Urinary biomarkers of physical activity: candidates and clinical utility

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Abstract / Summary: (122 words)

Chronic physical inactivity is a major risk factor for a number of important lifestyle diseases, while inappropriate exposure to high physical demands is a risk factor for musculoskeletal injury and fatigue. Proteomic and metabolomic investigations of the physical activity continuum, extreme sedentariness to extremes in physical performance, offer increasing insight into the biological impacts of physical activity. Moreover, biomarkers, revealed in such studies, may have utility in the monitoring of metabolic and musculoskeletal health or recovery following injury. As a diagnostic matrix, urine is non-invasive to collect and contains many biomolecules which reflect both positive and negative adaptations to physical activity exposure. This review examines the utility and landscape of biomarkers of physical activity with particular reference to those found in urine.

Key Words: Urine, Physical Activity, Biomarker, Proteomics, Metabolomics

Introduction

Throughout human evolution regular moderate to vigorous physical activity was essential for the maintenance of relative health through the provision of adequate access to nutritional sources, avoidance of physical threats and protection of territory. Today, poor health and injury, that occur as a result of too little, too much or otherwise inappropriate physical activity, represent major obstacles to the future economic security of most modern societies [1-5]. It is now clear that chronic exposure to physical inactivity (sedentariness) is a major risk factor for lifestyle diseases (Figure 1) including cardiovascular disease, type II diabetes and obesity-related disorders including a number of highly prevalent cancers [1,6-13]. Indeed, physical inactivity is now responsible for 6% of deaths globally [2]. Conversely, inappropriate prescription of exercise such as exposure to high physical demands is a major risk factor for musculoskeletal injury [3-5,14]. Importantly, in these contexts, an individual's response to physical activity (or lack of) is determined by their genome (predisposition) and its potential disturbance and adaptation relative to exposure (i.e. frequency, intensity and duration) to the physical activity continuum (extreme inactivity to extreme activity; Figure 1). The integrated interaction between the genome and the physical activity continuum results in constant alterations to gene expression; protein abundance and post-translational modifications; and, metabolite flux in almost every organ system in the body [15]. The increasing application of proteomic methodologies has been successful in the analysis of protein changes and providing new insights into cellular mechanisms [16]. Such insights have the potential for early detection of chronic disease conditions which is critical as a tool for primary prevention and evaluation of the efficacy of clinical interventions. Thus, understanding and monitoring these processes in order to manage exposure to the continuum of physical activity may play a potentially important role in health maintenance, injury prevention and recovery strategies.

Currently, physical activity is typically monitored through self-report or by utilisation of devices for measuring heart rate, blood pressure, number of steps, or metabolic energy expenditure etc. While these provide important information regarding the perception of effort and / or actual physiological responses

to given levels of activity, there is growing interest in developing non-invasive biomolecular measures of activity level. The hypothesis being that such biomarkers could provide clinically valuable information about the beneficial or detrimental responses to particular types and intensity of physical activity.

The search for physical activity biomarkers, typically in sport, has focussed mainly on blood plasma or serum as the diagnostic matrix. However, from an end-user perspective it is important to understand that people interested in monitoring their biological response to physical activity are unlikely to be willing to provide blood samples on a regular or semi-regular (daily or weekly) basis. Urine, on the other hand, is non-invasive to collect and pathways to utilisation of urinary diagnostics are already established for a number of urinary diagnostic modalities such as the pregnancy test and for drug testing in elite sport or on large industrial worksites. In this regard a key driver for the utilisation of urine as a diagnostic matrix for monitoring of the biological impact of physical activity is simply one of end-user-uptake.

Recent trends in biomarker development have moved toward utilisation of multiple markers to improve diagnostic sensitivity and specificity. This is also the case for the development of diagnostics of physical activity which extends along the continuum between extreme sedentary behaviour and extremes of human physical performance (Figure 1). For example, large scale data acquisition and systems biology approaches have recently been utilised to monitor over all wellness and health generally [17,18] and will no doubt continue to be developed into the future.

This review will outline the findings from recent studies which have used proteomic and / or metabolomic techniques to investigate biomolecular responses to physical activity and physical inactivity in human subjects (unless otherwise specified). The potential utilisation of these techniques as a tool in the analysis of modifications in protein profiles associated with physical activity and its impact on health and chronic disease will also be discussed.

Biomolecular profiling in MS-based omics

The birth of proteomics corresponded with the proliferation of computational and MS-based technologies in the post-genomic era. During this period it was clear that the number of genes did not coincide with the number of gene products observed in nature. As such the combination of gene and protein sequence databases along with increased computational power brought about the introduction of peptide sequence database searching, also termed "bottom-up" proteomics. Bottom-up workflows attempt to identify and measure the amount of a protein based on its constituent peptides. These peptides are produced through enzymatic digestion in a laboratory. The discontinuous nature of bottom-up workflows can be disadvantageous. Primarily because they require complex and computationally intensive analysis methods along with expert interpretation of results. For example, consideration in the handling of shared peptides between different proteins or understanding which subsets of proteins carry post translational modifications on a particular moiety requires an understanding of 1) appropriate analytical workflows, 2) the peptide modelling process and ultimately protein assignment, two crucial components in bottom-up workflows. Alternatively, there are proteomic methods that assess intact proteins or poly-peptides. These workflows remove some of the difficulties associated with the peptide spectrum matching procedure in bottom-up pipelines, but have caveats of their own. Top-down methods require specific analytical platforms such as MALDI as well as the applications of specialised fragmentation methods e.g. electron-capture-dissociation (ECD) or electron transfer dissociation (ETD). Furthermore, specific biologically relevant proteins can be accurately measured using targeted proteomic methodological approaches, the caveat being that the biological target of interest needs to be known prior to assay design.

A lot of these pitfalls have been and continue to be studied and addressed. The current difficulties facing the "omics" community are in the experimental design and analytical methodology post

acquisition primarily. This has come about due to the incredible amounts of information obtained from a single LC-MS acquisition. Certainly the field is mature enough that these methods are able to be accurately applied to a range of scientific questions and biological samples.

Urine as a diagnostic matrix for physical activity

Even though sport and exercise physiology research efforts have been the most active in the investigation of biomarkers associated with exercise and physical activity, blood remains the predominant matrix of choice for these studies. However, the invasive nature of blood collection is a key disadvantage [19] and analyte concentrations (proteins / metabolites) have a large dynamic range (approx. 14 orders of magnitude) which can hamper the discovery of useful biomolecular markers [20]. In addition, saliva has been proposed as a non-invasive diagnostic matrix; however, few of the known constituents of saliva reflect musculoskeletal tissue specific responses to physical activity [21].

The most common utilisation of urine as a diagnostic matrix is in the assessment of pregnancy status. Rapid point-of-care technologies are also in routine use for the quantification of leukocytes, glucose, urobilinogen, pH, haemoglobin, specific gravity, ketone, bilirubin and nitrite. In clinical research, the use of urine has mainly focussed on the discovery of biomarkers of kidney disease, allograft rejection following kidney transplantation [22-24] and early detection of cardiovascular disease and cancer [25-28]. While pregnancy testing seeks to report the simple presence or absence of specific analytes, these types of clinical investigations and investigations of the biochemical impact of physical activity are complicated by the need to interpret an increase or decrease of potentially thousands of molecules.

It is now clear that small proteins, along with potential degraded, cleaved or modified protein products, are capable of entering the blood-stream from the interstitial environment following soft

tissue damage. They are then circulated to the kidney with the ability to pass through the renal filtration process into the urine [29]. Within each kidney there are around one million nephrons which carry out the blood processing requirements of the organism. Each nephron consists of a structure called the glomerular apparatus which selectively filters proteins, peptides and metabolites from the adjacent blood source [30] in a manner that favours the filtering of low to medium molecular weight (MW) constituents as detailed in [31]. Once past the glomerular barrier the filtered components pass through a series of tubules (proximal convoluted tubule, loop of Henle and distal convoluted tubule) where some proteins and metabolites are resorbed by the tubule cells. The remainder of the filtered constituents are excreted into collecting ducts and expelled as urine.

The hypothesis that proteins, peptides and metabolites originating from peripheral tissues, such as muscle, are deposited in the kidneys is supported by the reported incidence of rhabdomyolysis, as a result of crush injury or extreme eccentric exercise induced muscle tissue remodelling [32]. Similarly, the cumulative micro-trauma of musculoskeletal structures due to over-exertion leads to protein degradation and metabolite accumulation which may be excreted via the kidney, into the urine [33]. This excreted low molecular weight (LMW) fraction of urinary protein and metabolite is thought to contain substantial amounts of valuable diagnostic and potential prognostic information [34]. As such, these molecules should be detectable using modern analytical profiling strategies. Indeed, due to several key urinary proteomic studies, the diverse range of proteins and polypeptides which are filtered into the urine by the kidneys is now becoming clear. These include extracellular, plasma membrane and lysosomal proteins, along with an abundance of soft tissue-specific polypeptides [35-39].

These findings support the potential for using urine as a source of protein, peptide and metabolite biomarkers which will reflect the natural homeostatic flux of soft tissue turnover during tissue

loading scenarios associated with physical activity and impact of activity or inactivity on the progression of chronic disease states.

