Detection and Correlation of Salivary Biomarkers to Burnout in Athletes

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The candidate confirms that the work submitted is their own and that appropriate credit has been given where reference has been made to the work of others.

Abstract

Competition is an important part of an athlete's career, creating opportunities for advancement and income. Research indicates that this can increase an athlete's risk of burnout. With its major diagnostic symptoms emotional and physical exhaustion, depersonalisation and reduced sense of accomplishment being measured using the self-reported athlete burnout questionnaire, false positives are common. Past studies have identified immune biomarkers as possible targets for more accurate detection of burnout. For more research to be performed to identify the optimal biomarker a simple analytical method for detecting these biomarkers is needed. Therefore, this study set out to develop enzyme-linked immunosorbent assays (ELISAs) for the subclasses IgA1, IgA2 and IgAsc. Optimisation methods exhibited high specificity and low cross-reactivity with other biomarkers in the saliva. Validation results showed that the IgAsc ELISA was optimised for use within an athlete population and the IgA2 ELISA could accurately measure all samples with future testing on larger populations desirable, showing all reagents were optimised. The IgA1 ELISA was too sensitive for the range of concentrations found in the saliva samples and requires further optimisation. Once the saliva samples were paired with self-reported questionnaires both IgA2 and IgAsc showed weak, non-significant correlations with total burnout and all three dimensions measured. Reduced sense of accomplishment showed the strongest correlation with both markers, highlighting the future research uses of the IgA2 and IgAsc ELISAs for identifying whether they are useful for early burnout detection once larger sample sizes can be investigated. Overall, the ELISAs developed in this study show a promising initial step in developing a reliable detection method for IgA subclasses, especially with further optimisation of the IgA1 ELISA.

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1. Introduction

Competition is an inherent aspect of sport, providing athletes opportunities to attend higher-level events, gain sponsorships, and prize money all of which are critical for funding the training and equipment needed for continued success (Konings et al., 2018). Unlike internal practice matches, true competition provides unique pressures and higher stakes that significantly increase motivation and performance intensity, pushing athletes to reach their physical and mental limits (Silveira Coswig et al., 2016). Due to the nature of competition, there will always be a winner and a loser, or in some sports, many losers. The anxiety to perform and win leading up to competitive events can therefore compound feelings of loss, especially with added pressure from teammates, coaches, and fans (Gustafsson et al., 2007).

The added demands of competitive events which cause them to be so useful to athletic development, are also known to take a toll on athletes. Mental and physical health can be damaged even if the athlete wins, leading to an inability to continue to perform and impacting their overall well-being (Gallo-Salazar et al., 2017; Post et al., 2017; Purcell et al., 2019). Athletes reaching the peak of their sport may face significant stress to maintain these performance levels. This increased pressure to perform during winning streaks, and to improve after losing, may encourage further exercise intensity, pushing them past their realistic capabilities and creating a higher risk of injury (Sabato et al., 2016). As athletes push themselves, negative feedback may occur as the body becomes fatigued and cannot perform as expected. This may lead to more effort being applied, exacerbating the physical toll and creating a cycle that can result in chronic fatigue and stress, culminating in mental burnout (Purcell et al., 2019).

Burnout is characterised by emotional and physical exhaustion, depersonalisation and reduced personal accomplishment (Schaufeli, 1996). If burnout is not detected early, it can lead to recurring injuries and repeated episodes of burnout may also increase the risk of depression (Chen et al., 2021). Therefore, early detection of burnout is crucial in preventing long-term physical and mental health damage by allowing coaches and medical teams to provide them with the tools to break the cycles of burnout. Identifying burnout is primarily done via psychometric surveys, relying on self-reported symptoms, such as the Maslach Burnout Inventory (MBI; Schaufeli, 1996) and MBI-General Survey (Maslach & Jackson, 1981) in the general population and The Athlete Burnout Questionnaire in athletes (Raedeke & Smith, 2001). However, these questionnaires have been considered possibly unreliable due to pressure to deny or understate levels of exhaustion especially due to fears of intervention or stigmatisation (Isoard-Gautheur et al., 2018). Therefore, the subjective nature of these diagnostic tools highlights the need for a more objective method of diagnosis, such as biomarkers.

Biomarkers, which measure biological processes, are shown in many fields to be promising options for the detection of burnout. They can provide more objective data

that complements the self-reporting method of questionnaires, providing a more comprehensive picture of the athletes' health. The hypothalamic-pituitary-adrenal (HPA) axis has been investigated as a key component of the body's stress response that may aid in the detection and prediction of burnout with many biomarkers being identified as potential indicators (Mommersteeg et al., 2008). Cortisol, testosterone, and DHEA-S have shown potential success as clinical markers of the chronic stress levels associated with burnout (Dutheil et al., 2021).

However, these HPA axis-based biomarkers have yielded inconsistent results in studies due to uncontrollable factors influencing their levels. Variations in age, gender and the time samples are collected can significantly affects levels, complicating their reliability as markers of burnout (Sjörs Dahlman et al., 2021). The diurnal nature of these hormones is seen across the board as cortisol, testosterone, dehydroepiandrosterone sulphate (DHEA-s) and Adrenocorticotropic hormone (ACTH) all peak after waking and gradually decline throughout the day (Kamin & Kertes, 2017). Sleep quality and quantity can also affect the HPA axis and changes frequently depending on the person, especially with athletes due to regular travel between competitions, adding to the complications of these biomarkers (Van Cauter et al., 2010).

For athletes, exercise has also been shown to elevate cortisol levels, especially during periods of high intensity such as competition (Dote-Montero et al., 2021). This may make comparing levels before, during and after competition unreliable. Diet and nutrition can also impact the HPA axis, with higher sugar intake increasing cortisol (Zänkert et al., 2020). Competitions may affect this due to reduced access to balanced meals and a change in the timings of meals, making the concentrations of HPA axis biomarkers at set times change day by day (Follenius et al., 1982).

Given these limitations of HPA axis biomarkers, other biological pathways and systems are also being investigated as biomarkers of burnout. Various immune molecules have been identified as potential biomarkers (Mommersteeg et al., 2006). Chronic stress is known to alter the overall immune function, leading to increased inflammation and dysregulation of immune responses. This provides a more stable and reliable indicator of burnout compared to biomarkers linked only to the HPA axis (Segerstrom & Miller, 2004). Putative immune biomarkers would also reflect longer-term physiological changes so may better reflect burnout over time (Dhabhar, 2014). These immune molecules, measured in saliva, serum, blood, and hair may also provide a more varied range of collection methods with saliva and hair presenting less invasive options (Giacomello et al., 2020). However, like the HPA axis biomarkers, their relevance to burnout still requires more research.

Identifying which immune biomarkers may work independently, in tandem with other immune biomarkers or even in tandem with HPA axis biomarkers is therefore crucial in the detection of burnout in athletes (Moore et al., 2024). The literature review which follows will critically examine the current research across immune biomarkers.

It studies those associated with athletes and burnout as well as non-athletic burnout to identify those with the most potential as reliable markers. Those with strongest correlations will be highlighted with their role in the physiological mechanisms of burnout outlined. Finally, gaps in the literature found will be identified and biomarkers which require further investigation will also be highlighted.

2. Literature Review

Previous studies have identified links between possible immune biomarkers and burnout, suggesting potential for early detection. Biomarkers, measured in saliva, serum, blood, and hair, have shown relevance across the sexes and offer a range of less-invasive options for monitoring the symptoms of burnout over time (Deneva et al., 2019). Whilst many promising biomarkers for burnout have been identified, further research is required to validate and optimise their reliability in diverse athletic populations both in laboratory and real-world applications (Moore et al., 2024). With a large age range, high levels of activity and symptomatic overlap of anxiety and depression with burnout, identifying which of the many theorised biomarkers are accurate for use with athletes is complicated.

Burnout shares many symptoms with anxiety and depression, including emotional and physical exhaustion, depersonalisation, and reduced personal accomplishment, complicating detection (Johnson et al., 2020). These overlapping symptoms affect the activity of physiological biomarkers, potentially resulting in inaccurate results. Additionally, if burnout develops alongside pre-existing anxiety or depression, biomarker changes may not be detectable, leading to a false negative (Bianchi et al., 2015). Therefore, diagnostic specificity of these biomarkers is critical, and each biomarker must be investigated to identify if they can be used only on those without pre-existing conditions.

To explore these issues, this review will explore the relationship between potential immune biomarkers and burnout by examining various molecules known to be affected by the immune response seen during chronic stress and burnout. Beginning with general inflammatory markers, pro-inflammatory cytokines, and natural killer cells this review then investigates antibodies and their possible interactions with other biomarkers. Overall, this review of possible immune biomarkers provides a comprehensive overview of a lesser investigated source of potential indicators of burnout, highlighting gaps in the current literature where further investigation could improve burnout diagnostics.

2.1 C-reactive Protein

C-reactive protein (CRP) is a complex protein produced in the acute phase of reaction to inflammation from a major trauma or infection (Toker et al., 2005). This is a reliable marker of inflammation and can be detected at low levels allowing tests to be sensitive (Dixon et al., 2008). Chronic stress, a major predictor of burnout, can dysregulate the body's stress response systems. Prolonged activation of these

systems can lead to increased production of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α), promoting the synthesis of CRP in the liver, raising levels circulating in the body (Dahlman et al., 2021).

Burnout is, however, not consistently correlated with changes in CRP. It is found to significantly positively correlate with increased CRP in women with Toker and colleagues finding a 1.6-fold increase in women with burnout compared to those without (Toker et al., 2005). This increase is, however, not seen to be significant in men, especially once depression is present (Langelaan et al., 2007). In other studies, there is no significant correlation amongst either gender (Bargellini et al., 2000).

CRP can also be significantly affected by various levels of exercise and physical activity due to it reacting to micro-inflammation (Langelaan et al., 2007). Moderate levels of exercise are seen to significantly lower levels of CRP, especially when contributing to a decrease in body mass index (BMI) (Fedewa et al., 2016). Higher intensities of exercise, in contrast, increase CRP, possibly due to the increase of IL-6 found after high levels of physical activity (Gokhale & Chandrashekara, 2007). This would require the test to be performed under very strictly controlled variables and thus would not be possible to accurately reproduce amongst athletes.

2.2 Interleukin-6

Interleukin-6 (IL-6) is a multifunctional cytokine involved in regulating immune responses, inflammation, and stimulating acute phase protein synthesis. As previously mentioned, IL-6 is a precursor to CRP, stimulating its production by the liver. IL-6 changes in concentration during times of inflammation such as during infection and chronic stress (Girotti & Donegan, 2013). This increase in levels of IL-6 is seen in studies with environments that cause burnout such as high levels of work demand with a lack of resources (Falco et al., 2017).

