Title: Realising the Potential of Urine and Saliva as Diagnostic Tools in Sport and Exercise Medicine: A Review

Running Title: Urine and Saliva as Diagnostic Tools in Sport and Exercise Medicine

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Abstract

Accurate monitoring of homeostatic perturbations following various psychophysiological stressors is essential in sports and exercise medicine. Various biomarkers are routinely used as monitoring tools in both clinical and elite sport settings. Blood collection and muscle biopsies, both invasive in nature, are considered the gold standard for the analysis of these biomarkers in exercise science. Exploring non-invasive methods of collecting and analysing biomarkers which are capable of providing accurate information regarding exercise induced physiological and psychological stress is of obvious practical importance. This review describes the potential benefits, and the limitations, of using saliva and urine to ascertain biomarkers capable of identifying important stressors which are routinely encountered before, during or after intense or unaccustomed exercise, competition, over-training, and inappropriate recovery. In particular we focus on urinary and saliva biomarkers that have previously been used to monitor muscle damage, inflammation, cardiovascular stress, oxidative stress, hydration status, and brain distress. Evidence is provided from a range of empirical studies suggesting that urine and saliva are both capable of identifying various stressors. Although additional research regarding the efficacy of using urine and/or saliva to indicate the severity of exercise induced psychophysiological stress is required, it is likely that these non-invasive biomarkers will represent 'the future' in sports and exercise medicine.

Key Points

1. Urine and saliva offer a non-invasive alternative to blood and muscle collection when monitoring various psychophysiological stressors.
2. Due to their practicality additional research addressing the diagnostic capabilities (sensitivity and specificity) of using these non-invasive biomarkers is required.

1. Introduction

Understanding the impact of exercise induced psychophysiological stress is essential in sports and exercise medicine. Historically, the analysis of exercise induced stress relied on the measurement of functional outcome measures including strength, speed, power and subjective scales to assess recovery status, the effectiveness of training programs, and symptoms of overreaching. Recently, the use of biomarkers, which in a clinical setting are considered the gold standard [1-6], has become the quintessential monitoring tool in the field of sport and exercise medicine. This is primarily related to the ability of biomarkers to provide a quantitative individualistic snapshot of the homeostatic response of an individual at a certain time point. Therefore, the purpose of this review is to examine the use of urine and saliva as non-invasive diagnostic media for the quantification of "stress" through biochemical markers in the field of sport and exercise medicine. A broad literature search was performed using PubMed which included the following terms: urine, saliva, exercise, sport, medicine, stress, muscle damage, inflammation, immune system, oxidative stress, brain stress, hydration and cardiovascular stress. Biomarker article inclusion criteria included research on active and healthy humans only.

1.1 What is a Biomarker?

In order to review the use of urine and saliva as diagnostic tools in sports and exercise medicine the nature and function of a biomarker must be explored. The National Institute of Health (NIH) defines a biomarker as "*a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention*" [7]. There are two types of biomarker; those used in risk prediction, and those used to screen, monitor and diagnose [8]. Consequently, for an analyte to be considered as a prognostic, diagnostic and acceptable marker, it has to go through five stages of evaluation:

1) analytic (precision and accuracy).
2) diagnostic (sensitivity and specificity).
3) patient outcome efficacy (medical decision-making).
4) operational (predictive value and efficiency).
5) cost/benefit (societal efficacy) [9].

Through providing a quantifiable characteristic of a biological process, biomarkers can be used as a clinical surrogate endpoint [10], or provide objective information regarding the effect of an exercise protocol [11-14] or clinical treatment [15-17]. In addition, they can aid in understanding the prediction, cause, diagnosis, progression, regression, or outcome of treatment of disease [8], or in the context of this review, the acute and chronic psychophysiological stress response to exercise.

Biomarkers have several added advantages that can include and are not limited to: the provision of a full spectrum of disease, recovery and injury progression, instantaneous and initial stress analysis that reduces the degree of misclassification [8] and separate individual physiological variations amongst individuals. These can isolate the individual variations in perceived soreness or fatigue that to help facilitate the coaches and medical staff in making objective decisions regarding recovery and re-entry into training and playing. Three measurements have been suggested which include the degree to which the biomarker reflects the biological phenomenon (content), the degree to which a marker measures what it claims (construct), and the extent to which it correlates with a specific disease (criterion) [18].

2. Invasive and Non-invasive Methods

While different invasive and non-invasive procedures offer various benefits, consistent sample collection in recreational and professional athletes requires an approach that is practical and non-disruptive to the playing and coaching staff alike. Whilst exercise research is in abundance, the general analysis consensus still revolves around an invasive approach: likely a result of the wealth of potential biomarkers available in plasma, serum and cerebrospinal fluid (CSF) and the extensive literature pertaining to exercise stress and the ability to make comparisons. Implementing a practise that incorporates the use of non-invasive methods that allows for the accurate evaluation of exercise induced physiological and psychological stress is important for continued and sustained participation. Moreover, the examination, development and identification of potentially useful biomarkers are often completed in a controlled laboratory setting which may not always successfully translate to an elite sport setting.

For the purpose of this review, an invasive procedure is defined as a diagnostic technique that requires entry of a body cavity (eg: skin) or interruption of normal body functions [19]. Typical invasive procedures in exercise science and medicine include blood (serum and plasma), CSF and muscle biopsies. A non-invasive technique is

strictly defined when no break in the skin is created and there is no contact with the mucosa or skin break or internal body cavity beyond a natural or artificial body orifice [19]. Procedures can include collection of urine, saliva, sweat and imaging techniques such as magnetic resonance imaging (MRI), electrocardiogram (ECG) or computerised tomography (CT).

