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Redox-related biomarkers in physical exercise

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ABSTRACT

Research in redox biology of exercise has made considerable advances in the last 70 years. Since the seminal study of George Pake's group calculating the content of free radicals in skeletal muscle in resting conditions in 1954, many discoveries have been made in the field. The first section of this review is devoted to highlight the main research findings and fundamental changes in the exercise redox biology discipline. It includes: i) the first steps in free radical research, ii) the relation between exercise and oxidative damage, iii) the redox regulation of muscle fatigue, iv) the sources of free radicals during muscle contractions, and v) the role of reactive oxygen species as regulators of gene transcription and adaptations in skeletal muscle.

In the second section of the manuscript, we review the available biomarkers for assessing health, performance, recovery during exercise training and overtraining in the sport population. Among the set of biomarkers that could be determined in exercise studies we deepen on the four categories of redox biomarkers: i) oxidants, ii) antioxidants, iii) oxidation products (markers of oxidative damage), and iv) measurements of the redox balance (markers of oxidative stress). The main drawbacks, strengths, weaknesses, and methodological considerations of every biomarker are also discussed.

1. Introduction

Physical activity has been called a miracle cure by the Academy of Medical Sciences [1]. This statement from the Academy is supported by solid scientific evidence that demonstrates the role of physical training in the prevention and treatment of several diseases: psychiatric, neurological, metabolic, cardiovascular, pulmonary, musculo-skeletal disorders and even cancer [2]. Paradoxically, during their contraction skeletal muscles generate reactive oxygen (ROS) and nitrogen species (RNS) and depending on the duration and intensity of the exercise it can result in oxidative damage to cellular constituents [3]. Exhaustion is a main future when discussing the relation between exercise and oxidative stress or damage [4]. In most of the studies in which redox biomarkers are determined the measurements are performed after the animals, or humans, meet the criteria for exhaustion. An objective definition of exhaustive exercise is difficult but in general terms it is measured, in humans, by the subjects' inability to maintain the exercise regimen through the quantification of the maximal oxygen consumption, or $VO_{2\ max}$ [5]. In animal models, exhaustive exercise has typically been evaluated by forced treadmill running or swimming. In these protocols, animals are considered exhausted when they refuse to continue running on a motorized treadmill equipped with a shock grid or when they sink to the bottom of the pool while swimming [5]. In this regard, our research group demonstrated that a single exercise bout caused oxidative stress only when exhaustive [4]. We found a linear correlation between a well-known marker of oxidative stress, oxidized to reduced glutathione (GSSG to GSH ratio), and lactate to pyruvate ratio. Rising blood lactate during exercise indicates that an individual is not in a metabolic steady state and predicts diminished performance [6]. Such changes in indicators of oxidative stress occur independently of the absolute intensity of exercise are directly associated with exhaustion [7].

Research from the past 40 years has shown that reactive oxygen and nitrogen species (RONS) generated during muscle contraction, depending on their source, levels or training status, apart from causing damage, can modulate several cell signaling pathways and regulate the expression of genes in our cells [3]. This is the basis of our adaptations to exercise training. As a result of the accumulation of exercise bouts the

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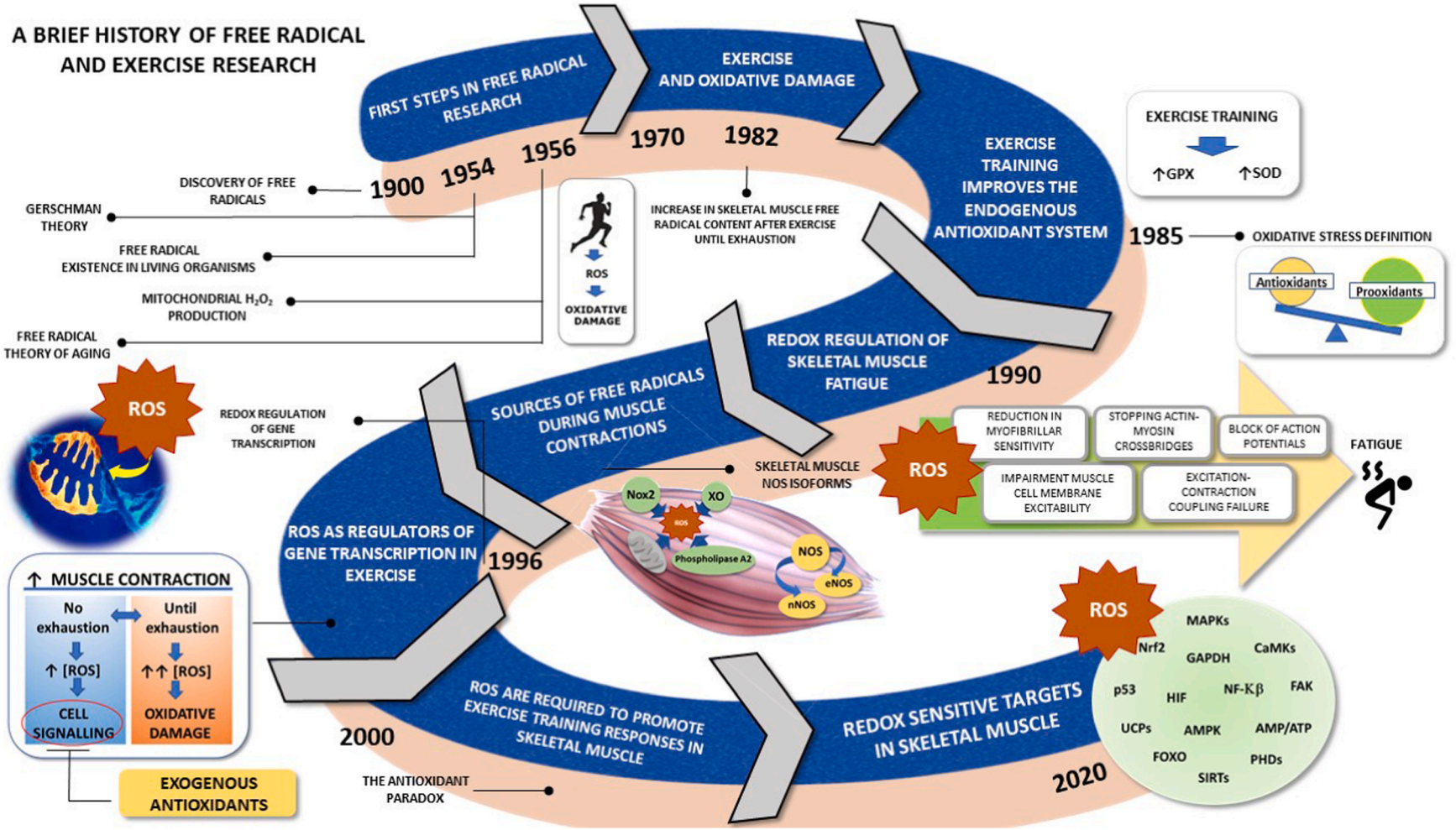