Application of urinary physical activity markers

Sport science has the most comprehensive experience in the use of biomarkers for the evaluation of physical activity (typically in blood) in elite athletes. The monitoring of biomarkers in athletes is an attempt to optimise training thresholds to achieve a 'functional over-reached' state, in order to promote positive adaptation and increased performance following rest; and to prevent development of a 'non-functional over-reached' state which is a consequence of intense training leading to decrement in ability even with adequate rest [40]. In particular, the monitoring of recovery from intensive training is likely to be of particular importance to coaching staff for effective programming of training regimes and schedules to better emphasise positive adaptation [41]. This is critical as extreme non-functional over-reaching is associated with overtraining syndrome [42]: the long term impairment of performance even with adequate rest between training sessions. The syndrome is characterised by depression, fatigue, sleep disturbances and hormonal alterations and has also been demonstrated to result in tissue catabolism [43]. However, the most common means of evaluation of an athlete's need for adequate recovery is by psychometric instruments in the form of questionnaires, since this approach provides rapid diagnostic feedback [41]. However, a recent review by Finsterer (2012) suggests that molecular biomarkers may be indicative of over-reaching states. Such markers include protein carbonyls, glutathione and thiobarbituric acid-reactive substances. In addition to these, the review also indicated that markers derived from ATP and purine metabolism, serum lactate, ammonia, isoprostanes, glutathione peroxidase, catalase, interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) had all been associated with various levels of exercise in both trained and normal or sedentary control subjects [44]. With the exception of the isoprostanes the rest of these molecules were almost exclusively investigated in serum. While monitoring of biomarkers of muscle injury or fatigue using serum samples may have utility at the elite sport level it

is more likely that such markers will be broadly utilised in sub-elite sports and workplace environments if measurements can be made non-invasively such as in urine, saliva or sweat.

Irrespective of the setting various protein structures are affected during the soft tissue damage process and throughout the repair and tissue remodelling process [33]. Therefore it may be possible to detect fluctuations in these proteins, degradation products or metabolites in the early stages of injury in order to pre-emptively introduce interventions designed to prevent progression of the injury toward a catastrophic failure in both sport and the workplace.

Previous research indicates that the manifestation of work-related musculoskeletal disorders (WR-MSDs) involve many factors [14]. With this in mind, the momentum gained through the identification of single markers is now being re-directed to techniques that enable investigation of multiple markers. The combination of multiple markers in combination with a systems approach to analysis in order to assess a given trait affords a far more powerful approach with an increased ability to discriminate between experimental subgroups (e.g. diseased / injured from control). Thus urine analysed using biochemical profiling strategies may potentially reveal biochemical markers that are present due to soft-tissue damage, which will aid in a greater understanding of the patho-mechanisms of musculoskeletal injury, along with repair, and their relationship to the onset and incidence of WR-MSDs and over-training syndrome in athletes. These same markers may also have application in the analysis of inactivity.

It is clear that chronic physical inactivity results in significant pathological maladaptation and disruption to homeostasis [15]. The physical demands of daily activities contribute substantially to an individual's ability to maintain biochemical homeostasis. Biomarkers of this biochemical range may be considered as informative of basal health – a reflection of the requisite minimal activity for acceptable wellbeing. Such markers would be influenced by the frequency, duration and intensity of

activities required for individuals to maintain homeostasis. A combination of these basal and sub-basal activity markers would be useful in the quantification of an individual's engagement in beneficial activity or indeed their individual biological response to inactivity. Given the establishment of sufficient health data, individuals could be prescribed, or self-prescribed, specific frequency duration and intensity of physical activity to maintain internal biochemistry within homeostatic, and importantly, disease-preventing margins. Exercise & Sport Science Australia (ESSA) recently launched the Exercise is Medicine® (EIM) Australia campaign [45]. This campaign is an international initiative of the American College of Sports Medicine in which clinicians are encouraged to design exercise into treatment plans for all patients [46-50]. The aim is to improve the overall health and wellbeing of the public and thereby in the longer term reduce the cost burden to health care systems [51]. Thus clinicians might use biomarkers of physical activity to evaluate the physiological impact of prescribed exercise or activity regimes on their patients in a similar way that they currently monitor the impact of drugs when treating various diseases.

Biomarkers of Physical Activity

The literature is a rich source of candidate biomarkers associated with a variety of physical activity types and interventions (Figure 2A; Supplementary Table S1). Cumulatively, this landscape is dominated by metabolites, chiefly identified in serum, followed by those identified in both serum and urine, urine alone and those found in other sample types (Figure 2A). The detection overlap between urine and serum metabolites is difficult to gauge from the overall biomarker catalogue due to an experimental bias towards serum analyses; however, two studies conducted by the same investigators demonstrate both the overlap and complementarity of urine and serum metabolite detection following a short, intense interval training intervention [52,53] (Figure 2D).

In general, many of the physical activity markers are components of diverse metabolic pathways (Supplementary Figure S1). As such these markers may provide limited insight into the detail of

physical activity. This is evident in the cumulative metabolite data where each of the 20 common amino acids has been separately associated with physical activity in at least one study (Figure 2B; Supplementary Table S1). This suggests that physical activity may generally alter amino acid abundance. Such a finding, while valuable, has limited utility in isolation. This likely extends to many of the other markers which are frequently reported in the literature (Figure 2A). However, a portion of the metabolite biomarkers are not associated with classical metabolic pathways and may therefore be found to be informative of more specific disorder or disease with the generation of sufficient data in the future.

Importantly, analysis of the biomarker catalogue demonstrates that a range of proteins have been identified, in urine and serum, following physical activity intervention (Figure 2A). Ontological analyses of these proteins provide information that is relevant to tissue structures and the molecular functions that are consequential to the physical activity (Figure 2C). In this regard protein biomarkers may provide additional insight beyond the activation or dysregulation of metabolic pathways; however, this depends on the degree of prior knowledge available for a particular protein.

One of the major factors that may influence the value of any particular marker lies in knowledge of its origin in the body. In this respect, there are two classes of biomarker relevant to monitor soft tissue flux, defined as systemic (indirect) and tissue specific (direct) biomarkers. Direct markers originate from the affected tissue itself and provide information on irregular anabolic or catabolic events within the tissue under investigation. Indirect markers on the other hand may be components that are not necessarily derived from the tissue itself, but are involved with its metabolism or homeostasis. These are generally systemic markers, and as such, are involved in multiple pathways within the body. These biomarkers will tend to change in abundance as a result of a range of internal or external factors which may not be directly related to exercise. For example, inflammation can be present due to over exertion but can also be present as a result of infection.

The following text explores a cross section of indirect and direct biomarkers and their roles in metabolism and physical activity.

Indirect biomarkers of physical activity

Acute Markers of energy metabolism during exercise

Multiple energy pathways are coordinated during exercise to provide requisite adenosine-triphosphate (ATP) supply, including the phosphagen, glycolytic and oxidative pathways. The activation of any one pathway is dependent upon the system's need, which is directly related to the exercise protocol under observation. Consequently, metabolomic studies using both serum and urine have identified markers associated with these energy production pathways. Some of these markers include adenosine-diphosphate (ADP) [54], ATP [54], creatinine [53,55-60] and creatine kinase (CK) [61-65]. Importantly, the initial supply of ATP is generated via the phosphagen / phosphocreatine pathways which involve utilisation of phosphocreatine (CrP) to convert ADP into ATP via a CK catalysed phosphorylation event which also results in creatinine production: CrP + ADP $+H^+ \rightarrow$ ATP + Cr. This is important because investigators are inconsistent with respect to the use of creatinine as a normalisation strategy in urinary 'omics' study designs. While some groups have demonstrated no differences in creatinine levels related to exercise [55], others report large increases in this biomarker due to short term intensive exercise protocols [66]. Serum creatinine has also been shown to increase after strenuous aerobic exercise and to correlate with an increase in urinary creatinine levels [67]. Irrespective of these findings creatinine levels seem to be largely dependent on the particular physical exercise routine employed, diurnal variations, diet as well as muscular mass [68-70]. Such findings demonstrate a requirement to unify the approach to sample normalisation in urinary omics investigations.

When the energy requirements of the system are at a level that is greater than the phosphagen energy pathway can sustain, the system switches from aerobic to anaerobic metabolism through the

glycolytic pathway. This involves the utilisation of carbohydrates and the production of both pyruvate and lactate which both show marked increases in the urine of participants who have undergone aerobic exercise using an ergocycle at 75% of $VO_{2\ max}$ [55]. Interestingly lactate is well known to interfere in metabolomic studies, particularly when using nuclear magnetic resonance spectroscopy [71]. As such, resonances associated with lactate are often removed as they can influence the downstream analysis.

Citrate is another energy pathway metabolite that has been identified as a marker of physical activity [52,53,60]. This metabolite is involved with energy conversion from carbohydrates, fats and protein via the Krebs cycle as part of the aerobic energy system. It is a commonly cited metabolite in studies investigating exercise and has been found to differ significantly from baseline measures under a range of exercise schedules including strength and endurance training [72] and structured sprint training regimes involving altered rest intervals [52]. Interestingly, fumarate and 2-oxogluterate were also shown to increase in the urines of the same study, thus highlighting the involvement of the aerobic pathways during exercise.

