

## ***Redox Status for Health and Athletic Monitoring***

### **Introduction**

Physiological homeostasis is a well established prerequisite for optimal biological function (1). To this end, various commercial products and scientific techniques exist to purportedly monitor longitudinal and acute changes of internal parameters which reflect homeostatic perturbations that impact biological function. The monitoring of internal metrics is commonplace within clinical populations with well established population reference ranges for various biomarkers that predict and classify pathologies (2). The use of internal monitoring techniques is also increasingly commonplace within competitive sport and with recreational athletes (3). The ultimate goal of internal monitoring for athletics is optimizing physiological homeostasis for exercise performance and adaptation.

Various analytical techniques and biomarkers exist for monitoring general health or exercise performance with the vast majority existing in one but not both of these contexts. Descriptive redox biomarkers that function to give a general snapshot of redox status are some of the most highly researched biomarkers in both health and performance contexts (4,5,6,7,8,9). This is because redox status (henceforth used interchangeably with redox homeostasis) is highly linked to physiological function (21) and alterations in redox status are linked to the pathogenesis of various diseases (22, 23) as well as decreased exercise performance and impaired exercise adaptations (24, 25). Despite the importance of redox homeostasis, measurement of the *in vivo* redox environment is extremely complex. Redox status is constantly in flux and varies across tissues and cellular compartments. Compounding this difficulty is the fact that most oxidants have extremely short half lives ranging from  $1 \times 10^{-3}$  s to  $1 \times 10^{-11}$  s making direct measurement difficult (26). Most attempts to examine redox status have focused on measuring endogenous antioxidants and products of oxidative damage with the general assumption that a decrease in oxidative damage markers and an increase in antioxidants is good. Within certain disease contexts this can be appropriate. As cystic fibrosis progresses for example, an increase in oxidative damage and decrease in antioxidant expression is directly related to increased disease severity (10). However, a negative interpretation of oxidative damage is reductionist as oxidative damage can be positive (eustress) or negative (distress) in different contexts (27). In the same vein, reductive stress resulting from unwarranted antioxidant supplementation or dysregulated increases in antioxidant expression may negatively impact health and performance (21).

Numerous redox related biomarkers exist (6), however many popular redox assays are technically flawed (i.e. TBARS, TAC analogues) and their discontinuation is recommended. Appropriate profiling and monitoring of biomarkers to assess redox homeostasis has applications within health and performance as a diagnostic criterion (4), in the assessment of disease progression (10), and in determining fatigue, readiness, and overtraining in athletes (28). Still, underappreciated opportunities exist to expand the assessment of redox status to include the monitoring of physiological signaling, cell function, adaptive capacity, and establish redox signatures specific to pathological and performance contexts. The aims of this review are threefold: 1) To summarize the biochemical components of redox homeostasis and outline its importance as it relates to both general health and athletic performance; 2) highlight how appropriate exercise benefits and maintains redox homeostasis in general, clinical, and athletic cohorts; 3) discuss the current state of redox biomarkers used both clinically and within exercise/performance contexts and describe the potential utility of improved redox monitoring tools.

## 1. Redox Homeostasis

### 1.1. Biochemical Components of Redox Homeostasis

The maintenance of redox homeostasis is a dynamic continuous process. Notable parent free radicals superoxide ( $O_2^-$ ) and nitric oxide ( $NO^-$ ) are continuously produced at rest, namely via electron leakage from the mitochondria and nitric oxide synthase (NOS) respectively, though oxidants are produced from various cellular compartments (11). The species created from these parent molecules can be generally categorized as reactive oxygen species (ROS) and reactive nitrogen species (RNS) which can be combined and referred to as reactive species. Reactive species is a collective term that includes the radical parent molecules, other free-radicals, and non-radical oxidizing agents (12). Reactive species (used interchangeably with oxidants henceforth) have diverse chemical properties and use of the term should be restricted to when the species involved in the chemical process is unknown (11). As such, when appropriate, the exact species involved in the biochemical processes described will be identified. Reactive species interact with various molecular targets including but not limited to antioxidants, transcription factors, proteins, DNA, lipids, and thiol groups on cysteine residues.

The production of reactive species and subsequent interaction with antioxidants contribute significantly to redox homeostasis and transient redox status. The endogenous antioxidant system is comprised of enzymatic (SOD, CAT, Gpx, PRDX, TRX) and non-enzymatic (Vitamin C, E, bilirubin, albumin, GSH) antioxidants which collectively function to maintain basal steady state redox status by preventing aberrant production of reactive species (31). The unregulated production of reactive species can lead to various negative outcomes including mitochondrial dysfunction, cell death, and various disease states (29, 30). Though many molecules in the body function as antioxidants, the primary molecules responsible for protection against aberrant reactive species production are *enzymatic* antioxidants. Some antioxidants react specifically with only one reactive species (SOD converts  $O_2^-$  to  $H_2O_2$ ) or exist in only one organelle (CAT is localized exclusively to peroxisomes). The extremely kinetically efficient biological reactions ( $2 \times 10^9 M^{-1} s^{-1}$  for SOD and  $O_2^-$ ) and localization of certain reactive species and antioxidants is a hallmark of redox biology: spatiotemporal specificity (14, 26). Both rapid kinetics and localization contribute to the difficulties in monitoring redox status, as to date it is not possible to get a total systemic assessment of redox status from a single tissue or blood sample (21, 26).

The production of antioxidants is, ironically also largely regulated by the production of reactive species and their interaction with the transcription factors nuclear factor erythroid 2-related factor 2 (Nrf2) and Nuclear factor kappa-light-chain-enhancer of activated B cells (Nf-kb) (30). Nrf2 is the master regulator of endogenous antioxidant defense and together with Nf-kb they are responsible for the induction of the endogenous enzymatic antioxidant system and work synergistically to maintain adequate antioxidant status and redox homeostasis (31). Both Nrf2 and Nf-kb are bound to cytosolic inhibitor proteins that tag them for ubiquitination and subsequent degradation by the proteasome. Oxidants (namely  $H_2O_2$ ) can react with the inhibitor proteins bound to Nrf2 and Nf-kb to induce conformational changes that release the transcription factors from their respective inhibitors. This allows Nrf2 and Nf-kb to translocate to the nucleus and act on their respective promoter regions to upregulate endogenous antioxidant defenses (32, 33). Oxidant induced activation of antioxidant systems represents a genetically regulated homeostatic feedback mechanism and is only one example of some of the critical cell processes reactive species regulate (11). It should be noted that Nf-kb also plays a large role regulating inflammation and innate immunity and can be inhibited by Nrf2. In this way, redox status is intricately linked to inflammatory homeostasis and immunology. For further reviews on the interaction between Nrf2 and Nf-kb the reader is directed elsewhere (30).

Not all reactive species are scavenged and neutralized by antioxidants, reactive species inevitably react with cellular lipids, proteins, and DNA both at rest and during periods of increased reactive species production (e.g. disease, exercise) (19). The interaction between reactive species and macromolecules can lead to oxidation products, namely lipid peroxidation (MDA, Isoprostanes), protein oxidation (protein carbonyls), and DNA oxidation products (8-oxoguanine). *Macromolecular damage* was originally thought to be exclusively negative and it was believed that the accumulation of reactive species and oxidation products directly resulted in aging (15). Indeed, increased macromolecular damage is often observed in cancer, neurodegeneration, psychiatric conditions, and in response to viral pathogens (17, 29, 34, 35); however it is unlikely macromolecular damage directly results in aging but instead contributes to the pathogenesis of aging-related diseases (20). It is now clear that oxidation products are not biologically inert and are also transducers of cell signals by directly reacting with kinases, phosphatases, and transcription factors (16). In addition, significant repair mechanisms exist *in vivo* to mend macromolecular damage (36). For example, 8-oxoguanine (8-oxog) is one of the most common DNA lesions that occurs from reactive species interaction with DNA and is frequently measured in relationship with aging (38), cancer (17), and exercise (37). 8-oxo-g base lesions are repaired by 8-oxoguanine glycosylase, (OGG1) and decreased OGG1 activity increases risk for every form of cancer (17).

The interaction of reactive species with cysteine-based thiol groups, methionine, tyrosine, and iron-sulfur (Fe-S) clusters all contribute to what is referred to as redox signaling (11). Redox signaling is the transduction of signals coding for cellular processes in which the key signal is the transfer of electrons (19). Oxidant signals can be transduced by direct oxidation of a target protein or indirectly via redox relays. Hydrogen peroxide ( $H_2O_2$ ) is the most critical reactive signaling molecule (17), and is involved in direct signal transduction by reaction with thiolate groups (-SH) on cysteine residues of specific proteins and via indirect redox relays involving peroxiredoxin (PRDX) antioxidant enzymes (19). Redox signals transduced by  $H_2O_2$  and other reactive species play critical roles in maintaining physiological homeostasis by inducing beneficial adaptations for health and performance (21). The induction of positive redox signals for beneficial adaptation is referred to as *oxidative eustress*. This is in contrast to *oxidative distress* which can result when overproduction of reactive species supersedes antioxidant defenses and results in indiscriminate unspecific damage to macromolecules.

The paradoxical duality of oxidants, on one hand critical for cell signaling and physiological function yet cytotoxic and deleterious in certain concentrations, is another hallmark of redox biology and underscores why previous attempts to monitor redox status simply by measuring antioxidants and oxidation products is reductionist and inadequate. In addition, the intracellular redox landscape is *dynamic*, meaning physiological redox status is open and susceptible to exogenous challenges and homeostatic adjustments (21). Exercise (discussed in depth later) is the best example of an exogenous challenge that positively disrupts redox status and results in an oxidative eustress and homeostatic adjustment that is beneficial for health. Exogenous environmental challenges like exposure to toxins, pollution, or behaviors like smoking or sedentariness negatively impact redox homeostasis and result in oxidative distress that is subsequently deleterious for health (39). The determinant of whether or not a redox insult is positive or negative depends on intracellular factors like the subcellular location of reactive species production and the type of reactive species, and environmental factors like if the stressor or stimulus is maintained for too long. The maintenance of appropriate redox signals and redox homeostasis is intimately linked to cellular and molecular events that determine physiological function and organism health (21). Physiological redox homeostasis is therefore characterized by a delicate balance of reactive species production, their subsequent scavenging by antioxidants, the damage of macromolecules, repair of

reversibly oxidized macromolecules, and oxidation of thiolate groups for cell signaling. All of these redox activities are coupled and simultaneously occur within a physiological system and the pleiotropic nature of reactive species means the maintenance of redox status has widespread systemic consequences for health and performance.

### *1.2. Health Consequences of Altered Redox Homeostasis*

The divergence from basal steady state redox homeostasis can interfere with cell signaling, result in excessive macromolecular damage, induce cell death, and contributes significantly to the physiology of aging and pathophysiology of aging-related diseases. This section will discuss health consequences of altered redox homeostasis as it relates to specific pathological contexts. Of course, this is not an exhaustive list of redox-related pathologies. In many cases it is unclear if the disruption in redox homeostasis precedes the clinical development/manifestation of symptoms or if it is in fact a consequence of the disease progression. Where known it will be identified.

#### *1.2.1. Aging*

Aging is defined as “a progressive deterioration of physiological functions ultimately leading to systemic dysfunction and death” (40). Whether or not aging is in fact a disease, or rather a risk factor for disease is still debated (57, 58, 59). Although aging is an inevitable and natural process of life, the molecular mechanisms that underpin aging are poorly understood. Harman first hypothesized the “Free Radical Theory of Aging” in 1955 and suggested a causal relationship between reactive species and aging (16). Ever since, the production of reactive species and accumulation of macromolecular damage have been a leading hypothesis to explain the aging process. Increased macromolecular damage and the accumulation of oxidation products are hallmarks of the aging process. Increased levels of malondialdehyde (MDA) and isoprostanes, as well as increased protein carbonyls, are found in the elderly (46, 47). There are marked increases in both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) damage measured by 8-oxo-g in older individuals (49). Increased oxidation of guanine base pairs during aging is linked to a dysregulation of Ca<sup>2+</sup> homeostasis which may further exacerbate reactive species production and increase macromolecular damage (48). Compounding this, the oxidation of guanine base pairs may also occur within RNA, leading to dysfunctions in protein translation and protein synthesis in the absence of direct damage to the DNA sequence. Notably, the oxidation of RNA is recognized as an early event in the pathogenesis of various human diseases (50). Nucleotide oxidation, and guanine oxidation specifically, is repaired by OGG1 whose activity is known to decline with age (51). Increased loss of functional protein -SH groups due to protein oxidation during aging directly influences redox mediated cell signaling and can result in a loss of redox homeostasis (52). Genomic regulation of redox homeostasis is also lost during aging as a general decrease in Nrf2 activity is observed throughout the lifespan (54, 55). The evidence of increased macromolecular damage, decreased repair mechanisms, and decreased transcriptional activity of Nrf2, coupled with a general shift to a pro-oxidant redox status in various ratios (i.e. NADPH:NADP<sup>+</sup>, NADH:NAD<sup>+</sup>, GSH:GSSG) during aging strongly implicate oxidative distress in the molecular mechanism of aging (52, 53).

Though it is clear that reactive species play a role in aging, exactly to what extent is still a matter of debate (57, 58). It is still unclear if loss of redox homeostasis precedes and causes the various hallmarks of aging: loss of proteostasis, telomere shortening, genomic instability, epigenetic alterations (54) or is a consequence of a generalized loss of homeostatic regulation. If indeed the production of reactive species directly causes the aging process as initially postulated by Harman (1955), then

antioxidant supplementation should be effective at attenuating aging. Antioxidant supplementation may prevent an increase in oxidation products, and in some instances helps protect against age related dysfunction (44, 45). However, interventions to increase longevity through antioxidant supplementation have largely been unsuccessful (42). Generally evidence to support the use of exogenous antioxidant supplements to extend longevity or slow aging is scarce and equivocal. Instead, it is recommended to consume a diverse diet rich in dietary antioxidants from fruit and vegetable sources (56). Given the nuanced and complex interactions of the redox landscape, it is not surprising that antioxidant supplementation fails to extend longevity and is equivocal at best in preventing age-related dysfunction. Further research is needed to clarify the underlying molecular mechanisms but it is clear altered redox homeostasis is a key feature directly related to the various tenets of aging.

### 1.2.2. *Sarcopenia/Dynapenia*

Sarcopenia is an age induced loss of skeletal muscle mass (quantity) (62). Dynapenia, in contrast, is an age induced loss of skeletal muscle force producing capacity (quality) (65). Sarcopenia contributes to dynapenia and up until recently both phenomena were collectively referred to as sarcopenia (63). Regardless, losses in both muscle quantity and muscle quality directly contribute to reduced quality of life and increased risk of mortality (60, 61). Unlike the general loss of systemic function with aging, it is known that the production of reactive species precedes and directly alters several mechanisms that influence skeletal muscle quantity and quality (66).

