The Effects of Caffeine on Salivary SIgA Concentration During Exercise and the Influence of the CYP1A2 Genotype rs762551

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Declaration

The following thesis has been submitted to the University of Kent only in order to fulfil the requirements for an MSc in Sports and Exercise Science (By Research and Thesis). The following work is my own and information that has been taken from previous researchers and authors have been cited. Descriptions for Statistical Tables in Appendix K are taken from IBM SPSS Statistics Version 27.0 (Armonk, NY: IBM Corp).

Abstract

Salivary secretory immunoglobulin A (saliva SIgA or salivary SIgA) is an integral part of mucosal immunity and is often the first to encounter antigens and foreign invaders. Should an individual's salivary SIgA concentration decrease, they could be more susceptible to an upper respiratory tract infection(s) (URTI). Exercise may induce increases or decreases in salivary SIgA concentration depending on the nature of the exercise. Few studies have investigated as to whether caffeine supplementation can influence exercise-induced changes in salivary SIgA. Nine male and two female healthy, recreationally active (VO_2 max: 52.18 ± 10.89 ml/min/kg, mean ± standard deviation) participants exercised for 30 minutes on a treadmill comprised of a 2.5 minute warm up, 5 intervals of 4 minutes at 70% VO_2 max and 1 minute at 40% VO_2 max, and a 2.5-minute cool down 1 hour after consuming either a placebo, a 2 mg/kg of body mass, or a 4 mg/kg of body mass dose of anhydrous caffeine. Saliva samples were taken upon arrival to the lab (initial), 1 hour post caffeine ingestion (pre-exercise), post-exercise, and 30 minutes post-exercise. Saliva was also used to measure osmolality. During analysis, saliva concentration was measured by both absolute concentration and concentration relative to osmolality. Capillary samples were taken to determine participant genotype for CYP1A2 rs762551. A three-way mixed ANOVA showed no significant main effect of condition, condition x time interaction, or genotype interaction ($p > 0.05$ for all). However, there was a significant main effect for time ($p < 0.05$). Post hoc analysis revealed a significant difference between salivary SIgA concentration relative to osmolality post-exercise and 30 minutes post-exercise (1.45 ± 0.63 vs. 2.05 ± 0.38 mg/mOsmol; $p < 0.05$). Post hoc analysis also revealed a significant increase in absolute salivary SIgA concentration post-exercise compared to initial and pre-exercise levels (153.03 ± 18.46 vs. 125.49 ± 3.15 and 153.03 ± 18.46 vs. 114.24 ± 10.88 mg/L). These findings suggests that caffeine does not have any influence on the exercise-induced salivary SIgA response.

Key words: **salivary SIgA, caffeine, exercise, genotype**

List of Figures and Tables

Chapter I: Introduction

Exercise has been known to prevent a number of noncommunicable diseases (NCDs) across all stages of life (Saqib et al. 2020). Lower rates of NCDs brought on by an active lifestyle can also contribute to a delay in all-cause mortality (Blair et al. 1989; Macera et al. 2003). In addition to its physiological benefits, physical activity can also improve self-esteem, decrease anxiety, and ameliorate overall mood (Mikkelsen et al. 2017). However, participating in extreme levels of exercise over an extended period of time can negatively impact overall health (Armstrong et al. 2015). The respiratory system is especially prone to URTIs following bouts of intense endurance exercise (MacKinnon and Jenkins, 1993). Epidemiologic data suggests that the risk for developing an URTI is greatest during heavy training and 1-2 weeks following prolonged and or strenuous competition events (Nieman, 1997). The association between the risk of URTI and exercise workload has been described as 'J' shaped by Nieman (1997). As seen in Figure 1., this pattern suggests that while high intensity exercise may contribute to the development of an URTI, moderate exercise can help minimize the risk of an URTI. The relationship described in Figure 1. by Nieman (1997) is also supported by Gleeson and Pyne (2000).