In a sports and exercise medicine setting the non-invasive nature of urine and saliva collection allows for personalised timing of sample collection, limited increases in stress hormone concentrations, rapid sample collection and reduced risk of cross-contamination which does not require a specific professional for obtaining a sample (e.g. phlebotomist). Moreover, the added advantage of saliva and urine over blood collection can be attributed to the simplicity of the collection devices. Blood collection requires sample tubes containing anti-coagulating compounds, clot activating factors, and ligand binding compounds to safely collect and stabilise the blood components whilst urine and saliva collection simply involves a sterilised, often pre-weighed tube [20].

Invasive procedures are also associated with pain and considerable distress of the athlete or participant. This may affect secretion of certain stress hormones like cortisol [21, 22]. This becomes especially important in studies quantifying stress following exercise that may lead to false-positive results and an over-estimation of the stress imposed by the exercise. Similarly, venepuncture can become overly obtrusive and time consuming especially in a team sport where large numbers of athletes are involved. Previous research has clearly demonstrated the unpleasant connotations associated with venepuncture. Comments ranging from “extremely distressing” in children [23] to “dread” in >10% of patients surveyed [24] indicate the general population’s preference for non-invasive procedures in sample collection.

2.1 Analyte Availability

Analyte availability can often cause certain restrictions in the assessment of exercise induced stress. Improvement in diagnostic methodologies however has led to the development of specific and sensitive immunoassay [25], chromatographic [26] and spectrometric assays [27] capable of providing an overview of an individual’s molecular profile through saliva and urine. Haematological parameters have been investigated extensively in exercise research [28-30] that provide a comprehensive overview of stress relative to acute and chronic exposure. Whilst this offers an effective representation, individual biomarkers often overlap providing

unnecessary and time consuming analysis. Non-invasive alternatives such as urine and saliva collection offer reliable indicators that do not restrict the ability to quantify "stress". Certain urinary biomarkers have been shown to repeatedly provide a precise quantification and analysis of exercise induced stress and disease or illness that does not require the use of an invasive procedure [26, 31-37]. For example, providing a measurement of the total free (un-bound) hormone concentration in the case of saliva more accurately reflects the active form in the body [38].

3. Saliva

Saliva is an exocrine solution [39] made of 99% water which can be considered as gland-specific or whole saliva. Differentiated by the four types of individual glands, analysis is typically completed on whole saliva secretions. It contains a wide spectrum of oral fluids, secretions from both the major and minor salivary glands, and several constituents of non-salivary origin such as gingival crevicular fluid (GCF), expectorated bronchial and nasal secretions, serum and blood derivatives from oral wounds, bacteria and bacterial products, viruses and fungi, desquamated epithelial cells, other cellular components, and food debris [40-44].

3.1 Correction of Measurements for Salivary Flow Rate

Typically, saliva compounds are expressed in four different forms;

1) Absolute concentration (µg/mL, nmol/L).

2) Secretion rate (µg/min) to account for those biomolecules (IgA, DHEAS) [45] affected by flow rate. Some evidence suggests that exercise induced changes in flow rate are psychological and related to parasympathetic withdrawal rather than sympathetic activation [46, 47]. Exercise is established as causing a decrease in saliva volume [48] that may cause a concentration effect of a marker. This correction has been presented following a 160 kilometre run where IgA concentration had not changed, but a 50 % decrease in secretion rate was observed [49].

3) Concentration relative to total protein content (µg/mg protein) to account for oscillations in protein content relative to changes in biomolecule concentration. The assumption is that total protein content does not change in response to exercise. A decrease in sIgA was observed following cross country skiers and cyclists which further decreased when corrected for total protein [50, 51]. However it has been suggested that correction with

total protein is inappropriate and misleading [52]. Studies have identified no change in sIgA or flow rate following intense exercise, but an increase in total salivary protein which indicates an evaporative loss of saliva when breathing through the mouth during exercise [53, 54].

4) Concentration relative to saliva osmolality (mg/mOsm) to account for low salivary flow rates [55]. Exercise is detrimental to saliva flow quantity but not quality, and with the total protein content in saliva less than one percent [52], expressing a salivary biomarker as a ratio to osmolality may be an appropriate means of expression.

The presentation and reference range determination of salivary biomarkers is an extremely important aspect of exercise induced stress analysis. This may explain some of the variation involved when comparing and contrasting results within and between sports. While most exercise studies will suggest the intensity of the protocol or game is the determining factor in changes in salivary biomarkers of physiological and psychological stress [33, 56, 57], it is possible the difference may be attributed to either the collection protocols in place and mishandling of samples, or the expression of the marker in the processes described.

3.2 Production and Composition

Saliva originates mainly from four pairs of glands; parotid, sublingual, submandibular and minor [58], with relative contributions ranging from four to 65 % [59]. Each gland can produce a variable amount of salts, ions and proteins [60, 61] that can be influenced by factors including psychological and hormonal status, physical exercise, flow rate, circadian rhythm, type and size of the gland, size and type of the stimulus, blood type, smell and taste, drugs, age, hereditary influences, and oral hygiene [59, 62-64] with composition variability dependent upon basal secretion or ANS stimulation [61].

Containing a variety of enzymes, hormones, antibodies, antimicrobial constituents, and growth factors [65, 66], saliva is an ideal medium for the analysis and diagnosis of exercise stress. It is primarily composed of water with an abundance of weak and strong ions where their concentration is dependent on secretion stimulation type [67]. Organic non-protein compounds such as uric acid, creatinine, bilirubin, glucose, amino acids, fatty acids, amines and lactate are also detectable [59, 68-73]. Other constituents include up to 2290 proteins [74]

comprised mainly of amylase, sIgA, carbonic anhydrase and proline-rich proteins, catecholamines [75] and hormones such as cortisol and testosterone whose concentrations represent serum-free levels [76].