Glycerol is a further energy pathway-specific metabolite that has been associated with physical activity [53,60]. Glycerol is irreversibly liberated through the lipolysis pathways [73] and has been previously found to reduce in concentration 60 min post exercise in plasma samples when compared to mid-exercise samples [60]. Additionally, this observation has been corroborated by others who found increasing levels from baseline following resistance exercise [74,75]. Interestingly, Lewis *et al.* (2010) hypothesised that glycerol might be indicative of general 'physical fitness' based on its lower levels within unfit participants resulting in impaired lipid utilisation as a response to exercise [60].

In terms of amino acid metabolism, Pechlavanis *et al.* (2013) observed a reduction in serum levels of the branch chain amino acids (BCAA) leucine, isoleucine and valine as a result structured sprint

interval training regimes [53]. The authors attributed this to an increased uptake of BCAAs in muscle tissue due to the tissue's propensity to degrade these amino acids during exercise [53]. Interestingly, they also observed a reciprocal increase in the downstream products of this BCAA catabolism in the urine samples of the participants. These products included 2-hydroxyisovalerate, 2-oxoisovalerate, 3-methyl-2-oxovalerate, 3-hydroxyisobutyrate and 2-oxoisocaproate [52]. Importantly, the author's previous work clarified that this observation is not a result of decreased branched-chain 2-oxoacid dehydrogenase activity but rather the increase in BCAA transamination leading to increased BCAA catabolism [52]. This observation highlights the importance of both observing metabolite abundance changes and understanding the relevant underlying protein biochemistry, although these proteins may not be biomarkers themselves.

Protein-based biomarkers have generally been observed in serum samples or tissue biopsies, and, to a lesser extent in urine samples. C-reactive protein (CRP), IL-1β, TNF-α and IL-6 are typical examples of indirect protein biomarkers of physical activity which have been specifically associated with musculoskeletal disorders (MSDs). In particular the serum concentration of CRP was observed to have a strong correlation between upper extremity overuse disorders while IL-1β, TNF-α, and IL-6 displayed moderate correlations [76]. IL-6 have also been found to be elevated following a series of muscle contraction-based studies, while α-actin has been observed to significantly increase within the plasma of individuals who had sustained musculoskeletal damage [77,78]. Furthermore, serum levels of lactate dehydrogenase (LDH), an enzyme that converts lactate to pyruvate under anaerobic conditions, has been used as an indirect indicator of MSD [79].

Fatty acid binding protein (FABP) has repeatedly been shown to increase following strenuous exercise. Indeed, FABP is suggested to be a better indicator of muscular stress than CK in a single training session due its immediate response following an exercise intervention [80]. This response appears to peak between 2 and 6 hours following exercise and eventually returns to basal levels

within 24 hours [65,81]. Typically it is measured in plasma but FABP can also be detected within the urine of healthy subjects making it more accessible for routine analysis [82].

The protein post-translational modifications ubiquitinylation and sumoylation have also been discussed as potential indirect biomarkers of physical activity, particularly in musculoskeletal injury based studies [83]. Ubiquitin plays a key role in targeted protein degradation [84] and may provide insight into the onset of increased musculoskeletal tissue catabolism [83,85]. Various oxidative stress biomarkers have also been investigated in studies looking at athletes [72], and have been suggested for application in a workplace context. Due to their lack of tissue specificity, these biomarkers can be considered indirect indicators of physical exertion [86-88]. Further detail of oxidative stress markers will be discussed below.

Direct markers of physical activity

Markers of physical activity-induced tissue flux

Tissue adaptation associated with muscle contraction will not only produce aberrations in skeletal muscle tissue but also adjacent connective-tissue structures. This includes the perimysium structures which package multiple muscle fibres, the endomysium which surrounds individual muscle fibres, and the outermost layer of epimysium tissue which is integrated into tendon as it becomes more collagenous. Thus physical exercise and overexertion injuries will alter the homeostasis of a range of soft tissues. Traditionally, analyses of protein degradation and turnover have incorporated the measurements of urea nitrogen [89,90], creatinine [66] and ammonia [91]. The drawback with measuring these variables, like the other indirect biomarkers discussed above, is that they provide little information regarding the type of tissue that is involved in the exercise / damage or the degree of damage. With this in mind, biochemical markers that are tissue specific would appear to hold more promise as a potential monitoring technology.

Collagen remodelling

In serum, type-1 collagen markers carboxy-terminal propeptide (PICP) and carboxy-terminal telopeptide (CTx) have been used to monitor the influence of load bearing exercise on the body [92]. In addition, these two markers have been used to compare the physical exertion of construction workers to that of sedentary workers [33]. It was found that alterations in serum concentrations of PICP and CTx appeared to be related to heavy physical work [33] highlighting their potential application to monitoring of collagen turn-over. Importantly, these two carboxy-terminal peptides of collagen are able to pass through the kidney and into the urine where they can be readily measured.

Post-translationally modified amino acids are commonly observed within the musculoskeletal system. Thus in addition to PICP and CTx, 5-hydroxylysine - which is a derivative of lysine that has undergone hydroxylation - is a target of interest in exercise related areas of research. 5-hydroxylysine is a major component of collagen and has been utilised previously as an indicator of collagen break-down. For example, Tofas *et al.* (2008) measured serum levels in a control and exercise group who underwent 96 plyometric box jumps to assess tissue loading and subsequent collagen breakdown. The authors observed an increase in serum levels after 48 hours which is thought to be due to an exercise-induced inflammatory response [93]. Additionally, Brown *et al.* (1997) reported a significant increase in urinary 5-hydroxylysine following voluntary eccentric actions of the knee extensors [94]. Similarly, hydroxyproline is specifically hydroxylated to stabilise and influence the structural arrangement of the triple helix of collagen. In models of connective tissue damage hydroxyproline has been shown to increase in both concentric and eccentric modes of contraction above the levels of age-matched controls [95]. Like hydroxylysine, hydroxyproline can also be readily assayed in urine and has been shown to increase following a bout of eccentric contractions [94].

Muscle remodelling

Two commonly used direct markers of muscle catabolism are 1-methylhistidine and 3-methylhistidine which are modified versions of histidine containing a methyl group ($R\text{-}CH_3$) located on the imidazole ring. This post-translational modification to histidine is specific to the muscle contractile proteins, actin and myosin. Moreover, urinary levels of 1 / 3-methylhistidine have been found to be increased in abundance following a range of exercise / physical activity parameters [52,54,55]. However, care must be applied when interpreting such results as dietary intake of meat can also influence urinary abundance of these biomarkers [96]. Elevated blood levels of myoglobin (Mb), the oxygen transporting protein found specifically in muscle cells, has also been discussed as a possible indicator of MSD [97]. Another common marker used to indicate muscle damage is CK which is involved with the regulation of intracellular ATP levels, particularly in tissues with high energy demands [98]. When muscle tissue is compromised the CK can 'leak' from muscle cells into the circulation. The CK enzyme consists of two subunits which reflect tissue specificity, either brain type (B) or muscle type (M). The CK-BB isoforms are expressed at low levels in many tissues whereas damaged skeletal muscle predominantly expresses the CK-MM isoforms (over 90%) and the CK-MB isoforms at <1%. Injured cardiac muscle releases about 25% of CK as CK-MB isoforms and 75% as CK-MM isoforms. Thus the relative abundance of circulating CK- MB isoforms have been used extensively as a clinical biomarker of myocardial infarction [99]. The CK-MM isoforms have been used extensively in serum samples as a direct marker of skeletal muscle damage [100]; however, it has not been described in urine samples as being associated with muscle damage.

Inflammation promotes tissue flux

The metalloproteinase family of zinc dependent endopeptidases have been shown to play a role in extracellular matrix homeostasis, along with injury repair and remodelling [101]. Recently the temporal expression of matrix metalloproteinases (MMPs) has been illustrated in and around muscle cells. For instance MMP-9 has been shown to be up-regulated following a single bout of exercise

(non-damaging), while MMP-2 and MMP-14 were elevated after 10 days following repeated bouts of exercise [102]. Along with MMP-2, -9 and -14, skeletal muscle tissue also contains MMP-3 and -7 [101]. Typically MMPs are related to ECM remodelling during normal tissue turnover, however, it has been demonstrated in humans that collagen metabolism is significantly influenced directly following eccentric exercise [103] indicating that MMPs play a role post injury during the remodelling phase.

Aside from MMP activity, other proteases may also be active following damage. The intracellular release of Ca^{2+} into skeletal muscle tissue of rodents has been well characterised following muscle damage in mice [104,105], and it is thought that this early release of Ca^{2+} into skeletal muscle tissue is a result of sarcolemmal ruptures. This has subsequently been shown to up-regulate calcium dependent proteases, notably calpain activity in human quadriceps following eccentric muscle damage [106]. Importantly, calpain is a protease that is spatially located very close to a range of filamentous muscle proteins at the I and M-bands of the sarcomere [107]. Due to its spatial proximity it has been implicated with the targeted removal of Z-lines from cardiac and skeletal muscle, which implies it may be involved in myofibrillar protein release during muscle damage [108,109]. An increase in Ca^{2+} mediated protein degradation via calpain correlates with the invasion of neutrophils following exercise induced damage in rats [110], in humans neutrophil infiltration is well documented, especially under supra-physiological conditions [111-113]. This suggests that protein degradation products act as damage associated molecular patterns (DAMPS) which attract these neutrophils to the site of injury, in turn causing additional damage themselves. Interestingly, sustained exercise studies such as that performed by Margonis and colleagues (2007) demonstrates an initial positive trend in leukocyte response after week one of the exercise protocol. This trend became significant at weeks two and three as the exercise volume increased [114]. Their work also undoubtedly highlights the large degree of redox biochemistry occurring during prolonged training / exercise which results in lipid peroxidation, protein degradation and protein modification.