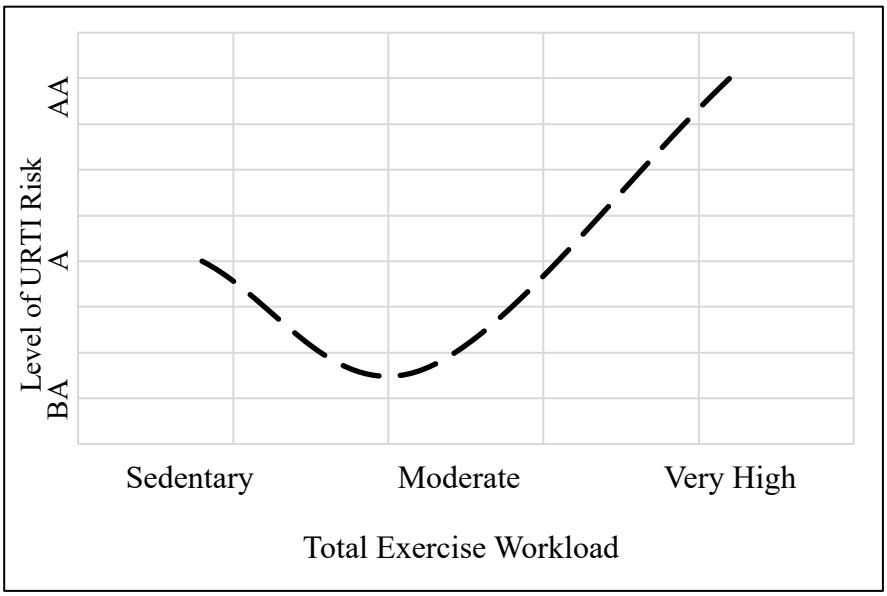

Figure 1. 'J' Shaped model relation between amounts of exercise and risk of respiratory tract infection risk. Adapted from Nieman, 1997. BA: Below Average level of risk for a URTI; A: Average level of risk for a URTI; AA: Above Average level of risk for a URTI

Habitual exercise at very high or intense levels can not only increase the risk of an URTI but can also cause the suppression of mucosal immune markers such as salivary SIgA (Gleeson and Pyne, 2000). Salivary SIgA is the most abundant antibody produced and is responsible for the defence of mucosal surfaces in the respiratory, gastrointestinal, and genitourinary

tracts (Jacob et al. 2008). It can therefore be considered an integral part of the immune system's first line of defence (Rico-Gonzáles et al. 2021).

Salivary SIgA has been extensively researched within exercise immunology partly due to its long association with URTIs (MacKinnon and Jenkins, 1993). During bouts of supramaximal exercise, decreases in salivary SIgA post-exercise has been associated with an increased risk of URTIs (Fahlman et al. 2005; Gleeson et al. 2012; MacKinnon and Jenkins, 1993). So much so that decreases in salivary SIgA secretion rates may serve as a useful clinical marker in predicting the extent of an URTI risk (Fahlman et al. 2005). Moderate bouts of exercise tend to have the opposite effect on salivary SIgA. Several studies have noted improvements in salivary SIgA levels post-exercise for both acute and chronic moderate exercise (Martins et al. 2009; Rosa et al. 2014; Sloan et al. 2013).

In order to establish the mechanisms behind the decrease of salivary SIgA following intense exercise, some researchers have analysed its response to supplemented exercise, particularly caffeine supplemented exercise. Caffeine is a well-established and effective ergogenic aid within the athletic industry and has been an ongoing subject of research since the early 1900's (Doherty and Smith, 2004; Grgic et al. 2020; Rivers & Webber, 1907). It is naturally derived from various species of tropical plants and then added to a multitude of foods, drinks, and medications (Durrant 2002; Griffiths & Vernotica 2000). Caffeine is an adenosine antagonist and can therefore influence the behaviour of the immune system (Haskó et al. 2007; Spriet, 2002). Although there have been several studies that have observed an effect of caffeine supplemented exercise on immune cells (Bassini-Cameron et al. 2007; Fletcher and Bishop 2011; Fletcher and Bishop 2012), to my knowledge there have been very few investigating the effect of caffeine supplemented exercise on salivary SIgA. One such study was conducted by Bishop et al. (2006). In response to exercise with caffeine supplementation, salivary SIgA concentration was found to increase after intense exercise. However, Dulson et al. (2019) observed opposing results as Bishop et al. 2006 when testing under similar conditions.