Although IL-6 levels reflect inflammation by a dramatic increase in response to stress it may not make an overall good biomarker for burnout (Girotti & Donegan, 2013). Exhaustion and depression are frequently seen to correlate to IL-6 levels and with depression and burnout being so closely associated and often argued to be one and the same this may pose a challenge when using this as a marker as it may be non-specific (Schonfeld & Bianchi, 2016; Sjögren et al., 2006). High levels of physical activity also effect levels, with up to a 100-fold increased circulatory amount released from the skeletal muscles (Del Giudice & Gangestad, 2018). It is also sometimes seen to act anti-inflammatory as well as pro-inflammatory with post-exercise IL-6 stimulating the release of anti-inflammatory IL-10. This may cause the test to be unreliable in athletes especially due to an inability to differentiate between the burnout-mediated release of IL-6 and the natural release locally by skeletal muscle after exercise (Pedersen & Steensberg, 2001).

2.3. Tumour Necrosis Factor Alpha

Tumour necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine secreted by activated macrophages and T helper 1 (Th1) cells during acute inflammation (van Loo & Bertrand, 2022). It is secreted before IL-6 and IL-1 so could facilitate an earlier detection of burnout (Papanicolaou et al., 1998). In women, TNF-α has been shown to significantly correlate with burnout, whereas in men no correlation is seen (Toker et al., 2005). This may be due to hormonal differences such as oestrogen's potential modulating effect on the immune response, which may make women more sensitive to inflammatory signals (Grossi et al., 2003). Additionally, unlike IL-6, TNF-α's effects are mainly autocrine or paracrine not endocrine. This may explain the lower or nonsignificant changes in the circulating amounts seen in some studies (Steptoe et al., 2001).

During burnout, the ratio of TNF-α to the anti-inflammatory Interleukin-4 (IL-4) and Interleukin-10 (IL-10) are seen to significantly increase. As the pro-inflammatory TNF-α increases and the anti-inflammatory IL-4 and IL-10 produced by TH2 cells decrease their ratio provides a more specific detection method as larger ratios are indicative of increased inflammation (Känel et al., 2008). TNF-α/IL-4 ratios are seen to be most significantly correlated to burnout at $p=0.021$ whereas IL-10 does not consistently show significant correlations at $p=0.996$ (Känel et al., 2008). TNF-α/IL-4 ratios are directly associated with total burnout, allowing it to possibly be a potential biomarker (Känel et al., 2008).

However, IL-4 levels can often be seen to decrease low enough that they are difficult to detect accurately in patients with burnout (Känel et al., 2008). IL-10 also decreases during illness which may cause inaccurate testing results due to the recorded higher levels of illness such as the common cold often seen in patients with burnout (Mommersteeg et al., 2006). Similarly, athletes may have a higher risk of infections so this marker would not be accurate in our target population (Gleeson et al., 1999).

2.4. Natural Killer Cells

Natural Killer (NK) Cells function as mediators at the beginning of the innate immune response, before adaptive immunity occurs. They may therefore act as an early indicator in burnout but may also show changes during acute stress or infection rather than specifically burnout (Nakamura et al., 1999). Levels of IL-6, which were previously seen to increase in burnout patients, have also been found to act as an inhibitor of NK cells (Cifaldi et al., 2015). This decrease in NK cells may further explain the process behind increased illnesses and chronic diseases seen in burnout patients as they cannot efficiently prevent infectious and chronic illnesses (Toker et al., 2012).

Using reduced levels of NK cells as a burnout biomarker has not been commonly studied and does not always show significant changes in burnt-out patients

compared to healthy patients (Mommersteeg et al., 2006). Patients with significant depersonalisation and low sense of personal accomplishment as measured on the MBI do show some negative correlation (p=-0.50 and p=-0.25) with NK cells, whilst emotional exhaustion does not show the same significance (p=-0.09) (Nakamura et al., 1999). This shows that total burnout is not accurately detected by the level of NK cells.

2.5. Immunoglobulin G

With IgG acting as the body's main systemic antibody defence against infection it has been extensively studied during chronic stress, showing some relation to burnout. During times of stress the body's immunity is reduced, therefore, it is theorised that there may be a correlation between the levels of IgG and burnout (Matos-Gomes et al., 2010). Previous studies have found that specific dimensions of burnout such as depersonalisation correlate significantly with reduced salivary IgG levels (H. Tian et al., 2016). This is further seen in other studies with other dimensions such as emotional and physical exhaustion and overall burnout which increases the efficacy as a biomarker as it is sensitive to more than just one dimension (H. E. Tian et al., 2016).

However, initial studies found that this decreased level of IgG may reverse after many years under the same job stress, causing IgG to only be clinically significant on new cases of burnout (H. Tian et al., 2016). With this adapted or normalised immune response, an individual who has already experienced burnout may not be accurately detected when a new case of burnout occurs. This may be hard to accurately screen for as burnout can occur gradually and does not have quantitative diagnostic boundaries to exclude when this biomarker is appropriate (Raedeke & Smith, 2001a; Schaufeli, 1996).

2.6. Secretory Immunoglobulin A

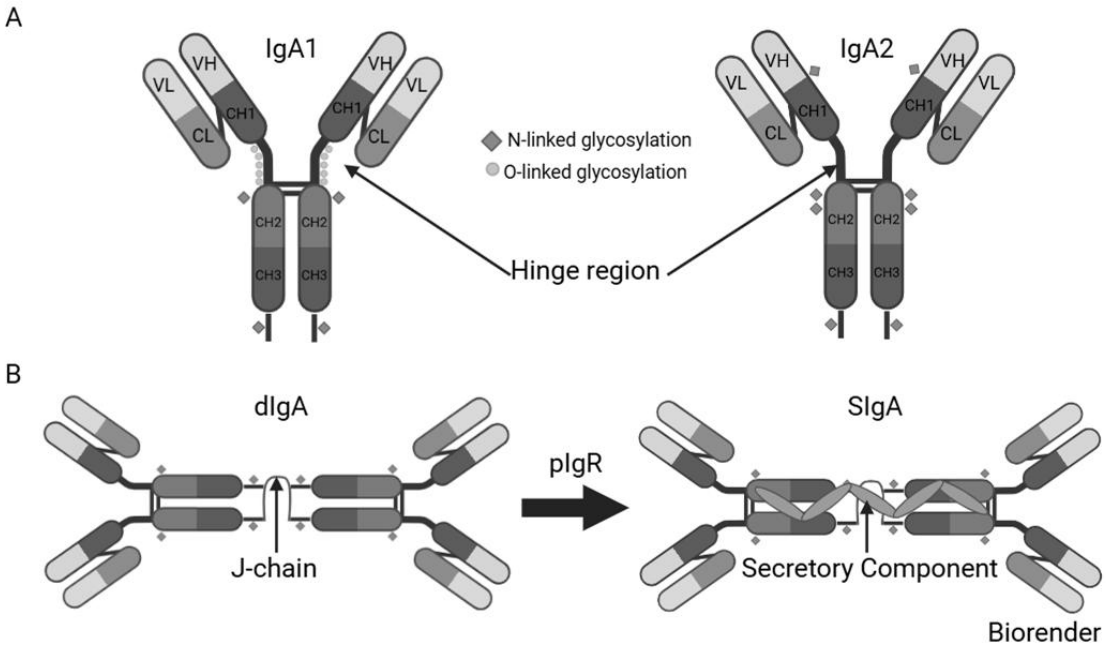


Figure 1 Schematic structures of IgA1, IgA2, and SIgA. A) IgA1 has a longer hinge region with O-glycosylation sites whilst IgA2 has a shorter hinge region which lacks the O-glycosylation sites. They both have N-linked glycosylation sites. B) Dimeric IgA (dIgA) becomes secretory IgA (sIgA) when it binds to the polymeric immunoglobulin receptor (pIgR) on epithelial cells. Once transported across the cell, the pIgR's extracellular portion (secretory component) is cleaved and remains attached to dIgA, forming sIgA. Created using (Scientific Image and Illustration Software | BioRender)

IgA is an antibody crucial to the mucosal immune function. It can be distinguished depending on where it is in the body with monomeric forms of IgA, figure 1a, found in the serum and secretory Iga (sIgA), the polymeric form shown in figure 1b, secreted from the plasma cells in the mucosae following antigen exposure (Hansen et al., 2018). Serum IgA occurs in much lower proportions than sIgA, with only 10-15% of IgA being found in serum (Pietrzak et al., 2020). This makes sIgA an ideal candidate for testing in the field as saliva is safer to handle than blood and serum. As its secretion and defence against pathogens make sIgA an essential mediator of mucosal immune defence it was identified as a possible biomarker for burnout (Castro-Quintas et al., 2023).

Physical symptoms seen during burnout such as increased gastrointestinal distress have also been theorised to link burnout, IgA and mucosal immunity (Li et al., 2020). Elite swimmers have been tracked across a 7-month training period in previous studies and it was found that lower levels of IgA significantly correlated with higher occurrences of respiratory illnesses (Gleeson et al., 1999). This study did however identify a suppression of IgA1 and exercise, with samples at the end of the training period having a lower concentration which did not correlate with infections. Other studies have found that small, recurrent mouth ulcers are seen to correlate with higher levels of IgA and stress from academic exams was also theorised to be associated with the ulcers (Saluja et al., 2012).

These studies found correlations with total IgA but also the individual isotypes of sIgA. Both isotypes of sIgA, IgA1 and IgA2 are found in serum and mucosal secretions, but IgA1 is predominantly found in the serum and may therefore reflect more systemic responses to the stressors in mental burnout compared to the more localised immune response reflected by the more mucosal secretory prevalent IgA2 (Delacroix et al., 1982). Past literature primarily measures total IgA, putting a limitation on this biomarker due to the two forms of IgA being known to act separately to each other. IgA1 is particularly effective at neutralizing viruses and toxins due to its ability to form larger immune complexes whereas IgA2 exhibits greater resistance to bacterial proteases found in the gut (Gleeson et al., 1999). Acute stress has already been seen to decrease levels of IgA1 whilst IgA2 is not significantly affected, this suggests their secretions are controlled different physiological mechanisms, especially in the saliva (Bosch et al., 2001).

IgA1 has more recently been seen to decrease in patients with long-term depressive symptoms and can be compared to the increase of IgA secretory component (IgAsc) in a ratio that significantly increases with symptoms commonly associated with burnout (Engeland et al., 2016). This IgAsc is a protein that facilitates the transport of IgA across the mucosal epithelial cells by binding with the antibodies to form a complex which can now be transported transcytotically, it is shown in figure 1b on both isotypes of sIgA (Corthésy, 2010). Levels of the secretory component may be seen to increase during stress and burnout suggesting higher transport activity whilst actual supply of IgA1 and IgA2 may not meet this demand (Engeland et al., 2016).