3.3 Use in Exercise Stress

Saliva represents an increasingly useful auxiliary means of diagnosis due to its relative ease and stress free collection protocol, especially when blood or urine sampling is not feasible. Its role and connection with several pathological [77, 78] and physiological states [79, 80] enables suitable analysis and predictions. Whilst saliva diagnostics have been predominantly utilised in the detection of oral diseases such as Sjögren [81] and Beçhet syndrome and oral tumours [82], they have been proposed as reliable and accurate predictors of several other illnesses, diseases and exercise induced stress changes as previously reviewed [83].

Exercise induced stress provides a similar avenue for the use of salivary biomarkers in prediction and evaluation. These biomarkers offer potential in both an acute assessment of physiological and psychological stress (e.g. a competitive game) [84] and chronic assessment (e.g. symptoms of over-training or non-functional overreaching) [35]. Salivary markers have also identified fatigued and over-trained swimmers during the course of a competitive season [85], those at risk of a upper respiratory tract infection (URTI) in elite rowers, yachtsmen and soccer players [86-89], marathon runners who become immnocompromised [90], elite rugby union and league players suffering from over-reaching, over-training or post-match fatigue [91, 92], psychophysiological and catabolic stress associated with high intensity or intermittent exercise [93-95], changes in the circadian rhythm of trained athletes following strenuous exercise [96], and monitored rugby based performance [97]. Thus saliva offers exciting potential in athletic settings.

3.4 Biomarkers

The development of more sophisticated detection techniques has allowed for an increasing concentration of salivary biomarkers capable of providing diagnostic capabilities in exercise research. Whilst the list of potential biomarkers is not quite as exhaustive as its serum and plasma counterparts, its combination with urinary biomarkers provides a similarly reliable alternative. Table 1 lists some of the most popular biomarkers found in saliva that have shown precision and accuracy for quantifying exercise stress. Although not exhaustive, Table 1 highlights the most commonly used salivary biomarkers in research on active individuals in exercise and sport medicine.

3.4.1 Salivary Immunoglobulins

Salivary immunoglobulins, in particular IgA, are the most well studied markers of the mucosal immune system due to their relative ease of collection and extensively investigated response in relation to exercise. A selective deficiency in sIgA has been noted in those with a high incidence of infection [98] or poor saliva flow rate [99]. This is in contrast to Ammann and Hong [100] who stated IgA deficiency is relatively common and not usually associated with a markedly impaired resistance to infection. Even though several diseases are associated with an increased sIgA secretion rate [101-103] and low incidence of URTIs [104], the common immunosuppression observed in athletes does not necessarily mean they are immunocompromised in their response to common antigens [105]. Equivocal evidence surrounds this topic with suggestions the antiviral defence mechanisms of athletes may be compromised which leads to a decrease in performance [106, 107].

The research on the effect of exercise on sIgA is expansive and extensively reviewed elsewhere [108]. Salivary immunoglobulin A is unquestionably exercise intensity dependent, with moderate intensity protocols failing to elicit any significant post-exercise changes [53, 109-111]. Similar studies have also identified no change in sIgA following an elite soccer match [112], intensive tennis training [113], a collegiate rugby game [114], jiu-jitsu matches [115] or resistance exercise [116, 117]. Participating in ultra-endurance events and rugby union however result in an immediate post-exercise suppression [20, 118-120]. Similarly, prolonged cycling at 70% $\dot{V}O_2$max [34], cross country skiing for 50 km [50], and competing in a triathlon [121] all cause an individual athlete to become immunocompromised.

sIgA monitoring may also be useful in determining the risk of infection [122, 123] and excessive training in athletes [124, 125]. Decreased levels of sIgA have been associated with stale, underperforming and over-trained athletes [126-129]. Elite kayakers have showed a 27 – 38 % decrease in sIgA secretion rate after each session of training over three weeks [130]. In contrast, no change in sIgA secretion rate was observed in 20 female division one soccer players during a 13 week season [131], which is similar to the lack of change in pre-race sIgA in competitors completing 10 marathons in 10 days [132] and professional rugby players during a competition [133].

There current literature appears to be in agreement that decreased levels of sIgA are associated with an increased incidence of URTIs [134-136]. An increase in URTIs has been linked to decreased sIgA concentrations following a soccer match [88], basketball game [137], at least three hours a week endurance training [79],

American Football [138], swimming [85] and rugby union [35, 139]. In contrast, no relationship between URTI incidence and sIgA following a 84 km ultra-marathon was observed [140], while similar observations have been seen in soccer [131], military training [141] and yachting [87].

3.4.2 Cortisol and Testosterone

Cortisol and testosterone are steroid hormones synthesised in the zona fasciculata layer of the adrenal gland and Leydig cells of the testes, respectively. Their secretion is controlled by the direct stimulation of the HPA and hypothalamic-pituitary-testicular axis. Cortisol specifically has several biological properties including stimulating gluconeogenesis and glycogen synthesis in the liver and its ability to inhibit protein synthesis and stimulate protein degradation in peripheral tissues [142]. Cortisol is extensively studied in professional and recreational athletes due to its immunosuppressive, catabolic and protein synthesis inhibitory effects. It has been shown to suppress both CD4 and CD8 T-cells [143] as well as regulating degranulation [144], ROS production [145] and mobilization of neutrophils [146]. Furthermore, cortisol also has the ability to inhibit DNA replication and mitosis and repress the formation of antibodies and lymphocytes [142, 147]. This was evident following three hours of intense exercise where a strong correlation ($r = 0.63$) was observed between post-exercise cortisol concentrations and lymphocyte apoptosis [148]. Testosterone meanwhile, has both anabolic [149] and andronergic effects [150] that are used in a medical context for hormone replacement therapy [151] and illegally to improve athletic performance [152].