Fig. 1. Exercise redox biology, an overview of the main research findings in the last 70 years. AMP: Adenosine Monophosphate, AMPK: AMP-Activated Protein Kinase, ATP: Adenosine Triphosphate, CaMKs: Ca2+/Calmodulin-Dependent Protein Kinase, eNOS: Endothelial Nitric Oxide Synthase, FAK: Focal Adhesion Kinase, FOXO: Forkhead Box Protein O, GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase, GPX: Glutathione Peroxidase, HIF: Hypoxia-Inducible Factor, MAPKs: Mitogen Activated Protein Kinases, NF-κB: Nuclear Factor KB, nNOS: Neuronal Nitric Oxide Synthase, NOS: Nitric Oxide Synthase, Nox2: NADPH oxidase 2, Nrf2: Nuclear Factor E2-Related Factor 2, PHDs: Prolyl Hydroxylases, ROS: Reactive Oxygen Species, SIRTs: Sirtuins, SOD: Superoxide Dismutase, UCPs: Uncoupling Proteins, XO: Xanthine oxidase.

organism adapts. Exercise adaptations refer to the long-term changes that occur in our body as a consequence of training. Skeletal muscle hypertrophy, mitochondrial biogenesis, angiogenesis and the improvement of our antioxidant defense are well-known examples of these adaptations [8]. Our daily physical activity, through different stimuli apart from ROS levels, is going to regulate molecular and physiological responses that have an impact on health-related processes and on the prevention of disease [9,10]. These stimuli include, but are not limited to, acidosis, hyperthermia, changes in Ca^{+2} homeostasis, and changes in the oxidized/reduced Nicotinamide Adenine Dinucleotide (NAD^+/NADH) and Adenosine Monophosphate/Adenosin Triphosphate (AMP/ATP) ratios [11]. These ideas will be discussed in the first section of the manuscript including the role of ROS in normal force production and in skeletal muscle fatigue [12].

The second part of the manuscript is devoted to the review of the main redox biomarkers in exercise. Redox biomarkers are redox sensitive molecules that respond to oxidative stress in biological systems. They can be classified in different categories that include the measurement of oxidants production, levels of antioxidants, oxidation products, and the antioxidant/pro-oxidant balance [3]. Their usefulness and reliability in sport sciences and the methodological problems associated to its determination are discussed.

We would like to finish this introductory paragraph by clarifying the term ROS and how we have used it in the review. ROS is a general term that refers to not only oxygen-centered radicals but also includes non-radical but reactive derivatives of oxygen. Free radical ROS include: superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$), peroxyl radical ($ROO^{\cdot}$), and alkoxyl radical ($RO^{\cdot}$). Non-radical ROS include: hydrogen peroxide (H_2O_2), organic hydroperoxides (ROOH), singlet molecular oxygen ($^1O_2$), electronically excited carbonyl (R–C═O), ozone (O_3), and hypochlorous and hypobromous acid (HOCl and HOBr) [13].

1. Exercise redox biology, an overview of the main research findings in the last 70 years.

1.1. First steps in free radical research

The discovery of electrons by Dr. Thomson in 1897 provided the bases to Moses Gomberg to propose the existence of free radicals, atoms or molecules that contain one or more unpaired electrons and are capable of independent existence [14,15]. The discovery of the Haber-Weiss reaction that showed that hydroxyl radicals can be generated by the interaction between hydrogen peroxide and superoxide brought additional acceptance to the concept that free radicals can exist in solution [14,16].

In 1954, Rebeca Gerschman and Wallace O. Fenn, pioneer researchers in the study of free radicals, published in Science what was later known as the "Gerschman Theory" in which they postulated that free radicals were responsible for the deleterious actions of both X-irradiation and oxygen toxicity [17]. The radiation-induced mutations resulting from the detonation of atomic bombs during World War II stimulated scientific attention toward understanding the mechanisms responsible for the illness associated with exposure to high levels of radiation [14]. Dr. Gerschman also studied the mutagenesis induced by the production of hydrogen peroxide by high oxygen pressures and she was able to confirm that the deleterious effects of oxygen in animals were also seen in plants and could be prevented by antioxidants [18]. Dr. Gerschman's work was considered so important to the scientific community that she was proposed for the Nobel Prize for Physiology or Medicine in the 1980s.

Coincidentally, the same year that Dr. Gerschman published her study in Science, George Pake's research group, in Nature, using a paramagnetic resonance absorption technique found high free radical levels in metabolically active tissues and estimated that their content was ~20 mol x 10^8/g dry weight in rabbit skeletal muscle in resting conditions [19]. This seminal paper provided the first proof showing that free radicals exist in living organisms.

Two years later Britton Chance and co-workers first reported that mitochondria produce H_2O_2 [20] and Harman and collaborators proposed the 'free radical theory of ageing' [21].

A major ground-breaking finding in the free radical biology field was the discovery, in 1969 by McCord and Fridovich, of the dismutation of superoxide radicals by superoxide dismutase (SOD) [22]. The demonstration of the existence of SOD1 provided the first convincing evidence that biological free radicals play an important role in cell biology [23] (See Fig. 1).

1.2. Exercise and oxidative damage

In the 1970s two research teams found that exercise is associated with oxidative damage both in human and animal studies [24,25]. Oxidative damage occurs when the organism is exposed to prolonged oxidative stress, i.e. to a high RONS production that cannot be managed by antioxidant defences.

Dillard and co-workers found that endurance exercise increased the levels of expired pentane (a marker of lipid peroxidation) in breath collected into a spirometer, and that supplementation with vitamin E reduced the exercise-induced increase in pentane production. Brady and collaborators reported an increase in thiobarbituric acid reactive substances (TBARS), an indicator of lipid peroxidation, both in liver and in muscle of rats as a response to swimming stress. In this study this increment in skeletal muscle oxidative damage was not affected by dietary E or dietary selenium [24].