IgA2 however, is seen to correlate less to overall burnout with only depression having a significant correlation, seen strongest when in ratio with salivary component (Engeland et al., 2016). These different isotypes of IgA are also seen to change during the course of a day with IgA concentrations following the natural circadian rhythm, peaking just after waking and reaching its lowest just before a person sleeps (Shirakawa et al., 2004). This would therefore need to be managed to accurately test for changes associated with burnout (Nader et al., 2010). There is also a significant change by age with levels gradually rising until 20 years since the first detection at 1 week old (Castro-Quintas et al., 2023). This is a major concern when testing, as adolescent athletes are seen to have burnout ranging from mild to high and many high-level athletes start their competitive careers young (Gustafsson et al., 2007; Odin Layla & Odi Lila, 2017).

Overall, IgA, and especially IgA1 is a promising biomarker candidate due to its consistently identified decrease during burnout. Being so prevalent in saliva allows it to be an ideal candidate, especially in athletes as this allows for safe sample taking without compromising sensitivity. Consideration of the circadian rhythm and age-related changes is required but its utility as a novel biomarker can be seen throughout previous studies.

3. Present Study

This review has identified multiple immune biomarkers as showing promise for identifying burnout in athletes in relation to chronic stress, inflammation and the body's immune response. Biomarkers such as CRP, IL-6, TNF-α, NK cells, IgG, and IgA demonstrate varying degrees of correlation with burnout symptoms. However, CRP and IL-6 show inconsistencies due to common factors such as sex, physical activity, anxiety and depression (Langelaan et al., 2007; Schonfeld & Bianchi, 2016; Toker et al., 2005). Similarly, TNF-α also faces challenges with consistency, especially when detected alongside IL-4, which compromises its practical application (Känel et al., 2008). Finally, NK cells are under-researched, but this is primarily due to their lack of consistent findings (Nakamura et al., 1999).

Immunoglobulins, particularly IgA, have therefore emerged as the most promising biomarker for burnout in our review. IgG showed fluctuating levels in response to chronic stress and social support requiring longitudinal studies to assess its reliability (H. Tian et al., 2016). In contrast, IgA was found to be most promising as it significantly decreases with reported burnout and chronic stress in general. The subclasses IgA1 and IgA2 are specific interests for further research as these could provide a more targeted insight into the changes in physiological processes during burnout, especially in athletes.

As IgA and its subclasses correlate with mucosal immunity these biomarkers could be used to develop non-invasive, field-based tests using saliva (Mak & Saunders, 2006). Unlike blood-based tests, saliva tests would be more accessible, easier to administer and better suit athletes who regularly travel for competition. Currently there are no commercially available saliva-based tests for any of these isotypes. Therefore, developing such tests could allow for more effective detection and prediction of burnout in athletes, facilitating early intervention.

3.2. Aims

The aims of this study are to develop ELISA tests based on saliva as a matrix for the detection of IgA1, IgA2 and IgAsc as potential biomarkers for burnout in athletes. This study will assess how these components of total IgA correlate with dimensions of burnout, such as emotional and physical exhaustion and devaluation, using samples from a three-wave longitudinal study of athlete burnout. The different responses to stress between IgA1, IgA2, and IgAsc will be investigated and their potential to provide a screening method for burnout will be identified. Through this, our research aims to provide a comprehensive understanding of IgA and its isotype's roles in burnout detection based on longitudinal data and their further application into lateral flow tests as non-invasive, accessible testing methods.

4. Materials and Methods

4.1. Design

This study is structured with three phases: assay development, assay validation, and analysis of sIgA levels in a longitudinal athlete burnout study. The first phase, assay development, outlines the design and optimisation of the three sIgA subclass ELISA assays. This includes the selection and optimisation of reagents and their optimal conditions when coating and washing, with sensitivity and specificity identified within the target range. An iterative approach was used as preliminary tests were conducted and adjustments were made as each reagent was optimised individually and together. The aim of this phase was to develop a reliable set of assays.

The second phase, assay validation, outlines the performance of the three developed assays when tested on the target population. Sensitivity and specificity were once again tested along with reproducibility and robustness in the target matrix.

In the third phase, sample analysis, sIgA levels were compared to the psychometric questionnaire results of burnout to establish correlations between the biomarkers and burnout characteristics. These methodologies along with the final ELISA protocols and statistical analysis are detailed in the sections below.

4.2. Reagent Preparation and Storage

Reagent	Storage Temp (°C)	Reference
Unconjugated Mouse-anti-Human IgA1 Antibody	2°C	(Aviva Systems Biology, Wembley, UK)
Unconjugated Mouse-anti-Human IgA2 Antibody	2°C	(Aviva Systems Biology, Wembley, UK)
Monoclonal Anti-Secretory Component (IgA) antibody produced in mouse	-20°C	(Sigma-Aldrich, Gillingham, UK)
Native Human IgA1 Protein	−80 °C	Stratech, Ely, UK
Human IgA2 Subclass Control: anti beta-Gal antibody	−80 °C	(Invivogen, Toulouse, France)
Native Human IgA	−80 °C	(Bio-Rad, Watford, UK)
Mouse anti Human IgA1 (subclass specific) conjugated with Biotin	2°C	(Nordic MUbio, Maastricht, Netherlands)
Mouse anti Human IgA2 (subclass specific) conjugated with Biotin	2°C	(Nordic MUbio, Maastricht, Netherlands)
Mouse anti Human secretory component (free and bound), conjugated with Biotin	2°C	(Nordic MUbio, Maastricht, Netherlands)
Streptavidin-HRP	2°C	(R&D Systems, Abingdon, UK)
TMB Solution VII	2°C	Biopanda Diagnostics, Belfast, UK

Table 1 Reagents and their storage methods

4.3. Assay Development

A systematic approach was used to develop three ELISA assays, each detecting a single IgA subclass. Each reagent was individually optimised for sensitivity and specificity before being combined to ensure the final ELISA procedure was reliable. Using an optimal signal range for our plate reader of 1.0-2.0 IU and negative controls to identify background noise through signal-to-noise ratios (SNRs), calculated as the absorbance signal of the sample divided by the signal of the negative control, ensured reliability whilst duplicates being used throughout ensured reproducibility. This was repeated with saliva samples to ensure reliability in the target matrix.

4.3.1 Dilution Buffer

Phosphate Buffered Saline (PBS) buffers were prepared using three methods to compare efficacy. The first method diluted a 10X PBS stock solution (Fisher Scientific, Waltham, USA) using Deionised water (DiH_2O) to achieve a 1X working solution. The second method utilised commercial PBS tablets (Sigma-Aldrich, Gillingham, UK), dissolved per the manufacturer's specifications to make a 140 mM NaCl, 3mM KCL, 10 mM phosphate buffer. Finally, an in-house 1X solution was made from individual components (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4) and adjusted to a pH range of 7.2-7.4. Buffers were freshly prepared on the day of use to avoid pH drift due to the large range of air temperatures in the storage area, ensuring reliability (Phillips et al., 1963). Each buffer was evaluated using total IgA (0.01-1ug/mL) (Bio-Rad, Watford, UK), comparing signals at target absorbance (1.0-2.0 IU) and SNRs to assess cross-reactivity.

4.3.2 Blocking Buffer Preparation

To block non-specific binding sites in the capture coated ELISA plate wells, a 1% (w/v) ≥98.0% purity Bovine Serum Albumin (BSA) blocking buffer was prepared by completely dissolving BSA in 1X dilution buffer (Sigma-Aldritch, Gillingham, UK. The same dilution buffer then had 1% glucose or 1% sucrose dissolved. These buffers were chosen to explore the protective properties of glucose and sucrose on capture stability during cold storage (Duralliu et al., 2020). The buffers were freshly prepared on the day of experiment and used immediately to avoid degradation and contamination.

4.3.3 Blocking Buffer Validation

To assess the blocking buffers efficacy, wells coated with IgAsc capture were blocked using either 1% BSA in 1X PBS dilution buffer, 1% BSA and 1% glucose, 1% BSA and 1% sucrose or 1X PBS dilution buffer without any additions. Total IgA standards, detector, Streptavidin HRP and TMB were applied as usual, and absorbance was measured. The effectiveness of the blocking buffers was determined by comparing SNRs, with a successful blocking buffer showing a higher SNR, indicating a significant reduction in no-specific binding and background noise.

4.3.4 Sample Dilution

To optimise the assays range and sensitivity for detecting IgA, initial tests evaluated sample dilution factors across a concentration spectrum. Due to known concentrations in saliva peaking at 974 µg/ml in studies from accredited laboratories (Salimetrics®) a range of 1 µg/ml to 1000 µg/ml was identified as being optimal sensitivity for the assay. Standards were available from 1000 µg/ml or lower in stock so a 1/1 dilution was not possible with reagent costs. Therefore, 1/10, 1/100 and 1/1000 dilutions were tested for optimal sensitivity within the optimal signal range (1.0-2.0 IU).

4.3.5 Capture Sensitivity

To optimise the capture antibody, immunoglobulin-specific capture antibodies were serially diluted within a known concentration of total IgA. Monoclonal Mouse-anti-Human IgA1 (Aviva Systems Biology, Wembley, UK) and Monoclonal Mouse-anti-Human IgA2 (Aviva Systems Biology, Wembley, UK) capture antibodies were diluted 1/125 as recommended by the supplier, then further to 1/250, 1/1000 and 1/2,000 to maximise resource usage. The IgAsc capture (Sigma-Aldrich, Gillingham, UK) was diluted 1/1k as recommended, then reduced to 1/3k, 1/6k, 1/12k, 1/24k and 1/48k to attain the optimal assay concentration.

Absorbance was measured for each, with an optimal target absorbance range defined between 1.0 and 2.0 IU for reliable SNR. Once optimal dilution ranges were identified using total IgA, the same series of dilutions were applied to a range of saliva samples. This confirmed that the captures remained specific in our target format.

4.3.6 Detector Sensitivity

To evaluate detector sensitivity, subclass-specific detectors were serially diluted from 1/10 as recommended by the supplier and then reduced to 1/100, 1/1000 and 1/10000 to optimise absorbance whilst minimising resource use. Total IgA was initially tested for optimal absorbance with SNRs used to identify the dilution factor that yielded consistent and reliable results for each subclass of detector.

Once the optimal dilution range for each detector was identified using total IgA, the same series of dilutions (1/10, 1/100, 1/1000, and 1/10000) were applied to subclass specific standards. It was hypothesised, based on past studies, that IgA1 would yield the highest signal, followed by IgAsc and then IgA2 the lowest so this trend was identified for sensitivity (Romero-Ramírez et al., 2023). A range of saliva samples was then tested, confirming that the detectors remained specific in our target format.