Oxidative Stress markers and Post-translational Modifications

Skeletal muscle is a metabolically active tissue. As such its protein constituents are prone to damage through oxidative stress and oxidative damage [115]. Oxidatively modified proteins have a propensity to lose their physiological activity and may as a consequence be sensitive to digestion by endogenous proteases [116,117]. Such modifications consist of carbonylation which occurs through the oxidation of proline, arginine, lysine and threonine residues, or reaction of cysteine, lysine and histidine residues with aldehydes or other reactive carbonyl species [118,119]. In hamster cell lines, others have demonstrated an augmented superoxide release response from within the muscle into the extracellular space which is accompanied by a reversible oxidation of muscle protein thiols [120,121]. Such adaptations in carbonyl derivatives have been reported previously using western blot analysis of rat gastrocnemius muscle tissue following a series of daily 60+ minute bouts of swimming over a period of 9 weeks [122]. Endurance studies have also illustrated an increase in protein carbonyls measured by ELISA within the blood of resistance trained men [123]. Factors influencing the rate of protein modification due to oxidative stress depend on the rate of protein degradation and the degree of oxidative stress [122]. These small proteins and protein degradation products pass from the blood to the urine, via the kidney. Subsequently, levels of oxidatively modified proteins / peptides have been shown to increase in urine samples of ultra-marathon runners across a four day event [122]. However, based on this particular study it is difficult to determine exactly when levels of reactive carbonyl derivatives and nitrotyrosine return to baseline, such information might inform future study designs by allowing estimations of clearance rates. Although, from this evidence it is clear that oxidative markers of physical activity are detectable within urine samples obtained from subjects exposed to physical activity, this area is yet to be truly explored in urinary proteomics with current technologies.

Oxidative modifications to proteins may come about due to the increased presence of metabolic by-products produced during physical exertion, or they may come from an oxidative burst which

contributes to secondary injury following initial invasion by neutrophils and macrophages [124,125]. Neutrophils lyse muscle cell membranes, presumably to begin the repair process by removing unusable cellular debris. This occurs via a superoxide dependent mechanism [126], hence with the invasion of neutrophils there is an assorted increase in superoxides which have been shown to increase muscle cell lysis in rodents [127]. Superoxides can be converted readily into a stronger oxidizing agent, hydrogen peroxide, which can naturally be converted *in vivo* into hydroxyl radicals or under enzymatic conditions to hypochlorous acid by myeloperoxidase, a hemeoxido-reductase which is increased within one hour of eccentric exercise [63]. Additionally, hydrogen peroxide has the ability to induce lipid peroxidation, causing degradation to polyunsaturated lipids which can cause the subsequent rupture to cell membranes [128]. The oxidation of polyunsaturated lipids results in the reactive electrophile bi-product, malondialdehyde (MDA), which is commonly used as an indicator of oxidative stress [129,130] and can be measured accurately in urine samples [131]. Others have correlated increasing abundance of urinary F_2-isoprostane in a healthy male cohort who performed a series of multi-joint exercises at varying degrees of intensity from 'light' to 'over-training' [114]. Additionally, an acute eccentric bout of exercise has also been shown to increase urinary isoprostane levels [132]. These data suggest cell membranes are oxidatively damaged during exercise and possibly following exercise due to leukocyte infiltration.

Clearly muscle tissue is under oxidative assault due to normal homeostasis during high energy tasks and as a result of muscle damage. Such an environment lends itself to an increased incidence of oxidatively modified proteins and subsequently, an increase in protein digestion and degradation due to an oxidation event. As is the case with protein degradation products, these post-translational modification events may provide additional routes to further characterise diagnostic targets of inactivity, exercise and soft tissue damage. Combined with the propensity for musculoskeletal protein to break down following strenuous bouts of exercise, targeting these degradation products

within urine in conjunction with oxidatively modified amino acids within them may hold even more specificity for a particular physical activity or tissue state.

Other markers

We recently found that the urinary concentration of the Laminin G-like Domain 3 or 'LG3' peptide, a C-terminal bioactive fragment of the extracellular matrix-derived protein endorepellin, was elevated in mining workers who were exposed to a more physically demanding work environment compared to more sedentary co-workers [133]. Endorepellin is otherwise known as domain-V of the heparin sulphate proteoglycan, perlecan [134]. Perlecan, in turn, is a major extracellular matrix (ECM) constituent of vascular basement membranes, articular cartilage and neuromuscular junctions [135-138]. Within the latter, perlecan regulates the localisation of acetylcholine esterase in neuromuscular junctions, and is thus critical for proper muscle function [139]. Stress on the microvasculature, muscle tissue and articular cartilage during physical activity results in minor tissue damage and remodelling [140-142]. These processes may induce the release of endorepellin and / or the LG3 peptide from perlecan into the circulation [134,143] where the LG3 peptide may be cleared by filtration through the kidney glomerular membrane into the urinary filtrate. Others have observed that a similar matrix-derived protein called endostatin, which is derived from the C-terminal of type XVIII collagen, is also released into the circulation following vigorous physical activity [144-147]. Endorepellin / LG3 and endostatin have been shown to be anti-angiogenic and anti-tumourigenic, restricting tumour neovascularisation through their interaction with specific integrins on endothelial cells both *in vitro* and *in vivo* [148-150]. This interaction results in cytoskeletal disruption, cell detachment, and inhibition of cell migration and proliferation [151-153]. It is therefore plausible that the acute release of these anti-angiogenic proteins in response to physical activity could limit the blood supply in developing tumours. This may represent a novel and potentially very important biological mechanism contributing to the observed beneficial effects of physical activity on cancer risk or survival from cancer. Certainly others have highlighted the benefits

of exercise in this regard. Matthews *et al.* (2012) found that high levels of physical activity (>42+ metabolic equivalent hours / week) was positively correlated with reduced levels of urinary estradiol and specific estrogen metabolites, particularly in the 2-hydroxylation and 16-hydroxylation pathways. The authors suggest that their observations may be indicative of one of the mechanisms by which physical activity is thought to reduce the risk of premenopausal breast cancer [154].

In addition to protein/metabolite targets, microRNAs and mRNAs have shown the potential to have future outcomes in the domain of exercise physiology. While relative to metabolomic and proteomics this field is still in its infancy, some interesting incites have been made into the differential expression or microRNAs and mRNAs following a range of exercise bouts. Tonevitsky and collegues revealed a dynamic response in microRNA-mRNA following a short bout of aerobic exercise [155]. Likewise Uhlemann *et al.* (2012) showed a significant change in serum miRNA-126 due to aerobic exercise while miRNA-133 appeared to increase following a eccentric muscle damaging protocol demonstrating some degree of exercise dependency [156]. Investigation into the dynamic expression of microRNAs following exercise is an exciting area of research that might yield an important biological understanding. At a systems-level perspective, such information would complement proteomic and metabolomic datasets giving more of a global perspective on exercise physiology and disease.

Overall, the physical activity biomarker landscape is comprised of a suite of molecules observed from across a diversity of physical activities. A large proportion of these markers relate to the increase in metabolic demand that is generally associated with exercise; others however, appear more specific, likely as a result of a direct relationship to a specific activity class. Through further controlled investigations, across the spectrum of physical activity, the roles and utility and value of these markers will become more apparent. The breadth and impact of such investigations are discussed below.

Expert commentary

Technology:

Data acquisition is a key factor in the analysis of biochemistry. The evolution of mass spectrometry (MS) as a discovery technology is at a point where both qualitative and quantitative information surrounding thousands of peptides or metabolites can be measured in a single assay. These advances allow researchers to take more holistic views of multiple biological systems in each study. As such, modern MS technologies and workflows incorporating Data Independent Acquisition (DIA), such as SWATH-MS/MS ^{ALL} [157], will be invaluable for omic investigations of exercise and the emerging field of inactivity physiology now and into the near future. A particular advantage with this type of analysis is that the data can be continually re-interpreted after acquisition and upon discovery of new molecular features, thus demonstrating a new approach to data management.

The exponential increase in the sensitivity of analytical technologies over the last decade has resulted in drastic increases in the size and therefore richness of MS data files. This therefore, presents additional challenges in data handling and storage along with data analytics and informatics. In this regard, multivariate statistical approaches and systems-based analyses continue to be developed and refined to combine and interpret increasingly complex data sets which incorporate multi-omics information including the lipidome and glycome. The complexity of the bio-statistical model is illustrated when considering the convolution of physical activity in combination with the multiple factors involved in determining final outcome including genetic profile, age, lifestyle behaviours, health status and / or exercise prescription to name a few.