The biological importance of the reported findings was unclear at the time and it was not until the early 80s that researchers identified the first link between muscle function and the cellular oxidoreductive (redox) balance by finding an elevated free radical content in frog limb muscles stimulated with a tetanic contraction [26].

In 1982 Dr. Davies and co-workers showed for the first time, in an *in vivo* study, a two-to three-fold increase in free radical content of skeletal muscle in rats following exercise until exhaustion [27]. Exhaustive exercise also resulted in decreased mitochondrial respiratory control, loss of sarcoplasmic reticulum and endoplasmic reticulum integrity, and increased levels of lipid peroxidation products. All of the reported changes were similar in exercise exhausted control animals and non-exercised vitamin E deficient animals, suggesting the possibility of a common free radical dependent damage process.

Shortly afterwards, it was found that vitamin E deficiency in rats results in a greater susceptibility of cellular membranes to exercise-induced oxidative damage [28]. Moreover, the authors reported that a short-term training protocol (3 weeks) was enough to reduce the susceptibility of mouse skeletal muscle to lipid peroxidation using an *in vitro* approach [29] (See Fig. 1).

1.3. Exercise training improves the endogenous antioxidant system

Quintanilha and Packer provided the first evidence that the endogenous antioxidant systems of both cardiac and skeletal muscle are plastic and adapt in response to exercise training [30]. Dr. Ji's research team delved into this finding and demonstrated an increase in antioxidant enzyme activities in rat hindlimb muscle and diaphragm after an acute bout of exercise [31,32] and suggested a post-translational modification of antioxidant enzymes by exercise-induced free radicals. Powers and co-workers confirmed that exercise training promotes an increase in the activities of SOD and glutathione peroxidase (GPX) in muscle, proportionally to the exercise intensity and duration [33,34].

In 1985 two different research teams found that exercise-induced free radical production contributed to the down-regulation of the activities of key metabolic enzymes such as malate dehydrogenase and citrate synthase [35,36]. That same year, the term oxidative stress was first defined by Helmut Sies as "a disturbance in the prooxidant-antioxidant balance in favor of the former" in a book entitled "Oxidative Stress" [37]. This definition has been subsequently refined as

"an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage" [38] (See Fig. 1).

1.4. Redox regulation of skeletal muscle fatigue

During the 90s different researchers highlighted the role of exhaustive exercise in the regulation of glutathione redox status [4,39,40]. We found a linear correlation between GSSG/GSH and Lactate/Pyruvate ratios in human and rat blood before, during, and after exhaustive exercise. We took it to suggest that oxidative stress (evidenced by the oxidation of glutathione) is related to exhaustion (evidenced by an increase in lactate). Several studies during the decade of the 1990s established the role of free radicals in muscle fatigue in animal models [41–45] and in humans [12]. Fatigue in skeletal muscle, which is defined as a decrease in maximal force production in response to contractile activity [46] can occur through various mechanisms that can be modulated in the presence of free radicals. These mechanism include: (i) impairment of the muscle cell membrane excitability, (ii) block of action potentials from reaching the T tubules, (iii) excitation-contraction coupling failure which stops the voltage sensor form interacting with Ca^{+2} ion channels, (iv) reduction in myofibrillar sensitivity, and (v) stopping actin-myosin crossbridges from forming in response to the presence of Ca^{+2} ions [47].

Michael Reid and colleagues found that the redox status of muscle fibres played an important role in the modulation of muscle force production [48]. Specifically, their prediction was based on experiments indicating that maximal force production in skeletal muscle fibres occurred at an optimal redox state [49]. Based on their results, dietary administration of thiol-containing substances such as N-Acetylcysteine (NAC) or glutathione, started being recommended to the sport population to prevent cellular damage caused by exercise [50,51] and to inhibit muscle fatigue [12,45]. Further, numerous studies using isolated muscle preparations also confirmed that exposure of skeletal muscle fibres to oxidants results in impaired muscle force production [52] (See Fig. 1).

1.5. Sources of free radicals during muscle contractions

The finding that skeletal muscle fibres release superoxide radical [44] and hydroxyl radical [53] at a rate proportional to the percentage of maximal force generation [54] encouraged the study of the sources of free radical production in the contracting muscles.

Skeletal muscle generates RONS at multiple subcellular sites, several of which increase in activity during muscle contractions [3]. The mitochondria, NADPH oxidase enzymes associated to the sarcoplasmic reticulum, plasma membrane, and transverse tubules, together with xanthine oxidase contribute to the increase in whole body oxidation [3]. Moreover, both calcium-dependent and independent forms of phospholipase A_2 play a role in muscle ROS generation. Phospholipase A_2 is an enzyme that releases arachidonic acid, which is a substrate for ROS-generating enzyme systems such as the lipoxygenases, from membrane phospholipids [55].

Many studies have examined indicators of oxidative stress in exercise assuming that skeletal muscle, due to its relatively large mass compared with other tissues, provides the major source of RONS generation during exercise [3]. However, we should keep in mind that apart from skeletal muscle it is entirely feasible that other tissues such as lungs, heart, or white blood cells may contribute significantly to the total body generation of ROS during physical activity. The non-muscle sources of ROS have also their biological importance [3].

NO is also generated continuously by skeletal muscle, a production that is increased by contractions [55]. An important discovery that occurred during the decade of the 1990s was the finding that skeletal muscle expresses two isoforms of nitric oxide synthase (NOS), the neuronal (nNOS) and the endothelial one (eNOS) [3].

1.6. ROS as regulators of gene transcription in exercise

In 1996 the concept that ROS were not only damaging agents but could also be signal transduction messengers involved in the regulation of gene expression and homeostasis was proposed in a paper entitled "Antioxidant and redox regulation of gene transcription" [56]. In this study, it was suggested that protein phosphorylation and the activation of two redox transcription factors: Activator Protein 1 (AP-1) and Nuclear factor κB (NF-κB), were dependent on the thiol-disulfide balance. Since then, it has been very well demonstrated that RONS are used in physiological settings as signaling messengers with important regulatory functions [13,57,58]. The basic premise of redox signaling is that ROS regulate reversible posttranslational modifications to thiol moieties on target proteins implicated in cell signaling. The modifications include sulfenic acid, sulfoxide and disulfide formations, S-nitrosylation, S-glutathionylation [58]. This was an important fundamental change, especially in the exercise redox biology field [8]. The concept of oxidative stress was updated to include the role of redox signaling and adaptation [59,60] and it was highlighted that ROS are capable of exerting positive stress, eustress, as well as the well-known deleterious effects, distress in cells [13,58].