Researching the effects of caffeine on the immune system is especially important because of its widespread use within the sports industry. Caffeine has been shown to reduce fatigue and improve energy availability, concentration and focus, and exercise performance (Ganio et al. 2009; Glade, 2010; Smirmaul et al. 2017). In a systematic review of forty-six studies by Southward et al. (2018), moderate to high doses (3 mg/kg to 6 mg/kg) of caffeine were shown to improve exercise performance to some degree in the majority of the studies examined.

These properties have made it a popular supplement across a wide range of disciplines including running, football (soccer), cycling, and rugby (Desbrow et al. 2012; Gant et al. 2010; Graham and Spriet, 1995; Roberts et al. 2010).

However, not all athletes may respond to caffeine supplementation in a similar manner. Previous studies have shown that genetics may play a role in whether an athlete benefits from caffeine (Grgic et al. 2021). The gene that has been specifically targeted by many researchers is the CYP1A2 gene (Grgic et al. 2021). CYP1A2 is found on chromosome 15 in a cluster with the genes CYP1A1 and CYP1B1 (Zhou et al. 2009). It is a part of a family of drug-metabolizing enzymes called cytochrome P450 (CYP) (Thorn et al. 2012). There are over 100 substrates for the CYP1A2 gene, ranging from clinically vital medications and procarcinogens, such as benzopyrene, to endogenous substrates such as arachidonic acid and steroids (Zhou et al. 2009). This extensive list also includes caffeine and its derivatives: paraxanthine, theobromine, and theophylline (Gu, et al. 1992). The P450 enzyme is responsible for metabolising approximately 95% of all ingested caffeine (Carswell et al. 2020). Depending the CYP1A2 allele, an individual may metabolize caffeine ‘slow’ or ‘fast’ (Nehlig and Alexander 2018). This distinction may determine whether or not an individual responds to caffeine supplemented exercise. Wong et al. (2021) found that the CYP1A2 genotype significantly influenced performance during caffeine supplemented exercise. Participants with the homozygous CC allele experienced a 12.8% decrease in handgrip strength after a 4 mg/kg caffeine treatment compared to the placebo.

Despite the large body of evidence emerging on the role this gene might play in many aspects of exercise performance, there is a scarcity of research on other physiological responses, such as immune responses, to caffeine. There has been no previous research investigating whether the CYP1A2 gene may influence salivary SIgA’s response to caffeine supplemented exercise and thus will be one of the main focuses of this study.

Chapter II: Review of Literature

2.1 An Introduction to Immunity

The immune system is an interactive and complex network of organs, tissues, proteins, humoral factors (immune components transported via blood), cells, signalling molecules called cytokines, and a host of other physiological elements (Parkin & Cohen 2001).

Immunity is divided into two classifications dependent on the speed and specificity of its reaction to foreign bodies, or antigens: the adaptive response and the innate response (Parkin and Cohen, 2001). The adaptive response involves the use of antigen specific B and T cells, which carry out antibody and cell-mediated immune responses respectively (Alberts, et al. 2002). While both B and T cells are produced in the bone marrow, they mature and are exported at separate locations: B cells in the bone marrow and T cells in the thymus (Kumar et al. 2018; Zhao et al. 2012). During cell-mediated immune responses, an activated T cell will kill an infected host cell that has presented a foreign antigen on its surface, thus eliminating the opportunity for pathogen replication (Alberts, et al. 2002). During antibody immune responses, activated B cells secrete antibodies, or immunoglobulins, which circulate throughout the bloodstream and bind to foreign antigens, thus preventing them from infecting host cells. While their mechanism for eliminating foreign invaders is different, both B and T cells are able to differentiate and 'remember' a specific antigen (Parkin & Cohen, 2001). They accomplish this by producing antigen specific memory cells following an infection (Omiluski and Goldrath, 2017; Weisel et al. 2017). If the infection returns, these longer-lived memory B and T cells are able to release antibodies that have a higher binding affinity and respond more rapidly and with a greater magnitude than non-memory or naïve B and T Cells (Marshall et al. 2018; Vitetta et al. 1991). The adaptive response is precise, but can take several days or weeks to develop (Parkin and Cohen, 2001).

