS* (insulin) were differentially methylated in response to exercise-activity. Voisin et al., also showed that DNA methylation decreased with exercise (60% of loci), suggesting increased gene transcription [117]. Studies conducted on human monocytic cells, granulocytes and peripheral blood mononuclear cells, demonstrate an increase, induced by moderate exercise, in the methylation of protein apoptosis-associated speck-like protein (ASC) a critical mediator of the cytosol-type inflammatory signalling pathway. Variations in the methylation pattern during exercise are related to levels of pro- and anti-inflammatory cytokines responsible for the lymphocyte activation and differentiation. These mechanisms reduce the basal level of inflammation preventing the development of diseases linked to a low-grade chronic inflammation [113].

Furthermore, exercise intensity benefits positive epigenetic changes regarding mitochondrial biogenesis [116].

It is well demonstrated that miRNA variations can be related to several diseases. Recently, an miRNA regulation on several metabolic processes in response to duration and strength of exercise was demonstrated [114]. For example, has been demonstrated in the heart that exercise modifies the levels of several miRNAs, such as miR-222 and miR17-3p, to avoid myocardial ischemia-reperfusion (I/R) injury; miR-29 to regulate fibrotic processes; miRNA-133 is involved in the modulation of the PI3K-AKT pathway phosphorylation acting on the IGF-I receptor, and its dysregulation leads to a decrease in the development of skeletal muscle. Studies on I/R injury and cardioprotection for pre- and post-ischaemic conditioning showed that some miRNAs such as miR-21, miR-144, miR-146b, miR-208b, miR-212, miR-214 and miR-335 were deregulated, suggesting a role in multiple cardio-protective mechanisms [114].

Further studies show that the physical exercise influences the expression of miRNAs involved in skeletal muscle tropism, such as myomiRs, and modulates the biogenesis of miRNAs by influencing the expression of proteins related to this process, for example, Drosha, Dicer and Exportin-5 [114, 116].

Identification of circulating miRNAs signatures characterising a particular exercise modality may substantially impact the optimisation of training, injury prevention and health status monitoring.

The relationship between epigenetics and the exercise-induced gene expression increases the study of candidate genes and their forms of epigenetic regulation in order to:

- clarify the different mechanisms on the effect of exercise
- identify a practical therapeutic approach, nutritional and training methods to preserve and improve health

Genetic risk of sudden cardiac death

Regular physical exercise has a beneficial effect on the general state of health and contributes to preventing chronic disorders, including CVDs [118]. In the last 50 years, different studies have shown a reduction in the development of coronary heart disease in subjects participating in sport [24, 119, 120], as well as a reduction in total mortality and cardiovascular mortality in cardiac patients undergoing appropriate training programs [121, 122]. In recent decades, however, it has also clearly emerged how physical exercise can act as a trigger to highlight the presence of underlying genetic risk for cardiac disease in asymptomatic athletes [123]. The experience of the last 30 years of study on athletes in the Veneto region in Italy, has shown that the combination of latent heart disease and physical exertion in some cases triggers an arrhythmic event, which may even lead to sudden death [124]. In other words, intense physical exercise acts as a risk factor in subjects with occult cardiomyopathies. In these athletes, physical exercise, especially if intense, can produce cardiovascular changes, with such an increase in the electrical instability of the heart to cause sudden cardiac death (SCD), which unfortunately becomes the first event of heart disease that the athlete unknowingly suffers [125, 126].

According to the most recent definition of the European Society of Cardiology (ESC), sudden death is defined as a “non-traumatic fatal event, unexpected, which occurs within an hour from the beginning of the symptomatology in a subject in apparent good health. The cardiac definition is added if the presence of cardiac pathology, congenital or acquired, potentially fatal, is known or if the autopsy examination shows a cardiac or vascular anomaly as a probable cause of the event, or when the post-mortem examination does not show any possible extra-cardiac cause” [127]. Often sudden death in athletes occurs during sporting competition or immediately after that; sometimes, it can also occur at rest or during sleep [128–131].

Although the SCD related to the sports activity is a rare event, recent studies report that it represents about 5% of total SCDs [132, 133], estimated in turn as approximately 15,000/year, corresponding to about 1/50,000 athletes/year [134]. It is believed that male athletes are affected more often than female athletes [124, 126], and that it is more likely for an athlete to die for a sudden cardiac event if the athlete is African-American, plays basketball or American football or plays soccer in Europe [134, 135]. Furthermore, most sport-related SCD happens in recreational settings, involving mostly amateur athletes: in fact, only 6% of SCDs related to sport are manifested in young competitive athletes, the remaining ones occurring in recreational settings [136].