We now know that redox signaling supports long-term remodeling and adaptation to contractile activity [61] including neuromuscular development [62] and regeneration [63], blood flow for physical activity [64], skeletal muscle hypertrophy [65], and mitochondrial biogenesis [66] among others. Moreover, it has also been shown that exercise-induced cardiovascular health is under redox control [67]. However, ROS are also related to disuse muscle atrophy [68,69]. Paradoxically, both skeletal muscle contraction and disuse or inactivity are associated with an increase in ROS generation leading to very different outcomes in the muscle cell [70]. An excessive ROS load is detrimental to muscle function and skeletal muscle sarcopenia, the decline in muscle mass and function commonly associated with ageing, is a phenotypic manifestation of underlying oxidative stress [71,72].

For ROS to act as physiologically essential signaling messenger, it is of the utmost importance that these species are produced in tightly regulated manner, that is, the location, time, and amount of production of particular species should be programmed and controlled [58].

As early as 1982, Packer research team suggested that ROS-induced damage could provide a stimulus to the skeletal muscle adaptations to endurance training and more specifically mitochondrial biogenesis. Since this early forecast, many investigations have provided evidence to support this prognostication (See Fig. 1).

ROS are required to promote certain exercise training adaptations in skeletal muscle. The antioxidant paradox.

A frequent approach in many studies performed in the free radical field has been to abolish the signaling effects of exercise-induced ROS production in skeletal muscle by treating animals or humans with antioxidants. For instance, we have found that the inhibition of xanthine oxidase activity *in vivo* during acute exercise prevents muscle damage in humans and animals [73–75] but also the exercise-induced activation of signaling pathways leading to adaptations in the active muscles [76–78]. This fact is in accordance with the 'antioxidant paradox', a term used to describe the observation that although ROS are involved in several human diseases, in several studies, giving large doses of dietary antioxidant supplements has not demonstrated preventive effect [79].

Using *in vitro* studies, it was shown that ROS production is a requirement for contraction-induced gene expression of peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) [80]. Similarly, and mainly using *in vivo* studies, we and others have found that administration of vitamin C prevents the exercise-induced increase in PGC-1α and mitochondrial biogenesis in rats trained for several weeks [66,81]. In accordance with these results, it was demonstrated that the exercise-induced expression of heat shock proteins (HSPs) in muscle is suppressed by antioxidant supplementation [82]. Four very valuable studies performed in humans confirmed the results previously published

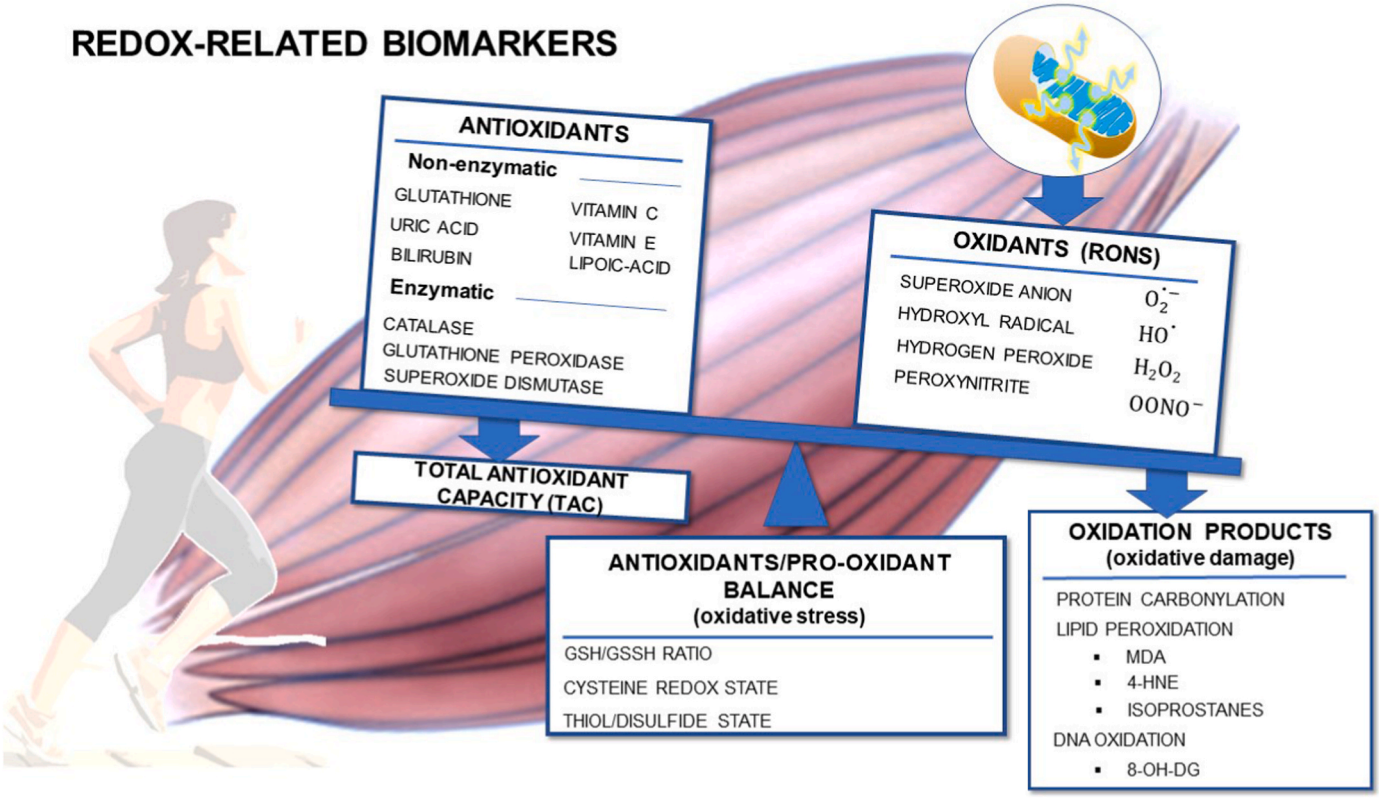

Fig. 2. Categories of redox biomarkers in exercise research.

in animal models. In the first study, Ristow and collaborators found that exercise-induced oxidative stress was involved in the amelioration of insulin resistance in humans. Daily administration of vitamin C and vitamin E precluded the health-promoting effects of exercise [10]. In the second study, a double-blind, randomized controlled study, supplementation with vitamin C and E hampered cellular adaptations to endurance training in young recreationally endurance-trained individuals [83]. Similarly, in the third study, it was found that Vitamin C and E treatment blunts the High-Intensity Interval Training (HIIT)-induced changes in calcium and in the mRNA expression of mitochondria-related proteins in recreationally active individuals [84]. Finally, in the fourth study, again a HIIT protocol was tested. Westerblad and co-workers found that the HIIT-dependent ROS generation induced an extensive fragmentation of the sarcoplasmic reticulum Ca^{2+} release channel, the ryanodine receptor type 1 (RyR1), in muscles of recreationally active subjects, thus resulting in changes in the expression of genes related to endurance exercise. However, the HIIT-induced RyR1 fragmentation did not occur in muscles exposed to NAC which explains why antioxidants blunt effects of endurance training.