Quantity of muscle mass is contingent on the balance of protein breakdown to protein synthesis (71). Protein synthesis is induced by various growth hormones and resistance exercise. Both of which activate the two major positive regulators of protein synthesis IGF1 and the Akt-mTOR pathway (67). Increases in reactive species can interfere with the activation of Akt-mTOR and directly inhibit protein synthesis (83, 84). Paradoxically, depending on the site and species of oxidant, reactive species may also be important in the induction of mTOR and protein synthesis (71). In contrast, the breakdown of skeletal muscle protein is regulated by various proteolytic systems, but mainly the ubiquitin-proteasomal system (UPS) and autophagy-lysosomal system. H<sub>2</sub>O<sub>2</sub> increases the expression of the catabolic family of transcription factors FOXO (71, 72) subsequently increasing expression of E3 enzymes such as Murf-1 which are necessary for protein breakdown by the UPS. The increased expression of FOXO also regulates the autophagy-lysosomal system (67, 70). Using a transgenic mouse model, researchers demonstrated that a mutant SOD that aberrantly increases H<sub>2</sub>O<sub>2</sub> levels resulted in significant skeletal muscle breakdown via the autophagy system and manifestation of a sarcopenia like phenotype (68). Similarly to H<sub>2</sub>O<sub>2</sub>, increased production of NO<sup>-</sup> from free cytosolic nNOS results in an upregulation of oxidative damage and increase in muscle loss (69).

Quality of muscle mass, or the force producing capacity per volumetric unit of muscle tissue (73) can be influenced by various factors independent of muscle size. Reactive species directly impact the force producing capacity of skeletal muscle by interacting with activation of the neuromuscular junction (NMJ), Ca<sup>2+</sup> release from ryanodine receptors (RyR), and myosin cross-bridge formation (66). Increased reactive species production in older mice decreases neurotransmitter release at the NMJ but also alters the excitability of skeletal muscle (74). Age related loss of redox homeostasis is also known to negatively alter the morphology of motor neurons and the NMJ (75). Age-induced accumulation of skeletal muscle oxidants can modify and oxidize RyR and associated stabilizing proteins leading to Ca<sup>2+</sup> leakage from the sarcoplasmic reticulum and a decrease in force production (76, 77). In this instance, antioxidant treatment with dithiothreitol (DTT) was able to reverse oxidation of RyR receptors and restore Ca<sup>2+</sup> homeostasis

(76). Findings that myofibrillar proteins are redox sensitive suggest that redox modifications of actin-myosin interactions may also impact muscle quality (79, 80). Though it appears that accumulation of myofibrillar oxidation products does not seem to be a major mechanism (79, 81, 82); oxidative modifications to nucleophilic cysteine residues on myosin inhibit efficient cross bridge formation and reduce force producing capacity (66).

In summary, the above evidence indicates a causal role for altered redox homeostasis in the pathogenesis of sarcopenia and dynapenia. Redox induced dysfunction in the mechanisms that control both skeletal muscle quantity and quality are relevant in both general health and sports performance. The monitoring of redox biomarkers (discussed in depth later) may assist in the identification/prevention of sarcopenia and optimization of skeletal muscle function for athletic performance.

#### *1.2.3. Neurodegeneration*

Loss of redox homeostasis plays a critical role in the pathophysiology of dementia (a generalized decline and loss of cognitive function) and specific neurodegenerative diseases (i.e. Alzheimer's, Parkinson's, Huntington's) (66, 85, 86). Mechanistically, various reasons contribute to the brain's increased susceptibility to oxidative damage (87). Some of these include neurotransmitter metabolism (56), a high demand for oxygen (568), and a comparatively low antioxidant defense relative to other tissues (569). Higher levels of lipid peroxidation and protein oxidation are associated with more significant cognitive decline in the elderly (88). Patients diagnosed with Alzheimer's show greater DNA (95), protein (92), and lipid (93, 94) oxidation than age-matched controls. Alzheimer's patients also have greater reduction in non-enzymatic antioxidants Vit E and Vit C compared to age-matched control which may further contribute to disease progression (89, 97). Ultimately, the increased oxidation of biomolecules in the neurodegenerative brain impacts critical cellular processes such as signal transduction and may induce cell death (96). In addition to reduced non-enzymatic antioxidant expression, increased oxidative damage is linked to a loss of Nrf2 function and decreased enzymatic antioxidant activity. As such, recent therapeutic approaches have been aimed at upregulating Nrf2 activation to treat both Alzheimer's and Parkinson's (100). Although the increased damage to macromolecules is a critical feature of neurodegeneration and Alzheimer's, it may not be universally observed (98). It is known that increased oxidative damage is one of the first events in the pathogenesis of Alzheimer's but may not persist into the latter stages of disease progression as patients with more advanced Alzheimer's present with lower oxidative damage than their early stage counterparts (99). Though further research is needed, this evidence suggests that a loss of redox homeostasis and increase in oxidative damage precedes significant deterioration and may play a causal role in development and progression of Alzheimer's disease (96). As a result some authors suggested the use of antioxidants in the prevention of neurodegeneration (90). However, evidence does not support the use of exogenous antioxidant supplements in the prevention of neurodegeneration or in the treatment of specific neurodegenerative pathologies (91, 96). Paralleling other redox related dysfunctions dietary consumption of antioxidants through a diet rich in fruits and vegetables is recommended to attenuate cognitive decline (96, 101, 102).

#### *1.2.4. (Cardio)Vascular Disease*

Loss of redox homeostasis contributes to vascular endothelial dysfunction, atherosclerosis, and cardiovascular disease (CVD) (62). The reactive species NO<sup>-</sup> is a critical modulator of vascular function (103), however the age-dependent increase in O<sub>2</sub><sup>-</sup> results in a loss of NO<sup>-</sup> homeostasis via the reaction between O<sub>2</sub><sup>-</sup> and NO<sup>-</sup>. The product of O<sub>2</sub><sup>-</sup> and NO<sup>-</sup> is the highly reactive peroxynitrite (ONOO<sup>-</sup>) radical

which can cause significant damage to DNA, lipids, and proteins resulting in oxidative modifications that disrupt vascular function (104). Increased reactive species production resulting in vascular remodeling and endothelial dysfunction are early events in the development of CVD (109). In fact, it is suggested that increased oxidation of low density lipoprotein (LDL) cholesterol by ONOO<sup>-</sup> plays a causal role in the development of atherosclerosis and subsequent CVD (62, 105). As such, oxidized LDL is an established and highly functional prognostic indicator of CVD. Other systemic biomarkers of lipid peroxidation such as plasma malondialdehyde, urine F2-isoprostanes, and myeloperoxidase are also elevated with increasing atherosclerotic and CVD progression (112). The increased oxidation of LDL cholesterol coupled with a decrease in endogenous antioxidant defenses (Gpx and SOD) in cardiac muscle with aging significantly increases the risk of CVD (110). As is the case with other pathologies, the use of exogenous antioxidant supplements currently does not have sufficient evidence to support their usage in the prevention of vascular dysfunction (56). Instead, increased consumption of dietary antioxidants through a balanced diet rich in fruits, vegetables, and whole grains is recommended by the American Heart Association to maintain vascular function and attenuate or prevent the development of atherosclerosis and CVD (111). Recent research has targeted the Nrf2 pathway in attempts to upregulate endogenous antioxidant defenses within the vasculature and prevent atherosclerosis and CVD (113). It is well known that Nrf2 exerts diverse protective effects against oxidative damage yet its exact role in maintaining redox homeostasis within the vasculature is unclear. Various animal models indicate Nrf2 may display antagonistic pleiotropy within the vasculature as both Nrf2 knockout (115) and Nrf2 overexpression (114) appear to prevent atherosclerotic progression. As such, the cardiovascular specific role of Nrf2 warrants further investigation.

#### 1.2.5. *Cancer*

There is a significant age-dependent increase in cancer risk and increases in oxidative damage and accumulation of oxidation products is a frequently cited explanation to this phenomenon. Indeed, significant research has demonstrated a causal link between loss of redox and inflammatory homeostasis and cancer (62, 118). The carcinogenic potential of increased reactive species results from their interaction with DNA and damage to nucleotide base pairs (123). Oxidative modifications to DNA can induce transcriptional errors and genomic instability, both of which contribute to carcinogenesis. Accordingly, increased levels of 8-oxo-g and 8-nitroguanine, two markers of DNA oxidation by oxygen and nitrogen reactive species respectively, are considered biomarkers of carcinogenesis (118). The oxidation of DNA recruits inflammatory cytokines, and though an important process of cellular repair, inflammatory cytokines can increase reactive species production and exacerbate oxidative damage, creating a vicious cycle that leads to a state of chronic inflammation and oxidative distress (62). Both inflammatory cytokines and reactive species activate Nf-Kb which increases the expression of genes necessary for carcinogenesis (119). In addition, excess oxidant production can increase the resistance of cancer cells to treatment and upregulate the expression of transcription factors necessary to induce angiogenesis within cancer cells (62, 122). High levels of reactive species are established initiators of hepatocellular carcinoma (121) and colorectal cancer (120).

It stands to reason that moderating oxidant-induced DNA damage may attenuate losses in redox and inflammatory homeostasis and subsequent carcinogenesis. Though some trials have found specific antioxidants effective in reducing risk of some cancer types (124, 125), this effect is mediated by various factors including age, gender, baseline antioxidant status, type of cancer, and cancer progression (56). As such it is not recommended that individuals supplement with exogenous antioxidants to reduce cancer risk

unless they have an identified dietary deficiency they are attempting to reverse (126, 127). Not long ago, Nrf2 was a highly attractive therapeutic target in cancer research as increases in endogenous antioxidant expression were postulated to attenuate aberrant oxidative distress and carcinogenesis (128, 129). Indeed, the protective role of Nrf2 in chemical or radiation induced carcinogenesis is well established and controlled intermittent activation of Nrf2 is chemopreventive (130). However, under instances of prolonged activation, Nrf2 may promote inflammation, proliferation, and survival of cancer cells (131, 132, 133). It is now clear that, much like reactive species themselves, Nrf2 is a highly pleiotropic transcription factor that may induce both tumor-suppressive and tumor-promoting effects depending on the context (130).

The above list is not an exhaustive review of redox-related pathologies or a comprehensive review of the many other postulated mechanisms that may underpin these conditions. Rather it is evidence to demonstrate the complex redox landscape at multiple organizational levels from cellular to organism and the consequences of redox dysfunction as they relate to human health. It is also meant to convey the complexity of redox homeostasis and how reductionist approaches to address redox dysfunction by exogenous antioxidant supplementation have been largely unsuccessful. As it stands, even increasing endogenous antioxidant production via Nrf2 manipulation does not adequately address redox dysfunction given the antagonistic pleiotropic nature of reactive species and antioxidants.

### *1.3. Performance Consequences of Altered Redox Homeostasis*

The role of reactive species within exercise physiology and performance has been an area of interest since seminal findings showed skeletal muscle contraction produced reactive species that resulted in oxidative damage (134) and an increase in reactive species was associated with muscle fatigue (135, 136). Paralleling the prevailing sentiment within health, it was initially believed that the production of reactive species was entirely deleterious for exercise performance. The production of reactive species and damage to macromolecules does significantly contribute to fatigue (137), muscle soreness (139), and strength loss after exercise (138); however, they are also critical cell signaling molecules that induce many of the beneficial and quintessential adaptations to exercise (24). The typical research approach within exercise redox biology has been to investigate performance or physiological outcomes following antioxidant supplementation. If the antioxidant supplement influences the performance/physiological outcome, then the outcome is deemed redox regulated. Though not entirely inappropriate, this approach often neglects the pleiotropic effects of various compounds which may contribute to the largely equivocal findings regarding antioxidant supplements to improve exercise performance (164, 165).

#### *1.3.1. Fatigue*

Muscle fatigue can be broadly defined as the inability of muscles to produce or maintain a certain force or power (140). Complex molecular mechanisms underpin muscle fatigue with both central (motor neuron drive) and peripheral (hydrogen ion accumulation) mechanisms involved. The first indications of redox regulated mechanisms of muscle fatigue came from animal studies that successfully used the antioxidant N-acetylcysteine (NAC) to attenuate reductions in diaphragm force production (141). Though not a limb muscle, the diaphragm is a skeletal muscle and contains the same internal physiology as limb muscles (144). During a standardized protocol of electrically stimulated fatiguing contractions, NAC pretreatment attenuated fatigue by about 25%. Subsequent analyses on skinned muscle fibers from rodents corroborated these findings and showed that NAC attenuates force reductions (142). NAC was also able

to increase time to exhaustion and contractile force of the diaphragm *in vivo* in healthy humans undergoing fatiguing contractions via inspiratory resistive loading (145). The role of oxidants in muscle fatigue was assessed in human limb muscle for the first time via isometric handgrip exercise and was in agreement with previous findings as NAC supplementation increased time to task failure by an average of 30% (146). The first double blind crossover studies on whole body exercise performance (cycling) to use NAC to attenuate muscle fatigue were also in agreement with animal and human studies (147, 148). The findings indicated that intravenous administration of NAC increased time to fatigue by about 26% in endurance trained individuals who performed cycling to exhaustion at 92% VO<sub>2</sub> max. A growing number of reports support that administration of NAC can attenuate muscle fatigue during submaximal exercise (149, 150). However, this effect is not observed for all antioxidant supplements (151, 152, 153) nor does NAC appear to be effective at attenuating fatigue during maximal intensity exercise (146). Regardless, the results from multiple performance studies indicate a causal role for increased oxidant production in muscle fatigue. Mechanistic studies using skinned muscle fibers confirm the role of reactive species in contributing to muscle fatigue and, interestingly, elucidated a more nuanced and complex relationship between skeletal muscle oxidant production and fatigue.