The prevention of this lethal complication of sports, in asymptomatic but affected athletes, is an important goal that involves many areas of the medical profession.

In most cases, SCD in athletes is due to genetic diseases with significant variability of clinical expression. Therefore, it is fundamental to identify the genetic defect responsible for cardiomyopathy, and also to know the individual susceptibility to developing an underlying cardiac pathology.

Genetically determined and life-threatening cardiac pathologies in young athletes (under 35 years of age) are essentially cardiomyopathies, i.e. diseases of the heart muscle sufficient to cause structural or functional myocardial abnormalities, in the absence of coronary heart disease, hypertension, valvular or inborn heart disease [137]. The cardiomyopathies involved in the sport-related SCD can be divided into four main categories, which, in descending order [138, 139] are represented by:

1. primary electrical disorders,
2. arrhythmogenic right ventricular cardiomyopathy,
3. hypertrophic cardiomyopathy,
4. dilated cardiomyopathy.

Primary electrical disorders

Primary electrical disorders also known as cardiac channelopathies are inherited genetic alterations of the cardiac ionic channels in the absence of structural cardiomyopathy [140]. Depolarising and repolarising ion currents engender the cardiac action potential. Alterations of these currents cause myocardium electrical vulnerability, which can produce polymorphic ventricular tachycardia or ventricular fibrillation carrying the risk of sudden death. Long QT syndrome (LQTS), Brugada syndrome (BrS) and catecholaminergic polymorphic ventricular tachycardia (CPVT) are the most commonly diagnosed

channelopathies. About 40 genes are associated with hereditary cardiac channelopathies, although each channelopathy has its most commonly related gene. Long QT syndrome is caused by mutations in at least 16 *LQTS* genes, even though the genes more frequently mutated are *KCNQ1*, *KCNH2*, sodium voltage-gated channel α -subunit 5 (*SCN5A*), *KCNE1*, *KCNE2*, coding for the α - or β -subunits of the potassium – or sodium-dependent voltage channels (Kv7.1, Kv11.1 and Nav1.5) [141]. Loss-of-function mutations in the *SCN5A* gene cause Brugada syndrome in about 30% of cases [142]. CPVT is caused by excessive calcium leakage from the sarcoplasmic reticulum, generally associated with mutations in *RYR2* or *CASQ2* genes, coding the ryanodine receptor and calsequestrin-2, respectively [143]. More rarely, the genetic basis of hereditary arrhythmic syndromes can also be found in genes encoding proteins associated with cardiac ion channels or their intracellular transporters, such as caveolin, calmodulin, calsequestrin, syntrophins and others, which can influence the activity of the channel itself.

Arrhythmogenic right ventricular cardiomyopathy

Arrhythmogenic right ventricular cardiomyopathy, currently better defined as arrhythmogenic cardiomyopathy (AC), is an inherited primary cardiomyopathy characterised by a partial or massive replacement of ventricular myocardium with adipose or fibro-adipose tissue. This replacement favours electrical instability that can trigger a ventricular arrhythmia and sudden death, especially in the young. AC is associated, in up to 60% of cases, to mutations in the *PKP2*, *DSG2*, *DSP*, *DSC2*, *JUP* genes, coding the desmosomal proteins plakoglobin, desmogleins, desmoplakin, desmocollins, plakophilins, respectively [144]. More recently AC has also been associated with mutations of α -catenin and cadherin 2 [145, 146], proteins located in adherent junctions of the intercalated discs. These new shreds of evidence suggest that AC may be envisioned as a disease of the entire area composite.

Hypertrophic cardiomyopathy (HCM)

HCM is the dominant monogenic cardiac disorder and a prevalent cause of SCD in young people. It is also called “sarcomeric” cardiomyopathy. At least 60%–70% of HCM cases are attributable to mutations in the genes encoding sarcomeric proteins as myosin heavy chain 7 (MYH7), myosin binding protein C3 (MYBPC3), troponin

T2 (TNNT2), troponin I3 (TNNI3), α -actin cardiac muscle 1 (ACTC1), tropomyosin 1 (TPM1), myosin light chain-2 (MYL2), myosin light chain-3 (MYL3) [147]. Less frequently, mutations in Z-disc proteins (such as titin, myopalladin, nebulin, obscurin, actinin, teletonin) or cytoskeletal proteins are associated with hypertrophic cardiomyopathy [148].