4.3.7 Calibration Standards

Calibration curves were prepared using known concentrations of purified proteins to compare to, and identify, unknown concentrations and in saliva samples. For IgA1, Native human IgA1 protein (LifeSpan BioSciences, Lynnwood, USA) was serially

diluted from stock using PBS 1X dilution buffer. A 1500ug/mL – 1ug/mL range was used as it includes the higher end of normal patients and the lower end of patients with illnesses that commonly cause increases in IgA (Saluja et al., 2012). The same range (1500ug/mL-1ug/mL), was used for Human IgA2 subclass control (Invivogen, Toulouse, France) and the IgAsc curve using Human Milk (Nordic MUBio, Susteren, Netherlands).

4.5. Assay Validation

Validation of the assays was performed using a range of saliva samples provided from a longitudinal athlete burnout study to test on a relevant population. These were tested the optimised assays using closely controlled incubation times, wash steps, and consistent dilutions across each subclass, ensuring the reliability and reproducibility of the assay results across all future samples. Their results were used to confirm all ELISA reagents remain within the optimal range (1.0-2.0 IU) in the target matrix with high SNRs.

4.6 Sample Analysis

4.6.1 Participants

Samples from a longitudinal burnout study were tested using the ELISAs with 82 athletes recruited in the first wave of data collection using convenience sampling. Of these, 57 participants completed the second wave and 55 completed the second wave. A subset of 30 participants were tested using the ELISAs as, with all three waves, this was the maximum capacity allowed by the plates used. Additional testing of the other 25 participants was not possible during this study due to depletion of reagents following a third repeat test assessing assay reproducibility.

The inclusion criteria for athletes to be eligible required them to be aged 18 years or older and to be actively competing in at least one sport. Participants were excluded if they had a diagnosis of a chronic health condition affecting the endocrine or immune system or if they had major depressive disorder.

Of our 30 samples (2 female) the athletes were on average 20.4 years of age (SD = 1.6) and all participated in team sports. Athletes competed at club (N = 4) and regional (N = 6) level and had on average competed for 11 years (SD = 3.7). Most athletes were at the beginning of their season at Wave 1 (N = 6), in the middle of their season at Wave 2 (N = 10) and at the middle of their season at Wave 3 (N = 9). Their training load in hours per week increased as the seasons progressed with Wave 1 averaging (M = 12.1, SD = 0.6), Wave 2 averaging (M = 12.5, SD = 1.0) and Wave 3 averaging (M = 13.3, SD = 1.1).

4.6.2 Procedure

Ethical approval for this study was received from the lead researchers' institutional ethics board. Athletes were provided with information about the study's purpose both verbally and in writing and were encouraged to ask questions prior to giving informed

consent. Participants then rinsed their mouths with water before completing the questionnaires followed by the collection of a saliva sample in a cryovial. During the second and third waves, participants repeated the questionnaires and provided a saliva sample. Each data collection wave lasted three weeks, followed by an eight-week break.

4.6.3 Saliva Sample Collection

Passive drool method was used to collect saliva samples, avoiding the parasympathetic stimulation of the salivary glands seen in active drool and contamination seen with swabbing (Bosch, 2014). Samples were collected over 4 minutes into 2mL polypropylene cryovials between 12pm and 3pm to account for natural circadian rhythm affecting salivary IgA. Smoking, eating, and exercising for 2 hours prior to the sample collection was prohibited to prevent their known effects on salivary IgA (Quelemes et al., 2023). The volume was recorded by weighing the cryovials before and after samples were taken to calculate the flow rate and samples were kept on ice until processing on the same day. Samples were centrifuged for six minutes at 10,000 x G to remove bacteria and mucins once in the laboratory. The supernatants were stored at -80°C until analysis. Concentrations were corrected for salivary flow rate by multiplying the absolute concentration by the saliva flow rate. This is recommended practice as the secretion of IgA which heavily depends on the salivary flow rate (Bosch, 2014).

4.6.4 Questionnaire-Based Data Collection

Athlete burnout was measured using the 15-item Athlete Burnout Questionnaire (Raedeke & Smith, 2001). The ABQ measures three dimensions: emotional and physical exhaustion (EPE), devaluation (DEV), and reduced sense of accomplishment (RSA), with five items in each category. Participants rated how often they experienced each symptom across each time point on a 5-point Likert scale, ranging from 1 (almost never) to 5 (almost always).

4.6.5 ELISA Procedure

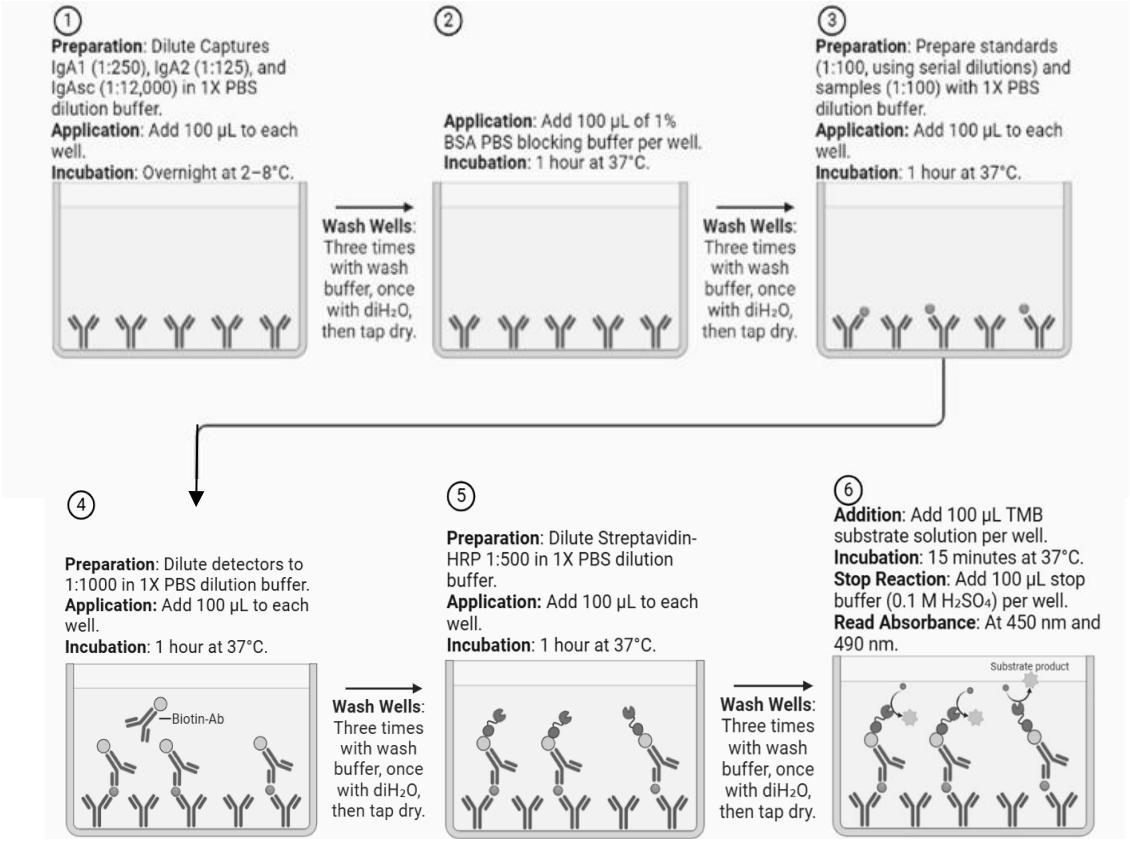


Figure 2 Final ELISA Procedure created using (Scientific Image and Illustration Software | BioRender)

Once all reagents had been optimised figure 2 shows the final concentrations and amounts used with the standardised procedure used for validation. 29 student athletes' saliva was used over 3 waves, with 87 samples from our target populations applied across 3 plates per isotype. These saliva samples were tested in duplicates and the mean of two independent runs was used, freezing between uses to test repeatability and possible issues in large scale testing.

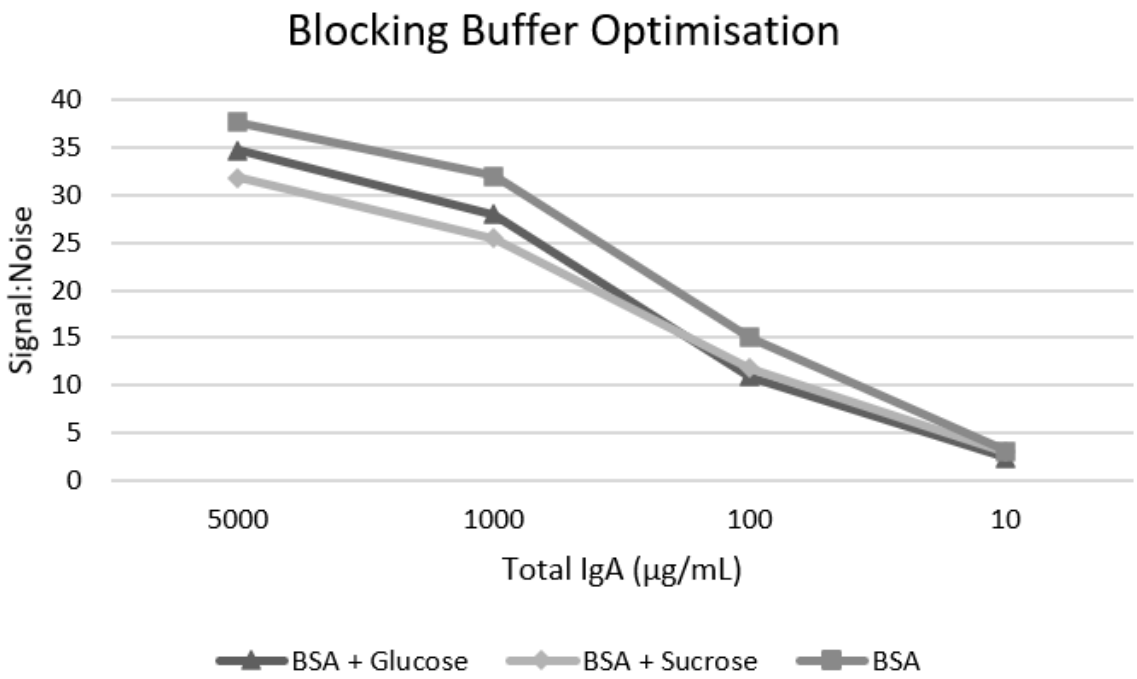
4.6.6 Statistical Analysis

Pearson's correlation coefficient (r) was used to analyse the linear relationship between IgA subclasses and burnout scores. This method identifies the strength and direction of correlations, ranging from -1 (perfect negative correlation) to +1 (perfect positive correlation. The strength of these correlations was interpreted using Cohens scale with 0.1-0.3 being regarded as a small correlation, 0-3-0.5 as medium correlation, and 0.5+ as large correlation (Cohen, 2013). Statistical significance was defined as $p<0.05$.