A series of studies exposed intact single mouse muscle fibers to the oxidant H<sub>2</sub>O<sub>2</sub> and demonstrated that prolonged exposure or high concentrations of H<sub>2</sub>O<sub>2</sub> result in decreased force production and fatigue (154). However, the force decline induced by H<sub>2</sub>O<sub>2</sub> was completely reversed by subsequent treatment with the antioxidant DTT. Mechanistically these findings support that antioxidant treatment can attenuate muscle fatigue in the presence of high oxidant production (i.e. prolonged or intense exercise). Surprisingly, brief treatment of muscle fibers with low levels of H<sub>2</sub>O<sub>2</sub> increased submaximal force production by 27% in the same contractions, indicating an oxidant induced facilitation of force production (154). In contrast, when muscle fibers were treated with DTT in the absence of H<sub>2</sub>O<sub>2</sub>, force production dropped by 40%. The results indicate that low-level oxidant production is critical for maximal force production but prolonged exposure to oxidant production reduces force production and contributes to muscle fatigue. Similarly, exposure to reductants (antioxidants) when oxidant production is high may restore muscle function and prevent fatigue, however, exposure to antioxidants in unfatigued muscles may reduce force production (154, 157, 158). As previously discussed, the redox status of skeletal muscle influences various elements of the contractile machinery. Myofibrillar Ca<sup>2+</sup> sensitivity is especially sensitive to intracellular H<sub>2</sub>O<sub>2</sub> concentrations. Force increases at low concentrations of H<sub>2</sub>O<sub>2</sub> are due to increased Ca<sup>2+</sup> sensitivity while fatigue and force reductions following increased exposure to H<sub>2</sub>O<sub>2</sub> are due to decreased Ca<sup>2+</sup> sensitivity (154). Beyond H<sub>2</sub>O<sub>2</sub>, it is known that increased O<sub>2</sub><sup>-</sup> levels can also induce fatigue and decrease force production by a different mechanism, that is, modifying SR function and altering Ca<sup>2+</sup> release (159). Endogenous production of NO<sup>-</sup> can also depress submaximal skeletal muscle force production (150, 160), however, it does not appear that NO<sup>-</sup> impacts maximal force production (154). It appears that NO<sup>-</sup> influences contractile function and may increase fatigue by decreasing myofibrillar Ca<sup>2+</sup> sensitivity, though this effect appears to be exclusive to fast twitch muscle fibers (161). Furthermore, ONOO<sup>-</sup> induced modifications of the RyR receptor influence Ca<sup>2+</sup> release and also contribute to fatigue (162, 163). It has also been suggested that oxidants may modulate fatigue by indirect modification of kinases and phosphatases that subsequently depress myofibrillar function, though it is unclear whether direct or indirect mechanisms predominate in these redox-regulated mechanisms of fatigue (144).

Collectively, the mechanistic insights from various research groups show that reactive species directly contribute to fatigue via direct modification of myofibrillar function (150). However, it is also

clear that basal levels of reactive species facilitate optimal force production (157) and an optimal redox balance exists to mitigate fatigue and optimize skeletal muscle function. This paradoxical duality mirrors the antagonistic pleiotropic functions observed in disease contexts and likely contributes to the equivocal findings regarding antioxidant interventions in exercise performance.

### 1.3.2. Impaired Training Adaptations

Exercise training is a powerful stimulus for physiological remodeling. The nature of the physiological remodeling will be contingent on the intensity, volume, frequency, and modality of the exercise stimulus. In a performance context, these exercise-induced training adaptations induce a shift in skeletal muscle phenotype that is critical for improving sports performance. Redox mechanisms regulate many of these beneficial exercise adaptations (24). It is clear that, even if antioxidant supplementation is effective in reducing transient fatigue and improving acute performance, supplementation may inhibit the activation of critical cellular signaling pathways necessary for exercise adaptations (166). Various elements of redox homeostasis including the source of oxidant production, the type of reactive species, the signaling properties (reactivity) of the reactive species, and the reversibility of oxidized molecules all contribute to redox regulated exercise adaptations (167).

Uequivocal evidence shows that regular endurance exercise upregulates mitochondrial size, number, and volume (168). This increase in mitochondrial content is coupled with increased mitochondrial enzymatic activity and improved ATP-producing efficiency thereby enhancing oxidative metabolism (168). As such, mitochondrial biogenesis is an invaluable adaptation for endurance performance. Canonically, all upstream targets and transcription factors involved in the induction of mitochondrial biogenesis converge on peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1a), the master regulator of mitochondrial biogenesis (168). PGC-1a is regulated by H<sub>2</sub>O<sub>2</sub> and various other activation factors. An acute bout of endurance exercise increases H<sub>2</sub>O<sub>2</sub>, PGC-1a expression, and other proteins involved in mitochondrial biogenesis (168). PGC-1a is also induced by various classical cytosolic signaling pathways including Ca<sup>2+</sup>/ calmodulin-dependent protein kinases (CaMKs), 5'-AMP-activated protein kinase (AMPK), p38 MAPK, and ERK (173). Unfortunately, the primary redox-sensitive mechanisms that regulate these classical signaling pathways are not fully understood (175). However, there is evidence that H<sub>2</sub>O<sub>2</sub> can activate AMPK by oxidative modifications to thiolate groups on its α and β subunits. These oxidative modifications cause conformational changes in the AMPK protein towards its active state, indicating a redox-regulated energy independent activation of upstream targets of mitochondrial biogenesis (177, 178). It was also demonstrated that both O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> formed within the mitochondria can activate p38 MAPK (179). Initial evidence that antioxidant supplementation impaired markers of mitochondrial biogenesis came from Ristow et al., (170) who demonstrated that an antioxidant cocktail of Vitamin E and Vitamin C impaired exercise induced improvements in insulin sensitivity which were coupled with a reduction in PGC-1a signaling following antioxidant supplementation. These findings were later confirmed by Gomez-Cabrera et al., (169) which found vitamin C supplementation in humans attenuated exercise-induced increases in PGC-1a expression and other markers of mitochondrial biogenesis. Similarly, Paulsen et al. (174) found that exercise induced increases in PGC-1a protein expression were blocked by a vitamin C and vitamin E cocktail in humans. Antioxidant treatment in mice also suppressed acute exercise signaling and longitudinal training markers of mitochondrial biogenesis (171). Interestingly, the reverse experimental approach which depleted endogenous glutathione pools (GSH) resulted in an exercise induced increase in PGC-1a mRNA expression (181) indicating the pre-exercise redox status dictates the magnitude of adaptive signaling. A

different experimental approach used NADPH oxidase-2 (NOX-2) deficient mice and found that NOX-2 derived reactive species are required for mitochondrial biogenesis and optimal exercise performance (176). Similarly, Wadley et al. (172) reported that the xanthine oxidase (XO) specific antioxidant allopurinol inhibits acute exercise-induced increases in p38 MAPK and ERK indicating that XO derived oxidants also play a crucial role in upstream activation of mitochondrial biogenesis. Interestingly, in this study, decreased activation of p38 MAPK and ERK did not impact PGC-1a activity, suggesting inhibition of upstream activators of mitochondrial biogenesis without subsequent decreases in mitochondrial protein content. Indeed, despite the strong evidence that antioxidant supplementation inhibits acute mitochondrial biogenesis signaling, it is still unclear to what extent this impairs exercise-induced increases in mitochondrial protein content (173, 175). This is likely a result of redundant mitochondrial biogenesis signaling pathways which allows for coordinated longitudinal training adaptations in spite of decreased reactive species production (173). As such, it is unclear if longitudinal antioxidant supplementation suppresses exercise induced mitochondrial biogenesis. Regardless, it is clear that reactive species have a regulatory role in mitochondrial biogenesis signaling. In a recent study, a novel methodological strategy showed for the first time, in the absence of antioxidant supplementation, that exercise induced aerobic adaptations ( $\text{VO}_2$  max, wingate, time trial performance) depend in part on the magnitude of redox response (230).

In contrast to endurance training, the archetypal adaptation conferred by resistance training is skeletal muscle hypertrophy. As previously mentioned, skeletal muscle hypertrophy is the result of a net positive protein synthesis:degradation ratio. Diverse physiological pathways including extracellular regulated kinase 1/2 (ERK1/2), IGF-1-PI3K, Akt-mTOR and its downstream initiator ribosomal protein S6 kinase (p70s6k)/eukaryotic translation initiation factor 4E (eIF4E), regulate protein synthesis in response to mechanical loading and homeostatic insults following resistance training (24). Both endogenous and exogenous  $\text{H}_2\text{O}_2$  production can activate IGF-1 leading to initiation of mTOR and subsequent activation of p70s6k and eIF4E (186). Treatment with both NAC and specific targeted antioxidants (SOD mimetics) abolished the  $\text{H}_2\text{O}_2$  mediated activation of IGF-1 and myocyte hypertrophy. NOX-4 knockout also attenuates IGF-1 signaling and subsequent mTOR activation indicating NOX-4 derived reactive species are involved in myocyte hypertrophy (186). Interestingly, a skeletal muscle specific SOD-knockout mouse model found that mice lacking SOD demonstrated greater muscle hypertrophy than their wild-type counterparts but decreased contractile force indicating a unique redox crosstalk influence on both hypertrophy and contractile function (186). Using various knockout and targeted antioxidants, Ito et al., (188) showed that production of  $\text{NO}^\cdot$  from nNOS plays a critical role in synergist ablation induced muscle hypertrophy via production of  $\text{ONOO}^\cdot$  (188). The proposed mechanism included  $\text{ONOO}^\cdot$  mediated activation of transient receptor potential cation channel subfamily V member 1 (TRPV1) leading to increased cytosolic  $\text{Ca}^{2+}$  and consequent activation of mTOR. Other synergist ablation models using traditional antioxidant treatment showed vitamin C supplementation reduced hypertrophy induced by mechanical overload and concomitantly reduced positive regulators of muscle protein synthesis ERK1/2 and p70s6k (182). In humans, combined vitamin C and vitamin E do not influence lean body mass or physiological cross sectional area but again reduce ERK1/2 and p70S6k signaling (183). This mirrors the influence of antioxidant supplementation on mitochondrial biogenesis. Supplementation appears to reduce redox sensitive adaptive signaling but may not necessarily equate to a change in protein content due to redundant adaptive mechanisms. However, Dutra et al., (184) did find reduced strength improvements following 10 weeks of strength training in subjects receiving a vitamin C/vitamin E cocktail despite no change in hypertrophy indicating the potential for antioxidant

supplementation to negatively influence strength performance outcomes. A recent meta-analysis on the effect of combined vitamin C and vitamin E supplementation on muscle mass and strength concluded that supplementation does not impact strength gains but may negatively impact muscle hypertrophy when performing regular resistance exercise (187).

### 1.3.3. Exercise Recovery

Various lines of evidence indicate a redox regulation of recovery of muscle function after exercise. Markers of oxidative damage increase significantly following unaccustomed exercise (203, 204, 205). It is believed that increased reactive species production helps clear debris from damaged tissue and simultaneously function as inflammatory signaling molecules which assist in the repair and regeneration of muscle fibers following unaccustomed exercise (206). A recent study demonstrated that the loss of muscle function following unaccustomed exercise is more closely related to oxidative damage biomarkers than to traditional muscle damage biomarkers creatine kinase (207). The findings indicate redox biomarkers may be an appropriate indicator of recovery of muscle function. Similar indicators of a redox regulation of muscle recovery were demonstrated by two studies supplementing with NAC. In one study subjects received NAC for 8 days following a bout of unaccustomed muscle damaging exercise (212). NAC attenuated loss of muscle function, inflammatory cytokine, and Nf-kb phosphorylation in the first two days following the unaccustomed exercise protocol. However, NAC also blunted increases in mTOR, p70s6k, and eIF4E in the recovery from exercise. In addition, only the placebo group fully recovered muscle function after 8 days indicating a redox-regulated recovery of muscle function and muscle adaptations (212). Following the same protocol, NAC was ingested for 8 consecutive days immediately following completion of an unaccustomed muscle-damaging exercise protocol (208). NAC supplementation was able to reduce acute losses (2-3 days) in muscle function but simultaneously prevented full recovery of muscle function to baseline levels following 8 days. In contrast, the non-supplementation group displayed greater acute losses in strength but superior recovery of strength and eventual supercompensation resulting in greater strength following 8 days (208). The reduced recovery in the NAC group was coupled with reduced immune cell mobilization supporting a redox regulation of immune function following exercise (210). This is noteworthy as immune cell mobilization following exercise is a known prerequisite for tissue healing following damaging exercise (210, 211, 213). Studies investigating antioxidants and polyphenols provide additional evidence of a redox regulation of muscle recovery. Polyphenols are a group of phytochemicals abundant in fruits and vegetables that have received extensive attention in the literature for their redox actions and potential to enhance muscle recovery (197). The use of tart montmorency cherries appears to accelerate recovery following both heavy resistance training in trained (199) and untrained subjects (198). Tart montmorency cherries also facilitate recovery after high intensity interval sprint training (200) and long distance running (201). Most frequently, the benefits of tart montmorency cherry supplementation have been attributed to reductions in post-exercise fatigue, distress and inflammation. However, more mechanistic studies that investigate this action *in vivo* are necessary (197). 8 weeks of curcumin supplementation was able to attenuate loss of muscle function and decrease soreness after a bout of muscle damaging (downhill running) exercise (196). Curcumin is frequently cited as an antioxidant with potential to reduce DOMS and decrease markers of muscle damage (164). However, due to the pleiotropic nature of polyphenols, it is not definitive that these compounds function as antioxidants *in vivo* following absorption within the GI tract (197). Many polyphenols demonstrate both pro-oxidant and antioxidant actions *in vitro*. The traditional belief was that polyphenols functioned by scavenging reactive species thereby attenuating

muscle damage and facilitating recovery. However, the contemporary view of polyphenols is that most, if not all, actually function as pro-oxidants, upregulating endogenous antioxidant defenses via interactions with Nrf2 (197). Ultimately, the mechanisms underpinning how certain polyphenols facilitate recovery from exercise are still unclear. Regardless, it is now clear that the production of reactive species is necessary to facilitate post-exercise increases in inflammation and immune cell mobilization. Attenuation of this process, though temporarily beneficial at reducing strength losses, negatively impacts full recovery of muscle function and adaptation (208, 212). Taken collectively, it appears that variable redox statuses may be beneficial in different recovery contexts. If rapid recovery is desired due to an impending need for superior acute performance (i.e. competition), decreased oxidant via antioxidant supplementation may restore muscle function more rapidly; however if adaptation is desired then reduced oxidant production may be detrimental.

#### *1.3.4. Substrate Metabolism*

Though it has long been established that redox signaling and reactive species interact with cellular metabolism, studies that specifically investigate exercise redox bioenergetics are scarce. Elevated levels of H<sub>2</sub>O<sub>2</sub>, ONOO<sup>-</sup>, and lipid peroxidation products can impair glycolysis by reacting with active site thiols on the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GADPH) (189). Mitochondrial produced oxidants can also impact two critical enzymes within the TCA cycle: aconitase and  $\alpha$ -ketoglutarate dehydrogenase ( $\alpha$ -KGDH). Aconitase catalyzes the conversion of citrate to isocitrate and contains a highly O<sub>2</sub><sup>-</sup> sensitive prosthetic group, indicating a potential redox control of the Krebs cycle. O<sub>2</sub><sup>-</sup> interactions with this prosthetic group can lead to diminished aconitase activity and decreased mitochondrial metabolism (189). Redox proteomic analyses have also found additional post-translational modifications of aconitase by H<sub>2</sub>O<sub>2</sub>, NO<sup>-</sup>, and glutathione (191, 192).  $\alpha$ -KGDH catalyzes the conversion of  $\alpha$ -ketoglutarate to succinyl-CoA and is a major producer and target of reactive species, specifically  $\alpha$ -KGDH is sensitive to inhibition by ONOO<sup>-</sup> and H<sub>2</sub>O<sub>2</sub> (190). Inhibition of  $\alpha$ -KGDH will result in decreased NADH availability to the respiratory chain and impair ATP production. This mechanism is highly relevant to neurodegenerative pathologies but has not been explored in an exercise context (190). Increased mitochondrial reactive species production may also react with fatty acids to create nitro-fatty acids that reduce the efficiency of beta-oxidation (190). The role of nitro-fatty acids from an exercise metabolism standpoint has yet to be explored. Performance related changes due to changes in substrate metabolism are well documented (570) yet the role antioxidant supplementation may have in altering substrate metabolism are unclear and likely depend on multiple variables (166). Chronic supplementation with astaxanthin decreases respiratory exchange ratio (RER), increases fat oxidation, and spares muscle glycogen in rodents (193) but does not appear to influence performance or fat oxidation in humans (194). Other antioxidant supplements, namely epigallocatechin gallate (ECGC), may be effective at increasing resting energy expenditure and decreasing RER (195), but its role on modifying substrate metabolism during exercise is equivocal (166). Reactive species are becoming increasingly recognized as an integral part of cellular metabolism as redox regulated pathways assist in the regulation of key metabolic pathways (24, 166). As such further explorations into the role of exercise redox bioenergetics and antioxidant supplementation in substrate metabolism on acute exercise and longitudinal training adaptations are warranted.