The HCM clinical manifestations are very heterogeneous, ranging from negligible to extreme heart hypertrophy [149], according to the presence of modifying factors such as sex, age, double mutations; however, physical activity could also modulate the HCM phenotype [150]. Therefore, an early HCM diagnosis is of the utmost importance in athletes, in which high levels of physical activity should be avoided, both to delay the onset of symptoms, and to prevent the worst complication of the disease, i.e. the sudden cardiac death.

Dilated cardiomyopathy (DCM)

DCM the most common cardiomyopathy worldwide, with a prevalence of 40/100,000. The most common DCM cause is ischemic heart disease. However, the advances in genetic diagnosis techniques made it possible to identify a genetic cause in about 30%–40% of DCM cases [151]. DCM genetic background is very heterogeneous. About 100 genes encoding cytoskeletal, sarcomeric and nuclear proteins have been linked to the pathogenesis of DCM [152]. In particular, mutations in the titin (*TTN*) gene, encoding the titin, the most abundant human protein, is present in 25% of the familial forms and 18% of the sporadic (or such) forms of DCM [153].

Most of the above cardiomyopathies are transmitted in an autosomal dominant fashion, although some forms show autosomal recessive, X-linked or matrilineal inheritance. Genetic heterogeneity is a phenomenon common to all cardiomyopathies, so that the same phenotype may be due to mutations in different genes, for example, hypertrophic cardiomyopathy is related to more than 100 different genes [148]. However, the phenomenon of allelic heterogeneity is also present, so that different mutations in the same gene can cause different diseases; for example, mutations in the *SCN5A* gene may cause LQTS, BrS, HCM or DCM [139] or even give rise to overlapping manifestations within the same family nucleus [154].

Another common feature of these cardiomyopathies is the variable expressivity and the incomplete penetrance when a certain proportion of individuals, despite having the disease genotype, do not manifest the corresponding phenotype [155]. In these asymptomatic carriers, physical

exercise can be the environmental factor that reveals the presence of a genetically determined latent cardiomyopathy [156–158].

Therefore, the inclusion of molecular analysis in the sports pre-participation screening in athletes with borderline symptoms/signs and/or with a family history positive for cardiomyopathy could:

- allow reaching a definitive characterisation of the underlying cardiac disease
- prevent the sudden cardiac death

Lipoprotein(a) and cardiovascular risk

The regular physical exercise implicates changes in lipoproteins metabolism [159]; the positive effects on HDL-C values and the reduction of LDL-C and TG values are established, while the role on lipoprotein(a) Lp(a) values is still under discussion.

Lp(a) is a macromolecular complex found in human plasma that merges structural elements from the lipoprotein and blood clotting systems, and that is associated with premature CVD [160]. The *Lp(a)* gene is located on chromosome 6, close to the plasminogen gene (*PLG*), with which it shares a high degree of homology [161]. Compared to other lipoproteins, Lp(a) blood concentrations are mainly determined by the *Lp(a)* gene [162] and are less influenced by lifestyle changes such as diet and exercise.

Lp(a) consists of a core of cholesterol and phospholipids and a component protein, apoB, bound with a

disulfide bond to apoprotein(a) apo (a), that gives it peculiar characteristics [163] (Figure 3).

Its role has been identified as an independent, causal risk factor for CVD [164].

The inter-individual range of Lp(a) concentrations is extensive. Lp(a) circulating levels are not distributed normally, like many biological variables, but have a clear prevalence of subjects with low values and few with very high values. Plasma levels of Lp(a) are genetically determined for a proportion varying between 20% and 60%. This variability also depends on the fact that the distributions of the levels plasma Lp(a) in Afro-American populations countries are different from the Caucasian ones [165]. The main determinant of the levels of Lp(a) is represented by the polymorphism of the length of the Kringle IV-2 of the apo(s) [166]. This ranges from less than 0.1 mg/dL to more of 300 mg/dL and is strongly unbalanced towards low levels in most of the population [167], with higher risk for values above 30 mg/dL [168], especially for values above 50 mg/dL [169]. In the Copenhagen City Heart Study, subjects at the 95th percentile for the concentration of Lp(a) (120 mg/dL) increased by more than 3 times risk of developing a myocardial infarction compared to those with lower values [170].