5. Results

5.1. Assay Development

5.1.1. Buffer Optimisation



The optimal dilution factor for saliva samples was identified as 1/100 and was able to detect the expected physiological concentration range (1–1000 µg/mL) and had a high SNR (34.65). A 1/10 dilution yielded lower signals than the 1/100 dilution, suggesting a hook effect, where the antigen concentrations exceed the binding capacity of the capture and detection antibodies, whilst 1/1000 dilution yielded weak signals below the optimal range and a lower SNR (3.96) with possible non-specific binding. Commercial PBS options (10X PBS stock or PBS tablets) did not yield significantly higher signals within the optimal range compared to in house. Therefore, the in-house buffer was used going forward due to reduced material cost. Figure 3 shows that ELISAs using a 1% BSA blocking buffer had a higher SNR than 1% BSA with added glucose or sucrose. This confirms that 1% BSA in 1X PBS provides reliable blocking.

5.1.2 Capture Optimisation

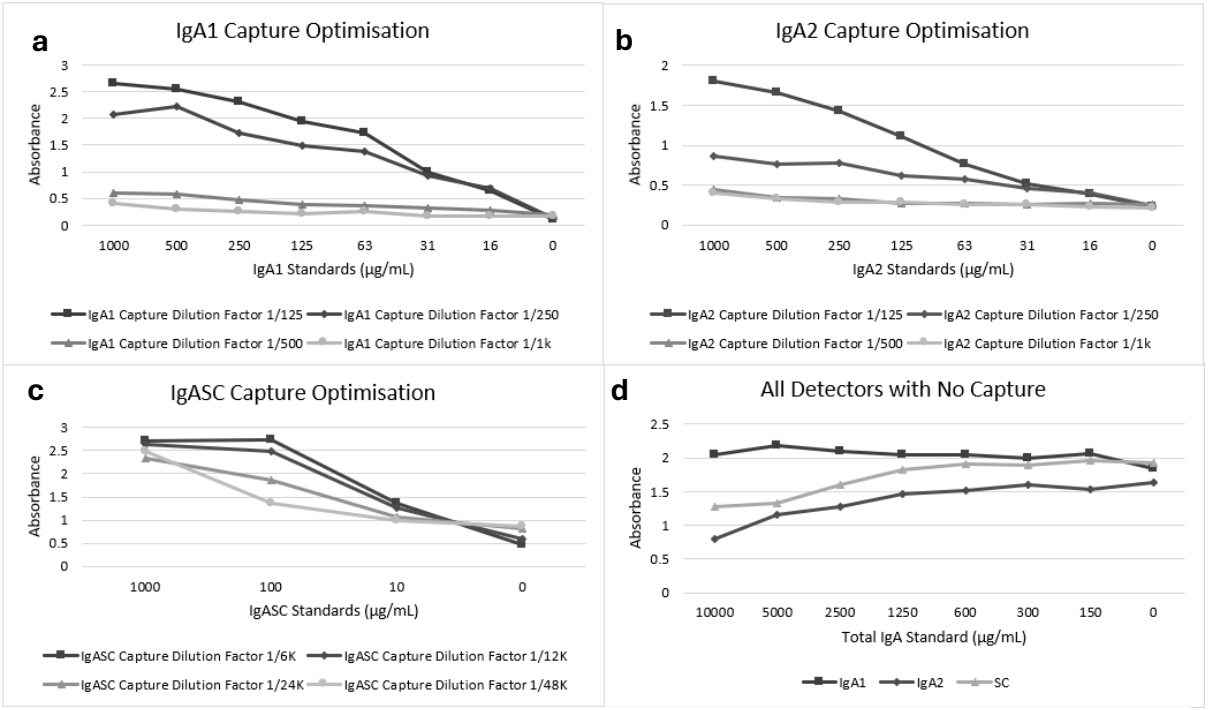


Figure 4 Optimisation of IgA subclass capture concentrations. a) IgA1 capture dilutions compared (1/125–1/1000), with 1/250 yielding the optimal absorbance within range; b) IgA2 capture dilutions compared (1/125–1/1000), with 1/125 yielding highest and optimal absorbance; c) IgA SC capture dilutions compared (1/6000–1/48000), with 1/12000 and 1/6000 yielding equivalent highest absorbance; and (d) specificity testing for IgA1, IgA2, and IgA SC without capture antibodies. All plots present absorbance (y-axis) against specific standard concentrations (x-axis), with graph (d) using total IgA concentration.

Optimal dilutions for subclass-specific captures were identified using serial dilutions. IgA1 capture (1/250) maintained absorbance within the optimal 1.0–2.0 IU range with a high SNR of 25.4 at 100µg/mL, higher capture concentrations (1/125) exceeded the optimal range seen in Figure 4a. For IgA2, however, a 1/125 dilution yielded optimal signals as seen in Figure 4b. IgAsc capture was optimised at 1/12000, achieving the target absorbance whilst higher concentrations shown in Figure 4c showed minimal improvement. These dilutions remained sensitive across saliva samples.

Specificity was also assessed in figure 4d by identifying the SNRs when captures are not used. Capture-free tests showed low SNRs consistently across all detectors and standard concentrations, never yielding above 2.2, indicating elevated background noise and non-specific binding.

5.1.3 Detector Optimisation

	IgA1 Standard (µg/mL)			IgA2 Standard (µg/mL)			IgAsc Standard (µg/mL)		
	1000	100	10	1000	100	10	1000	100	10
1/500 Detector SNR	4.82	1.64	1.12	2.21	1.03	1.02	1.34	0.85	0.86
1/1k Detector SNR	7.49	1.98	1.12	3.81	1.21	1.21	1.17	0.74	0.91
1/2k Detector SNR	8.68	2.12	1.05	2.85	1.46	1.20	0.87	0.57	0.89
1/4k Detector SNR	6.14	1.91	0.95	1.91	1.02	1.18	0.99	0.75	1.01

Table 2 Detector optimization for IgA subclasses shown as the signal-to-noise ratio of the absorbance at standards within and above the expected range to the negative control.

Each detector's specificity was evaluated with the expected signal strength order seen in table 2; IgA1 yielding the highest signal at 1000µg/mL with a 1/500 detector of 1.05IU, followed by IgAsc at 0.19IU and IgA2 at 0.15IU. Negative controls showed higher non-specific binding at 1/500 detector, with SNRs at 1000µg/mL and 100µg/mL seen in table 2 as 4.8 and 1.6 at 1/500 compared to 7.5 and 2.0 at 1/1k detector for IgA1, 2.2 and 3.8 at 1/500 and 3.8 and 1.2 at 1/1k for IgA2. IgAsc had small differences between detector concentrations so 1/1k was used for consistency. Saliva samples yielded the same order of signal yields showing consistent detector performance across matrices.

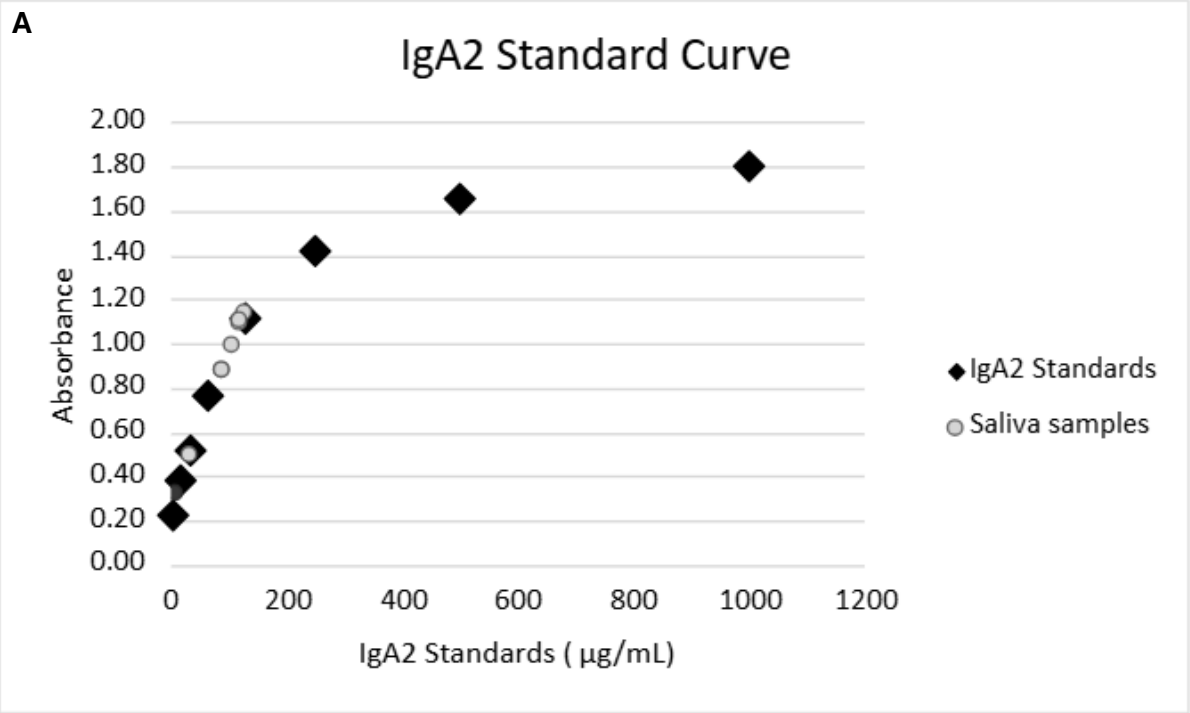
Detector sensitivity was optimised using serial dilutions (1/10 to 1/10000) against specific standard concentrations (0-1000µg/mL). Optimal absorbances and high SNRs were seen at 1/1000 dilution, with 1/500 dilution showing lower SNRs and non-specific binding with the negative control. The 1/1000 dilution maintained strong signals and high SNRs in saliva matrices.

Low SNRs at lower, expected concentrations were identified to be due to a cross-reactive streptavidin HRP which was replaced.