#### *1.3.5. Overtraining*

Overtraining (OTS) is a rather enigmatic pathology characterized by persistent underperformance, mood changes, lack of motivation to train, and a prolonged recovery period lasting months or years (214). Research investigating overtraining is understandably difficult to pursue given the ethical constraints with inducing overtraining in human subjects. Furthermore, it is virtually impossible for early diagnosis of OTS given that all other possible endocrinological or psychological disorders must first be ruled out (214). Though various hypotheses exist to explain OTS, a recent review highlighted how intramuscular redox mechanisms are the most likely cause of the prototypical skeletal muscle symptom of OTS, prolonged decreased force producing capacity (215). As previously discussed, transient alterations in redox homeostasis reduces skeletal muscle contractile function via a shift in the “optimal redox” balance. Chronically elevated oxidants resulting from prolonged improper exercise training and psychosocial stressors supersedes endogenous antioxidant defenses resulting in a shift towards a “pro-oxidant” status resulting in a homeostatic adaptive shift towards a deleterious pro-oxidant status. This new “pro-oxidant” status perpetuates skeletal muscle dysfunction via previously described mechanisms: oxidant induced modifications of RyR, altering myosin-cross bridge formation, and inhibiting EC coupling (215). Without adequate removal or modification of the oxidative stimulus, the system is unable to revert back to an optimal homeostatic state. The same physiological mechanisms that induce positive exercise adaptations under normal conditions (i.e. increase in reactive species) hinder adaptation during chronic oxidative distress in OTS given the abnormally high basal levels of oxidative distress. Additional evidence to support this hypothesis comes from Nrf2 levels in diseased and healthy populations following exercise (216). In response to an exercise stimulus, reactive species increase Nrf2 signaling in the mouse heart (218), brain (219), and lung (220), and human skeletal muscle (221), and peripheral blood mononuclear cells (PBMCs) (222). The increase in Nrf2 signaling is an oxidative eustress that results in improved endogenous antioxidant expression and protection against subsequent oxidative insults indicating that Nrf2 mediates cytoprotective responses following exercise. However, individuals with higher resting oxidative distress have a decreased acute Nrf2 response to a single exercise session (222) indicating reduced capacity for a eustress response. This is coupled with higher basal Nrf2 levels under conditions of chronic oxidative distress. Higher basal Nrf2 and lower acute exercise induced Nrf2 in chronic oxidative distress indicates a ceiling effect of Nrf2 inducibility (216). In contrast, younger or healthy individuals show lower basal Nrf2 and higher increases in Nrf2 in response to an acute exercise session (222) supporting increased capacity for oxidative eustress. Taken collectively it appears that basal Nrf2 levels mirror the levels of resting oxidative distress (216). Though the role of Nrf2 has not been investigated within OTS, the evidence suggests that following prolonged intense exercise loading with insufficient recovery, coupled with non-exercise psychosocial stressors, a state of increased resting oxidative distress is induced, resulting in increased basal Nrf2 levels. Though initially adaptive, without removal of the oxidative stimulus Nrf2 can not maintain adequate cytoprotective responses due to the ceiling effect leading to the increased oxidative damage observed in overtrained athletes (223, 224, 225). Within skeletal muscle oxidative distress damages structural proteins resulting in contractile dysfunction, the major peripheral symptom of OTS. Dysregulated redox state also explains the primary central symptom of OTS, dysregulated mood state. A wide range of psychoneurological symptoms have been reported with OTS including anxiety, agitation, lack of focus, depression, loss of motivation, and restlessness (226). Studies consistently show that redox dysregulation is a consistent feature across a wide range of neuropsychiatric conditions including schizophrenia (227), anxiety (228), OCD (229), and depression (228). Though the underlying mechanisms and pathogenesis of OTS are largely speculative, it is clear that athletes experiencing OTS demonstrate increased oxidative damage and altered redox homeostasis can

explain the prototypical symptoms of OTS. As such, it may be beneficial to monitor redox status in the progression of OTS.

To summarize, the performance consequences of altered redox homeostasis are profound and systemic. The monitoring of redox status to mitigate redox dysfunction has practical applications for athletes attempting to optimize performance, minimize fatigue, maximize training adaptations, recover from exercise, and monitor the pathogenesis of OTS. In addition, given redox regulation of training adaptations, monitoring of exercise redox status within the general population may be an effective strategy to assess if individuals will be “non-responders” to exercise (5). Considering the diverse redox heterogeneity observed between individuals and that basal redox status influences redox responses (231), further investigations into exercise redox biology may characterize redox signatures indicative of performance and physiological outcomes that can be used in the development of personalized exercise prescriptions to maximize health and performance.

## **2. Appropriate Exercise in the Maintenance of Redox Homeostasis and Prevention of Redox Dysfunction: Exercise Redox Biology**

The efficacy of regular exercise to improve health, functionality, and longevity is unequivocal. The Academy of Medical Royal Colleges went as far to describe 30 minutes of moderate intensity aerobic exercise five times a week as a “miracle cure,” more effective than many pharmaceutical drugs given to treat non-communicable disease (NCD) (238). There is robust evidence from clinical trials and observational studies that exercise reduces risk of cardiovascular disease (292), cancer (314), diabetes (315), and all-cause mortality (300). Regular exercise training enhances neurogenesis and combats age-induced losses of brain function and dementia (219, 291). Similarly, the use of structured exercise training to improve physical performance for sport is not only ambitious, but also invaluable for success (316). Despite the unequivocal benefits of regular exercise for health and performance, the molecular mechanisms that underpin disease preventing and performance enhancing adaptations to exercise are still poorly understood. Unraveling the molecular mechanisms that underpin the health benefits of exercise is the purpose of the recently founded Molecular Transducer of Physical Activity Consortium (MoTrPAC) initiative supported by the National Institute of Health (NIH) Common Fund (317). Similarly, a few years prior to the induction of the MoTrPAC Initiative, the Athlome Consortium was started with the goal of unraveling the molecular mechanisms that underpin elite athletic performance (318). Given the widespread pleiotropic functions of redox molecules, increased interest has been placed on redox mechanisms that may potentially explain the health and performance benefits of exercise (24, 246).

Alterations in redox homeostasis are an established consequence of transient exercise (137). Unequivocal evidence demonstrates that exercise increases the production of reactive species (290, 302, 303) and increases measures of oxidative damage in various tissues including erythrocytes (304, 305), plasma (306, 307, 308), and skeletal muscle (309, 310). These observations are somewhat enigmatic given the diverse negative consequences of altered redox homeostasis and yet unequivocally beneficial effects of regular exercise. It is now clear that exercise functions as a powerful oxidative *eustress*, upregulating systemic endogenous antioxidant defenses to protect against subsequent oxidative insults, and downregulating resting levels of oxidative damage, and protecting against oxidative *distress* (247). Though still a field in its infancy, exercise redox biology is growing rapidly and exercise-derived redox

molecules are increasingly becoming recognized as critical modulators of gene transcription, cell signaling, and protein abundance and function (248).

### *2.1. Hormesis and Exercise Induced Oxidative Eustress for Health*

As previously discussed, the initial belief was that an increase in reactive species and oxidative damage was exclusively harmful and contributed to performance decrements, such as fatigue and a state of OTS. Furthermore, increased oxidative damage is causally linked to tumorigenesis, cancer, CVD, Alzheimers, and Parkinson's (45). Reconciling the evidence that clearly showed exercise increased reactive species and oxidative damage yet simultaneously improved health status and prevented disease required an improved theoretical framework (246). The concept of hormesis, which was adopted from toxicology, and applied to exercise helped explain the enigmatic and divergent roles of reactive species following acute exercise (290). Hormesis can be graphically displayed by an inverted parabola and is firstly characterized by a bi-phasic response in which a transient low level of a stressor induces a beneficial adaptive response whereas chronic or excessive exposure to a stressor induces cytotoxicity (288, 289) (**Insert Figure Here**). This concept parallels the antagonistic pleiotropic function of reactive species, on one hand critical for optimal physiological function, performance, and health adaptations, yet simultaneously contributors to muscle fatigue, aging, and various pathologies. Whether exercise derived oxidants are ultimately damaging or beneficial to health then depends on the magnitude of oxidative damage location of cellular production, the specific reactive species produced, and capacity of antioxidant defenses. There is some debate as to whether or not exercise induces a "true" hormetic effect (278). There are multiple physiological factors during acute exercise that will ultimately limit the intensity or duration of exercise and may, consequently, prevent the increase in oxidants necessary to produce toxic effects (264). Regardless, within a redox context, the concept of exercise-induced hormesis is widely accepted (246, 278, 290).

The second characteristic of hormesis that helps explain the health promoting benefits of exercise is that transient repeated exposures to a stressor induces positive adaptations that protect against future stresses (290). In other words, acute exercise is an oxidative *eustress* that, when repeated throughout the lifespan, induces redox adaptations that protect against future oxidative *distress*. The acutely altered redox cellular milieu activates adaptive pathways including Nrf2, Nf-kb, PGC1a, and FoxO which all help maintain redox homeostasis throughout the lifespan and contribute significantly to the health protective effects of exercise (266). Exercise induced activation of Nrf2 increases endogenous antioxidant expression which can help prevent cognitive decline in neurodegenerative diseases (86), vascular dysfunction contributing to cardiovascular diseases (109), and tumorigenesis and metastasis in cancer (118). Exercise induced activation of Nrf2 also regulates proinflammatory cytokine release through inhibition of Nf-kb (30). In an animal model of Alzheimer's, physical exercise was able to reduce inflammation via decreased Nf-kb activation in the hippocampus (291) and this anti-inflammatory process is modulated by exercise-derived reactive species (290, 292). Redox-mediated activation of PGC-1a was previously discussed in the context of mitochondrial biogenesis and aerobic performance adaptations. PGC-1a has additional functions in health and antioxidant defense as increased mitochondrial content reduces the ATP demand per mitochondria, thereby reducing potential O<sub>2</sub><sup>-</sup> leakage and age associated oxidative distress (295). PGC-1a also regulates the expression of antioxidants CuZn SOD, Gpx, and CAT as PGC-1a knockout mice present with significantly reduced antioxidant capacity (293). Furthermore, it appears exercise induced protection against neurodegenerative disorders is in part dependent on redox mediated activation of PGC-1a (294). The FoxO family contributes to antioxidant defenses and the

cellular stress response. FoxO induces SOD-2, PRDX-3, and PRDX-5 within the mitochondria, and CAT within peroxisomes. Exercise induced redox down-regulation of FoxO transcription factors is involved in the maintenance of skeletal muscle mass (269). As a key modulator of protein breakdown, FoxO regulation by exercise-derived reactive species is involved in muscle homeostasis and directly combats muscle loss from sarcopenia (269, 270) and cancer induced myopathy (271). Redox dysregulation of FoxO is reportedly involved in the pathophysiological events leading to metabolic syndrome and diabetes (272). Chronic exercise ultimately reduces basal oxidative damage and maintains redox homeostasis through the aforementioned increases in antioxidant expression, improvements in inflammatory and muscle homeostasis, improved mitochondrial function, and increased oxidative repair methods (296, 298).

Quite paradoxically, a lack of exercise also increases reactive species production and subsequent oxidative damage. Sedentariness is a greater risk factor than smoking, high blood pressure, or high cholesterol for all cause-mortality (247, 319) and is strongly linked to the development of CVD, diabetes, cancer, and neurodegeneration (320, 321). Sedentariness is shown to increase measures of plasma oxidative damage independent of age (322), while sedentary and obese individuals demonstrate greater resting oxidative damage than age matched active healthy weight controls (323). Redox dysregulation appears to be the primary cause of vascular dysfunction in sedentary post-menopausal women compared to active age matched controls (324). Exactly how exercise induced increases in reactive species promote health benefits but sedentariness induced oxidants promote redox dysfunction and disease is not entirely clear. However, it is likely that the transient nature of exercise with moderate increases in reactive species, followed by removal of the stressor induces a positive hormetic response whereas sedentariness induces chronic increases in reactive species and oxidative distress. In addition, the subcellular location of oxidant production and the specific species that are produced in response to either exercise or sedentariness both contribute to the physiological outcomes (167).