The ESC guidelines recommend measuring Lp(a) levels in selected patients at high risk of CVD and consider the cut-off of 50 mg/dL as an additional factor that indicates a very high cardiovascular risk [171]. The Lp(a) is capable of enriching cholesterol in the atheromas plaques. The pathogenic mechanism of Lp(a) regards its atherogenic and prothrombotic characteristics. Lp(a) possesses proatherogenic mechanisms concerning its intrusive capacity. LDL-R does not internalise it, therefore

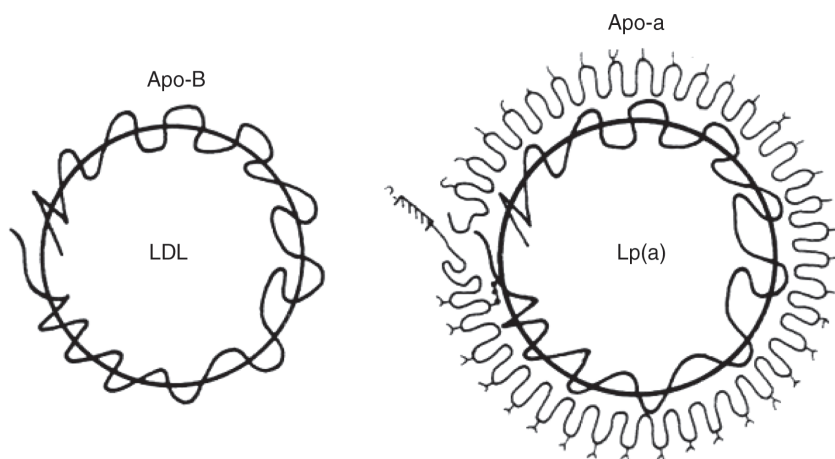


Figure 3: Lipoprotein (a) structure.

also in the subject with low LDL or normal LDL the accumulation of lipoproteins in the arterial wall can occur equally and cause atheroma plaque [172]. Once it has penetrated Lp(a) stimulates profiling and migration of the smooth muscle cells from the medium tunic to the intima.

Regarding the levels of Lp(a) in the athletes, the results that emerge from the literature are conflicting. No large studies are comparing Lp(a) levels in physical exercise individuals with a matched control subject. Several studies show no influence of physical activity on Lp(a) plasma levels [159, 173–176]; Hubinger et al. [175] compared runners and control subjects revealing no significant correlation between Lp(a) levels and any other variable, thus confirming previous reports that indicated Lp(a) to be unrelated to the other lipoprotein levels. One possible exception may be an increase of Lp(a) serum concentration in adult endurance and power athletes [173], although a study dedicated to endurance athletes and power athletes, showed small effects on Lp(a) concentrations. Conversely, Ponjee et al. [177] studied the effect of long-term physical exercise on Lp(a) levels showing a significant increase after 24 and 36 weeks, both in male and in the female group. The work hypothesised that progressively Lp(a) concentration raises as the stress increases together with other elements such as fibrinogen, strongly correlated with Lp(a). Rostami and Zafari [178] have investigated changes in Lp(a) levels in different patient settings: speed, semi-endurance, endurance runners and sedentary females, highlighting that the differences between the groups of athletes and with the control group were not significant. In the same direction, the most recent results of the study by Sponder et al. [179], which conducted a prospective observational trial evaluating the effect of long-term physical activity on PCSK9, HDL-C and LDL-C, and lipoprotein(a) levels. The results show Lp(a) levels: 37.9 (51.9) nmol/L to 43.3 (60.6) nmol/L; $p=0.218$, with no significant differences. On the other hand, some studies show a decrease in Lp(a) levels in those who practice physical exercise [180–182]. In particular, Mohammadi et al. [183] studied a population of middle-aged men, assessing the effect of 12-week aerobic exercise on plasma levels of some biochemical parameters, including Lp(a). The results of the study showed a statistically significant reduction in Lp(a) levels compared to the control group. Rigla et al. [182] investigated effect of physical exercise on Lp(a) modifications in type 1 and type 2 diabetic patients highlighting the decrease in Lp(a) in patients with higher Lp(a) concentrations. Some researchers have focussed on the

influence of the degree of glycaemic control on serum Lp(a) concentrations [184, 185].

Although the abuse of androgen-anabolic steroids (AAS) in young healthy athletes have been associated with premature cardiovascular events [186], AAS may selectively reduce Lp(a) concentrations [187]. Treatments with AAS showed a substantial lowering effect on Lp(a), while only nandrolone decanoate induced a non-significant reduction in lipoprotein concentration. The use of these drugs also negatively modulates the serum concentrations of apolipoproteins and HDL-C [187], and their effect on atherogenesis remains to be investigated.