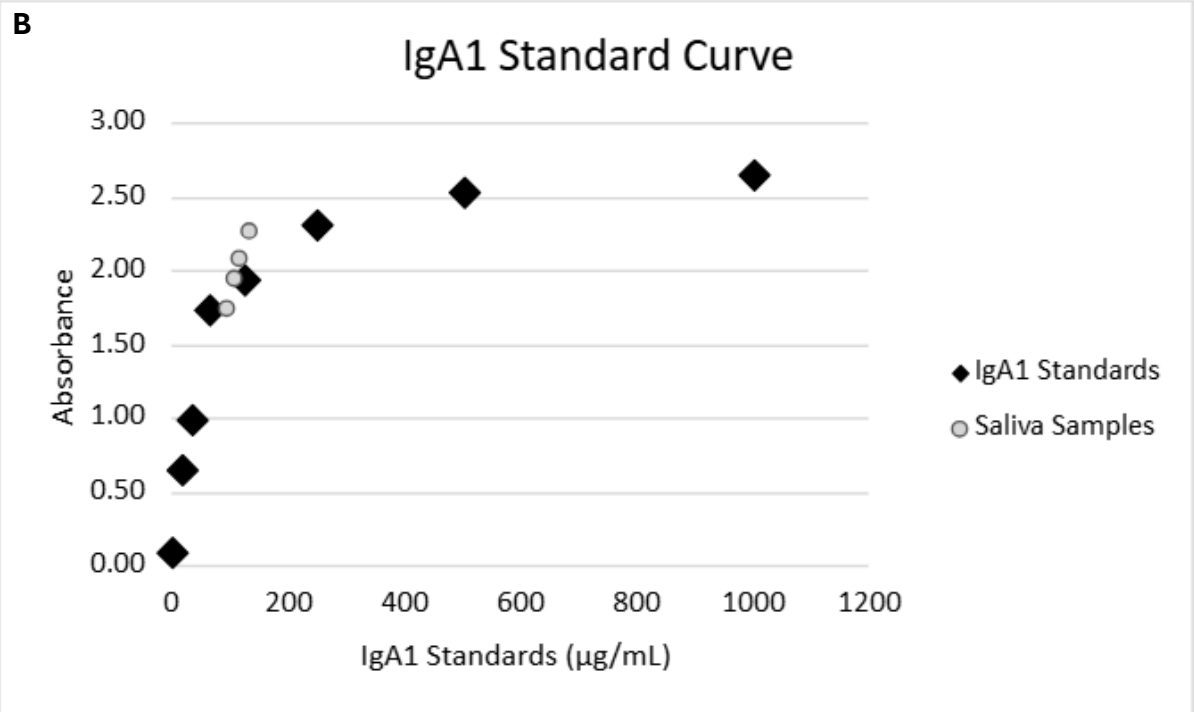
5.1.4 Calibration Curves

Standard curves were identified and compared with non-athlete saliva to confirm the physiological concentrations of IgA1, IgA2 and IgAsc would fall within the detectable range. Commercial standards were spiked into the PBS buffer at a range of expected concentrations (15µg/mL to 1000 µg/mL), diluted by 1/100 as determined earlier, were used to generate the calibration curves.

A


IgA2 Standard Curve
Absorbance
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
200
400
600
800
1000
1200
IgA2 Standards (µg/mL)
IgA2 Standards
Saliva samples

B


IgA1 Standard Curve
Absorbance
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0
200
400
600
800
1000
1200
IgA1 Standards (µg/mL)
IgA1 Standards
Saliva Samples

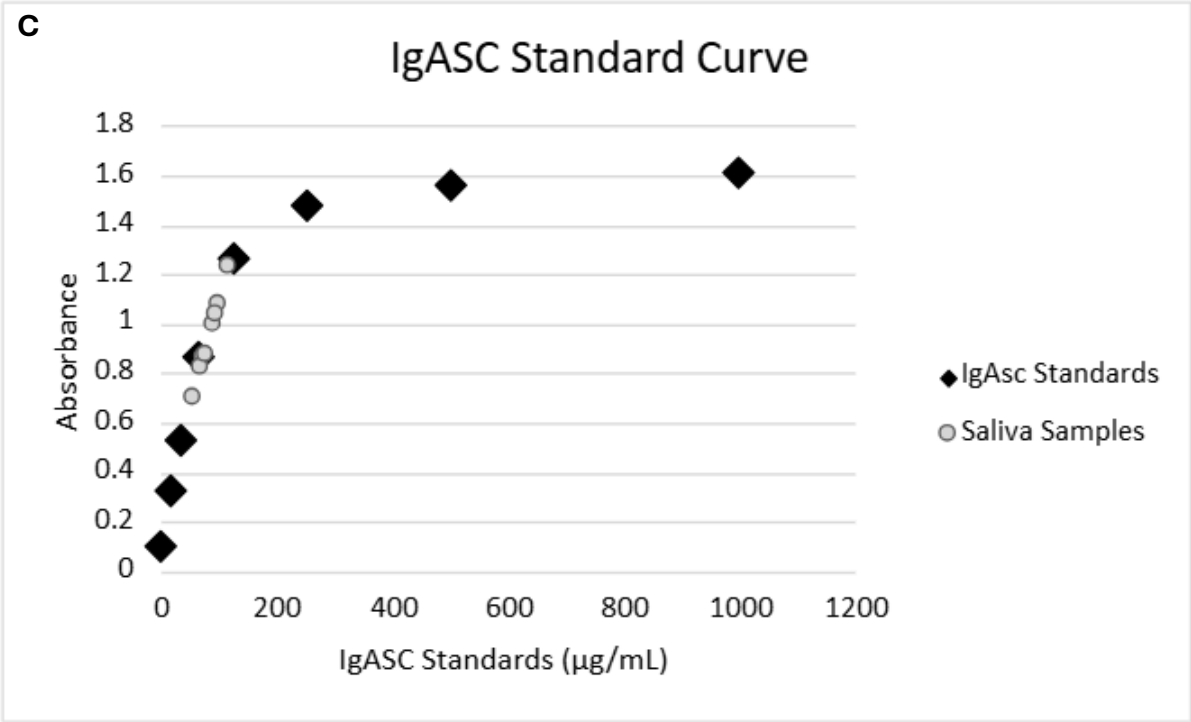
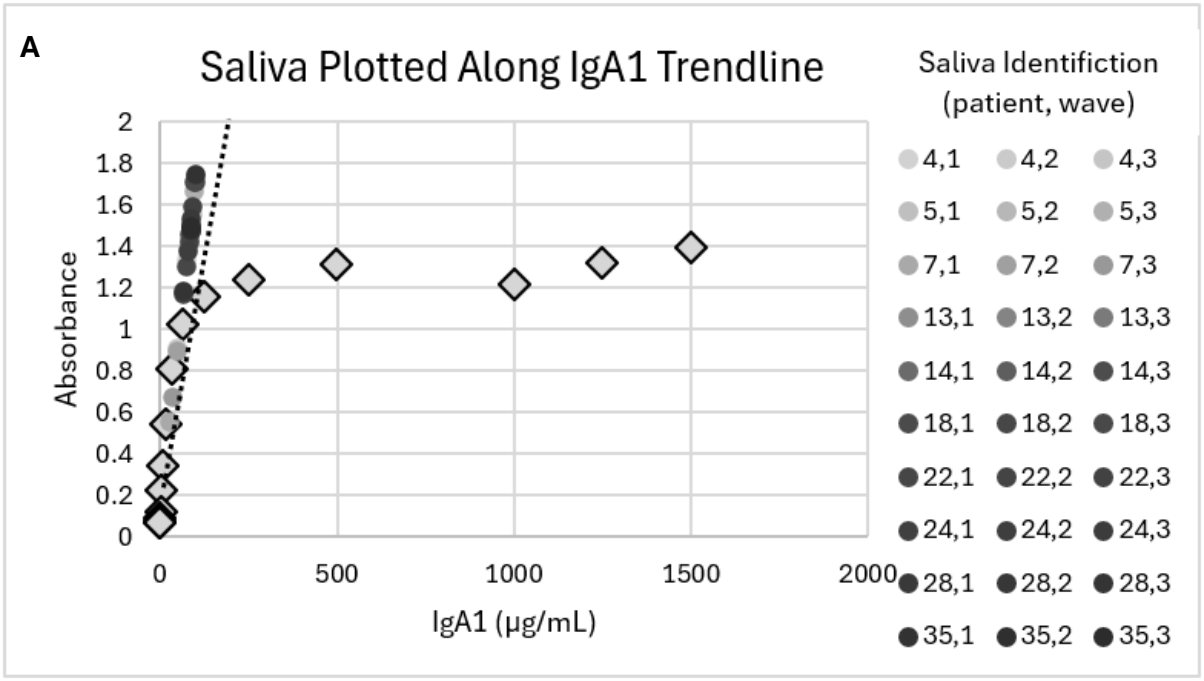
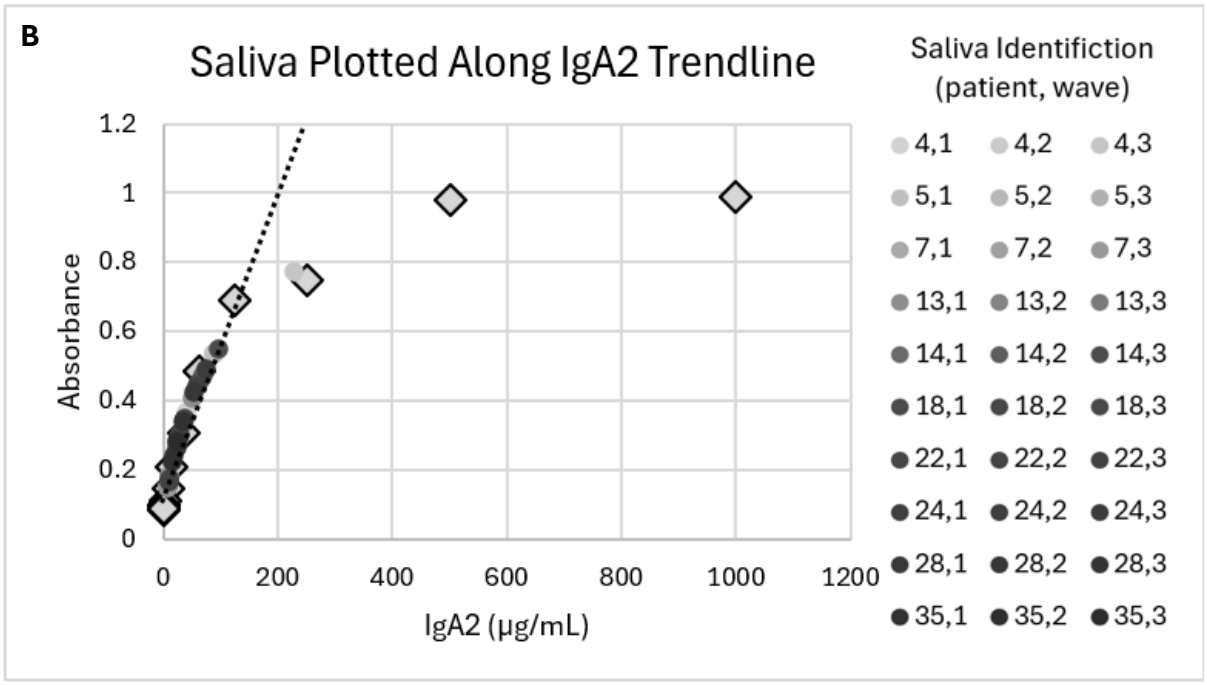


Figure 5 Standard curves for IgA subclasses with saliva sample data. a) The standard curve for IgA1, using specific IgA1 standards; b) the standard curve for IgA2, using specific IgA2 standards; and c) the standard curve for IgA SC, using specific IgA SC standards. Saliva samples are included in each graph to demonstrate if the standard curves are sensitive to the population tested and identify if all samples fall within the detectable range of the respective standard curves. Saliva samples are labelled to indicate they fall out of the detectable linear range, none labelled sampled are within the detectable range.