Biology:

Molecular biomarkers of physical activity, including those found in urine, will provide an important means of monitoring the biological impact of physical activity and exercise prescription. Given that

these markers have been primarily observed in small sample studies, additional research is required with larger sample sizes to verify current findings and identify stronger relationships between the multiple variables involved.

Research into the efficacy of monitoring exercise-related aberrations in the urinary proteome is relatively limited. This is somewhat surprising considering current knowledge of the propensity for post-exercise musculoskeletal protein degradation and the opportunity to investigate this mechanism using urine. However, when examining the links between the release of biomolecules into urine and physical activity, omics researchers need to recognise the complexity of exercise / inactivity and the difficulty in the determination of optimal and individualised prescription models. These and other exercise-related factors should be carefully considered during study design in order to control for variability and provide more reliable and meaningful outcomes. These studies will clearly add to our knowledge of the biochemical responses to physical activity but they are not without challenges. For instance, exercise intervention studies involving human subjects are often time consuming and expensive to perform. Moreover, there are no relevant *in vitro* models that cover the breadth of co-variates / risk factors known to influence the pathology of tissue adaptation or mal-adaptation. The need to control for physical activity in research must also extend beyond investigations of activity / inactivity.

Physical activity status must be accounted for in disease versus control cohort cross-sectional studies. Through this review we have cite the breadth of research demonstrating the direct influence of physical activity on biochemistry. As such, the discovery of biomarkers which characterise one disease cohort from another may in some instances be attributed to the relative difference in physical activity levels. In addition, because of the significant temporal variance in exercise outcomes and molecular profiles, more emphasis should be placed on the design of multiple time point analyses in exercise intervention studies in contrast to cross-sectional or case study designs.

Renal function must be accounted for when examining urinary levels of proteins or metabolites. Given inconsistency in observations of creatinine response to across a range of exercise studies, urinary analyte normalisation to this metabolite should probably be avoided. In proteomic studies normalising to total protein is a common practice; however, this will likely result in a potential bias since a fraction of the proteome will derive from the blood filtrate and a fraction from the lower urinary tract. If the ratios between these fractions differ between subjects then this may introduce a bias between the two urinary protein sources. Other options include normalising to estimated glomerular filtration rate based on theoretical equations which account for a range of covariates such as age, gender, ethnicity etc. Importantly, such equations still require the measurement of creatinine and typically do not take into account activity status. Another promising method could be to normalise to Cystatin-C which does not appear to be influenced by confounders like muscle mass or energy requirement of muscle tissue in the same way that creatinine is [158].

Utilisation:

Physical activity is a highly effective, non-invasive and inexpensive therapy for the prevention and management of major chronic health conditions. Accordingly, any advances in our understanding of the mechanisms involved in are extremely important. This not only includes the uptake of exercise but the avoidance of inappropriate physical activity which may have adverse effects exacerbated by individual differences in health status such as chronic diseases, medication usage etc. When applied, the potential to determine the effects of various exercise interventions on individual health status will be extremely valuable in monitoring the efficacy of an intervention as well as appropriate modification if required. Therefore, the ability to continuously monitor biological and physiological adaptations to an exercise intervention using non-invasive procedures, such as urine analysis, will reduce the time required for individual participation in the monitoring process and lead to improved compliance.

Chronic health conditions may progressively change over time, being asymptomatic and undiagnosed in the early stages before progressing to more serious disorders. The knowledge generated from urinary proteomic and metabolomic physical activity research has the potential to underpin tools for the early diagnosis and guidance in the early intervention that will avoid the onset of chronic health conditions. Importantly, the very high levels of inactivity in the community are recognised as a major cause of significant chronic health conditions such as cardiovascular disease, diabetes, obesity and cancer. The methodologies being developed that link physical activity and individual biomolecular profiles will play a major role in increasing our understanding of the consequences of a sedentary lifestyle and evaluation and efficacy of strategies to modify this behaviour.

Finally, exposure to risk factors for chronic MSDs can be evaluated by models of muscle and tissue damage. As in other chronic conditions, MSDs generally occur over long periods of time. As such, the early detection of tissue micro-injury and adaptation is highly desirable so that levels of exposure to the harmful activities can be modulated for the prevention of longer term damage. Again, in this respect, urinary biomarker research will provide important tools for the early detection and modification of high risk exposures.

Five-year view:

Research into the molecular processes mediated by physical activity is well established and will continue to be an area of significant research effort. Additionally, the molecular processes underpinning the physiological consequences of physical inactivity will also be a key emerging area of research interest as governments struggle with the increasing healthcare burden of chronic lifestyle diseases. The application of the 'omics' disciplines to understand these molecular processes will continue to provide opportunities for the development of biomarker diagnostics to evaluate the

maintenance of or progression toward health due to prescription of physical activity. As such, there is an increasing prioritisation towards health monitoring versus disease diagnosis. This represents a continuing drive toward preventative personalised medicine of which initiatives such as the 'Exercise is Medicine®' campaign is a clear example.

Looking towards the future, it is unlikely that single target biomarker diagnostics will be developed for particular classes of physical activities. Instead the application of systems biology approaches will identify panels of markers which will offer potential for tissue specific diagnostic classification. Large amounts of data are now relatively easy to generate but having to use analytical software which is restricted to single users or to computers with limited processing power restricts the flow of information arising from increasingly more complex studies. To address this, High Performance Computing facilities will increasingly be utilised in analytical workflows which may mean software vendors need to adopt alternative licensing models. Alternatively, with the increasing uptake of open-source software (e.g. Skyline, Cytoscape etc.) by researchers for data analysis and interpretation, proprietary software may well become increasingly redundant in the near future.

In summary, physical activity and physical inactivity are major factors influencing morbidity and mortality across the globe. Investigations into the biochemistry of the physical activity continuum have demonstrated molecular signatures, in several biofluids - including urine, with potential clinical utility across a range of health contexts. With an increase in the knowledgebase around the biochemical responses to physical activity these biomarkers are sure to become an integral means to prevent injury and promote health in the future.

Key issues:

- Poor physical health threatens economic security of developed nations and this can be largely attributed to inappropriate levels of physical activity.

- Biomarkers of physical activity offer a means of monitoring maintenance of or progression toward health.
- Urine is an ideal matrix for proteomic and metabolomics studies of physical activity.
- Urinary biomarker diagnostics hold potential for broad end-user-uptake due to the non-invasive nature of sample collection.
- Sport science has the most experience in identifying biomarkers of physical activity. Various metabolites and proteins have been identified as biomarker of physical activity or of injury to musculoskeletal structures as a result of over-use or over-training.
- Systems biology approaches will yield panels of markers with increased diagnostic resolution.
- Larger sample sizes are required to provide improved statistical rigour in physical activity studies.
- There remain limitations in technology, analytics and computing power.

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Figure Legends

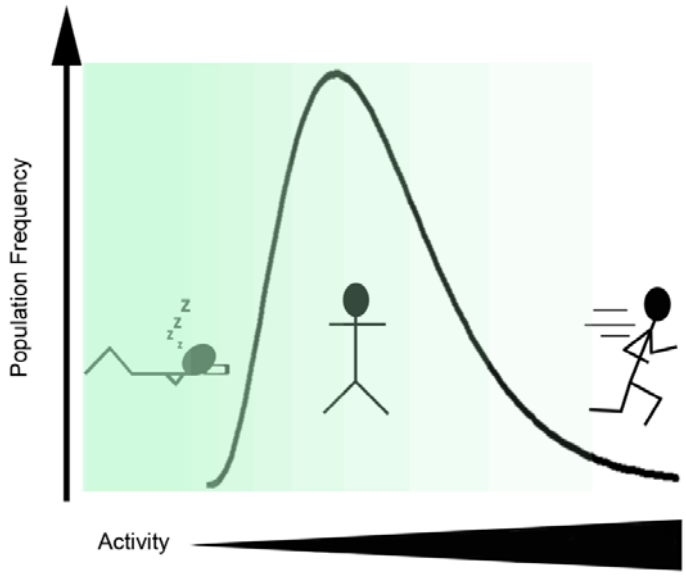


Figure 1. The distribution of physical activity and disease risk. A. The distribution of an adult (>18 years of age) Australian population as distributed by hours of physical activity undertaken. Frequencies are approximated from the 2011-2012 Australian Bureau of Statistics Health Survey: Physical Activity - document 4364.0.55.004 [159]. The distribution captures the very inactive to the very active which forms a Gaussian distribution skewed towards inactivity. Disease risk, as it relates to physical activity [1,6-13], is shown by a colour gradient whereby individuals in the darker region of the graph are at higher risk of morbidity and mortality than individuals in the lighter region of the graph.