Salivary cortisol has been used in exercise related studies due to its ease of collection and general reflection of the free fraction of the free component in blood [153, 154]. Most serum cortisol is bound to proteins including cortisosteroid binding protein and albumin, whereas free cortisol passes relatively obstruction free through plasma membranes [155] allowing for rapid availability and ease of measurement. It has been used to study the acute effects of exercise [156, 157], identify athletes in a state of over-training [158-160], the recovery of an athlete [91], or used simultaneously with testosterone to predict performance [161-163]. Participation in professional rugby league matches has been associated with significant increases in salivary cortisol [164], which is similar to the post-exercise increases following soccer [112], ultra-endurance marathon [120], synchronized swimming [165], golf [166], rock-climbing [167], and resistance training [168-170]. However there is conflicting evidence regarding the salivary cortisol response and some studies have reported no

alterations in the biomarker following a professional soccer match [171], resistance exercise [172], repeated high intensity sprints [173] or a professional rugby season [133].

Testosterone has been used extensively in physical impact sports such as rugby union and league to monitor training programs [174, 175] and predict performance [176, 177]. It has also been used in sporting competitions [178], cross country running [179], resistance exercise [180], body building training [169], elite triathlete training camps [181], high intensity interval cycling [182] and as a direct marker of overtraining in elite volleyball players [183].

3.4.3 Other Biomarkers

Several other biomarkers have the precision and accuracy to provide quantifiable data pertaining to exercise stress. Alpha-amylase is responsible for starch and glycogen degradation [83] and is a marker of psychophysiological stress and sympathetic nervous system activation [184]. Its measurement provides information regarding sympathetic adrenal medullary axis activation, given that α-amylase concentration has found to be lower in athletes who are more experienced and confident [185]. It has also shown to change in response to acute down-hill running [186] and cumulative tennis training [160].

Lactoferrin and lysozyme, antimicrobial compounds found in saliva secretions [187, 188] have been used to monitor longitudinal changes in elite weightlifters [189] and basket-ballers [190], measured in endurance exercise in which hydration has an effect on secretion [191] and shown to increase in response to running at 75% VO_2max [192]. Intense exercise however, may reduce their concentration which may subsequently increase risk of infection and incidence of a URTI [83]. Other useful biomarkers include TBARS and uric acid, both of which have been shown to change in response to resistance training [193] and provide a reliable indication of oxidative stress. Protein carbonyls and 8-oxo-2'-deoxyguanosine (8-oxo-dG) also have potential; while not yet measured in an exercise context they have been used as key indicators of oxidative stress in diabetes mellitus [194] and periodontitis [195]. Saliva osmolality can provide hydration status in exercise research [196], whilst S100B has shown promise as a non-invasive biomarker of brain injury in various sportsmen during competition [197]. More recently, nanoparticle-enabled protein biomarkers (proteomics) have been proposed as sensitive indicators of brain injury/concussion with further developments required [198].

Salivary CRP and neopterin have not been measured in exercise research but have been shown to provide information pertaining to inflammation in a clinical context [199-201]. Similarly, cTnT and CK-MB have also been used in a clinical context to provide information regarding cardiovascular stress [202] and muscle damage [203] respectively, that may become useful in exercise medicine as non-invasive alternatives. Furthermore, specific interleukins have been identified in saliva [204, 205], although their relationship to blood concentrations remains unclear.

3.5 Limitations

As a diagnostic medium, saliva has some limitations; specifically in the medical field where diagnostics rely heavily on combinations of biomarker panels used as screening tools to improve overall reliability. Its practicality, in addition to the development of modern and more precise technologies, has however expanded its efficacy in the assessment of exercise-induced stress.

As a consequence of the diurnal and circadian variations of certain biomolecules present in saliva [206], concentrations do not always reliably reflect the concentrations of these molecules in serum. Therefore it is imperative when analysing exercise stress to plan a precise collection protocol before the experiment to understand the individual biological variation of a subject, or take multiple samples at the same time to provide meaningful results [38].

Biomolecules in saliva that rely on active transport are generally flow rate dependent meaning exercise-induced changes have to be corrected for saliva flow rate. Studies have identified differences in post-exercise changes in sIgA in cyclists completing an exercise protocol in a cold environment. Saliva flow decreased significantly following exercise resulting in an increase in sIgA concentration, however when corrected for flow rate, the protocol seemingly caused immune suppression calculated by the secretion rate of IgA [34].

Salivary composition can also be influenced by the method of collection and the degree of stimulation of salivary flow [40, 45]. Careful consideration has to be given to the methodology of sample collection due to the risk of over-stimulation providing a false-positive or negative result (analyte dependent) using cotton absorbent materials, hydrocellulose or acidic based stimulation (citric acid) [207-211]. However, some devices

such as Sarstedt-Salivette® have been shown to reflect total and free steroid concentrations more accurately than the traditionally accepted passive-drool technique [212]. Furthermore collection time duration and position of collection can significantly affect concentration and secretion rate of specific analytes [213].

Saliva contains analytes in concentrations that are several-fold lower than blood [67, 75, 214, 215] which reduces the risk of danger or infection when dealing with potentially hazardous antigens. It is for this reason advanced technologies are required to quantify any changes in analyte concentration. The development of affordable ELISAs can detect concentrations of salivary steroids as low as 2 pg/mL with a sensitivity limit of 1 pg/mL, while liquid-liquid extraction by either diethylether or dichloromethane provides an added detection step extracting polar substances from the saliva [216]. This allows the detection of any small changes following exercise stress that may represent a significant change. As a result of the large concentration differences between blood and saliva, contamination through use of mouth guards or impact related gum trauma causing bleeding can significantly alter the concentrations of specific analytes [45, 217]. Consequently, the need for robust saliva collection protocols is imperative for precise and accurate interpretation of results.

4. Urine

Urine is an aqueous solution of an organism that is used to excrete the by-products of cellular metabolism through kidney filtration; its appearance is based on colour, foaming, odour and clarity [218]. The filtration process and non-invasive nature of collection involved in urine sampling makes it an ideal matrix for the detection of exercise-induced stress.