For IgA1 and IgAsc, seen in figure 5a and 5c, all saliva samples yielded absorbance values within the standard concentrations testable range. A plateau was reached at the higher range of concentrations tested, indicating the assays' peak absorbance had been covered by this range. This showed all samples were within the assay's sensitivity range. IgA2 however had a sample below the lowest standard (15µg/mL), marked in figure 5b, showing that some samples in the population could not be detectable accurately with the current standard curve. Therefore, for future assays the standard curve for all assays was lowered to include 0.25µg/mL to ensure all saliva samples would be detectable within a linear range.

5.2 Assay Validation





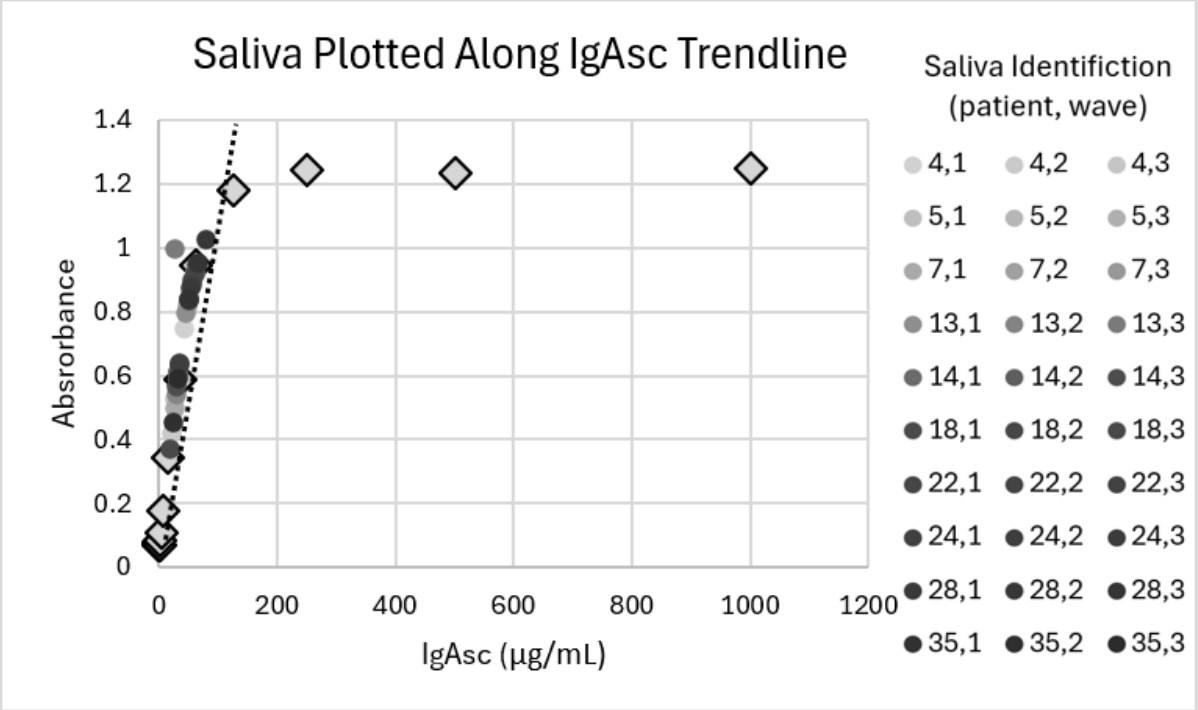


Figure 6 Standard curves validated by determining the concentrations of longitudinal saliva samples. a) IgA1 standard curve, with a linear range between 0.25 µg/mL and 250 µg/mL, with only 13% of saliva samples falling within this range and able to be accurately identified; b) IgA2 standard curve, with a linear range between 0.25 µg/mL and 125 µg/mL, with 97% of saliva samples falling within this range and all samples able to be accurately identified using a 4 parameter logistic (4PL) curve (4PL) and; c) IgA SC standard curve, with a linear range between 0.25 µg/mL and 125 µg/mL, with all 100% of saliva samples falling within the range and able to be accurately identified using a 4PL curve.

All athlete saliva samples yielded absorbances above the negative control as seen in tables 6a, b and c showing the assay's sensitivity can detect low levels in the population. The ranges of SNRs are indicative of the expected difference in signal strength across each isotype with IgA1 having the highest (M=20.41 SD=4.28), followed by IgAsc (M=9.57 Sd=2.47) and IgA2 (M=3.99 SD=1.60) indicating that background noise can be differentiated from all our targets.

IgAsc showed a linear range from 0.25µg/mL-125µg/mL (R2=0.93) in figure 6c, with signals plateauing beyond this concentration, indicating maximum absorbance. All saliva tested (87) were within this range, allowing concentrations to be determined using a 4-logistic parameter (4PL) curve. This validates the assay to detect IgAsc in this population.

Similarly, IgA2 in figure 6b shows a linear range of 0.25µg/mL-125µg/mL (R=0.98), with signals plateauing beyond this concentration. One sample (3.33% of the total) fell outside of this range, yielding a 30% higher signal than the next highest sample. Duplicate testing confirmed consistency, suggesting biological variability or a sampling error. The concentration could still be determined using a 4PL curve showing this assay was accurate even amongst unexpectedly high samples.

For IgA1, the linear range extended from 0.25µg/mL-62.5µg/mL (R=0.93) shown in figure 6a, however only 4 of the 30 samples (~13%) were within this range. The hook

effect may be causing this, as seen at 1000 µg/mL where the absorbance is 1.22, but it then unexpectedly increases to 1.31 at 500 µg/mL, before decreasing again to 1.24 at 250 µg/mL. This indicates that the current test format would not accurately detect most samples (87%), limiting the assay's current applicability.

During repeat testing on new plates, the IgA1 standard absorbances increased by an average of 164.88%, whilst IgA2 and IgAsc standards decreased by 42.8% and 19.23% respectively. This suggests significant issues with the repeatability of the assay when reagents are subject to rounds of freeze-thawing. Saliva absorbance values were also affected, with IgA1 decreasing by 46.48%, IgA2 increasing by 335.93% and IgASc increasing by 106.57%.

5.3. Correlation to Burnout

	Emotional and Physical Exhaustion	Devaluation	Reduced Sense of Accomplishment	Total Burnout	IgA2	IgAsc
Emotional and Physical Exhaustion	-	0.56 (p=0.001)	0.68 (p=<0.001)	0.9 (p=<0.001)	-0.02 (p=0.929)	-0.20 (p=0.299)
Devaluation	0.56 (p=0.001)	-	0.61 (p=<0.001)	0.78 (p=<0.001)	-0.19 (p=0.320)	-0.05 (p=0.781)
Reduced Sense of Accomplishment	0.68 (p=<0.001)	0.61 (p=<0.001)	-	0.89 (p=<0.001)	-0.25 (p=0.183)	-0.30 (p=0.105)
Total Burnout	0.9 (p=<0.001)	0.78 (p=<0.001)	0.89 (p=<0.001)	-	-0.15 (p=0.416)	-0.23 (p=0.219)
IgA2	-0.02 (p=0.929)	-0.19 (p=0.320)	-0.25 (p=0.183)	-0.15 (p=0.416)	-	0.44 (p=0.014)
IgAsc	-0.20 (p=0.299)	-0.05 (p=0.781)	-0.30 (p=0.105)	-0.23 (p=0.219)	0.44 (p=0.014)	-

Table 3 Matrix of Pearson's Correlation Coefficients displaying the strength and direction of linear relationships between burnout characteristics (Exhaustion, Devaluation and Reduced Sense of Accomplishment), Total Burnout and IgA subclass biomarkers (IgA1 and IgAsc). Correlation values range from -1 (perfect negative correlation) to +1 (perfect positive correlation), with 0 indicating no linear relationship.

Correlations between self-reported total burnout levels and burnout symptoms with the different IgA subclasses were evaluated overall as individual timepoints had too low sample sizes to estimate correlations. As shown in table 3, none of the differing correlations seen amongst the IgA subclasses, total burnout and burnout symptoms reached statistical significance. IgA2 exhibited a weak negative correlation with total burnout $r=-0.15$ ($p=0.416$), whilst IgAsc showed a weak positive correlation $r=0.23$ ($p=0.219$) though neither of these reached statistical significance.

When investigated further, the individual dimensional scores of burnout similarly showed only weak, non-significant correlations with IgA2 and IgAsc. Reduced Sense of Accomplishment had the strongest, though still non-significant, correlations with both IgA2 at $r=-0.25$ ($p=0.183$) and IgAsc at $r=-0.30$ ($p= 0.105$) being negative. Other dimensions shown in table 3, like Emotional and Physical Exhaustion and

devaluation also showed non-significant correlations. Notable whilst both were negative, devaluation was the only symptom stronger in IgA2 than IgAsc although this difference was minimal and non-significant.

Due to insufficient samples being within the detectable range for IgA1, statistical analysis could not be performed.

6. Discussion

This study aimed to address the lack of studies examining IgA subclass profiles in saliva in the context of athlete burnout. As all subclasses play critical roles in mucosal immunity, their responses to stress make them compelling targets for research (Li et al., 2020). IgA1 is found predominantly in serum and is theorised to reflect systematic responses to burnout with IgA2 by comparison correlating more to individual dimensions of burnout and being seen most in gut and mucosal immune defence (Gleeson et al., 1999). IgAsc facilitates transport and protection of IgA1 and IgA2 so changes in tandem with them both which can be measured as a individual marker and in ratios with the other subclasses (Engeland et al., 2016).

Initial tests were performed to develop and optimise ELISAs for the three identified subclasses IgA1, IgA2 and IgAsc in a singleplex sandwich format. These specific ELISAs will facilitate future research into their theorised uses as biomarkers for athlete burnout. After optimisation, these tests utilised longitudinal saliva samples across athlete's competitive seasons to identify any relevant correlations. Non-significant correlations were seen between IgA2, IgAsc and burnout and its' dimensions. The key findings of this study are discussed in the following after which limitations and possible future applications are considered.