Lp(a) is an atherogenic protein, identified as an independent cardiovascular risk factor. The impact of sport on its production and plasma levels is controversial:

- some studies show a positive effect of physical activity on Lp(a) levels, as is the case for the total and fractionated cholesterol profile; other studies even show an increase in levels. Others, on the other hand, show no effect on its levels
- new randomised trials would serve to study the issue in depth and define the effect of physical activity on Lp(a) levels, given the conflict of information that emerges from the studies carried out so far

Antioxidant supplementation

Aerobic metabolic processes are responsible in living organisms for the production of reactive species of either oxygen or nitrogen, collectively indicated as RONS which are involved in several physiological events [188–190]. Oxidative stress is caused by an imbalance between pro-oxidant and antioxidant species in the cellular milieu, which can be either provoked by endogenous causes or triggered by external sources [191, 192]. Exercise and oxidative stress are partners in a complicated relationship whose molecular determinants and details have not been completely elucidated yet. Physical exercise undoubtedly induces an increase in the production of RONS due to the increased metabolic rate and oxygen consumption by muscle fibres [193, 194]. Other tissues have been considered as potential sources of RONS during exercise including heart, lungs and blood constituents such as leukocytes, which are activated as part of the systemic inflammatory response to intense, prolonged exercise [195]. However, the effect of exercise on redox balance is far from being simple and straightforward and differs when considering age, sex, training level and specific features of the exercise performed such

as duration and intensity. An incorrect intake of anti-oxidants may even partially suppress what appears to be an adaptive mechanism of the organism dealing with exercise-induced repeated increases in ROS production. Most importantly, important methodological and technical issues remain, encompassing, for example, the use of out-dated assays and/or inappropriate sample preparation techniques, which complicate the effective biochemical redox status assessment of an individual [196]. Moreover, aspects such as the use of multiple biomarkers to assess the oxidative damage are often not taken into account in several studies and relying upon measurements of individual markers might result in an incomplete evaluation of an individual's redox balance.

Interestingly, the use of the determination of thiobarbituric acid reactive substances assay to assess lipid peroxidation or the evaluation of the total antioxidant capacity (TAC) have shown several limitations, which strongly suggests that their informativeness might be reconsidered shortly [196].

Nonetheless, antioxidant integration has become a common practice among athletes and exercisers to reduce global oxidative stress, promoting muscle recovery and improving performance. In this framework, vitamins C and E, CoQ10, polyphenols, astaxanthin are among the most commonly used antioxidants (Figure 4).

Data in the literature suggest that vitamin C and E supplementation have no significant or minimal beneficial effect, for example, in athletes of ultra-endurance sport; in this case antioxidants requirements can be fulfilled by dosages equivalent or close to the recommended daily allowance, which can be afforded merely by a balanced diet. Most importantly, extremely high dosage of these compounds, both alone or in combination, appear to be even harmful [197]. CoQ10 is a lipophilic, antioxidant vitamin-like quinone, usually referred to as ubiquinone, which acts as an essential cofactor in mitochondrial oxidative phosphorylation and has proven to prevent lipid peroxidation [198]. Studies concerning the potential beneficial effect of CoQ10 on either performance or exercise-induced injury and oxidative stress have shown mixed and sometimes conflicting results, independently from the type of exercise considered (aerobic, anaerobic) [199, 200]. Polyphenols are a different and important class of antioxidant molecules that include compounds such as quercetin, curcumin, resveratrol and catechins, compounds endowed with immunomodulatory, anti-inflammatory, cardioprotective, antitumoral and mitochondrial stimulatory activities [201]. Resveratrol is a polyphenol frequently used in sports practice and is a natural polyphenolic flavonoid present in grain and grain skins, red wine, mulberry,