4.1 Correction of Measurements for Urine Volume

Whilst measurement of salivary biomarkers requires correction for flow rate, urine requires volume correction. Hydration levels are known to affect the concentrations of urinary biomarkers. Hypo-hydration has a concentration effect while hyper-hydration has a dilution effect. In exercise specifically, the consumption of water and carbohydrate-electrolyte formulations is known to influence temperature regulation, physiological strain and endurance during exercise [219-222]. This is a key indication of how vital hydration status is for exercise performance and therefore the challenges associated with urinary biomarker correction and hydration status.

It is critical when presenting the concentration of a selected marker in relation to a reference range to correct for the hydration level of a subject, patient or athlete. This is especially important when dealing with a severe intensity exercise or analysing the urine of suspected doping athletes in a controlled competition. The two most commonly utilized methods for urine volume correction involve creatinine and specific gravity (SG).

4.1.1 Creatinine

Creatinine is a spontaneous breakdown product of creatine and creatine phosphate [223] during muscle cell metabolism present in serum, erythrocytes, cerebrospinal fluid and all bodily secretions [224, 225]. Originally identified in 1847 [226] and produced initially through heating of creatine in 1885 [227], creatinine is now recognised and utilised as a principal compound for urine volume correction and hydration status.

Creatinine's constant secretion rate [228] and easy quantification are two of its key properties that allow it to be used as an indicator of hydration status [229, 230], while lean body mass [231, 232], age [233] and renal function are contributing factors in its clearance that have to be considered.

Modern quantification methods include the reaction with o-nitrobenzaldehyde [234], reverse phase [235-237] and cation-exchange HPLC [26], cation-pairing HPLC [238], mass-spectrometry and gas-chromatography [239]. The advantage of chromatography is the simultaneous determination of creatinine with the analyte that requires creatinine correction. Usually analysed at 234 nm by ultra-violet detection, the method has been used effectively in the quantification correction of NP [26, 240, 241], desmosine [242], catecholamines and metanephrines [243].

There are a number of limitations associated with urinary creatinine correction. Evidence has identified higher levels of urinary creatinine in the urine of men or lean individuals than women and obese individuals respectively [231, 244, 245]. This indicates lean body mass as a major factor in its excretion. Furthermore, those of African-American descent excrete 5 % more creatinine per weight than those of European descent, and older individuals and those with renal impairment excrete less urinary creatinine than the young and healthy [246]. Individuals who are subjected to a creatine-free or excess creatine diet show a gradual decline and increased excretion respectively [247, 248]. The combination of all these variables identifies the need to

ascertain the normal excretion of creatinine for each individual, even though the co-efficient of variation (CV) for individual creatinine excretion can vary from 4 to 50 % [249-251].

Exercise provides another layer of complexity for creatinine volume correction. Urinary creatinine has been demonstrated to increase by as much as 50 - 100% following a strenuous 6-mile run, 100 km marathon and 70 – 90 km cross country ski race [252-254]. It is difficult to ascertain whether observed changes in a selected analyte are negatively affected by this change. A study [255] investigating the excretion of the muscle damage marker 3-methyl histidine following resistance exercises showed a decrease in concentration in conjunction with an increase in serum myoglobin and CK. It is unusual that a change in muscle tissue damage occurs without skeletal muscle protein degradation. This may be a result of the likely increased urinary creatinine concentration that has been shown to increase following resistance training; which presents the potential difficulties associated with urine volume correction by creatinine. In rugby union, the large aerobic and anaerobic component of the game may stimulate large increases in creatinine that could theoretically affect the interpretation of any meaningful urinary biomarker. However, the World Anti-Doping Agency (WADA) (www.wada-ama.org) has adapted the use of SG in their quantification of urinary steroids and doping analysis [256].

4.1.2 Specific Gravity

Specific gravity is the ratio of the density of a substance to the density of a reference substance measured optimally through refractometry [257]. Urine specifically can be compared to distilled H_2O as a reference and is known to increase in SG with solute concentration [257]. This varies with the total mass of solutes that depends not only on the number of particles present, but also on their molecular weight [258]. The advantages of urinary SG include its cost and time efficient measurement using a simple hand-held refractometer in comparison to the time consuming and costly assays associated with creatinine.

Since 1908, creatinine has been used as the method for correcting analyte concentrations in urine due to its "constant rate" [228]. As a result of the known variations in creatinine excretion, SG has been sought after as a viable alternative and used in recent exercise research [20]. The minimal studies that have compared creatinine and SG have shown SG performs as well as creatinine in correcting urinary analyte concentrations [26, 229,

258-260] and assessing patient and pre-exercise hydration status [261-263]. Its limited use is a result of finite research that has not elucidated reference ranges which are critical for diagnosis.

In the field, the Levine-Fahy equation is used to correct fluctuating urinary concentrations and adjust them to a reference value for a specific population [264].

$$Concentration_{SG\ normalized} = concentration_{specimen} \bullet (SG_{reference} - 1) / (SG_{specimen} - 1)$$

The WADA uses a SG of 1.020 [265] for normalisation of testosterone precursors, metabolites, and other endogenous steroids in urine [256]. Other reference values ranging from 1.018 to 1.024 have also been used [258, 264, 266, 267] that are population dependent, while values < 1.0010 or > 1.020 indicate relative hydration and dehydration respectively [268]. However, these guidelines are not without complications as measuring SG before two different marathons in a large cohort identified 46 % of the runners would be considered dehydrated which seems unlikely considering the magnitude of the impending exercise [269].

Similar limitations are associated with urinary SG quantification. As a correction method, it may not be appropriate for individuals with diabetes mellitus and nephrotic syndrome. These diseases cause high concentrations of heavy molecules known to affect SG and therefore introduce the potential for underestimation of a urinary analyte [257, 270, 271]. The effect of exercise on SG however is not well understood, although it can be assumed the aerobic, anaerobic and physical impacts of contact sports would significantly affect the density of urine. Furthermore, the contraction and expansion of urinary constituents due to temperature and pressure requires the measurement of SG to be conducted in a uniform manner [272], while values < 1.002/1.003 or > 1.003 are considered too “dilute” or “concentrate” for accurate correction [230, 272-274].