## *2.2. Performance Strategies to Maintain Redox Homeostasis and Prevent Oxidative Distress*

Within sports performance, the prescription of appropriate exercise variables is a fundamental element of athlete readiness to optimize performance and adaptations. In contrast to the general population where only a small minority reach recommended exercise guidelines (239), athletes have significantly higher training volumes that supersede the recommended exercise prescriptions for general population (325). This is due to the clear dose-response relationship between exercise training volume and physiological adaptations that are beneficial for sport performance such as VO<sub>2</sub> max and muscle strength (571, 572). Improved sport performance requires physiological overload, consequently the difficulty arises to manage and prevent the negative consequences of altered redox homeostasis and potential oxidative distress resulting from high training volume (i.e. excess fatigue, impaired adaptations, OTS). As such, athletes can find themselves on the right side of the exercise induced hormesis curve. Frequently, athletes consume antioxidant and anti-inflammatory supplements (164), which in the days leading up to a competition may be beneficial in quickly reversing a pro-oxidant or pro-inflammatory state to improve performance in lieu of increased physiological signaling (197). In contrast, if maximal adaptation is desired, it is recommended that athletes avoid antioxidant and anti-inflammatory supplements due to their role dampening the physiological responses necessary for adaptation (326). From an exercise prescription standpoint, the use of specific strategies such as tapering prior to a competition or “de-load” weeks following periods of intense training help manage training workload, allow for supercompensation, and superior performance (214). Unfortunately, only one study has examined alterations in redox homeostasis following a period of decreased training load. Margaritis et al., (330) found that a two week taper in

triathletes attenuated oxidized glutathione (GSSG) increases and improved Gpx activity. Despite only one study examining decreased training load, multiple studies have demonstrated that redox status is responsive to increased training load throughout a season (214, 327, 328, 329). Significant increases in plasma and erythrocyte lipid peroxidation (TBARS) were observed during periods of increased training load in elite handball players (327). The erythrocyte GSH:GSSG ratio decreases (indicative of a pro-oxidant state) during periods of higher training load measured by weekly RPE in elite soccer players (328). In another group of elite soccer players, significant increases in leukocyte derived reactive species production were found during periods of increased training load (329). Despite heterogenous outcome measures, the available data indicates that training volume does significantly impact redox homeostasis, with increased training load resulting in increases in oxidative damage that are associated with fatigue and reduced performance (214). Following 12 weeks of very high training volume, increased levels of F-2 isoprostanes were strongly correlated to a decline in counter movement jump ( $r = 0.78$ ), 1-repetition maximum ( $r = .92$ ), and mean power ( $r = .77$ ) (332). Two additional studies found strong correlations between post-exercise lipid peroxidation (MDA) and time trial performance ( $r = -0.81$ ) (331) and pre-exercise GSH status with triathlon race performance ( $r = .65$ ) (333). However, due to the acute nature of these two studies, no relationship with training load can be discerned. Altogether, the available evidence indicates that periods of decreased training load result in improved antioxidant responses and superior performance (214), and may subsequently be an effective strategy to restore redox homeostasis and prevent oxidative distress. There is clearly an optimal balance between increased training load leading to superior adaptations, and decreased training load for faster recovery and improved performance. The beneficial response of exercise within a performance context again mirrors a horoptic curve, but it is unclear where, when, and by how much training variables should be adjusted to maximize eustress adaptations, prevent oxidative distress, and optimize performance *(figure)*. Very heterogenous redox responses, including reductive stress, are observed following exercise (334) and while appropriate exercise prescriptions induce oxidative eustress, mismanaged exercise prescriptions can result in oxidative distress, derailing both acute performance and performance adaptations. Though it may be tempting to attribute superior exercise performance to associations with a single redox biomarker, this neglects the various biochemical components involved in redox homeostasis and furthers the reductionist dogma that pro-oxidant status/oxidative damage increases are negative, and antioxidants positive. Further attempts to characterize the redox status that facilitates superior exercise performance under various conditions and the redox status that is most advantageous for superior exercise adaptations is necessary.

### 2.3. *Mechanisms of Exercise Induced Oxidative Eustress*

Though reactive species are likely produced in various tissues during exercise, it appears as though skeletal muscle is the predominant source of reactive species production during exercise (250). Multiple intracellular sources likely contribute to exercise-induced oxidant increases. Primary intracellular targets under investigation include the mitochondria, phospholipase A2 (PLA<sub>2</sub>), and oxidant producing enzymes NOX-2 and NOX-4 located within the mitochondria, sarcolemma, sarcoplasmic reticulum (SR), and T-tubules (246, 251, 252, 253, 254). It was initially believed that mitochondria were the primary source of exercise-derived oxidant production. This was hypothesized because initial reports estimated 2-5% of O<sub>2</sub> consumed during basal oxidative phosphorylation converted to O<sub>2</sub><sup>-</sup> from electron leakage (255). It was reasonably predicted that increases in oxidative metabolism would result in increases in O<sub>2</sub><sup>-</sup> leakage. However, it is now clear that increased oxidative metabolism and ATP demand actually decreases electron leakage and subsequent O<sub>2</sub><sup>-</sup> production (256, 257). There is increasing interest around PLA2, a membrane

phospholipid cleaving enzyme that produces arachidonic acid, a substrate for many reactive species producing enzymes (258). Increased activity of PLA2 may also modulate reactive species production in the cytosol and mitochondria (246, 259, 260, 261). However, current evidence seems to indicate that the primary producers of reactive species in skeletal muscle are NOX-2, located in the sarcolemma and T-tubule, and NOX-4 located in the mitochondria and SR (246, 253, 262, 263). Even more specifically, NOX-2 is the primary producer of reactive species production in contracting skeletal muscle following activation by agonists including mechanical stress (264).

Intracellularly, reactive species can induce cell signaling through direct modification of transcriptional inhibitory proteins and oxidation of amino acids in phosphatases and kinases (266, 267). It appears that intracellularly, exercise induced oxidative eustress is a complex and highly coordinated process determined largely via H<sub>2</sub>O<sub>2</sub> (267). H<sub>2</sub>O<sub>2</sub> is the primary reactive signaling molecule and thanks to its relatively low reactivity compared to other radical reactive species, H<sub>2</sub>O<sub>2</sub> can function as a messenger to carry a redox signal from the site of production to a target site within the cell. Diverse intracellular H<sub>2</sub>O<sub>2</sub> concentration gradients allow it to travel throughout the cell via simple diffusion and even extracellularly via designated aquaporin (AQP) membrane channels (267). During exercise H<sub>2</sub>O<sub>2</sub> concentrations begin to increase, low concentrations of H<sub>2</sub>O<sub>2</sub> are associated with physiological responses like angiogenesis, a critical adaptation to endurance exercise. As exercise intensity and H<sub>2</sub>O<sub>2</sub> concentrations increase, the Nrf2 stress response is initiated (267). At higher concentrations H<sub>2</sub>O<sub>2</sub> induces Nf-kb and subsequent inflammation, and at very high concentrations H<sub>2</sub>O<sub>2</sub> acts as an apoptotic signal inducing cell death (267). However, this is an effect observed *in vitro* and authors have questioned whether or not it is possible that exercise could produce the levels of reactive species needed to induce this apoptotic response (278). Given basal H<sub>2</sub>O<sub>2</sub> concentrations are kept at extremely low concentrations (474), even small increases during exercise have the potential to interact with target proteins and induce redox signaling. Though conceptually simple, extremely kinetically efficient intracellular antioxidants exist specifically to prevent excess accumulation of H<sub>2</sub>O<sub>2</sub> that would lead to these deleterious consequences. The PRDX family of antioxidants interact with H<sub>2</sub>O<sub>2</sub> at a rate constant (*constant*) many orders of magnitude faster than thiol groups on target proteins (i.e. Nrf2, Nf-kb). When considering this biochemical efficiency, it is not entirely clear how H<sub>2</sub>O<sub>2</sub> appreciably react with their targets in the presence of *in vivo* endogenous antioxidants (277). Recently, the role of the PRDX family of antioxidants has been expanded beyond simply buffering against H<sub>2</sub>O<sub>2</sub> induced toxicity to include the propagation of redox signaling and oxidative eustress (279). The interaction between PRDX and H<sub>2</sub>O<sub>2</sub> forms an intermediate sulfenic acid (-SCH<sub>2</sub>) group on PRDX which can transfer the redox signal to a -SH group on a cysteine amino acid located on a target protein (279). The transfer of an electron from H<sub>2</sub>O<sub>2</sub> to PRDX to a target protein is known as a redox relay and is increasingly recognized as a critical mechanism for which reactive species and H<sub>2</sub>O<sub>2</sub> in particular exert their physiological effects.

One of the difficult tasks in elucidating the molecular mechanisms that underpin the health and performance benefits of exercise is understanding how skeletal muscle contraction produces systemic effects that are beneficial in various peripheral tissues. Because reactive species have extremely short half-lives, it is highly unlikely that oxidants produced in skeletal muscle can diffuse out of the cell, into circulation and induce systemic effects. Recently, Olstrom et al., (275) used an elegant intra-animal design to control for variability in redox homeostasis between mice and demonstrated that high intensity exercise induces a redox-dependent systemic Nrf2 increase in the non-exercising limb and peripheral tissues. In contrast, low-intensity exercise did not induce a systemic Nrf2 increase indicating a “intensity gated” regulation of systemic Nrf2 inducibility which is critical for widespread cytoprotective responses (275).

The authors suggested an unknown redox exerkine was responsible for propagating the redox signal from the contracting skeletal muscle through systemic circulation and into peripheral tissues. Various signaling moieties to date have been identified as exerkines and include factors released from skeletal muscle (myokines), heart (cardiokines), and adipose tissue (adipokines) (249). Interestingly, it was recently shown that the skeletal muscle from old mice show reduced oxidation of PRDX-2 compared to adult mice (280). The authors hypothesize that this reduction in oxidation of PRDX-2 contributes to the reduced cell signaling responses and attenuated adaptations that are observed following exercise in older individuals. In support of this, there is evidence of increased PRDX secretion from skeletal muscle in response to muscle damage (281, 284) and increased oxidation of PRDX dimers are in erythrocytes (282, 283) and immune cells (285, 286) following exercise. Though limited, the available evidence supports a role for PRDX as a redox exerkine transmitting redox signals from increased skeletal muscle intracellular H<sub>2</sub>O<sub>2</sub> production to peripheral tissues and attenuation of this process is associated with decreased adaptive responses in aging.

In addition to PRDX, lipid peroxidation products 4-HNE and HODE are known mediators of cell to cell communication during and following exercise and can induce transcription factors in peripheral tissues such as Nrf2 (287). Additional exercise metabolites such as lactate can cross the sarcolemma and induce physiological responses in peripheral tissues. Interestingly, many of the physiological responses in peripheral tissues are also contingent on reactive species production. For example, lactate increases in an intensity dependent manner during exercise and is subsequently released into circulation. Lactate can then be taken up by various tissues including the liver (573), kidneys (574), and brain (575). Once in the liver, lactate stimulates PGC-1a and mitochondrial content in a redox dependent manner (577). It was recently shown that lactate mediated increases in PGC-1a and mitochondrial content were inhibited by antioxidant NAC treatment, indicating redox regulation of PGC-1a in peripheral tissue following exercise (576). Similarly, well studied myokines such as interleukin-6 (IL-6) are known to upregulate reactive species production in a variety of tissues and their release during exercise are inhibited by pre-exercise antioxidant treatment (578). IL-6 release from skeletal muscle following exercise has well established systemic effects including increased insulin sensitivity and improved beta-oxidation efficiency. It also appears that these functions of IL-6 are contingent on reactive species activation of AMPK in remote tissues (579). In this manner, secondary reactive species production in peripheral tissues following the release of exercise metabolites and myokines appear to be involved in the beneficial *eustress* adaptive response following exercise. The primary production of reactive species from contracting skeletal muscle followed by a secondary production in peripheral tissues was summarized by Louzada et al., (287) as the Hypothetical Wave Model of Reactive Species Production. Here it is hypothesized that an initial increase in skeletal muscle oxidants from sources such as NOX-2 activate intracellular targets such as Nrf2 and PGC1a; this is followed by an increased oxidant production in peripheral tissues due to the release of myokines, exosomes, and metabolites following exercise which stimulate signaling pathways in remote tissues.

In summary, the production of intramuscular reactive species during exercise has various potential consequences. Within skeletal muscle, excess production of reactive species may have negative consequences such as oxidation of myofibrillar proteins or alterations in calcium handling. However, skeletal muscle-derived reactive species are also a local stimulus for muscular glucose uptake, mitochondrial biogenesis, skeletal muscle hypertrophy, and increased endogenous antioxidant capacity (267). In this manner, skeletal muscle derived oxidants induce local oxidative eustress by activating signaling pathways needed for health and performance adaptations to regular exercise training (24). In

addition, exercise-induced reactive species function as paracrine and autocrine signalers, activating multiple cell signaling pathways within and on adjacent skeletal muscle fibers primarily via H<sub>2</sub>O<sub>2</sub> (265). Multiple redox molecules including antioxidants and oxidation products may function as exerkines responsible for transmitting redox signals or inducing signaling pathways in peripheral tissues. Myokine and metabolite induced secondary production of reactive species in remote tissues following cessation of exercise are also likely implicated in many of the systemic health benefits of exercise.

Though there is still much to uncover regarding the underlying molecular mechanisms, the existing evidence clearly indicates that exercise of appropriate intensity and duration has systemic health and performance promoting effects which mirror a hormetic response and undoubtedly involve oxidative eustress and redox homeostasis. The activation of transcription factors and induction of physiological signaling cascades by reactive species following exercise are an oxidative *eustress*. Exercise induced oxidative eustress upregulates antioxidant protection, improves repair mechanisms, and helps maintain protein, inflammatory, and redox homeostasis which inhibit the development of aging related dysfunction and pathologies (295). In a performance context, redox homeostasis facilitates optimal exercise performance and oxidative eustress promotes the positive muscular and physiological adaptations necessary for competitive sport. True to the hormesis hypothesis, transient exercise of extreme duration or intensity may induce significant oxidative damage without concomitant benefits in physiological signaling (247). Longitudinally, chronic high volume training with insufficient rest and recovery may induce oxidative distress and OTS. In a similar manner, there is some evidence that after a certain threshold, additional exercise over the lifespan does not induce additional health benefits (300, 301). The exposure to repeated transient oxidative eustress via exercise upregulates defenses against future oxidative *distress* which confers protection against various redox NCDs including CVD, cancer, metabolic syndrome, Alzheimer's, and Parkinson's (45). Accordingly, athletes demonstrate upregulated endogenous antioxidant defenses and reduced oxidative damage to repeated exercise bouts. Following clinical diagnosis, exercise is also effective at slowing the progression of Alzheimer's (311), decreasing the likelihood of metastasis in various cancers (312), and decreasing the likelihood of a second heart attack (313). Not surprisingly, exercise helps improve and restore redox homeostasis in each of these clinical populations (96, 292, 312). Unfortunately, quite soberingly, less than 5% of American adults meet the recommended prescription of 150 minutes of moderate intensity aerobic exercise per week (239). There is both a desperate need to increase general population engagement with exercise and underappreciated opportunities to optimize exercise prescriptions for health and performance. Exercise, redox homeostasis, and oxidative eustress lie at the crossroads of health and performance and as such the reliable quantification of physiological redox responses to exercise is of paramount importance.