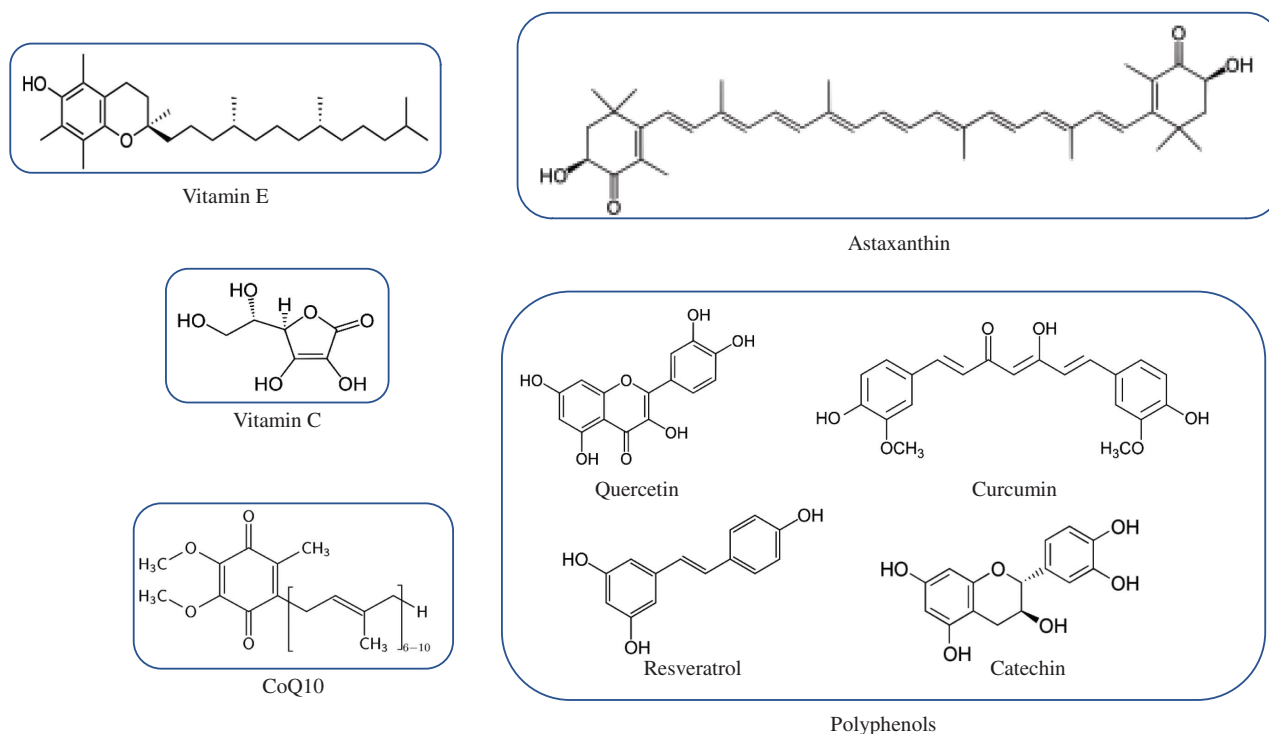


Figure 4: Chemical structure of common antioxidants used in dietary supplements for exercise and athletic performance.

peanut and rhubarb and freely commercially available as dietary supplements [202, 203]. Astaxanthin is a natural compound found in algae, fish and birds, which seems to act on carnitine palmitoyl transferase 1 (CPT1) function, thus leading to an improved fatty acyl-CoA uptake into the mitochondria and increased fat oxidation. In several mice studies, astaxanthin supplementation has been reported to improve the running time of swimming and treadmill use until exhaustion [204]. A recent study on young football players has shown that astaxanthin may be suitable for athletes that are more susceptible to oxidative stress [205].

All the compounds described share the ability to interfere with oxidative changes positively; however, they usually fail in improving performance when tested on humans. Noteworthy, attention has been focussed on the use in sports nutrition of reduced thiols donors, such as purified nitrate salts and nitrate-rich foods, which have been shown to delay fatigue or increase endurance in different experimental conditions [206].

It is worth noting that the clinical expectations of antioxidant-based therapies have been frequently disappointed. The discrepancy among a large number of studies published on this topic derives from several aspects [207]. Training, training status and type of supplementation (type, dosage, natural extracts or pure compounds) used in the studies are entirely different from each other. Besides, also endpoints (endurance, performance, oxidative stress biomarkers) and analytical methods used are not homogenous.

All these aspects hamper a precise evaluation of this highly debated topic:

- the clinical studies are considering the effect of the same antioxidant on equal groups of subjects
- although it is becoming more and more evident that a balanced and diversified diet is sufficient in many cases, the possibility to give a moderate and timely limited antioxidant supplementation during intensive training or energy restriction/weight loss regimens cannot be ruled out at the moment