4.2 Composition and Biomarkers

As an aqueous solution, urine is predominantly water (> 95 %), electrolytes, metabolic excretory products and other organic and non-organic compounds [218] controlled by the kidneys in a series of events; filtration, reabsorption, secretion and excretion [275]. While urine includes nitrogen containing compounds and specific elements [276], it also contains several compounds (Table 2) capable of providing useful information regarding certain illnesses and psychophysiological changes following exercise induced stress. The subsequent list is a

compilation of the most popular biomarkers found in urine that have been used to evaluate "stress" in sport and exercise medicine research.

4.2.1 Myoglobin

Myoglobin is an O_2 transportation heme protein, is specific to muscle and its detection in urine is diagnostically relevant and indicative of trauma induced muscle damage commonly seen following strenuous exercise [277]. The extremely rapid elimination kinetics of myoglobin [278] in comparison to more commonly utilised plasma markers such as creatine kinase [279, 280], make it an ideal biomarker that provides an immediate evaluation of severity. It has been used extensively in several forms of exercise [20, 240, 241, 281-285] and is quantifiable using both ELISA [241] and RP-HPLC [284].

4.2.2 3-Methylhistidine

3-Methylhistidine is a commonly used muscle damage marker that directly represents myofibrillar degradation [286]. It has been demonstrated to increase considerably following exercise [287-289]; although careful consideration is required based on the influence dietary meat can have on its abundance [290].

4.2.3 Total Neopterin and Inflammation

Total neopterin (neopterin + 7,8-dihydroneopterin) are compounds released by activated macrophages [291] following γ-interferon stimulation by T-cells [292]. They are up-regulated following resistance training [26] and indicate acute and cumulative changes in immune system activation following games of rugby union [20], Olympic rowing [293] and mixed martial arts [285]. Careful interpretation of neopterin data is essential however due to the large disparity in the presentation of neopterin as either neopterin or total neopterin; the former of which is the oxidation product of 7,8-dihydroneopterin and therefore may represent a change in oxidative status [240, 294]. Other inflammatory biomarkers such as interleukin-1β (IL-1β), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-12 (IL-12), tumor necrosis factor-α (TNF-α), monocyte chemotactic protein-1 (MCP-1) and interferon-γ (IFN-γ) have also been used to identify a physiological response to various forms of exercise [295-297].

4.2.4 Stress

'Stress' has been assessed in urine through cortisol [298], testosterone [299], epitestosterone [300] and tetrahydrobiopterin (BH_4) [240]. Steroidal compounds including natural and synthetic varieties are filtered through the glomerulus without any limitation. Identification of these compounds within the urine can provide useful information regarding doping; the primary identification system used by the WADA. The testosterone:epitestosterone ratio is often used in the detection of doping athletes [152] with concentrations exceeding one to four considered above normal. BH_4 meanwhile has been used to assess synthesis of monoamine neurotransmitters such as epinephrine and nitric oxide following rugby union matches [240], while the quantification of steroidal compounds is traditionally measured in saliva to avoid the need for 24 hour collections and because of its non-invasive nature [301].

4.2.5 Cardiovascular Stress

Assessment of cardiovascular stress is often neglected when monitoring the internal load of athletes despite being a pertinent marker of exercise performance, cardiovascular fatigue and subsequent performance potential. NT-proBNP is the cleaved inactive fragment of brain natriuretic peptide (BNP) synthesized by cardiac myocytes [302] and fibroblasts [303] in response to ventricular wall tension/stress. It is increased in the plasma of patients with cardiac dysfunction [304] and healthy individuals following strenuous endurance exercise [305]. Although not currently measured in urine following exercise, it has been quantified repeatedly in patients with cardiac failure [306, 307] and could potentially provide an assessment of cardiovascular stress in exercise and sports medicine.

4.2.6 Other Biomarkers

Urinary pterins [240] and isoprostanes [308] have increased in response to professional rugby union and resistance training respectively. Moreover, urine also provides a reliable diagnostic medium for the assessment of hydration through creatinine [254, 297] and specific gravity [26] from endurance exercise to body-building resistance training, as well as showing promise as a medium for the detection of exercise induced brain injury [309]. Although S100B has only been measured in people suffering traumatic brain injury [309], the increasing concern for the welfare of athletes in contact related sports may require a non-invasive biomarker capable of providing instantaneous information regarding concussion or damage severity. However, careful consideration

is required when interpreting S100B levels because of conflicting data about the specificity of this biomarker to brain related injury [310, 311].

5. IMPLICATIONS AND RECOMMENDATIONS FOR RESEARCH

When designing exercise or sports medicine research requiring the capability of biomarkers, investigators should consider urine and saliva as a similarly reliable alternative to traditionally utilised invasive procedures. Whilst the literature on urine and saliva as a diagnostic medium is progressively emerging in exercise and sports medicine, their employment in a clinical setting warrants further investigation. Specifically, developing sensitive and specific urine and saliva assays capable of quantifying both the physiological and psychological stress response which provide a stress-free, non-disruptive and non-invasive approach is required. Where recruitment and sustained participation of athletes or patients is required, the ability to effectively identify "stress" through urine and saliva may also allow provide a cost-effective approach. Moreover, the developing cascade of biomarkers within urine and saliva has the potential to not only alleviate any safety concerns and specific training associated with invasive techniques, but also provide a comprehensive methodology that offers similar efficacy with the likes of blood and CSF for an accurate snapshot of a biological process. Providing urine and saliva are effectively utilised in the future for assessing both the intra- and inter-individual psychophysiological stress response to exercise and sports medicine research, a substantial database incorporating reference ranges applicable to various exercise protocols and sporting codes can be developed. This will allow data between the extensive array of studies to be comparable and positively influence the direction of prospective exercise and sports medicine research.