### **3. Redox Monitoring: Physiological Insights from Healthcare to Exercise and Sport**

Within a clinical setting, monitoring can be generally defined as the “periodic measurement that guides the management of a chronic or recurrent condition” (232). Comprehensive monitoring techniques are regularly used in clinical settings to assess overall health and disease progression (237). Within an exercise context, monitoring can be defined as any measurement(s) that are taken to quantify the stress imposed by an exercise intervention, and to assess the efficacy of an exercise intervention in inducing a physiological outcome (outcome referring to both acute responses and longitudinal adaptations to exercise). Exercise monitoring then essentially guides the prescription of exercise workloads and assesses

the relationship between workload variables and health and performance outcomes. Exercise monitoring has rapidly expanded in recent years due to technological advancements that have produced a plethora of biosensors and commercially available products. These products can monitor heart rate, heart rate variability, respiratory rate, steps, calories, and blood glucose with non-invasive or minimally invasive techniques (235). These tools are giving individuals the resources that were once reserved for clinical settings allowing for improved physiological monitoring and greater control over individual health. Sports performance is one setting benefiting greatly from increased access to physiological monitoring tools. Monitoring within sports performance is usually applied to the training load and/or the fatigue/recovery status of the athlete. High performance athletes have comprehensive teams of support staff that utilize various monitoring strategies to profile the athlete's status and manipulate training prescriptions with the goal of inducing maximal performance, adaptations, and monitoring overall athlete wellbeing (234). Common physiological monitoring strategies in sport performance include measuring heart rate variability, neuromuscular function through performance tests, subjective wellness questionnaires, and biochemical/hormonal/immunological data in blood (233). Due to the burgeoning biotechnology industry that is consistently developing new biomarkers and physiological monitoring methods there is now an overwhelming volume of potential markers to analyze within exercise and performance (335). However, many commercial products and scientific techniques still require further large-scale validation and may not be as strongly associated with the physiological outcome they purportedly monitor (235). Given the irrefutable role exercise plays in health and performance, physiological monitoring tools are critical at this juncture to improve self-efficacy surrounding exercise for health, and optimize training prescriptions for health and performance. An overview of all the potential physiological monitoring techniques used in exercise and sport performance are beyond the scope of this review and for such a review the reader is directed elsewhere (citation). Instead, the aim of this section is to review the current state of redox monitoring through redox biomarkers and discuss their current and potential utility within both health and performance contexts.

Redox biomarkers provide measures to calculate redox homeostasis and putatively communicate important information regarding the functional and physiological status of individuals at rest and in response to exercise. Though overlooked in the past, recent evidence indicates significant translational value of redox biomarkers in health, disease, and exercise (173). Generally redox biomarkers can be classified into four categories depending on what it is that they measure i) levels of reactive species, ii) antioxidants, iii) oxidation products, and iv) reduced to oxidized ratio (i.e. GSH:GSSG, cysteine:cystine). Most of these molecules and their relationship with redox homeostasis, exercise, health, and performance have been discussed throughout this review and their value is well recognized. However, the potential of redox biomarkers in exercise, health, and performance is stymied by technological limitations, neglect of the biochemical complexity of redox molecules, improper methodological approaches, vague terminology, and non-quantitative analyses (36, 230, 474). For example, terms used throughout this paper such as reactive species, though helpful conceptualization terms, are nebulous and include divergent functioning molecules that may be highly reactive and indiscriminately damaging to macromolecules ( $\text{OH}^-$ ), compartmentalized signal transducers ( $\text{H}_2\text{O}_2$ ), or easily capable of diffusing through cell membranes ( $*\text{NO}$ ). Similarly, the term antioxidant does not effectively denote the biological function of a molecule. The term antioxidants have been used to refer to direct free radical scavengers (SOD), enzymes responsible for the repair of damaged macromolecules (OGG1) (473), and pro-oxidant compounds that induce endogenous antioxidant defenses (197). The role of exercise induced oxidative eustress has been largely overlooked until recent years and therefore there is a lack of effective biomarkers to quantify

oxidative eustress (26). Despite these limitations, the current body of literature suggests that with appropriate methodological and analytical consideration, redox biomarkers have the potential to provide robust physiological insights necessary to differentiate between eustress and distress, describe the acute physiological stress of exercise, predict the magnitude of exercise adaptation, and inform practitioners of the functional value of specific exercise interventions for health and performance.

### *3.1. Clinical Functions of Redox Biomarkers*

The use of redox biomarkers within clinical settings has been historically tethered to the assessment of oxidative distress, understandably so given the fact that the pathophysiology of so many diseases are redox regulated. Redox biomarkers are most frequently measured in blood or urine given the difficulty in collecting tissue samples in human subjects (476). There is also a recent increase in the usage of salivary redox biomarkers due to their ease of collection and improved validity and reliability (477, 478). Various pathophysiologically relevant redox biomarkers exist with prognostic value in cardiovascular medicine (4, 475), neurodegenerative disorders (8, 9), metabolic disease (479), insulin resistance (480), cystic fibrosis (10) and frailty (481). Redox biomarkers reflect redox homeostasis within cells or tissues and may be used in combination with other information to diagnose a disease or as a marker that reflects the progression or severity of disease by indicating the extent of altered redox homeostasis. For example, oxidized LDL is an established biomarker used in the diagnosis of CVD (106). In contrast levels of myeloperoxidase (MPO), a reactive species producing enzyme, in blood plasma are associated with the progression of atherosclerosis. MPO shows strong clinical evidence in predicting adverse events including mortality in patients with atherosclerosis however the diagnosis of atherosclerosis does not usually include MPO (475). In patients with inflammatory bowel disease, it was recently shown that serum free thiols are a superior indicator to fecal calprotectin, the current gold standard biomarker, in predicting disease severity and progression (481). The salivary ferric ion reducing antioxidant potential (FRAP) is a redox biomarker that can stratify patients with mild to extreme renal kidney dysfunction as well as differentiate between healthy and ill patients (482). Lipid peroxidation markers 4-HNE and MDA in plasma are significantly elevated in patients with Alzheimer's (580) compared to age-matched controls supporting the potential for peripheral redox biomarkers to be used as diagnostic criterion supporting or replacing current more invasive diagnostic measures done in cerebrospinal fluid. The use of salivary redox biomarkers has gained popularity in the 'clinical' assessment of neurodegeneration due to their non-invasiveness and accurate reflection of redox status in plasma and cerebrospinal fluid though more work is needed in this area (8). Given loss of redox homeostasis and increases in oxidation products are inextricably linked to aging, the assessment of endogenous antioxidant systems such as GSH and Trx systems may present a potential biomarker to assess biological age independent of chronological age (483). Overall, the evidence from clinical settings indicates that redox biomarkers can be used as complementary diagnostic criteria and have additional utility in disease management as assessments of pathological severity and disease progression. A recent review highlighted the potential importance of longitudinal and acute redox monitoring in the absence of clinical symptoms as an important early indicator of disease risk (471). Common lifestyle behaviors that predispose individuals to disease such as smoking (521), alcohol intake (523), or poor sleep habits (522), increase oxidative distress and push individuals towards redox dysfunction. It appears that increased oxidative distress induced by lifestyle habits in otherwise healthy individuals is detectable by conventional redox biomarkers. When plasma redox status was assessed by total antioxidant capacity (TAC) and hydroperoxide (HPX) concentration in 100 apparently healthy middle aged men and women, multiple significant associations were observed

between the TAC/HPX ratio and sleep quality ( $P = 0.019$ ), red meat intake ( $P = 0.005$ ), and body fat percentage ( $P = 0.011$ ) (524). As altered redox homeostasis plays an important role in the initiation of NCD and aging related pathologies, assessment of altered redox homeostasis in the absence of clinical markers of disease may be able to provide an independent indicator of disease risk. The findings indicate that redox biomarkers may be used as a disease prevention tool in the prodromal phase of redox related dysfunctions in addition to disease management (471).

### 3.2. Redox Biomarkers in Response to Exercise

In previous decades the use of redox biomarkers in exercise was largely focused on the assessment of oxidative damage or antioxidants following exercise to determine to what extent exercise impacts redox status. All three classes of oxidative damage biomarkers: lipid peroxidation, protein oxidation, and DNA oxidation, as well as enzymatic and non-enzymatic antioxidants have been extensively researched in response to acute and chronic exercise. Redox biomarkers are repeatedly presented as one of the most promising, and yet enigmatic, biomarkers of exercise status in the literature (335, 393) given their differential responses to acute and chronic exercise. The assessment of redox status is most frequently done via biomarkers in bodily fluids such as blood, saliva, or urine given the invasive nature of collecting tissue samples in humans (476). Acute exercise generally results in an increase in oxidative damage markers in bodily fluids immediately after exercise regardless of the population, though the magnitude of increase is dependent on multiple variables such as diet, age, and fitness level. This occurs alongside a variable directional change of antioxidant content contingent on the antioxidant, tissue sampled, subject characteristics, and timing of sample (494). In contrast, redox responses to chronic exercise are usually more subtle, with less pronounced changes in both oxidation products and antioxidant content. What is clear is that all exercise, regardless of specific training variables, functions as an antioxidant, increasing resting endogenous expression of antioxidants and decreasing resting levels of oxidative damage in accordance with the concept of hormesis (247) (*enter in a custom figure demonstrating with arbitrary units, the changes in oxidation products/antioxidants following acute & chronic exercise*). From this evidence, one can reasonably assert that redox responses follow a pattern similar to a repeated bout effect, as in following chronic exercise the same acute exercise intervention results in reduced oxidative damage due to greater resting antioxidant expression. Though oxidative damage may be reduced in trained individuals compared to untrained individuals following the same acute exercise intervention intervention (581); evidence was recently published that Nrf2 signaling, which regulates the endogenous antioxidant response, is more sensitive to acute exercise after regular endurance training (222). The findings suggest mechanisms beyond a basic negative feedback and that our implicit assumptions surrounding the functional significance between redox responses and exercise may be flawed.

#### 3.2.1. Acute Exercise

In response to acute exercise, thiobarbituric acid-reactive substances (TBARS), a common non-specific marker of lipid peroxidation, increases by up to 50% following a competitive soccer match, remains elevated for 48 hours, and their levels are associated with knee ROM and muscle soreness (453). TBARS also increases in response to an incremental cycling test, isometric contractions, and acute resistance training (487, 488, 224). The sensitivity of TBARS to exercise of different modalities and intensities, in addition to a linear relationship between TBARS and resting fatigue, led some to suggest TBARS as a marker of fatigue following exercise (453). Similarly, F-2 Isoprostanes, another marker of

lipid peroxidation widely regarded as the “gold standard” of redox biomarkers, appears to increase significantly in response to acute exercise with proportional increases relative to exercise intensity (484). Isoprostanes are derived from the oxidation of arachidonic acid and reflect the oxidative damage to cell membranes following exercise. F-2 Isoprostane levels increase rapidly immediately following cessation of exercise (0-60 minutes) before returning to baseline and therefore may be used to communicate the acute stress of the exercise intervention (484). In contrast to the rather transient nature of some lipid peroxidation markers, protein oxidation markers such as protein carbonyls (PC) are a stable and irreversible oxidation product that are highly abundant. Previous research indicates significant increases in PC concentrations following high intensity exercise in both men and women (489, 490). High intensity eccentric contractions induce marked increases in PC in both trained and untrained individuals, though the increase is greater in untrained subjects (581). Following 40 minutes of cycling at 70%, 75%, and 80% of VO<sub>2</sub> max, increases in PC were observed only in the 80% group indicating that exercise intensity is a modulator of PC levels (491). In addition, it appears that exercise duration and training status both influence PC concentrations following exercise (492). Surprisingly, various studies indicate decreases in PC levels following exercise despite concomitant increases in markers of lipid peroxidation (283, 494). These findings appear to be indicative of increased proteasomal activity leading to increased clearance of pre-existing PC levels supporting a role for exercise in the regulation of proteasome activity, though this mechanism requires further exploration (492). Markers of DNA oxidative damage following exercise are most frequently assessed via 8-oxo-g, a biomarker that is the product of reactive species modification of guanine; or the comet assay, a single cell gel electrophoresis technique that can measure single and double strand DNA breaks (37). Acute aerobic exercise increases DNA damage 2 hours to 1 day following cessation of exercise and may result in DNA damage lasting beyond 5 days through the magnitude of DNA damage beyond 5 days is unclear. Exercise intensity again appears to be a primary determinant of DNA oxidation. In a recent systematic review and meta-analysis, aerobic interventions of high intensity (>75% VO<sub>2</sub> max) induced significantly greater oxidative damage compared to interventions of long durations (>42 kilometers) (37). The influence of resistance training on markers of DNA damage is less clear though it was reported that acute resistance training increases 8-oxo-g in urine immediately post and 24 hours post exercise (582).

Plasma GSH is a routinely assayed antioxidant that is typically reduced following acute exercise (494). Concomitantly, increases in GSSG and reductions in the GSH:GSSG ratio are frequently observed following acute exercise, indicating a pro-oxidant state. These changes in glutathione rapidly return to baseline around 30 minutes following cessation of exercise. Antioxidant “capacity” assays (TAC, ORAC) which consist of some combination of non-enzymatic antioxidants, usually vitamin C, vitamin E, uric acid, bilirubin, and GSH, generally decrease immediately in response to acute exercise followed by a rebound period in which they exceed basal levels (494, 496) though this effect is not always observed. The acute exercise TAC response appears rather transient as TAC collection at 24, 48, and 72 hours following an ultramarathon no maintain race is variable in direction and magnitude (533) and may be a reflection of diet over the 72 observation window rather than post exercise non-enzymatic antioxidant mobilization. Conflicting evidence exists for the four primary enzymatic antioxidants SOD, CAT, Gpx, and glutathione reductase (Gr) in regards to their directional change following acute exercise as increases (497, 498), decreases (499, 500), and no change have been reported (501). Age, fitness, and basal health/redox status all influence the magnitude of antioxidant responses following acute exercise. In age-matched older adults, individuals with higher initial fitness levels present with greater antioxidant enzyme expression and subsequently decreased oxidative damage to acute exercise (550). Between young

and old adults, significant increases in SOD activity were observed following acute exercise only in young adults (551). Certainly, age and fitness both influence redox status, however, in a rather clever study, researchers exposed subjects of identical age and training status to either a pro-oxidant (passive smoking) or antioxidant (Vitamin C) stimulus and compared redox response to acute exercise before and following 12 days of exposure to their respective stimulus (552). Subjects exposed to a pro-oxidant stimulus increased resting oxidative damage, decreased resting GSH levels, and subsequently showed no decrease in GSH or increase in oxidative damage following exercise. In contrast, those exposed to an antioxidant stimulus decreased oxidative damage and increased GSH at rest, resulting in a marked redox response following acute exercise. The experimental setup clearly exposes how basal redox status, independent of age or fitness, influences the antioxidant response to acute exercise.

The significance of acute post-exercise changes measured by systemic redox biomarkers in health and performance is not entirely clear. The consistently observed reduction in GSH and GSH:GSSG ratio following exercise may be used as an indicator of fatigue from aerobic exercise (393). Furthermore, strong relationships between resting GSH and aerobic exercise performance have been reported (333). However, acute resistance exercise does not appear to significantly influence GSH levels (536, 537). Greater post acute exercise SOD activity following chronic training in elite runners (539) was reflective of decreased running speed post-training compared to runners who showed no change in post acute-exercise SOD activity and increased running speed post-training. It is unclear exactly what mechanisms relate changes in systemic antioxidant status to performance though the findings indicate that acute changes in redox biomarkers may be reflective of performance capacity. Farche et al., (525) reported significantly greater TBARS production in untrained runners than in trained runners in response to the same graded Bruce treadmill protocol, potentially indicating greater fatigue following the same exercise in untrained individuals. However, training status was not the only stratifying variable in this study, untrained low aerobic fitness runners produced lower post exercise TBARS relative to untrained high aerobic fitness runners. The authors concluded the findings supported previous results that a greater acute post-exercise lipid peroxidation response is predictive of superior aerobic exercise performance (526). However, this conclusion conflicts with other results that greater aerobic fitness levels are protective against acute exercise induced lipid oxidation (27, 528). In elite endurance athletes, it appears that the plasma TAC response immediately following acute aerobic exercise is related to aerobic fitness measures lactate threshold, VO<sub>2</sub> max, and velocity at lactate threshold (531). These findings are in accordance with Braakhuis et al., (532) who reported the largest increase in TAC approximately 1 hour following acute exercise in rowers with the greatest aerobic capacity. However the two studies did not report agreement on resting TAC levels, which may be a result of different sport types, runners vs rowers (531, 532). The TAC assay is also sensitive to fluctuations in training load and one of its derivatives, the Free Oxygen Radical Defense (FORD), is minimally invasive taking measures from a finger prick blood sample making it an appealing practical measure of exercise workload following acute training (531). However, the basal response to chronic exercise training of TAC and its analogues is variable meaning it may not be an effective indicator of a redox adaptation (335).