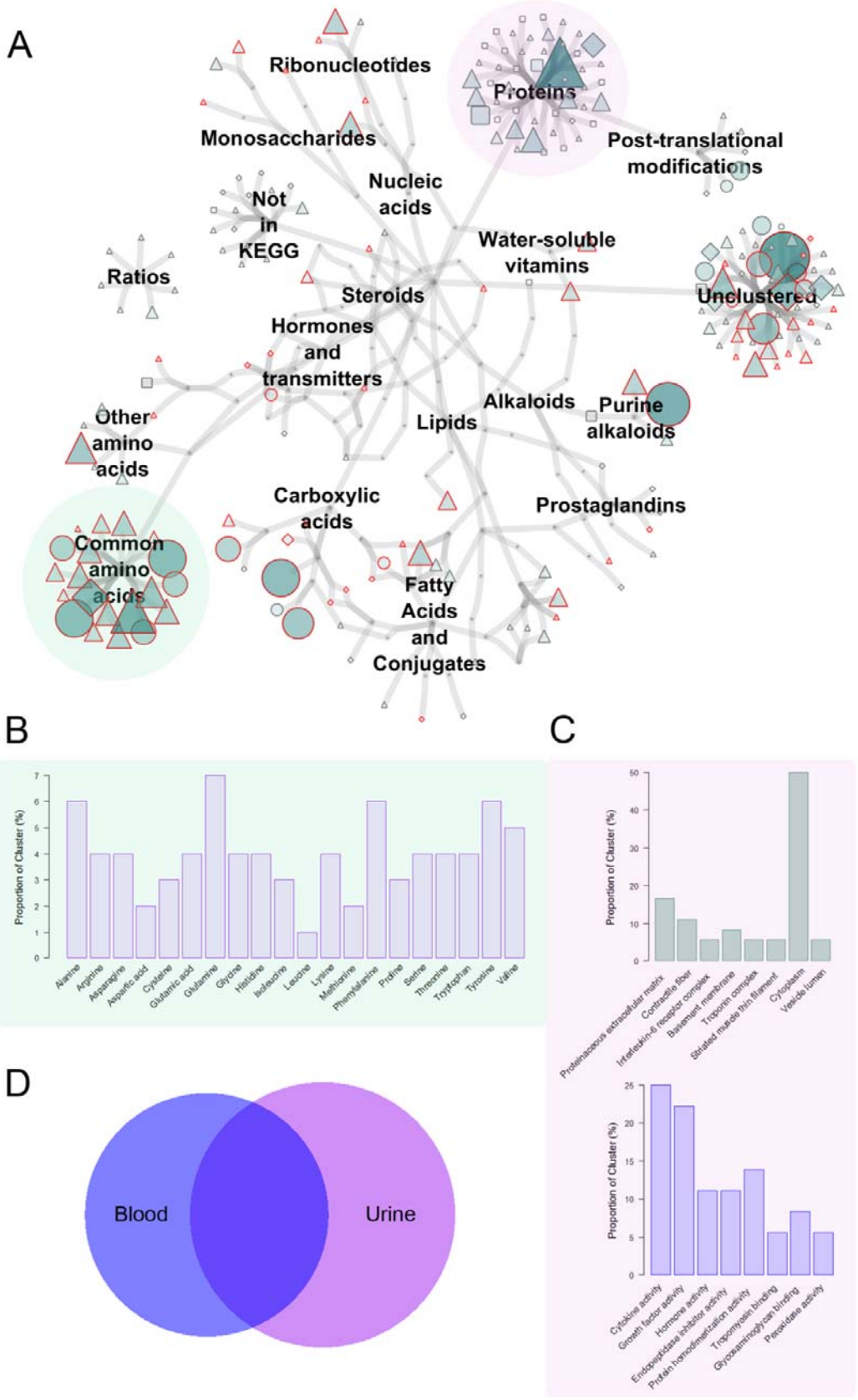
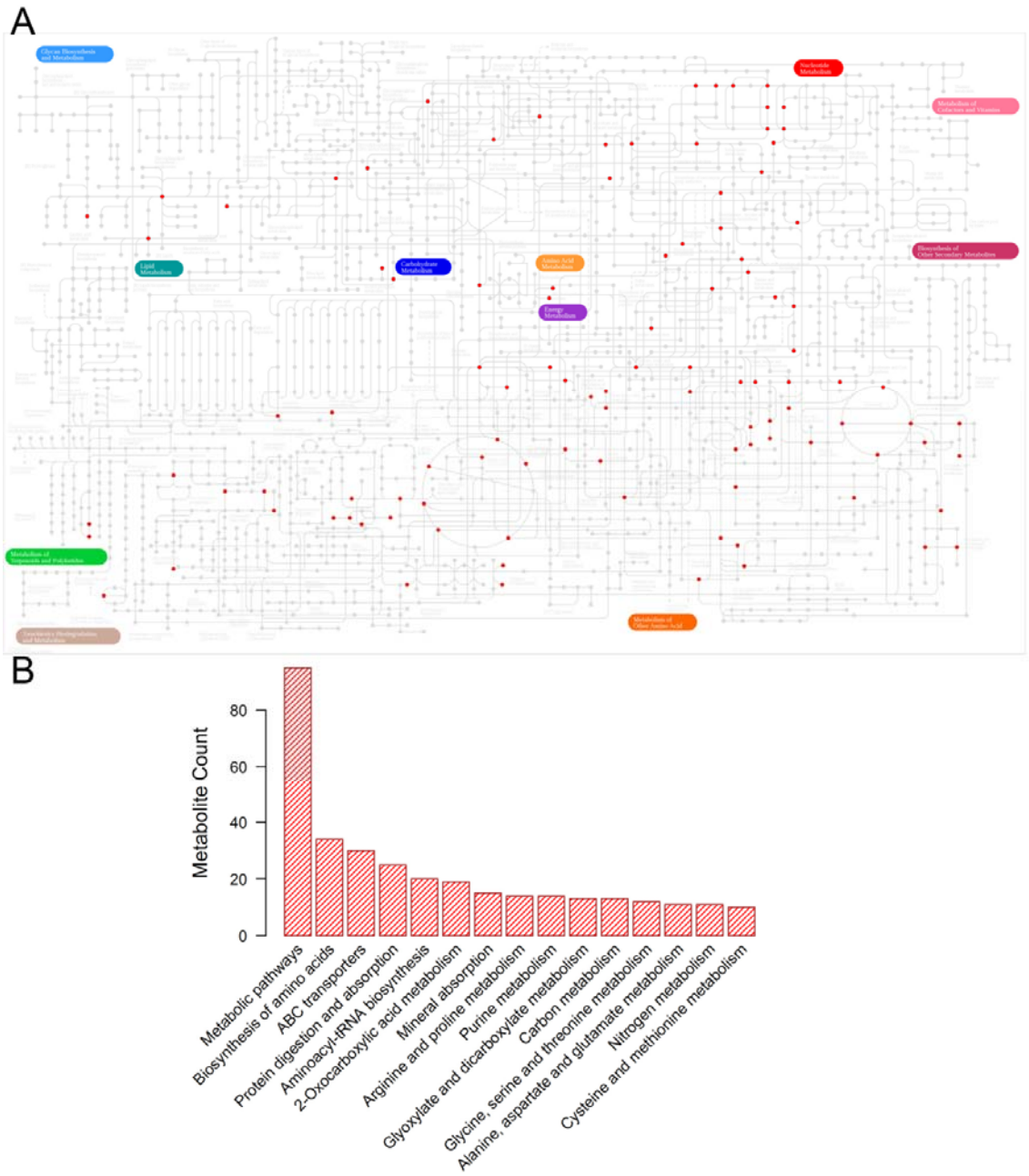

A
Ribonucleotides
Proteins
Monosaccharides
Post-translational modifications
Nucleic acids
Not in KEGG
Water-soluble vitamins
Ratios
Steroids
Unclustered
Hormones and transmitters
Alkaloids
Purine alkaloids
Other amino acids
Lipids
Carboxylic acids
Prostaglandins
Common amino acids
Fatty Acids and Conjugates
B
Proportion of Cluster (%)
Alanine
Arginine
Asparagine
Aspartic acid
Cysteine
Glutamic acid
Glutamine
Glycine
Histidine
Isoleucine
Leucine
Lysine
Methionine
Phenylalanine
Proline
Serine
Threonine
Tryptophan
Tyrosine
Valine
C
Proportion of Cluster (%)
Proteinaceous extracellular matrix
Contractile fiber
Interleukin-6 receptor complex
Basement membrane
Troponin complex
Striated muscle thin filament
Cytoplasm
Vesicle lumen
Proportion of Cluster (%)
Cytokine activity
Growth factor activity
Hormone activity
Endopeptidase inhibitor activity
Protein homodimerization activity
Tropomyosin binding
Glycosaminoglycan binding
Peroxidase activity
D
Blood
Urine

Figure 2. The physical activity biomarker landscape. A. Network graph of 226 unique candidate biomarkers of physical activity based on current evidence generated in Cytoscape [160]. Metabolites are clustered under the Kyoto Encyclopaedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/kegg2.html; [161]) BRITE compound classification hierarchy. Annotated KEGG molecules that are not found in the BRITE hierarchy are shown as "Unclustered" and where absent from the KEGG altogether as clustered under the term "Not in KEGG". Protein-related biomarkers are clustered under "Proteins" and "Post-translational modifications". Small black nodes correspond to the KEGG BRITE Compound hierarchy classifications while molecule nodes, including proteins, are coloured with a gradient of white-through-green and vary in size with darker and larger nodes corresponding to physical activity biomarkers that are observed with higher frequency in the literature. Markers found in urine, serum, both, or neither/unreported are shown as diamond, triangle, ellipse and square nodes, respectively. Nodes with red boarder colour are annotated in the KEGG Metabolic Pathways Global Metabolism map (hsa01100; see Supplemental Figure S1). B. Bar chart depicting the frequency that each of the 20 common amino acids is observed as being associated with physical activity. C. Bar charts showing the enriched cellular component (upper) and molecular function (lower) ontology terms as a percentage of the "Proteins" cluster ($p < 0.01$; Modified Fisher's Exact Test; DAVID Bioinformatics; http://david.abcc.ncifcrf.gov/; [162]). D. Quantitative Venn chart demonstrating the overlap between detected metabolites in urine and serum as demonstrated by the observations of Pechlivanis *et al.*, (2010) and Pechlivanis *et al.*, (2013), respectively, in two cohorts of sprinters with rest intervention. See supplementary information for further details of biomarker classification and analysis.