Genetic variations

Athletic performance is a complex phenotype influenced by a myriad of environmental and genetic factors, and variation in human physical performance and athletic ability has long been recognised to have a strong heritable component [208]. Both the scientific and sporting communities acknowledge that genetic factors contribute to athletic performance. In order to identify new genetic variations,

high throughput molecular diagnostics tools, such as array comparative genomic hybridisation (a-CGH) and next generation sequencing (NGS) technology [209–211], are useful tools. In fact, since 2009, more than 200 genetic variants had been associated with physical performance, with more than 20 variants being associated with elite athlete status. Each sport has unique physical requirements, and these can be dramatically different between sports. Therefore, any study of the genetic influence on performance must consider the performance components most appropriate for the sport of interest. Considering the number of systems that must interact (musculoskeletal, cardiovascular, respiratory, nervous, etc.), athletic performance is one of the most complex human traits. Perhaps the first noticeable difference between athletes of different specialties is in body composition. Beyond body morphology, endurance, strength and power are primary factors underlying athletic performance. Additional components of athletic performance include cognitive factors and injury susceptibility. It is critical to remember that the environment (e.g. training, nutrition) also influences many of these traits. Elite athletic status, therefore, results from the interaction of an optimal combination of genetically driven physical and mental traits with the ideal environment for athletic success [212]. Different studies suggest that genetic variability may play a fundamental role in this context. Some genes have common variations in sequence, known as polymorphisms, which, depending on where this polymorphism occurs within the gene, can directly affect gene expression and ultimately the amount of protein produced or can modify the protein product, thus potentially altering function. Sequence variations may be represented by a single nucleotide polymorphism (SNP), in which a nucleotide is replaced by another nucleotide or an insertion/deletion polymorphism (indel) [213]. In athletes with tendon lesions were identified gene variations in genes encoding collagen (*COL1A1*, *COL5A1*, *COL12A1*, *COL14A1*), in a gene encoding tenascin-C (*TNC*), in a gene involved in the repair of connective tissue injuries matrix metalloproteinase 3 (*MMP3*) and in genes encoding growth factors as growth factor- β 1 (*TGFB1*) and growth differentiation factor 5 (*GDF-5*). The most studied include angiotensin I-converting enzyme (*ACE*) and α -actinin-3 (*ACTN3*) genes. The ACE, I/D polymorphism, was the first genetic factor to be associated with human performance. The *ACE* gene codes for angiotensin-1 converting enzyme, part of the renin-angiotensin system responsible for controlling blood pressure by regulating body fluid levels. The ACE I/I genotype is consistently associated with endurance performance and higher exercise efficiency while the D/D genotype is associated

with strength and power performance. The *ACTN3* gene codes for the protein α -actinin-3, a structural sarcomeric protein found exclusively in the fast type II muscle fibres, which are used during dangerous activities. A polymorphism leads to a premature stop codon (X) rather than an arginine (R) at position 577. The R allele represents an advantage in power-oriented events, as the RR genotype is overrepresented in elite power athletes while the XX genotype is associated with lower sprinting ability and muscle strength [214]. Other genes whose variants are differently associated to athletic performance are: *CK-MM*, myosin light chain kinase (*MLCK*), adenosine monophosphate deaminase 1 (*AMPD1*), *IGF-1*, insulin-like growth factor II (*IGF-2*), peroxisome proliferator-activated receptor α (*PPARA*), peroxisome proliferator-activated receptor δ (*PPARD*), peroxisome proliferative activated receptor- γ , coactivator-1 α (*PPARGC1A*), β 2-adrenoceptor (*ADRB2*), nuclear respiratory factor 1 (*NRF1*), nuclear respiratory factor 2 (*NRF2*), hypoxia-inducible factor-1 α (*HIF1A*), cholinergic muscarinic receptor 2 (*CHRM2*), uncoupling protein-2 and -3 (*UCP2*, *UCP3*), interleukin-6 (*IL6*), interleukin-1 β (*IL1B*), CC chemokine ligand 2 (*CCL2*), adrenoceptor α 2A (*ADRA2A*), bradykinin receptor B2 (*BDKRB2*), nitric oxide synthase 3 (*NOS3*), superoxide dismutase 2 (*SOD2*), methylenetetrahydrofolate reductase (*MTHFR*) tumour necrosis factor (*TNF*) [208, 215, 216].

The study of genes associated with athletic performance and their variants would contribute to:

- know the possible response to a particular type of exercise
- help the coaches in customising the physical exercise of their athletes
- maximise the recovery and adaptation and reducing the risk of injury associated with overload