6. CONCLUSION

Urine and saliva have the ability to provide a quantitative physiological and psychological "stress" assessment in sport and exercise medicine using a non-invasive approach. Like all biological media both saliva and urine have some limitations. In comparison to plasma or serum, salivary and urinary biomarkers have not been used extensively in sport and exercise medicine; however the markers currently being used are capable of providing a comprehensive overview of physiological perturbations that mirror the extensive literature conducted on invasive procedures. If collection, handling, storage, analysis and correction of salivary and urinary biomarkers are standardised, it may be feasible to determine their sensitivity and specificity as a diagnostic medium in

exercise trials. Although additional research regarding the efficacy of using urine and/or saliva to indicate the severity of exercise induced psychophysiological stress is required, it is likely that these non-invasive biomarkers will represent 'the future' in sports and exercise medicine.

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Table 1. List of the most popular non-invasive salivary biomarkers used in sports and exercise science. Selection criteria included studies on healthy, active humans only.

Stress type	Biomarker	Method of analysis	Study design	Participants (total n; M:F)	Setting	Outcome
Muscle damage	CK-MB[203][a]	Autoanalyzer	Observational	Cardiac patients (32; 22:10)	Myocardial infarction	Sig. ↑ in patients vs healthy controls
Inflammation	CRP[199, 200][a]	ELISA	Observational	Healthy adults (61; 18:43)	Medium to study inflammation	Sig. correlated with serum CRP
		ELISA	Observational	Cardiac patients (28; N/A) Controls (55; N/A)	Cardiac disease	Sig. ↑ in patients vs healthy controls
	Neopterin[201][a]	ELISA	Observational	Patients (16; 10:6) Controls (13; 7:6)	Periodontitis	Sig. ↑ in patients vs healthy controls
Cardiovascular stress	cTnT[202][a]	ELISA	Observational	Cardiac patients (30; 20:10) Controls (30; 23:7)	Myocardial infarction	Sig. ↑ in patients vs healthy controls
Stress	Cortisol [20, 156, 312]	ELISA	Observational	Rugby player (24; 24:0)	Three consecutive rugby games	Sig. ↑ post-games
		Radioimmunoassay	Observational	Strength trained (28; 28:0)	Resistance training	Sig. ↑ post exercise
		ELISA	Observational	Professional rugby league players (17; 17:0)	Rugby league game	Sig. ↑ post exercise
	Testosterone [156, 181]	Radioimmunoassay	Observational	Strength trained (28; 28:0)	Resistance training	Sig. ↑ post exercise
		ELISA	Observational	Professional rugby league players (17; 17:0)	Rugby league game	Sig. ↑ post exercise
	Alpha-amylase[14, 185]	Kinetics method	Observational	College athlete (42; 21:21)	Rowing	Sig. ↑ at exhaustion
		Kinetics method	Observational	National athletes (12; 12:0)	Cycling to exhaustion	Sig. ↑ post exercise
	DHEA[312, 313]	Radioimmunoassay	Observational	Elite athletes and controls (24; 0:24)	Handball	Sig. ↑ in controls vs athletes

		Radioimmunoassay	Observational	Strength trained (28; 28:0)	Resistance training	Sig. ↑ post exercise
Immune system	sIgA, sIgM, sIgG [20, 51, 314]	ELISA	Observational	Professional rugby players (24; 24:0)	Rugby game	Sig. ↓ post-game
		ELISA	Observational	Trained cyclists (8; 8:0)	Endurance exercise	Sig. ↓ post-exercise
		ELISA	Observational	Healthy men (12; 12:0)	Interval exercise	Sig. ↓ post-exercise
	Lactoferrin[190]]	ELISA	Observational	Elite athletes (8; N/A)	Basketball season	Sig. ↓ during training and competition
		ELISA	Observational	Elite rowers (17; 8:9) Sedentary group (17; 8:9)	Rowing season	Sig. ↑ post exercise Sig. ↑ in rower
	Lysozyme[315]	ELISA	Observational	Elite rowers (17; 8:9) Sedentary group (17; 8:9)	Rowing season	Sig. ↑ post exercise No differences between groups
Oxidative stress	8-oxo-dG [195]*	ELISA	Observational	Patients (34; 17:17) Healthy controls (17: 11:6)	Periodontitis	Sig. ↑ in patients vs healthy controls
		ELISA	Observational	Patients (215; 105:110) Controls (481; :217:264)	Diabetes mellitus	Sig. ↑ in patients vs healthy controls
	Protein carbonyls[194]*	ELISA	Observational	Patients (215; 105:110) Controls (481; :217:264)	Diabetes mellitus	Sig. ↑ in patients vs healthy controls
	Uric acid[193]	ELISA	Observational	Trained men (11; 11:0)	Resistance exercise	Sig. ↑ post-acute training
	TBARS[193]	Spectrophotometric test	Observational	Trained men (11; 11:0)	Resistance exercise	No change post exercise
Hydration	Osmolality[196, 316]	Freezing-point depression	Observational	Healthy males (12; 12:0)	Endurance cycling	↑ linearly with dehydration
		Freezing-point depression	Observational	Healthy active volunteers (24; 17:7)	Extracellular dehydration Treadmill walking	Sig. ↑ post dehydration
		Freezing-point depression	Observational	Soldiers (8: 6:2)		Sig. ↑ post dehydration
Brain distress	S100B[197]	Immunoluminometric assay	Case–control study	Professional athletes (25; 25:0) Controls (50, 50:0)	Before and after vigorous exercise	Sig. ↑ post exercise Sig. ↑ in athletes vs controls at rest and after exercise

[a] Have not been used as markers in exercise research, but show promise in clinical contexts with potential use in sport and exercise medicine.