More recently Hamilton's research team have made a relevant contribution to the area. They have confirmed that vitamin C interferes with redox-sensitive proteostatic mechanisms and mitochondrial biogenesis in skeletal muscle. Moreover, they have also found that treatment with bioactive phytochemicals that activate the Nuclear factor E2-related factor 2 (Nrf2) are able to maintain redox balance [85]. Treatment with protandim during training did not interfere with redox-sensitive proteostatic mechanisms in skeletal muscle. This study for the first time suggests that targeting the endogenous antioxidant network facilitates adaptations to exercise (See Fig. 1).

1.7. Redox sensitive targets in skeletal muscle

Research from the last twenty years has shown us that RONS are important factors within the list of signals generated during muscle contraction. RONS are purposefully generated and harnessed during exercise to regulate a diverse array of physiological processes. They are involved in the activation of molecular events leading to physiological responses and subsequent adaptations [86]. The list of targets of redox signaling is growing at accelerating pace. Among the main redox sensitive targets that serve as hubs to support biological functions we can highlight: Nrf2, a transcription factor responsible for the antioxidant response; protein kinases such as Mitogen Activated Protein Kinases (MAPKs) and AMP-Activated Protein Kinase (AMPK) involved in autophagy and mitochondrial biogenesis; NF-κB a mediator of the inflammatory response, and the Hypoxia-Inducible Factor (HIF). It is also worthy to mention the enzyme Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), which is related to metabolic adaptations; the nuclear transcription factor Forkhead Box Protein O (FOXO), involved in the stress adaptation; p53, another nuclear transcription factor that regulates apoptosis, and the Uncoupling Proteins (UCPs) which regulate mitochondria energetics [8,13]. The main adaptations in which RONS are involved during exercise are: increased mitochondrial mass, enhanced antioxidants defences, and myofiber hypertrophy. They affect protein function, enzyme activity, gene transcription, and membrane and genome integrity.

Other important signals that have been identified during exercise include: Ca^{+2} that activates Ca^{2+}/calmodulin-dependent protein kinase (CaMKs), (NAD^+/NADH) that activates sirtuins (SIRTs), (AMP/ATP) that activates AMPK, partial pressure of inspired oxygen (PiO_2) that activates prolyl hydroxylases (PHDs), and mechanosensation that activates Focal Adhesion Kinase (FAK) [87].

Adaptations to exercise training result from the cumulative effect of the exercise bouts. They induce transient increases in mRNA transcripts that encode for various proteins. These repeated bursts in mRNA expression appear to be essential to drive the intracellular adaptive response to exercise training [11]. The specific effects of RONS are modulated in large part through the covalent modification of specific cysteine residues found within redox-sensitive target proteins. The

major mechanism by which ROS mediate their biological effects in redox regulation is through thiol-based modification of target proteins [13]. However, non-coding RNA or microRNAs, have also been shown to be redox sensitive and contribute to redox signaling [13] (See Fig. 1).

Based on the role of constant progressive physical activity in boosting resilience and increasing ROS detoxifying mechanisms, exercise is able to improve skeletal muscle function.

2. Redox biomarkers, usefulness in sport sciences and methodological problems

Sport scientists are very interested in the identification of biomarkers for assessing health, performance, recovery during exercise training, and overtraining [88]. The exercise prescription, both in top level athletes and for the promotion of health and cure of diseases, requires a functional assessment of the participants in the physical activity programs [89]. Among the different sets of biomarkers that could be determined in the sport population to accomplish their evaluation we could include the following: (i) nutrition and metabolic health (macronutrient and micronutrient metabolism), (ii) hydration status (plasma osmolarity and sodium, thirst, creatinine), (iii) muscle status (IGF-1, testosterone, dehydroepiandrosterone, amino acids, markers of muscle damage), (iv) endurance parameters (ferritin, transferrin, hemoglobin), (v) injury status and risk (S100 calcium-binding protein B, bone mineral density), and (vi) inflammation (interleukins, Monocyte Chemoattractant Protein, C-reactive protein).

Redox biomarkers are not determined regularly in the sport population. The lack of standardized values for these biomarkers and the technical difficulties for their determination are the main reasons why they are not usually reported in the athletes' functional assessment or even in routine clinical laboratory analysis.

Reliable redox biomarkers in exercise should have the following characteristics: 1) be chemically unique and detectable, 2) be increased or decreased after one bout of exercise or a protocol of exercise training, 3) have relatively long half-lives, and 4) not be impacted by other cellular processes (e.g., cell cycle, nutritional interventions, etc.) [3].

2.1. Redox biomarkers categories

Redox biomarkers in exercise usually fall into one of four categories (Fig. 2):

The *first category* involves the detection of oxidants. First evidence showing an increase in free radical content in rat skeletal muscle following exercise until exhaustion by using electron spin resonance, was reported in 1982 [27]. Further studies in humans have confirmed an increase in the production of radical species in the venous blood of healthy human after an acute bout of exercise [90] and in their skeletal muscle [91].