6.1 Key Findings

6.1.1. ELISA Development

The development and optimisation of the three specific ELISAs addressed key challenges in specificity whilst maintaining stability and cost-efficiency. All reagents then performed with low non-specific binding across matrices and in the target population. This mirrored the approaches of other assay developments which achieved high specificity in the saliva matrix through optimised antigen and antibody selections (Jiang et al., 2021).

Buffer optimisation identified cheaper, simpler options as superior. The in-house PBS buffer was the most effective for dilutions and the addition of 1% BSA created an optimal blocking buffer. Commercially available buffers and extra additions were not significant improvements. This enhanced the ELISAs specificity without compromising on cost-effectiveness despite studies having found additions like sugars can act as cryoprotectants for antibodies such as our captures (Gutka, 2018). This will be beneficial to the future use of these tests as lower overall costs for tests will allow them to be used in wider sports environments across age groups and

socioeconomic backgrounds. It will also encourage tests to be used more frequently, enabling burnout to be caught earlier and treated with higher efficacy.

When optimised, the IgAsc and IgA2 ELISAs were able to test the concentrations of 30 saliva samples, across 10 participants and 3 time points from a three-wave longitudinal study of athletes. The concentrations were identified against a standard control curve of known concentrations. All participant saliva absorbance values were consistently above the negative controls indicating that assay sensitivity has been optimised at the lowest end of the tested range present in this population. It had been expected that athletes may have lower than normal levels from findings in past studies, however all samples were within the expected range of concentrations (Ben-Aryeh et al., 1986; Gleeson et al., 1999).

6.1.2. sIgA and Burnout

Using self-reported questionnaires, burnout scores were paired with the IgA2 and IgAsc concentrations in saliva across the three-wave study. However, neither biomarker demonstrated statistically significant correlations with burnout. While both biomarkers both showed a weak negative correlation with total burnout and its individual dimensions, with IgAsc especially showing a stronger correlation in total burnout, these did not reach significance and should be interpreted with caution due to the small sample size. These weak correlations have been seen in previous studies with depressive symptoms in university students being negatively associated with the IgA2/IgAsc ratio with the weaker correlation when compared to IgA1 being theorised to show it is therefore less responsive to stress (Engeland et al., 2016). This suggests that these are regulated differently by mucosal signals with IgA2 more prominent in the gut due to its resistance to bacterial proteases and its modulation by the microbiota. In contrast IgA1 is more common in the respiratory tract and with chronic stress disrupting systemic immunity rather than microbiota interactions burnout is likely to affect IgA1 more (Pabst, 2012).

IgAsc showed a stronger, but still non-significant, negative association with total than IgA2 which may be due to its overall link to mucosal immune function as it transports both IgA1 and IgA2. As the secretory component aids in transportation of both forms of IgA and shields them from enzymatic degradation changes in its concentration in different parts of the body would accompany the changes in both IgA1 and IgA2 (Corthésy, 2010). With IgA2 only regulated by local microbiota it is less affected by systemic stress and if none of our participants had severe burnout it may not have had enough chronic stress to significantly reflect. This could be further investigated with a larger sample size and a more diverse population.

Devaluation had a weak negative correlation with both IgA2 and IgAsc, with these both being non-significant but stronger in IgA2. This may suggest a mechanism of action with devaluation affecting the gut and digestive system locally, influencing the IgA2 levels directly whilst the more systematically influenced IgAsc is not as reactive at this level. In contrast, EPE showed the reverse effects with IgAsc having the

stronger negative correlation than the IgA2. Although all correlations were weak and not statistically significant, this pattern may reflect a more systemic, physiological impact of EPE, including IgA1, and causing broader decreases in levels of IgAsc. EPE is also associated with increased inflammation due to being a physical factor which may further cause immune activation (Toker et al., 2005). The strongest correlations with both IgA2 and IgAsc were seen in RSA. This was non-significant but higher than total burnout in both suggesting it impacts both localised and systemic immune function but may also affect immune function in a more specific way than the broader total burnout.

As burnout is linked to higher cases of infection and slower recovery from injuries these negative correlations, though non-significant, are consistent with expectations. An increase in IgA would act as a protector against immune suppression so a decrease removes this protection and increases the cases of illness amongst athletes (Gleeson et al., 1999). With RSA being the strongest correlation across both isotypes and EPE being the next strongest correlated with IgAsc this also shows that IgAsc is best at detecting earlier symptoms of burnout. This would be beneficial for mitigation strategies to be applied earlier in the progression of burnout if it is found in larger scale studies to work well. However, as total burnout is not the strongest correlated when compared to its dimensions, RSA for both isotypes and devaluation for IgA2, these biomarkers may not be specific to burnout and are instead specific to earlier symptoms which has been theorised in past studies (Edú-valsania et al., 2022).

6.2 Technical Limitations

There was a lack of statistically significant findings which may be due to the small sample size used. With 10 participants over 3 time points if any participants became ill and did not identify this prior to giving a sample then 3.33% of our samples would be affected. Similarly, if any participants had exercised the day of the sample taking the levels of IgA1 would naturally be decreased, affecting the levels of IgAsc and increasing the chance of anomalous results. With such a small sample size this would have a proportionally large effect on results. As only 20% of our sample size were female and all were under 25 these results were also not representative of the population we are studying. Previous studies have found other immune biomarkers to be significantly correlated with burnout in women but not men so this lack of female participants may have affected our results (Langelaan et al., 2007).

Significant challenges were also identified due to timing and logistical constraints in a dual-use laboratory. The first round of testing yielded absorbance signals above the detectable range of the plate reader due to limited access of incubators causing plates to be coated for longer than designated in the procedure. This was solved in the second round of testing by ensuring equipment was available. During this testing the IgA1 ELISA was too sensitive to measure 87% of samples tested and we were not able to identify any trends with burnout and its dimensions. This is theorised to

be due to the hook effect, with the antigen concentrations exceeding the binding capacity of the capture and detection antibodies (Hoofnagle & Wener, 2009). Further dilution of both the standards and the saliva samples are therefore required in future testing to optimise this assay and allow it to be sensitive to the target population.

Unfortunately, during the final round of testing, reagent stability was highlighted as an issue as all standards and saliva tested changed in absorbance. The high viscosity of saliva samples may have contributed to human error due to precise pipetting of samples being difficult, affecting dilution (Porex Life Sciences Institute, 2020). However, the consistency in changes across all saliva and standards indicates a wider issue with the freeze-thaw process used on all reagents except streptavidin HRP. Aggregation and degradation of the proteins during freeze-thaw cycles from -10°C to 37°C may cause the range of changes seen (Paul & Hesse, 2013). These effects can impair binding and affect signal intensity, but previous studies have not found evidence of this at the extent we are seeing (Booth et al., 2009). As both IgA2 and IgAsc standards decreased in absorbance whilst the respective saliva sample absorbance levels increased, structural differences between the IgA subclasses cannot be the only difference in resistance to freeze-thaw damage (Göritzer et al., 2024). These results highlight the need for further testing to determine whether these changes are from human error during the procedure or instability of reagents after repeated freeze-thaw cycles.

During optimisation, the IgA2 ELISA identified a single sample outside of the linear range of the test. This was still within measurable range for the 4PL curve developed using the standard curve but was 30% higher than the closest sample and may indicate a current infection or allergic response (Gleeson et al., 1999). Whether this high concentration is an anomalous issue with sample collection, storage and processing or an expected change due to an immune response, the assay was still able to measure it within accurate and range and comparisons with other biomarkers such as IgA1 could help to identify the cause (Sánchez-Salguero et al., 2019). The variability must be further investigated using a larger sample size to identify if it is suitable for testing athletes whose regular travel can increase their risk of infection (Gleeson et al., 1999).

6.3 Future Applications

The ultimate goal of this study is to provide an accurate test for measuring the concentrations of IgA1, IgA2 and IgAsc to enable future testing of these biomarkers on athletes, identifying if they are suitable for early detection of burnout. This early detection could allow for mitigation strategies to be implemented, preventing total burnout and reducing the psychological and physiological effects it can cause (Gustafsson et al., 2008). A transfer from ELISA format to non-invasive lateral flow tests may also be possible for accessible, real-time screening in athletic and broader settings (O'Farrell, 2015). This could further improve the ability to catch early burnout

during times of travel and in communities without access to laboratories capable of performing full ELISAs.

Possible improvements, especially if future testing shows significant correlations between burnout, could also include multiplexing the tests, allowing for more efficient and convenient testing formats (Tighe et al., 2015). Combining these biomarkers if they are shown to have weak, but significant trends may increase reliability as the different biomarkers can reduce occurrences of false positive results as, for example, our study found IgAsc specifically is more reactive to devaluation than the other dimensions of burnout. Combining this with another biomarker which is more sensitive to the other dimension or total burnout may create an overall reliable test (Tighe et al., 2015).

7. Conclusion

The findings of this study are mixed yet promising in the further study of IgA subclasses for detection of burnout in athletes. Being closely associated with EPE, physical fatigue, and reduced performance, burnout poses a substantial threat to athletes' careers and well-being (Gustafsson et al., 2008). If these biomarkers can provide early and accurate detection and improve on current methods of detecting such as subjective self-reported questionnaires, mitigation of burnout may be more affective (Purcell et al., 2019). As these immunoglobulins are integral to mucosal immunity and respond dynamically to stress they will hopefully be found to be ideal targets for burnout detection in saliva, proving to be a reliable, easy to use and cost-effective test (Matos-Gomes et al., 2010).

Initial optimisation of reagents ensured specificity and eliminated reagents which caused cross-reactivity. The use of in-house buffers and identification of high dilution factors for both captures and detectors enhanced sensitivity and reduced costs. Further optimisation of dilution factors across both standards and saliva samples ensured that IgA2 and IgAsc samples were within the detectable range, increasing testing efficiency by using minimal sample volume. The IgAsc ELISA emerged as the most robust, with a reliable linear testing range and the IgA2 ELISA also showed a strong linear range, though outliers indicated the need for additional validation. In contrast, the IgA1 assay faced limitations, with most absorbance values being outside of readable range and a hook effect seen at higher concentrations, suggesting further dilution and optimisation is required.

These assays demonstrated high specificity and sensitivity; however, variability during repeat testing raised concerns for the stability of reagents during storage, especially across freeze-thaw cycles. Initial testing against self-reported questionnaires revealed non-significant, but present correlations between IgA2, IgAsc and burnout dimensions as well as total burnout. IgA2 showed negative correlations with total burnout whilst IgAsc showed positive correlations, however, once individual dimensions were compared IgA2 also showed some positive

correlations. With only 30 samples tested these weak, non-significant correlations may more susceptible to anomalous results as seen in IgA2 testing. These findings support the need for further validation in larger populations and further refinement for the IgA1 assay.

8. References

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