8-oxo-dG; 8-oxo-2'-deoxyguanosine, ↑; increase, ↓; decrease, CK-MB; creatine kinase-MB, CRP: C-reactive protein, cTnT; cardiac troponin T, DHEA; dehydroepiandrosterone, ELISA; enzyme linked immunosorbent assay, F; female, M; male, N/A; not available, S100B; S100 calcium binding protein B, sIg; secretory immunoglobulin, Sig; significant, TBARS; thiobarbituric acid reactive substances.

Table 2. List of the most popular non-invasive urinary biomarkers used in sports and exercise science. Selection criteria included studies on healthy, active humans only.

Stress type	Biomarker	Method of analysis	Study design	Participants (total; M:F)	Setting	Outcome
Muscle damage	Myoglobin [20, 240, 281-283]	ELISA	Observational	Amateur rugby players (24; 24:0)	Rugby game	Sig. ↑ post-game
		ELISA	Observational	Professional rugby players (37; 37:0)	Rugby season	Sig. ↑ post-games
		ELISA	Observational	Professional rugby players (25; 25:0)	Rugby game	Sig. ↑ post-game
		ELISA	Observational	Healthy students (119; 74:45)	Push-ups	No change
		Automated analyser	Observational	Duathlon athletes (14; 14:0)	Duathlon	↓ during and ↑post-exercise
	3-methylhistidine[286-288, 317]	GC/MS	Randomised double blind	Resistance trained (24; 24:0)	Resistance exercise	No change
		GC/MS	Randomised	Resistance trained (20; 20:0)	Resistance exercise	No change
		HPLC	Randomised double blind	Untrained healthy individuals (32; 32:0)	Resistance exercise	Sig. ↑ post-exercise
		HPLC	Randomised double blind	Strength trained (69; 52:17)	Resistance exercise	No change
Inflammation	Total neopterin [26, 241, 293, 318]	HPLC	Observational	Amateur rugby players (11; 11:0)	Rugby game	Sig. ↑ post-exercise
		HPLC	Observational	Amateur body-builders (10; 10:0)	Body-building	No change
		HPLC	Observational	Professional rugby players (37; 37:0)	Rugby game	Sig. ↑ post-exercise
		ELISA	Observational	Olympic athletes (27; 27:0)	Rowing camp	Sig. ↑ post-camp
	IL-1 β, IL-1ra, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, TNF-α, MCP-1, γ-IFN [297]	ELISA	Observational	Duathlon athletes (14; 14:0)	Duathlon	Sig. ↑ post-exercise
Cardiovascular stress	NT-proBNP [306][a]	Immunoassay	Observational	Cardiac patients and controls (116; 96 patients: 20 controls)	Heart failure	Sig. ↑ in patients vs control

Stress	Testosterone and epitestosterone[299, 300, 319-322]	ELISA	Randomised	Resistance trained (10; 10:0)	Resistance exercise/administration	Testosterone ↑ in admin group
		GC/MS	Observational	Professional cyclists (7; 7:0)	Cycling race	Testosterone ↑ compared to epitestosterone post-race
		GC/MS	Double-blind	Elite rugby league players (22; 22:0)	Resistance exercise/supplementation	No change
		GC/MS	Randomised cross-over	Healthy active individuals (13; 13:0)	Supplementation	No change
		GC/MS	Observational	Elite handball players (19; 0:19)	Handball game	Sig. ↑ post-game
		GC/MS	Observational	Professional weight lifters (19; 19:0)	Resistance exercise training	No change
	Cortisol[298, 323, 324]	HPLC	Observational	Healthy untrained and trained triathletes (19; 19:0)	Triathlon season	Seasonal variation
		HPLC	Observational	Trained adolescent tennis players (7; 0:7)	Tennis season	Sig. ↑ end of season
		Immunoassay	Randomised	Resistance trained (29; 0:29)	Resistance exercise	Sig. ↑ in concentric group post-exercise
	BH_4 and norepinephrine [240, 323]	HPLC	Observational	Professional rugby players (25; 25:0)	Rugby game	Sig. ↑post-game
		HPLC	Observational	Trained adolescent tennis players (7; 0:7)	Tennis season	Sig. ↑ end of season
Oxidative stress	NP, BP, XP [240]	HPLC	Observational	Professional rugby players (25; 25:0)	Rugby game	Sig ↑ post-game
	Isoprostanes[308]	ELISA	Observational	Recreationally trained (12; 12:0)	Resistance exercise	Sig. ↑ during overtraining
Hydration	Specific gravity [26, 241]	Refractometer	Observational	Amateur body-builders (10; 10:0)	Resistance exercise	No change
		Refractometer	Observational	Amateur rugby players (11; 11:0)	Rugby game	Sig. ↑ post-game
	Creatinine[241, 297]	Automated analyser	Observational	Duathlon athletes (14; 14:0)	Duathlon	Sig. ↑ post-race
		HPLC	Observational	Amateur rugby players (11; 11:0)	Rugby game	Sig. ↑ post-game
Brain distress	S100B[309][a]	Immunoassay	Observational	Patients (does not state gender)	Traumatic brain injury	Sig. ↑ post head injury

[a] Not currently measured in sport related concussion but has the potential to do so.

↑; increase, ↓; decrease, γ-IFN; gamma-interferon, admin; administrated, BH_4; tetrahydrobiopterin, BP; biopterin, ELISA: enzyme linked immunosorbent assay, F; female, GC/MS: gas chromatography/mass spectrometry, HPLC; high performance liquid chromatography, IL; interleukin, ra; receptor antagonist, M; male, MCP-1;monocyte chemoattractant protein-1 , NP; neopterin, NT-proBNP; N-terminal prohormone of brain natriuretic peptide, S100B; S100 calcium binding protein B Sig; significant, TNF-α; tumor necrosis factor-α, XP; xanthopterin