Gut microbiome

The gut microbiome is widely recognised to play an essential role in human health. Besides, microbiome composition and functions are recently becoming important biomarkers of health/disease state. The microbiome may be influenced by several factors, among which diet may be considered the most important. The long-term habitual diet seems to be the primary factor influencing gut microbiota, with regular diet having the most significant effect on microbiome composition and correlated release of microbial metabolites [217, 218]. The fact that regular exercise can be one of the drivers of a specific microbiome composition and related functions has recently

gained remarkable interest [219, 220]. Recent literature describes a higher level of microbial diversity, usually associated with a healthy gut, in elite athletes compared to sedentary cohorts [221]. Exploring the metabolic functions by metagenomics, the same authors recently found an enrichment of *Akkermansia* in athletes and increased the abundance of pathways such as the biosynthesis of organic cofactors and antibiotics, as well as carbohydrate degradation and secondary metabolite metabolism, which could be relevant to health benefits [222]. Higher levels of potential metabolic pathways from *Prevotella* and *Methanobrevibacter smithii* were recently found in cyclists compared to control populations, highlighting a remarkable different microbiome composition and functions in athletes [223]. Also, higher levels of faecal short chain fatty acids and other diet-related beneficial microbial metabolites were found in athletes compared to sedentary controls. Overall, data from the current literature suggest that athletes have a healthier gut microbiome composition and functions, with potential improved capacity of energy harvest from the diet by improved biosynthesis of carbohydrates and nucleotides, consistent with the energy demands associated with regular physical exercise. Although often protein-rich, athletes tend to have a healthy dietary pattern, very rich in fibres, which can trigger a healthier composition of the microbiome. Therefore, it is still to be clarified whether diet or physical exercise plays the most active role in gut microbiome composition and functions in athletes. In the meantime, gut microbiome evaluation remains a promising biomarker for the evaluation of overall health and systemic homeostasis in athletes.

Conclusions

The scope of this review was to focus on a series of biochemical markers, frequently tested in clinical biochemistry, that can be used to characterise and describe the health status of elite athletes.

In sports medicine it is necessary to gain a simple diagnostic strategy to prevent sports injuries. To date, no specific tests are available for elite athletes, apart for those recommended for the general population. Besides, an appropriate strategy will be helpful to determine whether an athlete is suitable to engage in a particular sport or event. Accordingly, there is a strong need to set up a screening strategy, in turn, to identify an asymptomatic pathologic condition and to ensure that health problems, when present, are adequately managed. The primary

purpose is to screen for injuries or medical conditions that may be risky for safe participation.

Each parameter described in this review essentially contributes to better defining the risk associated with a sport. The variation in haemostasis balance may contribute to enhance the risk for coronary artery thrombosis and to predispose to exercise-related profound vein thrombosis events.

Regarding hormonal adaptations in athletes, close monitoring of endocrine functions in elite athletes would be recommended in order to identify endocrine-related diseases at an early stage and to apply therapeutic and/or lifestyle interventions directed to resume physiological, hormonal condition.

Changes in haematological parameters in athletes may induce risky modification for healthy athletes, such as anaemia, suggesting information for the appropriate therapeutic strategy aimed at a quicker functional recovery; therefore, the haematological screening avoids any potential use of doping.

The screening of biochemical parameters can well define the metabolic organ status and characterise any modification and tissue and organ damage.

The urine test, a non-invasive and just investigation, is useful for the assessment of renal function and to diagnose and monitor the treatment for a disease or infection of the urinary tract. Also, urinary biomarkers variations, due to modifications in hydration status, may have corrective advice strategies to enhance performances.

The epigenetic mechanisms can modulate a variety of human diseases; exercise is a strong environmental stimulus by inducing an epigenetic regulation and variations in gene expression. The study of candidate genes and their forms of epigenetic regulation can contribute to defining the effective therapeutic approach, nutritional and training methods to preserve health and improve performance.

The presence of genetic cardiomyopathies and genetic variations responsible for silent and potentially fatal diseases can be identified by combining the ECG screening with DNA test and biochemical biomarkers. In particular, the Lpa evaluation is strongly recommended as an independent, causal risk factor for CVD.

Physical exercise is known to induce an increase in the production of RONS, a consequence of augmented metabolic rate and oxygen consumption by muscle fibres. Antioxidant integration represents a common practice among athletes in order to reduce global oxidative stress damage and to improve performance after muscle recovery.

In this contest, gut microbiota may play a pivotal role in controlling inflammatory responses and oxidative

stress, in improving metabolism and energy cost during intense exercise.

These appropriate prevention strategies could also explain why an individual can excel in one sports discipline and why an individual develops more injuries than another one. All the collected information will contribute to identify individuals with advantageous physiology, morphology and maybe psychology and to identify athletes who are most likely to benefit from nutritional and exercise programmes.

Anyway, some of the mentioned biological factors need to be further evaluated in a scientific environment taking into account for what group of athletes (age, sex, sport ...) the study pertains.

Such a strategy will allow elite athletes to optimise sports performances without increasing the risk of injuries, which could affect health over their lifetime.

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