Supplementary Figure S1. Mapping physical activity metabolite biomarkers to known metabolic pathways. A. Literature-derived metabolite names were manually parsed through the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database using the KEGG BDGET database retrieval form to obtain corresponding KEGG compound numbers. Compound numbers were searched against the KEGG metabolic pathway maps using the KEGG Mapper – Pathway Search which

assigned 95 of a possible 126 metabolites (75%) to the human Metabolic Pathways Global Metabolism map (hsa01100). Physical activity biomarkers are displayed as **red nodes** on the pathway. B. A bar chart showing the KEGG metabolic pathway maps that encompass 10 or more reported physical activity metabolite biomarkers. Cumulatively, 85% of the reported physical activity biomarkers map to known metabolic pathways in the KEGG database (data not shown).

Table Headings

Supplementary Table S1. Physical activity biomarkers reported in the literature.

Supplementary table 1: Physical activity biomarkers reported in the literature

	Biomarker	Sample number	Exercise details	Reference	Biological fluid
1	2-Hydroxyisovalerate	n=12	6 x 80 meter maximal sprint intervals, two block design with either 10s or 1min rest between every other pair of sprints.	[1]	Urine
2	2-Hydroxybutyrate				
3	2-Oxoisocaproate				
4	Leucine				
5	Valine				
6	Isoleucine				
7	3-Hydroxyisobutyrate				
8	3-Methyl-2-oxovalerate				
9	2-Oxoisovalerate				
10	3-Hydroxybutyrate				
11	Lactate				
12	2-Hydroxyisobutyrate				
13	Alanine				
14	Acetate				
15	Acetoacetate				
16	Pyruvate				
17	Succinate				
18	Citrate				
19	Dimethylamine				
20	2-Oxoglutarate				

21	Creatinine				
22	cis-Aconitate				
23	Malonate				
24	Carnitine				
25	Trimethylamine N-oxide				
26	Taurine				
27	Glycine				
28	N-Methylnicotinamide				
29	Glucuronate				
30	Allantoate				
31	Allantoin				
32	Inosine				
33	Fumarate				
34	trans-Aconitate				
35	Tyrosine				
36	Phenylalanine				
37	Hippurate				
38	Tryptophan				
39	Hypoxanthine				
40	Formate				
41	Histidine				
42	1-Methylhistidine				
43	3-Methylhistidine				
44	oxidized glutathione	n=1	Stenuous aerobic endurance cycling. 70% VO2peak for 45 min, was then increased to 90% VO2peak for 6.5 min until exhaustion.	[2]	Blood
45	reduced glutathione				
46	3-methylhistidine				
47	L-carnitine				
48	O-acetyl-L-carnitine				
49	creatine				

50	NAD+				
51	ATP				
52	ADP				
53	D-glucose-6-phosphate				
54	O-propionyl-L-carnitine				
55	tyrosine				
56	arginine				
57	L-phenylalanine				
58	L-histidine				
59	L-glutamic acid				
60	L-glutamine				
61	L-lysine				
62	L-aspartic acid				
63	L-proline				
64	LDL/VLDL	n=14	4 x 80 meter maximal sprint intervals prior to training session and 6 x 80 meter maximal sprint intervals post-training. either 10s or 1min rest between every other pair of sprints.	[3]	Blood
65	Leucine				
66	Valine				
67	Isoleucine				
68	3-Hydroxyisobutyrate				
69	Alanine				
70	Lactate				
71	Acetate/acetone				
72	Arginine/lysine				
73	Glutamate				
74	Glycoprotein acetyls				
75	Glutamine				
76	Acetoacetate/acetone				
77	Proline				
78	Pyruvate				

79	Citrate				
80	Methylguanidine				
81	Albumin lysyl				
82	Creatinine				
83	Malonate				
84	Dimethylsulfone				
85	Choline-containing compounds				
86	Taurine				
87	Trimethylamine N-oxide				
88	Glucose				
89	Glycine				
90	Glycerol				
91	Threonine				
92	Tyrosine				
93	Histidines				
94	Phenylalanine				
95	Formate				
96	2,3-dinor 11-beta-PGF 2alpha	n=15	15 triatheletes. Comparisons made between baseline samples, 2 weeks after intensified training and a recovery period were compared	[4]	Urine
97	2,3-dinor 8-iso-PGF 2alpha				
98	8-iso-PGF 2alpha				
99	Tetranor-PGEM				
100	11-beta-PGF 2alpha				
101	6-keto-PGF 1alpha				
102	Lactate	n=22	Cycling on an ergometer. Short sprints and prolonged cycling at 75% VO2 max until exhaustion	[5]	Urine
103	Alanine				
104	Acetate				
105	Acetoacetate				
106	Pyruvate				

Succinate					
Creatinine					
Malonate					
trimethylamine-N-oxide					
Hipurate					
Hypoxanthine					
MMP-9	n=10	45 min of one-legged exercise with either 20% blood flow restriction or no restriction	[6]	Biopsy
Cartilage oligomeric matrix protein (COMP)	n=58	Supervised weight-bearing exercises of the lower extremity (1 h). Performed at five stations, intensity ≥ 60% of maximum heart rate.	[7]	Serum
malondialdehyde	n=18	30s cycle ergometer test	[8]	Serum
Urea	n=1	Cycling 45 miles	[9]	Urine
Sulfuric acid				
Ammonia				
Phosphoric acid				
Mid region pro-adrenomedullin 45–92	n=12	Double-blind, placebocontrolled study investigating allopurinol effects on soccer players post game (markers increased in placebo group).	[10]	Serum
Growth differentiation factor 15				
Dihydroxy benzoates	n=9, n=17, n of exposures = 92	Open water, closed-curcuit diving	[11]	Urine
C-reactive protein				
N-terminal pro-brain natriuretic peptide				
hs-Troponin T				
Creatinine				
Cystatin C				

129	Aldosterone	n=9	A range of exercises across four days, including Bicycle ride, circuit training, running and calisthenics	[12]	Serum
130	Bilirubin				
131	Aldosterone				
132	Angiotensin II				
133	P68133, Actin, alpha skeletal muscle	n=10	45 min of downhill running	[13]	Muscle biopsy
134	P17661, Desmin				
135	P31930, Cytochrome b-c1 complex subunit 1, mitochondrial				
136	Q15286, Ras-related protein Rab-35				
137	O75112, LIM domain-binding protein 3				
138	P31415, Calsequestrin-1				
139	P68871, Hemoglobin subunit beta				
140	Isoprostanes	n=12	12-week multijoint resistance training	[14]	Serum
141	TBARS				
142	Carbonyl protein adducts				
143	Oxidized glutathione				
144	Reduced glutathione				
145	Glutathione peroxidase				
146	Catalase				
147	c-Telopeptide collagen II, mg/mmol creatinine	n=208	Comparison of levels measured in urine across multiple occupations	[15]	Urine
148	c-Telopeptide collagen I, mg/mmol creatinine				
149	neutrophil gelatinase associated lipocalin	n=16	60 km ultramarathon	[16]	Serum
150	neutrophil gelatinase				Urine

	associated lipocalin				
151	Creatinine				Serum
152	Creatinine				Urine
153	Estradiol	603 premenopausal women	Activity status based on questionaire data. Including type of activity, duration and intensity	Matthews, Endocrine Research, 2012 [17]	Urine
154	2-Methoxyestrone				
155	2-Methoxyestrone-3-methyl ether				
156	16-alpha-hydroxyestrone				
157	16-ketoestradiol				
158	cross-linked N-telopeptide, mg/mmol creatinine	n=76	A range of athletic disciplines including rowing, swimming, running. 16 non-athletic controls.	[18]	Urine
159	c-Telopeptide collagen II, mg/mmol creatinine				
160	8-hydroxy-2'-deoxyguanosine	n=18	60 min of cycling at 70% of VO2max	[19]	Urine
161	Acetone				
162	Propanal				
163	Pentanal				
164	Malondialdehyde				
165	Butanal				
166	o,o'-dityrosine				
167	Carbonyl protein adducts	n=8	4 day supermarathon consisting of 93, 120, 56 and 59 km in each day	[20]	Serum
168	Carbonyl protein adducts				Urine
169	Nitrotyrosine				Serum
170	Nitrotyrosine				Urine
171	Catalase	n=14	“all-out” 30-s anaerobic Wingate test performed on professional judokas	[21]	Serum
172	malondialdehyde				
173	HSP27	n=24	14 sets of 5 repetitions of eccentric actions with the elbow flexors.	[22]	Tissue
174	HSP70				
175	Insulin-like growth factor 1	n=862	Hand grip strength used as a proxy for overall	[23]	Blood