Direct measurement of radical production in living cells is difficult because radicals are highly reactive and have a short half-life. Therefore, exogenous molecules such as fluorescent probes or spin traps are commonly used to measure oxidant production in cells. When added to a biological system, the probe or spin trap is converted into a uniquely modified radical product with a relatively long half-life that can be quantified as a measurement of oxidant production [3]. The reaction of RONS with reporter dyes results in the generation of both specific, often less abundant, oxidized products and more abundant, non-specific products. Therefore, the detection of a specific molecule requires the identification, separation, and quantification of its oxidation product. Furthermore, proper utilization of fluorescent dyes requires performing a series of controls in conjunction with molecular or pharmacological inhibitors for the identification of the reactive species involved. For instance, Dichlorodihydrofluorescein (DCFH), Hydroethidine (HE), and Dihydrorhodamine (DHR) are being used frequently for measuring H_2O_2, $O_2^{\cdot-}$, and $ONOO^{-\cdot}$ in biological systems in exercise studies [92–94] but they have some limitations. Two potential drawbacks of using probes or spin traps should be mentioned in this section of the manuscript. The first one is that fluorescent dyes such as DCFH, HE and DHR do not directly react with a specific molecule and their fluorescence should not be used as a direct and specific measurement of either H_2O_2, $O_2^{\cdot-}$ or $ONOO^{-\cdot}$. The second potential drawback is that these molecules may be toxic for the cells and alter the biological system being investigated [95]. We recommend the following papers for a comprehensive review of the challenges and limitations of the most widely used fluorescent probes for detecting and measuring RONS [96,97].

The *second category of redox biomarkers* includes the measurement of antioxidants in tissues or in blood. Antioxidant biomarkers can be evaluated assessing: non-enzymatic antioxidants (GSH, uric acid, bilirubin, Lipoic-acid, vitamin C and E, etc.), enzymatic antioxidants (catalase, glutathione peroxidase, superoxide dismutase, among others), and total antioxidant capacity (TAC) [98]. The most common tests used to assess TAC are the trolox equivalent antioxidant capacity assay, the oxygen radical absorbance capacity assay, and the ferric ion reducing antioxidant power assay [98]. Some examples of exercise studies that have measured these category of redox biomarkers include the assessment of brain [99], muscle [77], and plasma [100] glutathione levels after an acute bout of exercise. Total antioxidant capacity has been also determined in exercise studies [101]. In any case the use of total antioxidant capacity is, in general terms, not recommended for lack of specificity.

Although the measurement of a decline in tissue antioxidant levels has been widely used [55], this approach is not without weaknesses. High levels of antioxidant enzyme activity may be protective against an acutely elevated oxidative stress status, but they may also reflect the up-regulation of defence mechanisms in response to chronically high levels of oxidants [102]. Other factors, apart from exercise, such as diet changes can influence antioxidant levels in cells. Another drawback associated with the measurement of tissue antioxidants is the potential for auto-oxidation during sample handling resulting in antioxidant depletion in the cells [95].

A *third category of redox biomarkers* involves the evaluation of oxidatively modified molecules mainly reflecting the assessment of oxidation products in biological molecules and including nucleic acids, lipids, and proteins [3]. Common measurements of biomolecules oxidation include the measurement of protein carbonyls as an indicator of protein oxidation; assessment of isoprostanes, malondialdehyde [MDA], and 4-hydroxyl-2-nonenol as signs of lipid peroxidation; and evaluation of DNA oxidation by assaying the levels of the oxidized deoxy nucleoside, 8-hydroxy-2-deoxyguanosine (8-OH-dG) [3]. Urine excretion of nucleosides, has been determined, most notably by the group of Dr. Poulsen, to estimate RNA oxidation [103]. Many laboratories have shown increases in these redox biomarkers in different tissues after exercise in animal [77,104] and humans studies [4,105,106].

Protein carbonyls represent an irreversible form of protein modification. They are relatively stable in contrast to lipid peroxidation products [107]. The most commonly used marker to assess protein oxidation is via the determination of protein-bound carbonyls and can be detected by various methods, all relying on the derivatization of carbonyl groups. The general drawbacks of the spectrophotometric assay are that the method is rather work-intensive, time-consuming and high throughput measurement is not possible. Regarding immunoblotting, an accurate determination of the carbonyl concentration is not possible. In Enzyme-Linked ImmunoSorbent Assay (ELISA) methods, the limitations include that the determination of the protein concentration is mandatory before the assay and the risk of loss of sample in washing steps [108]. Therefore, the semi-quantitative OxyBlot method is recommended because has shown the best sensitivity and specificity [109]. The exercise-induced increase in protein carbonylation in plasma [110, 111], liver [112,113], and skeletal muscle [114] has been widely demonstrated in the exercise field.

8-OH-dG, as DNA damage marker, has also been determined in exercise studies [115–117]. In a recent reported meta-analysis in which

the authors reviewed more than 35 studies, a significant increase in DNA damage immediately following acute high intensity exercise (≥75% of maximum rate of oxygen consumption) was found. This increase remained significant between 2 h and 1 day, but not within 5–28 days post-exercise. Such an increase however, is not found in long-distance protocols such as running a marathon [118].

The first reported analysis of 8-OHdG (or 8-oxodG) as a major oxidation product of DNA in urine was published by Bruce Ames' research group in 1989 [119]. Since then, several scientific groups have reported 8-OHdG analysis by using different methodologies [53,120,121]. However, methodological differences among scientific laboratories directly analysing the levels of 8-OHdG have resulted in a lack of consistency in the results, especially in the exaggeration of the background levels of 8-OHdG in human cellular DNA, sometimes by up to two or three orders of magnitude [122,123]. Such large differences can be attributed to inaccurate experimental protocols and isolation methodologies that give rise to overestimations, probably by artefactual DNA oxidation during the procedure. As a general trend, levels of 8-OHdG obtained by the indirect approach (enzymatic assays) show significantly lower values than those obtained by using direct methods of measurement. Direct measurements can identify the specific lesion using physico-chemical methods subsequent to DNA extraction and hydrolysis, such as High Performance Liquid Chromatography (HPLC) or Gas Chromatography-Mass Spectrometry (GC-MS) [124]. In order to resolve the methodological problems encountered in measuring quantitatively 8-OHdG, the European Standards Committee for Oxidative DNA Damage was set up in 1997 to resolve this issue, including the artefactual oxidation problems during the procedures of isolation and purification of oxidative DNA products [125]. The Liquid Chromatography with tandem mass spectrometry (LC-MS-MS) method, was found to be as reliable, sensitive, and precise as the best HPLC procedure and it had the added advantage of giving unambiguous information on the identity of the analytes [126].