### 3.2.2. Chronic Exercise

The influence of chronic exercise on specific redox biomarkers is less pronounced. Despite the general trend for all forms of chronic exercise to reduce markers of oxidative damage and increase expression of antioxidants at rest (274), the influence of specific exercise variables on individual redox biomarkers is unclear. Multiple studies (502, 503, 504) indicate a trend for decreased F-2 isoprostanes levels measured

in either plasma or urine following chronic exercise in different clinical populations (484). However, due to differences in sampling time following training and exercise protocol it is unclear what magnitude of change can be expected. Three weeks of aerobic exercise training (3x/per week) at a fixed dose of 16.5kcal/kg/wk can significantly decrease resting TBARS in patients with severe depression (505). In patients with osteoarthritis, chronic exercise of walking 50 min per day/3 times per week over 12 weeks at a non-standardized low-moderate intensity significantly decreased resting TBARS (506). A recent network analysis attempted to delineate how different exercise intensities influenced redox status in various clinical populations (507). In this analysis, most studies showed reductions in lipid peroxidation and increases in antioxidant activity following exercise training regardless of intensity. The analysis concluded that the greatest reductions in lipid peroxidation (MDA) and increases in SOD activity may be seen following moderate intensity aerobic training (508, 509) and low intensity training (510) though this effect is modulated by the clinical population analyzed.

The measured increase in basal enzymatic antioxidant expression and decrease in basal oxidative damage following chronic exercise training are redox adaptations that reflect improved redox homeostasis and can be quantified by redox biomarkers. The importance of redox homeostasis for health, as well as some of the mechanisms underpinning how exercise maintains redox homeostasis have been discussed throughout this review. This importance is further underscored by the increased popularity of exercise research targeted at improving redox homeostasis and treating redox related dysfunction. Recent reviews and meta-analyses have measured changes in redox biomarkers to highlight how exercise improves redox status and prevents the progression of CVD (518), modulates redox signaling in cancer and prevents metastasis/tumorigenesis (312), restores pulmonary function in patients with COPD (516), restores mitochondrial function and decreases oxidative distress in patients with Alzheimer's (583) and decreases markers of RNA oxidation and all cause mortality in patients with type 2 diabetes (519, 520). Unfortunately, low methodological quality and heterogeneous exercise protocols that neglect to quantify external training workload, and/or neglect to relate redox outcomes to physiological outcomes (553) means exercise prescriptions targeted to redox status/biomarkers for individuals with specific redox related pathologies do not yet exist (516, 517). More robust work is needed that considers the multitude of exercise variables in controlled randomized clinical trials in order to relate how exercise variables acutely influences specific redox biomarkers and ultimately longitudinal redox adaptations and improved physiological function. It stands to reason that if redox biomarkers are clinically used to assess disease progression and severity, improvements in redox biomarkers following chronic exercise can be used as an efficacy measure to assess the effectiveness of an exercise prescription at treating a specific disease.

Mirroring the redox response to chronic exercise in clinical populations, athletes and high fitness individuals demonstrate greater resting enzymatic antioxidant capacity and decreased oxidative damage parameters resulting from regular exercise training (529, 530, 535). Athletes demonstrate increased resting GSH levels following 23 weeks (510), 13 weeks (511), or 45 days (512) of chronic sport specific training; as well as increased resting enzymatic SOD and CAT expression, and reduced Gpx activity (511). Following a competitive season in elite kayakers, marked increases in plasma non-enzymatic antioxidants are observed which may explain the attenuated basal levels of TBARS at the end of the season (515). Some studies indicate an increase in oxidative damage markers such as TBARS or PC following chronic exercise training in athletes (513), however, these studies likely reflect the oxidative damage resulting from recent acute exercise sessions, not a redox adaptation to chronic exercise. Some indices of oxidative damage may remain elevated for up to 96 hours (244) and increased training load increases markers of oxidative damage (327, 328, 329). Indeed, when redox status is measured after

allowing for sufficient recovery time between the end of a training period and testing, there are marked improvements in antioxidant defenses and decreases in resting oxidative damage in both adults and adolescents (514). Redox biomarkers also provide practical physiological indicators of OTS in individuals with diagnosed and experimentally induced OTS following periods of prolonged intense exercise training. Clinical examination of redox biomarkers in an elite international rower with diagnosed OTS demonstrated significantly altered SOD, GSH, and plasma antioxidant capacity as assessed by FORD, in addition to increased measures of lipid hydroperoxides (25). In an experimentally induced resistance training model of OTS in recreationally trained males, a period of overtraining produced significant and sustained increases in PC, F-2 Isoprostanes, CAT, Gpx, and decreases in GSH (224). Certainly, from a diagnostic standpoint, understanding how and to what extent redox biomarkers are influenced during OTS will help in the management and prevention of OTS during periods of intensive exercise.

### 3.2.3. *Redox Biomarkers as Proxies for Physiological Adaptation*

An intriguing proposed role of redox biomarkers may be as indicators of cardiovascular, metabolic, and skeletal muscle adaptations to exercise. Following 8 weeks of aerobic exercise training at a moderate intensity (70% VO<sub>2</sub> max) in healthy untrained males supplementing with Vit C, lymphocyte SOD and CAT expression failed to improve and this reflected cytoprotective heat shock protein 70 (HSP70) and HSP60 content in muscle tissue (538). In contrast, subjects who did not receive supplementation saw positive adaptations in lymphocyte SOD and CAT concurrently with improvements in HSP70 and HSP60 within skeletal muscle indicating potential for peripheral redox biomarkers to reflect skeletal muscle cytoprotective adaptations. Ristow et al., (170) demonstrated that a 4-week exercise training program of combined biking or running and circuit training in healthy young men induces beneficial effects in glucose infusion rate and insulin sensitivity only in the absence of antioxidant supplementation. The benefits in insulin sensitivity following training that occurred in the non-antioxidant group paralleled increases in skeletal muscle expression of SOD1, SOD2, and Gpx irrespective of training status indicating potential for enzymatic antioxidant redox adaptations to reflect metabolic adaptation. However peripheral redox biomarkers were not collected in this study. Researchers also measured post-acute exercise (3 days into training intervention) TBARS levels in antioxidant and control groups and found reduced TBARS following acute exercise only with antioxidant supplementation, as expected. Interestingly though, post acute exercise TBARS levels prior to the training intervention were significantly correlated ( $r = 0.353$ ,  $P < 0.05$ ) with post-intervention insulin sensitivity across all 39 subjects irrespective of training status or antioxidant supplementation (170). It appears that the authors only performed statistical analysis on post acute exercise TBARS and the pooled post-intervention glucose infusion rates from both groups, instead of analyses for each treatment group. It stands to reason that perhaps a stronger association may have appeared between post acute exercise TPARS and post intervention glucose infusion rate had analyses been performed for each treatment group given glucose infusion rates only significantly improved in the non-antioxidant group. A similar finding was recently reported following an exercise intervention in the absence of any antioxidant supplementation. Researchers tested the F-2 Isoprostane response of 100 subjects following an acute aerobic exercise test and stratified subjects according to the magnitude of the F-2 Isoprostane increase (i.e. high post-exercise F-2 response, moderate post-exercise F-2 response, low post-exercise F-2 response) (230). Following a standardized, non-periodized, 6-week exercise intervention of 45 minutes of cycling at 70% W<sub>max</sub> in all subjects, the subjects with high and moderate pre-intervention F-2 Isoprostane responses to acute exercise demonstrated significantly greater improvements in various aerobic measures VO<sub>2</sub> max, time trial, and Wingate test (230). Taken together

with previous findings, it appears that not only can peripheral enzymatic antioxidant biomarkers reflect skeletal muscle performance and metabolic adaptations, but lipid peroxidation products both at rest (230) and in response to acute exercise (170, 230) may be indicative of post-intervention adaptation. It is unclear if additional redox biomarkers, such as markers of protein oxidation, DNA oxidation, or other lipid peroxidation markers may function as adaptation indicators.

#### *3.2.4. Limitations of Traditional Redox Biomarkers in Exercise*

For all the evidence demonstrating significant relationships between redox biomarkers and performance (333), fatigue (393), workload (214, 327, 328), and adaptation (170, 230). These biomarkers still come with numerous drawbacks. Namely, and preceding any discussion regarding their biochemical appropriateness, all redox biomarkers are assayed via blood, urine, saliva or tissue sample meaning they require at least some level of invasiveness in addition to lab analysis which comes with associated time and cost. In contrast, sweat readings or HRV measures can be immediately relayed to users via wearable technologies and biosensors. It is possible that less invasive means to test redox status become widely available. For example, given that GSH is found in every extracellular fluid (540), development of a wearable biosensor that provides real time feedback on sweat GSH status could give insights into non-enzymatic antioxidant status without the need for a lab or invasive testing. Indeed, sensors similar to this are already being developed (541). It is however important to note that depending on the selected biomarker, the redox status of peripheral fluids such as blood or plasma, may or may not always reflect the redox status of tissues such as the skeletal muscle, liver, or brain (542). A recent systematic review concluded that generally there is good agreement between most blood redox biomarkers and tissue redox status except for GSSG and the GSH:GSSG ratio (543), though the vast majority of the studies analyzed were done in non-human subjects. Furthermore, there is very little information on the relationship between sweat redox biomarkers and blood redox status. This gap in the literature must be addressed in order for the development of peripheral fluid based wearable biosensors of redox status.

Beyond the invasiveness of current redox biomarkers, multiple authors have previously addressed the biochemical limitations of certain redox biomarkers that directly influence potential results and interpretation of redox data (26, 35, 167). Namely, both the TBARS and TAC assays are considered flawed and their discontinuation in exercise research is recommended (35). The full extent of the chemical flaws of these two assays are reviewed elsewhere (545). Briefly, the TBARS assay does not measure a specific biological product but is rather a non-specific assay of lipid peroxidation. The TBARS assay purports to measure MDA which can react with thiobarbituric acid (TBA) forming a pink compound (TBARS) that is measured. However, the assay procedure itself produces additional MDA by heat induced lipid decomposition, and generates non-lipid (i.e. sugars and proteins) sourced MDA through extraneous TBARS reactions leading to inflated values (26, 35). TAC is equally flawed and purports to measure the total antioxidant capacity of a biological system. However, TAC and analogues (ORAC, FRAP, FORD) are only measures of non-enzymatic antioxidant capacity and evidence clearly demonstrates that the vast majority of biological *in vivo* antioxidant activity is from enzymatic antioxidants (246), as such it is hard to understand what is “total” about the assay (26). At best, TAC would be an indicator of a minority of the antioxidant activities occurring within a biological system, at most 25% according to previous research (546), and would not indicate the relative contribution of specific non-enzymatic antioxidants. The issue of non sample sourced spurious MDA production resulting from TBARS is clear, the assay may indicate increases in lipid peroxidation in the absence of an actual increase. Similarly, when aware of the interpretational drawbacks of TAC, it is difficult to ascertain the

biological significance of the frequently reported TAC increases following acute exercise (495, 496, 531, 532). For example, individuals with higher physical fitness have greater resting enzymatic antioxidant concentrations (547). Simultaneously individuals of highest aerobic fitness such as athletes reportedly demonstrate greater TAC increases post exercise (531, 532) which is usually discussed as a physiological response against exercise induced reactive species production (548). However, if the majority of antioxidant defense comes from enzymatic sources, and individuals with higher fitness have greater enzymatic antioxidants, what explains the need for greater non-enzymatic “mobilization” following exercise observed in individuals with high fitness? To better understand the nuance of antioxidant responses and make sense of conflicting literature, alternatives to TAC such as specific measurement of antioxidant vitamins (i.e vitamin C and vitamin E) following exercise is recommended.

In addition to proliferative use of flawed assays, exercise redox biology presents several interpretational difficulties. Redox biology is a fundamental process that has the potential to connect biochemical molecular interactions taking place at the lowest level of biological organization to physiological phenotypes. However, many previous redox biology studies within both clinical and exercise settings failed to collect physiological data (i.e. measures of muscle mass or function) and instead opted to collect only redox data. While redox data is unequivocally important, it represents information from the lowest level of biological organization and is difficult to connect to physiological outcomes without appropriate measurements. For example, a recent publication investigated the oxidative damage and enzymatic antioxidant responses to steady state cycling for 10, 20, and 30 minutes at 50, 60, and 70% VO<sub>2</sub> max (554). Though an important contribution certainly as more data regarding redox responses to changing exercise variables is needed, the researchers did not collect any additional physiological information to contextualize the observed redox changes with an acute physiological outcome. Experimental approaches such as this reduce the translational value of redox biomarkers and limit insights between redox biology and physiological outcomes. Even basic measures like sRPE post acute exercise can help relate and contextualize measures of oxidative damage and antioxidants to physiological outcomes and would ultimately assist in the practical application of redox biomarkers. In addition, pre-existing biases regarding the role of reactive species in physiology (i.e. belief they are primarily distress or eustress molecules) influence the discussion of redox data leading to different interpretations to the same redox response (553).