Bioavailable testosterone		strength. Associations made between strength and catabolic/anabolic markers over time		
Dehydroepiandrosterone sulfate				
IL-6				
Catalase	n=20	3 x 20min sesson on cycle ergometer	[24]	blood
superoxide dismutase	n=9	Incremental cycling on ergometer until exhaustion	[25]	Serum
glutathione peroxidase				
hydroxyproline	n=18	96 Plyometric box jumps over 50cm hurdles	[26]	Serum
hydrosylisine				
Alanine	n=28	30 total hours of technical and aerobic exercise across 2 weeks	[27]	Blood
Beta-D-Methylglucopyranoside				
Pyroglutamic acid				
Threonic acid				
Glutamic acid				
Uric acid				
Palmitic acid				
Linoleic acid				
Oleic acid				
Valine				
Phenylalanine				
Glutamine				
Tyrosine				
Omithine				
Butyric acid	n=29	65 min of ergometer cycling at 20 min on 55%, 25 min on 70%, 10 min on 55%, 2 min 'all-out' and final 8 min on 30% VO2max	[28]	Blood
2-Aminobutyric acid				
Valine				
Urea				

202	Phosphoric acid				
203	Isoleucine				
204	Glycine				
205	Picolinic acid				
206	Serine				
207	Threonine				
208	S-methycysteine				
209	Beta-alanine				
210	Malic acid				
211	Erythritol				
212	Pyrrolidonecarboxylic acid				
213	Asparagine				
214	Aspartic acid				
215	Non-identified				
216	Creatinine				
217	Cysteine				
218	Threonate				
219	Ornithine				
220	Phenylalanine				
221	Lauric				
222	Taurine				
223	Asparagine				
224	Ribose				
225	Lysine				
226	Ribitol				
227	Hypoxanthine				
228	Citric acid				
229	Arginine				
230	Tyrosine				

231	D-galactono-1,4-lactone				
232	Beta-Dmethylglucopyranoside				
233	Palmitelaidic acid				
234	Palmitic acid				
235	Inositol				
236	Uric acid				
237	Tryptophan				
238	Linoleic acid				
239	Elaidic acid				
240	Stearic acid				
241	Cystine				
242	Docosahexaenoic acid				
243	Inosine				
244	Gamma-tocopherol				
245	Adenosine-5-monophosphate				
246	Alpha-tocopherol				
247	Cholesterol				
248	Beta-sitosterol				
249	glucose				
250	insulin				
251	hepatonoic acid	n=24	4 x 90 min ergometer cycling at varying workloads,	[29]	Blood
252	Valine				
253	Ethanolamine				
254	Phosphoric acid				
255	Isoleucine				
256	Glycine				
257	Glyceric acid				

258	4-Deoxyerythronic acid				
259	Serine				
260	Threonine				
261	Erythrose methoxyamine				
262	Malic acid				
263	Methionine				
264	Pyroglutamic acid				
265	Hydroxyproline				
266	Creatinine				
267	Cysteine				
268	Threonic acid				
269	Glutamine				
270	Ornithine				
271	Phenylalanine				
272	Lauric acid				
273	Taurine				
274	Asparagine				
275	Ribose				
276	Suberic acid				
277	Lysine				
278	Arabitol				
279	Glycerol-3-phosphate				
280	Citric acid				
281	Arginine				
282	3-Methylhistidine				
283	Fructose				
284	Tyrosine				
285	Indole-3-acetic acid				
286	9-(Z)-Hexadecenoic acid				

287	myo-Inositol				
288	Uric acid				
289	Palmitic acid				
290	Tryptophan				
291	Linoleic acid				
292	Oleic acid				
293	Stearic acid				
294	Cystine				
295	Pseudouridine				
296	Arachidonic acid				
297	Docosahexanoic acid				
298	Inosine				
299	Sucrose				
300	Adenosine-5-monophosphate				
301	Cholesterol				
302	Cholic acid				
303	Lactate	n=70, n=8, n=25	Range of exercises: bicycle ergometry, exercise treadmill testing, Boston Marathon athletes	[30]	Blood
304	Malate				
305	Succinate				
306	Glycerol				
307	Fumarate				
308	Pyruvate				
309	Niacinamide				
310	Pantothenate				
311	Glucose-6-phosphate				
312	Alanine				
313	Inosine				
314	Hypoxanthine				

315	Citrulline				
316	AMP				
317	Isoleucine/Leucine				
318	Serine				
319	Glutamate				
320	Xanthine				
321	Cysteine				
322	Allantoin				
323	3-Phosphoglycerate				
324	Homocysteine				
325	Glutamine				
326	Malate				
327	Citric/Isocitrate				
328	Uridine				
329	Fumarate				
330	Aconitic acid				
331	Lactate				
332	Pyruvate				
333	Hypoxanthine				
334	Xanthine				
335	Methionine				
336	Alanine				
337	Succinate				
338	UTP				
339	Niacinamide				
340	Inosine				
341	Xanthosine				
342	Pantothenate				
343	Cystathione				

344	Glutamine				
345	Hippurate				
346	Uric acid				
347	Ornithine				
348	Allantoin				
349	Quinolinate				
350	Tryptophan				
351	Anthranilate				
352	Pantothenate				
353	Arginosuccinate				
354	Alpha ketoglutarate				
355	Niacinamide				
356	Serine				
357	Proline				
358	Ornithine				
359	Lysine				
360	Lactate				
361	Threonine				
362	Betaine				
363	Pyruvate				
364	Asparagine				
365	Glycerol				
366	Xanthosine				
367	Creatinine				
368	Dimethylglycine				
369	Glycerol-3-Phosphate				
370	Glutamine				
371	Xanthine				
372	Histidine				

Malate					
Glucose-6-Phosphate					
Hypoxanthine					
Succinate					
Kynurenate					
Aconitate					
Fumarate					
Citrulline					
Valine					
IMP					
Isoleucine/Leucine					
Creatine					
Homovanilate					
Citrate/Isocitrate					
Beta-hydroxybutyrate					
Hydroxyphenylpyruvate					
Arginine					
AMP					
troponin I	n=18	20 min down-hill running at 70%VO2max	[31]	Blood	
Myoglobin					
Creatine kinase (CK)					
Myosin heavy chain					
LG3	n=10	Comparison of Operators (active) vs Crew (less-active) mining workers	[32]	Urine	
Prostaglandin E2 (PGE2)	n=11	Bycicle ergometry at 50% VO2max for 2 h	[33]	Blood	

397	2,3-dinor6-keto-PGf1a	n=10	Bicycle ergometry incremental exercise test to failure	[34]	Urine
398	Lipoxin A4	n=9	Bicycle ergometry. Workload set at 25W and increased by 25W every 2 min. Exercise terminated when estimated max HR reached 83-98%. Duration was 13.3+/-2.3 min	[35]	urine
399	α1-antichymotrypsin	n=9	Eccentric exercise of the elbow flexors involving 3 sets of 10 curls at predetermined weight	[36]	blood
400	C-1 protease inhibitor				
401	Creatine Kinase				
402	Myoglobin				
403	Lactate dehydrogenase				
404	alpha1-antichymotrypsin				
405	Myoglobin	n=10	Eccentric exercise of the elbow flexors involving 6 sets of 5 curls at predetermined weight	[37]	Blood
406	Creatine Kinase				
407	G-CSF				
408	TNF-α				
409	IL-8				
410	IL-10 after second bout of exercise				
411	LDH	n=13	Eccentric exercise of the elbow flexors involving 6 sets of 6 curls on a dynamometer	[38]	Blood
412	CK				
413	Complement C3				
414	Platelet activating factor				
415	C-reactive protein	n=11	300 unilateral, maximal isokinetic eccentric contractions at 30 deg/s	[39]	Blood
416	Creatine Kinase				
417	monocyte chemoattractant protein-1				

macrophage colony-stimulating factor					
granulocyte colony-stimulating factor					
IL-6					
Growth hormone					
Endostatin	n=7	Graded treadmill exercise	[40]	Blood	
VEGF					
sIL-6R	n=18	Participants completed 6 sets of 10 repetitions of unilateral eccentric/concentric actions of the knee flexors	[41]	Blood	
CK					
IL-6	n=9	An incremental exercise test on a treadmill with the gradient set at 1%	[42]	Blood	
Hepcidin					
Creatine kinase M-type	n=5	6-wk interval training program	[43]	Muscle biopsy	
Beta-enolase					
Troponin T					
Heatshock protein beta-1					
CH60					
Albumin					
ATP synthase	n=10	Electrically stimulated isometric contractions	[44]	Muscle biopsy	
NADH-ubiquinone oxidoreductase					
Ubiquinol cyt C reductase					
beta-enolase					
Tubulin					
Superoxide dismutase					

440	Peroxiredoxin-3				
441	Alpha-beta crystallin				

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