Lipids are oxidized by enzymes, free radicals, and non-radical oxidants by distinct mechanisms to produce a great diversity of products [58]. ROS-mediated lipid peroxidation proceeds by a non-specific manner to produce multiple isomeric products. Lipid peroxidation products may induce alterations of integrity, permeability, and fluidity of membranes, modify lipoproteins to pro-atherogenic and pro-inflammatory forms, and generate toxic products. The lipid peroxidation of arachidonic acid produces cyclic endoperoxides and cleavage products such as isoprostanes and unsaturated aldehydes in addition to hydroperoxyeicosatetraenoic acids [58]. On the other hand, lipid peroxidation of polyunsaturated fatty acids produces multiple aldehydes being 4-Hydroxy-2-nonenal (HNE) the most reactive one [13]. The lipid peroxidation products of polyunsaturated fatty acids and their esters and also of cholesterol exert deleterious effects such as cytotoxicity and modification of proteins and DNA bases. However, the lipid peroxidation products also induce adaptive response to increase defence capacity by enhancing expression of antioxidant compounds and enzymes [58]. For instance, it has been shown that, although HNE itself is cytotoxic, treatment of cells with sublethal concentrations of HNE induces adaptive response and enhances cell tolerance primarily through induction of antioxidant enzymes [58]. It has been argued that lipid peroxidation products such as HNE act as physiological redox signaling messengers and exert hormetic functions [127]. This third category of biomarkers, as with the previous ones, is not exempt from certain methodological considerations [128]. For instance, the quantification of TBARS to detect the oxidation of pure unsaturated fatty acids has been questioned in complex biological systems. For more details see the following manuscript for expert recommendation on free radical research terminology and methodology [97].

The *fourth category of redox biomarkers* involves the measurement of cellular redox balance [3]. One of the most commonly measured markers of this balance is the GSH to GSSG ratio. This assay is useful because increased oxidant production results in an increase in the GSSG/GSH ratio, indicating lower levels of reduced GSH in favor of increased oxidized GSH (GSSG). Nonetheless, although this assay is conceptually simple, experimental artifacts are common and can occur during tissue removal and sample processing due to improper tissue handling permitting auto-oxidation [129]. Regarding thiol and disulfide analysis, the major artifact occurring during the analysis is represented by glutathione disulfide (GSSG) and S-glutathionylated proteins (PSSG) overestimation, due to artificial oxidation of GSH during sample manipulation [130]. The cellular levels of GSH are at least two order of magnitude higher than those of GSSG. Thus a 2% oxidation of GSH leads to an error in the estimation of GSSG of about 100%. Oxidation of GSH must be lowered to levels of about 0.1% [131]. This methodological problem can be solved by the addition of quenching agents such as N-ethylmaleimide (NEM), 2-vinylpyridine, or iodoacetic acid, that may be used to alkylate thiol groups immediately after sample collection. NEM is preferred because of its rapid reaction rate (1 min), in contrast to 2-vinylpyridine (20–60 min) or iodoacetic acid (5–15 min) [131]. Furthermore, alkylation with NEM can be achieved at 4 °C, whereas alkylation with 2-vinylpyridine or iodoacetic acid occurs at room temperature [131]. However, it should be noted that NEM is a potent inhibitor of glutathione reductase and it interferes with enzymatic assays of GSH and GSSG based on the activity of this enzyme. Therefore, NEM must be removed from the sample in these assays [130].

Glutathione has been widely quantified in exercise studies and in different tissues [4,51,132,133]. Glutathione oxidation increases during exercise both in humans and in animals and that it is proportional to exercise intensity [4]. GSSG levels decrease progressively after exercise returning to control values, minutes after exercise cessation. For this reason, a rapid sample processing is essential to guarantee a correct determination of this biomarker.

Taking into account that each category of redox biomarkers has limitations, the measurement of multiple biomarkers is required to confirm the presence of oxidative stress and or of oxidative damage in tissues during exercise [3].

2.2. Oxidative stress vs oxidative damage

Importantly, we would like to highlight at this point of the review the differences between oxidative stress and oxidative damage [134]. Oxidative stress includes reversible alterations of redox-status of cell compartments that may affect redox pairs (i.e. the GSH/GSSG ratio) but that do not cause damage to molecules such as DNA oxidation, lipid peroxidation, or protein carbonylation. However, oxidative damage is irreversible unless there are specific repair mechanisms like DNA repair systems (base excision, nucleotide excision or single and double strand break repair) or degradation of oxidized proteins by, for instance, the proteasome. Oxidative stress precedes oxidative damage which occurs when reducing systems cannot cope with the rate of the oxidation of cell components.

2.3. Biomarkers of muscle damage and inflammation in exercise

Redox biomarkers during exercise have traditionally been associated to muscle damage [73,74]. Muscle damage is an expected part of exercise training, as are the physiological and immune responses that occur during and after muscle tissue damage. In the next section of the manuscript, we will deal with the classical markers of muscle damage and inflammation during exercise.

2.4. Muscular biomarkers in exercise

The response of skeletal muscle to unaccustomed eccentric exercise has been studied extensively, yet it is incompletely understood [135]. Eccentric muscle actions are the primary source of muscle damage during exercise. Sarcoplasm, sarcolemma, and the extracellular matrix in the muscle fiber can undergo structural changes after one bout of

damaging exercise. These changes can be visualized by the translocation of some of their constituent proteins which can be determine by using antibody staining techniques [136]. HSPs are involved in the cellular protein quality control machinery [137] and are frequently used as biomarkers of damage in muscle tissue because their localization in the sarcoplasm reflect the fiber structural stability [138].

Different exercise regimes can modulate the expression of the HSPs. The association between muscle force generation and HSP70 levels has been widely described [139,140]. Physiological concentrations of ROS can transiently and reversibly activate HSP70 and HSP60 following non-damaging muscle contractions [141]. However, eccentric contractions, involves the phosphorylation and translocation of small HSPs [141]. It has been shown that only 3-h after one bout of high intensity eccentric exercise, Hsp27 is translocated and accumulated as stained clusters on cytoskeletal/myofibrillar structures [139,141]. Hsp27 is located close to the Z-disks and I-band [139] and stabilizes actin and actin-binding proteins such as tropomyosin and troponin T avoiding their aggregation [142].

Muscle damage is reflected in structural changes in sarcolemma too. Dystrophin is a cohesive protein that protects sarcolemma by transmitting force laterally from contractile filaments to extracellular matrix through a structural complex (dystrophin associated complex, DAPC) [143]. Dystrophin's C-terminus can be immunostained with the proper antibody and allows to identify changes in the sarcolemma due to muscle fiber damaging contractions [144].