The typical approach to redox biomarkers has been the measurement of antioxidant expression/activity and oxidation products in blood plasma/serum. Notwithstanding the previously discussed biochemical limitations of some of the most popular redox assays (TBARS and TAC), assessment of redox homeostasis via this method is inadequate as it cannot differentiate between meaningful production of reactive species involved in *eustress* redox signaling and aberrant production of reactive species resulting in *distress*. Measuring eustress redox signaling via systemic increase in antioxidant enzyme abundance/activity, chaperone proteins (i.e HSP70), or Nrf2 following exercise is inappropriate because each of these markers responds to other stimuli in addition to reactive species. Without direct measurement of reversible thiol oxidation of these specific proteins, it is not possible to quantify oxidative causes or fully attribute the changes in protein abundance or function to redox mechanisms. Similarly, concluding oxidative distress due to increases in oxidative damage is reductionist as oxidation products are not inert, may interact with redox signaling, and may be desirable and necessary to induce beneficial adaptations (230). However conventional assessment and interpretation of oxidation products largely neglects this. Furthermore, as discussed in reference to 8-oxo-g and OGG1, not all oxidation products are permanently oxidized, significant repair and efflux mechanisms exist that

ultimately control the concentrations of redox biomarkers (18). An increase in an oxidation product in blood may be a result of increased oxidative damage, but also decreased oxidative repair or efflux. Though it is unequivocal that exercise increases reactive species, the impact of exercise on oxidative repair and efflux is not fully clear. In the absence of additional redox data to contextualize changes in oxidation products at the systemic level, it is recommended that researchers refrain from attaching biological meaning and concluding functional significance to oxidation products (35). For example, a conclusion that lipid hydroperoxides are markers of fatigue because of increases following fatiguing exercise ignores both efflux and repair as well as the multiple reactions lipid hydroperoxides may undergo that could inhibit or induce additional oxidative damage (555).

Finally, the astute reader may have noticed certain elements missing from both the text and figures related to redox biomarkers. Namely, a lack of quantitative units when discussing redox measures. This is common practice within the redox literature and meant to reflect the ambiguity as it relates to actual quantitative measurements of reactive species. Though reference values for antioxidants and oxidation products exist (584, 585, 586), they are highly variable between individuals and different tissues. In addition, general population reference ranges may not be useful for individuals within a clinical or performance setting. The accurate quantification of actual reactive species, namely H<sub>2</sub>O<sub>2</sub>, remains one of the greatest logistical challenges within redox biology (549). Though various fluorescent probes exist that purport to measure H<sub>2</sub>O<sub>2</sub> and other reactive species, many of them fail to chemically react with their proposed target, are prone to artifactual oxidation, and only define relative concentrations, not absolute concentrations (26). Answering the basic question: what are the physiological concentrations of H<sub>2</sub>O<sub>2</sub>, will require superior analytical techniques and will certainly vary according to the tissue and analytical conditions. More deliberate attempts to quantitatively analyze steady state concentrations of reactive species and antioxidants are critical to delineate the border between oxidative stress and distress (549).

In conclusion, current redox biomarkers play an important role in exercise monitoring as they are differentially sensitive to acute exercise variables and chronic exercise training. The sensitivity of different redox biomarkers to alterations in acute exercise modality (244), volume (327), and intensity (554) means differential forms of exercise produce certain “redox signatures” that reflect the acute physiological responses to changes in workload. The cumulative effect of repeated acute transient redox changes from exercise result in redox adaptations that attenuate the development and progression of NCD. As such, current redox biomarkers may provide the necessary information to develop population specific exercise prescriptions that target redox related pathologies. In this sense redox biomarkers function as both an efficacy outcome measure and a workload monitoring tool. Acute redox responses may also reflect and predict the magnitude of skeletal muscle, metabolic, and cardiovascular adaptations to exercise. Further research is required to confirm this function of redox biomarkers and assess what variables may influence their predictive validity. Despite their multiple applications, various drawbacks hinder their translation into clinical and exercise settings. Firstly, no single redox biomarker can comprehensively characterize the physiological response to exercise, as such a multi-marker approach is best to confirm a redox outcome and lend greater validity to the relationship between redox outcome and physiological outcome. A lack of non-invasive techniques to measure redox status, use of flawed assays, inappropriate interpretation of redox data, and a lack of quantitative redox information stymie the potential of redox biomarkers within health and performance. Regardless of current limitations, under-appreciated opportunities exist to advance the utility and interpretation of redox biomarkers both within applied practice and laboratory settings.

### *3.3. Future Application of Redox Biomarkers*

While existing systemic redox biomarkers have undoubtedly proved useful in furthering our understanding of exercise, health, and disease, they fail to provide the comprehensive insights necessary to draw meaningful mechanistic conclusions or insights towards modifying applied practice. The fate of GSH following aerobic exercise will be used as an example. Firstly, GSH has various functions. GSH can donate an electron to reactive species via the oxidation of its thiol group on cysteine forming its corresponding disulfide GSSG. GSH provides a reducing equivalent for Gpx to return oxidized Gpx to its native state and can donate electrons to ascorbate reducing dehydroascorbate to ascorbic acid assisting in the  $\alpha$ -tocopherol redox cycle attenuating the development of excess lipid peroxyl radicals (556, 557). GSH also has a critical role in the conservation of protein function under conditions of increased reactive species production by glutathionylation, the addition of GSH to a thiol containing protein (558). Various proteins including peroxiredoxins (559), Nf-kb inhibitor proteins (561), and the 20S proteasome (560) are protected against oxidative damage during conditions of increased reactive species production by glutathionylation. Acute aerobic exercise consistently reduces plasma GSH levels and decreases the GSH:GSSG ratio immediately post exercise (494). This is frequently interpreted as evidence of a pro-oxidant shift and oxidative distress. Though it is not inaccurate that this change does indicate a pro-oxidant shift, it is a known fact that exercise increases reactive species, so a pro-oxidant shift is expected. This fact does not provide evidence of oxidative distress, explain which biological action of GSH was induced by the preceding exercise stressor, or necessarily provide any actionable insights. Concluding oxidative distress from a shift towards a pro-oxidant state ignores that an increase in GSSG can induce protective protein glutathionylation (558). Instead of concluding oxidative distress, from existing evidence one may be able to reasonably conclude that a significant decrease in the GSH:GSSG ratio following acute aerobic exercise is actually a eustress given higher resting pro-oxidant status attenuates GSH reductions after exercise (552) and attenuated redox responses inhibit adaptations to exercise (216, 230). Concluding a function of GSH within skeletal muscle from plasma samples is equally inappropriate since GSH and GSH:GSSG concentrations differ across cellular and tissue compartments (562). As such it is difficult to ascertain exactly what the biological significance of this change is without knowing the terminal fate of GSH. All to say, the insights provided by a change in peripheral fluid GSH following acute aerobic exercise are incomplete without some measurement of protein modification or output related to one of the numerous functions of GSH.

The rapid advent of omics based analyses provide the means to unravel the massive diversity of redox modifications following exercise and elucidate underlying mechanisms of protein modification (167). Redox proteomics is the gold-standard and recommended approach to quantify oxidative eustress redox signaling in thiol based proteins. Redox proteomics approaches essentially tag a redox process or protein of interest and analyze them on a proteome wide scale to assess the extent of this modification. Instead of assaying global LC products following exercise which does not provide information regarding the magnitude or location of carbonylated proteins, proteomics approaches analyzed via high-throughput mass spectrometry can identify and quantify the location and extent of protein carbonylation within specific tissues such as skeletal muscle or systemically (26). Through redox proteomics, one is able to analyze the extent and location of protein glutathionylation within skeletal muscle following acute exercise and relate it to systemic GSH measures to clarify how and if a systemic redox biomarker is related to a specific mechanistic action of a redox molecule (23). Proteomics approaches have identified multiple cysteine residues on antioxidant enzymes that are selectively oxidized in redox related

pathologies, negatively impacting functionality and contributing to the pathogenesis of cancer, neurodegeneration, and aging (7). Very few studies have applied redox proteomics following exercise. One such study showed for the first time that older individuals (64-79 years) demonstrate a lack of resilience to exercise induced reactive species production characterized by greater oxidation of cysteine proteins and loss of mitochondrial proteins not observed in younger counterparts (18-30 years) following the same high intensity cycling intervals at 80% VO<sub>2</sub> max (565). Though it is difficult to ascertain the biological significance of this difference, it is likely that it is related to the reduced adaptive response to exercise seen in older individuals. Beyond identification of oxidatively modified proteins, redox proteomics provides the analytical tools necessary to identify novel redox biomarkers for health, aging, disease, and exercise; connecting mechanistic insights to clinical and applied practice (23). Unfortunately, alongside their robust and comprehensive insights, omics workflows come with high costs, time, specialized laboratory equipment and resources making them a laboratory specific tool not easily accessible to most. To this end, recently published recommendations include workflow examples using novel immunological assays and step-wise guides to selecting and interpreting existing redox assays to achieve superior insights into the biological significance of exercise-induced redox perturbations (26).

From an applied practice perspective, the change in GSH and the GSH:GSSG ratio may be related to phenotype changes in response to an acute exercise stress (i.e. increased fatigue) (333) or chronic exercise (i.e. improved cardiovascular fitness) (510). However, due to redox variability and their sensitivity to multiple variables, in order to practically apply insights from the GSH response to acute aerobic exercise, one would need robust data sets that categorize the fluctuations of GSH in response to different training variables and according to different individualized inputs (i.e age, body fat, lifestyle habits). This is true for all redox biomarkers since they are sensitive to pre-existing individual status and exercise variables. The longitudinal monitoring of redox biomarkers may provide the necessary information to interpret the functional significance of redox changes (532) and predict an individual's fitness or disease trajectory (39). In order for effective longitudinal redox monitoring, consideration must be given to biological variation in redox outcomes which allows for the calculation of random variation in response to temporal changes (534). Given the vastly heterogeneous nature of basal redox status and redox responses to exercise, individualizing redox responses through calculation of critical difference values will also help in the interpretation of functionally significant redox responses (534). Furthermore, partitioning the variability in physiological outcomes related to basal redox status and redox responses from the variability caused by measurement artifacts and within-subject variability will assist in uncovering the true relationship between redox variables and physiological health and performance outcome variables (587). In the absence of relevant redox reference ranges for specific populations (534), and given the general consensus that redox responses to an exercise stressor and the response time course are more informative than baseline measures (230, 244, 563), implementation of these analytical considerations will help further redox biomarkers within applied practice.

An interesting hypothesis for future application of redox monitoring is to address exercise tolerance in the context of exercise participation. Exercise intolerance is the inability to perform exercise at a normally expected level and is associated with negative perception of exercise and significantly more negative exercise side effects such as excessive fatigue, post-exercise pain, and nausea which reduce exercise participation (591, 592). Evidence from clinical populations suggests that increased oxidative distress resulting from genetic mutations or disease progression contributes to exercise intolerance by inhibiting both vascular and skeletal muscle function (10). In clinical populations characterized by increased oxidative distress, antioxidant supplementation improves exercise tolerance via enhancing

vascular and skeletal muscle function following reduced oxidative distress (588). Given that sedentary and/or lifestyle habits may gradually drive redox status towards a state of oxidative distress (39), it is possible that increasing oxidative distress throughout the lifespan, in the absence of disease, also contributes to exercise intolerance in adults and aging populations. Improving exercise tolerance by reducing oxidative distress in non-clinical populations may assist in reducing the negative perception and side effects of exercise, ultimately leading to superior exercise adherence following that a positive affective response to acute exercise leads to greater motivation to exercise, greater self-efficacy, and more sustained motivation over time (589). There is already evidence in non-clinical populations with specific redox deficiencies that targeted antioxidant supplementation helps improve exercise tolerance and performance (165), whether or not this subsequently leads to more motivation and better compliance with consistent exercise over time remains to be seen. Since motivation is one of the primary reported barriers to exercise (alongside self-efficacy) (590), efforts to improve the positive affective response by targeting exercise intolerance via redox status may be a novel and relatively simple public health strategy that increases engagement with voluntary exercise. This hypothesis is purely speculative but given the reported difficulties in maintaining motivation over time (590), and failure of strategies like education (564) to maintain exercise motivation and adherence, it is clear additional strategies are warranted.

The monitoring of redox status is unique in its scope with both mechanistic and clinical application (citation). The molecular cross-talk and relationships between redox biology, exercise, and health is just beginning to be unraveled and certainly many more discoveries are expected in the coming years. Moving forward, novel redox biomarker discovery provided by omics based analyses will likely provide new targets for monitoring in aging, disease, and exercise that can provide more robust insights into redox homeostasis and physiological status. Exercise redox proteomics will also help unravel the vast and diverse array of oxidative post-translational modifications that occur following exercise and further mechanistic understanding of the beneficial health effects of consistent and appropriate exercise. In the absence of novel redox biomarkers or advanced laboratory techniques, significant value can still be obtained from existing redox biomarkers by applying recommended statistical and analytical approaches to individualize and contextualize redox biomarkers to the heterogeneous redox landscape. Given the extensive physiological processes related to redox biology, there is significant scope for novel hypotheses that test the relationship between redox status determined by existing redox biomarkers and physiological outcomes, such as the use of antioxidant supplementation to improve resting redox status and positive affective responses to exercise. To effectively move the monitoring of redox status forward and connect its clinical and mechanistic scope is an exciting scientific challenge that will require interdisciplinary work from exercise physiologists, biochemists, molecular biologists, clinicians, biostatisticians, and bioinformaticians.

#### 4. Conclusions/Future Directions

Redox homeostasis is the “golden mean of healthy living” (566) and its role in health and disease is unequivocal. The monitoring of redox homeostasis and transient redox status is therefore of paramount importance for human health. From a cellular to a systemic level, it is clear that redox homeostasis is a highly organized and tightly regulated physiological system that promotes specific phenotypes. The importance of exercise in maintaining health and optimizing physical performance is also unequivocal, however, many of the mechanisms underpinning the health and performance promoting effects of exercise

are still unknown. Despite knowledge that exercise increased reactive species and resulted in specific redox perturbations for more than 30 years, only recently has the redox landscape been appreciated as an integral part of exercise physiology (citation). Current evidence suggests that many of the health and performance promoting effects of exercise are governed by transient changes in redox homeostasis within cells and specific cellular compartments. The overall physiological adaptation and expressed phenotype to both exercise induced oxidative eustress and disease/aging oxidative distress results from redox homeostasis: the balance of reactive species production, antioxidant activity, macromolecular damage, repair of reversibly oxidized macromolecules, and oxidation of amino acid residues for cell signaling. However, relating information from low-level biological organization to high-level phenotype changes is a challenge. Current redox biomarkers provide snapshots of systemic redox homeostasis which can provide valuable information regarding the progression/severity of disease (citation), the trajectory of an individual's health in the absence of disease (citation), and functional performance information regarding fatigue status (citation), and overtraining (citation). Unfortunately, laboratory expertise and vast inter-individual variability limit their implementation and interpretation. Still, redox biomarkers have potentially vast utility beyond these aforementioned functions including the potential to develop pathology-specific exercise prescriptions that induce discrete transient redox perturbations that maximize beneficial redox adaptations to treat and slow the progression of specific disease. Similarly, characterizing the magnitude and signature of oxidative eustress for performance adaptations such as VO<sub>2</sub> max and muscle hypertrophy may provide the necessary information to individualize exercise prescriptions providing the optimal exercise workload for a desired outcome. Pre-clinical longitudinal monitoring of redox status will allow for early intervention of both exercise and other lifestyle habits aimed at reducing oxidative distress, improving health, and slowing aging, potentially saving millions in health care costs. The research is clear on the significant translational potential that exists regarding redox biology and when fully developed redox monitoring may provide the means to fully individualize exercise as medicine and achieve the "Golden Mean."