2.5. Local inflammatory biomarkers in exercise

Ultrastructural myofibrillar disruptions following intense exercise is accompanied by an infiltration of inflammatory cells in the muscle fiber [135]. Monocytes infiltration has been identified in skeletal muscle biopsies after a single bout of eccentric exercise [145]. Cluster of Differentiation 68 (CD68)-positive cells occupy the entire sarcoplasm of damaged skeletal muscle fibers. However, since CD68 antibody not only recognize monocytes/macrophages but also satellite cells and fibroblasts, this marker cannot be used for a quantitative analysis for inflammatory cells [146].

2.6. Systemic biomarkers in exercise

Eccentric muscle actions produce delayed decrements in force production, muscle soreness, swelling, and increased levels of muscle-specific proteins in blood [135]. When the loading exceeds a certain limit, muscle intracellular proteins leak into the interstitial fluid and finally reach the blood.

Otto Warburg was the first to detect enzymes in blood plasma which normally remain only within living cells [147]. He determined, initially, aldolase and lactate dehydrogenase. As they have no function to perform in the plasma. Warburg reasoned, that these enzymes were derived by a cells' leakage or disintegration. Warburg also found in that the aldolase content of plasma could rise in pathological states such in tumour-bearing rats. Based on these findings, enzymatic assays in plasma became established methods of clinical biochemistry [148].

As mentioned in the previous section the HSPs levels show a marked increase in muscle following a damaging contraction protocol [139,141]. Moreover, HSPs can be used as biomarkers of muscle damage also in plasma [149].

The variation of pre- and post-exercise CK (Creatin Kinase), LDH (Lactate Dehydrogenase), AST (Aspartate Aminotransferase), and ALT (Alanine Aminotransferase) levels in serum, are the most useful markers of muscle injury [150,151]. Serum levels of skeletal muscle enzymes depend on training, type, and intensity of exercise [152–154]. Eccentric muscular contractions associated to muscle injury induce the greatest increases in serum CK activity [155]. The three cytoplasmic CK isoenzymes, CK-MM, CK-MB, CK-BB, are increased after prolonged and strenuous exercise [156], being the total CK level up to 4-fold its normal value [157]. Total LDH serum levels also are increased after eccentric exercise (up to 2-fold) [157]. Although serum alanina aminotransferase (ALT) and aspartate aminotransferase (AST) values and the AST/ALT ratio is used to diagnose chronic liver disease, AST activity is also significantly increased after muscular exertion [157]. Other enzymes whose serum levels can be useful as biomarkers of muscle injury are aldolase and carbonic anhydrase. Aldolase A binds to the actin-containing filament of the cytoskeleton in the muscle. This enzyme regulates cell contraction through its reversible binding to the filaments [158]. Carbonic anhydrase III is also located in skeletal muscle and released after muscle damage [159].

Non enzymatic muscle proteins are frequently used to evaluate muscle damage too. This is the case of myoglobin, which is released when there is a degradation of protein structures after strenuous exercise. As consequence, myoglobin serum levels are increased up to 4-fold [157]. Troponin could be also a biomarker of damaging exercise since

Table 1
List of abbreviations.

8-OH-dG	8-Hydroxy-2-Deoxyguanosine
ALT	Alanine Aminotransferase
AMP	Adenosine Monophosphate
AMPK	Amp-Activated Protein Kinase
AP-1	Activator Protein 1
AST	Aspartate Aminotransferase
ATP	Adenosine Triphosphate
CaMKs	Ca^{2+}/Calmodulin-Dependent Protein Kinase
CD68	Cluster of Differentiation 68
CK	Creatin Kinase
cTnT	Cardiac Troponin T
DAPC	Dystrophin Associated Complex
DCFH	Dichlorodihydrofluorescein
DHR	Dihydrorhodamine
ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	Endothelial Nitric Oxide Synthase
FABP	Fatty-Acid-Binding Proteins
FAK	Focal Adhesion Kinase
FOXO	Forkhead Box Protein O
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GC-MS	Gas Chromatography-Mass Spectrometry
GPX	Glutathione Peroxidase
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
HE	Hydroethidine
HIF	Hypoxia-Inducible Factor
HIIT	High-Intensity Interval Training
HNE	4-Hydroxy-2-Nonenal
HPLC	High Performance Liquid Chromatography
HSPs	Heat Shock Proteins
IGF-1	Insulin-Like Growth Factor-1
LC-MS-MS	Liquid Chromatography with Tandem Mass Spectrometry
LDH	Lactate Dehydrogenase
MAPKs	Mitogen Activated Protein Kinases
MDA	Malondialdehyde
NAC	N-Acetylcysteine
NAD	Oxidized Nicotinamide Adenine Dinucleotide
NADH	Reduced Nicotinamide Adenine Dinucleotide
NEM	N-Ethylmaleimide
NF-κB	Nuclear Factor KB
nNOS	Neuronal Nitric Oxide Synthase
NOS	Nitric Oxide Synthase
Nrf2	Nuclear Factor E2-Related Factor 2
PGC-1α	Proliferator-Activated Receptor Γ Coactivator 1α
PHDs	Prolyl Hydroxylases
PiO_2	Partial Pressure of Inspired Oxygen
RNS	Reactive Nitrogen Species
RONS	Reactive Oxygen and Nitrogen Species
ROS	Reactive Oxygen Species
RyR1	Ryanodine Receptor Type 1
S-100B	S100 Calcium-Binding Protein B
SIRTs	Sirtuins
SOD	Superoxide Dismutase
TBARS	Thiobarbituric Acid Reactive Substances
UCPs	Uncoupling Proteins
XO	Xanthine oxidase

several studies have reported a pronounced increase in Cardiac troponin T (cTnT) serum levels after intense exercise compared with exercise at moderate level [160]. Fatty acid-binding proteins (FABPs) are useful as early biochemical markers of muscle injury. The ratio of the concentrations of myoglobin over FABP in plasma is different between heart (4.5) and skeletal muscle (20–70) and allows to discriminate between tissues injury [161]. (see in Table 1 the list of the abbreviations used in the text)

Finally, the levels of some metabolites in urine can be used as biomarkers of muscle injury. This is the case of titin fragments determination in urine in response to eccentric exercise-induced muscle damage [162]. After damaging exercise two amino acids from collagen, hydroxyproline and hydroxylysine, show increased levels in urine too. The evaluation of creatine/creatinine ratio can be also used to assess muscle damage [163].

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