The innate response is rapid, responding to foreign pathogens within minutes or hours of initial infection (Marshall et al. 2018). Unlike adaptive immunity, innate immunity has no immunological memory and is antigen-independent, meaning it responds to a broad range of pathogens that share common structures in a non-specific manner. Elements of the immune system that contribute to the innate response include but are not limited to neutrophils, which eradicate pathogens at the site of infection, eosinophils, which protect the body against parasitic infections, and immunoglobulins, which neutralize toxins and viruses and opsonize, or mark, microbes to be phagocytosed (Parkin & Cohen, 2001; Vaillant et al. 2021).

There are two distinct mechanisms by which the immune system can 'learn' a new antigen: active immunity and passive immunity (Baxter, 2007). Active immunity involves exposing the immune system to an antigen in order to instigate an adaptive immune response. The response may take several days or weeks to develop but has the potential to become permanent. Examples of active immunity include 'wild' infections that cause an adaptive response, and long-lasting vaccines such as the two dose Hepatitis A.

Passive immunity is short lived, only lasting a few weeks to a maximum of three to four months (Baxter, 2007). Examples of passive immunity include immunoglobulins transferred through breast milk from mother to baby and specific types of vaccines such as the human rabies vaccine, the tetanus vaccine, and the Hepatitis B vaccine.

2.2 Mucosal Immunity and IgA

The mucosal immune system is the largest contributor of immunoglobulin production and deployment of immune cells (Russell et al. 2020). Its primary role is to protect surface areas covered by external secretions in places like the upper respiratory tract, salivary glands, and nasal glands. (McGhee & Fujihashi, 2012). These and other areas are covered by a single layer of mucosal epithelial cells, a number of which contribute to innate immunity. For instance, secretory epithelial cells that line the respiratory tract are able to initiate an innate immune response through Toll-like receptors, an important pattern recognition receptor for foreign pathogens (Hewitt & Lloyd, 2021).

The mucosal immune system can be divided into two separate categories based on anatomical and functional properties: inductive sites and effector sites (McGhee & Fujihashi, 2012). Inductive sites are present near lymphoids and provide a continuous source of memory B and T cells. Effector sites can be found in secretory glandular tissues (salivary mammary, lacrimal, etc.) and the upper respiratory tract. They contain IgA producing plasma cells, or differentiated B-cells, that produce and secrete antibodies that protect against pathogens (D'Souza & Bhattacharya, 2019; McGhee & Fujihashi, 2012). The production of secretory IgA begins when polymeric IgA binds to the polymeric Ig receptor on the basal surface of the epithelial cell. It is then be transported across the cell becomes dimeric secretory IgA once it is released (McGhee & Fujihashi, 2012; Vaillant et al. 2021). While the dimeric molecular form of IgA is commonly found on mucosal surfaces, higher molecular weight species such as trimers and tetramers can also be present (de Sousa-Pereira & Woof, 2019).

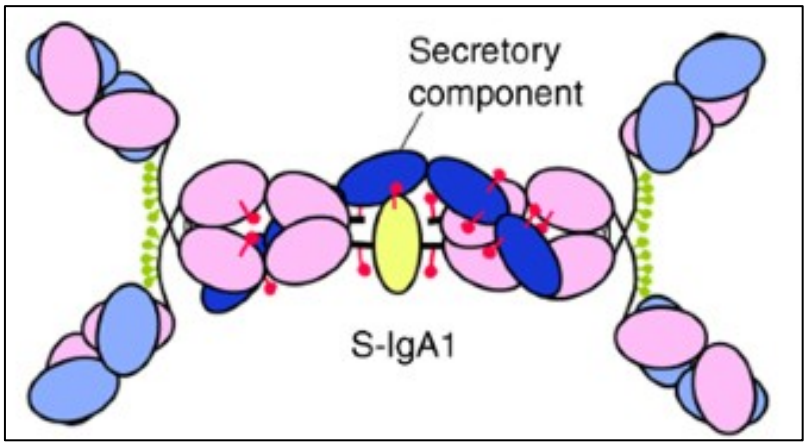

Figure 2. Structure of Salivary SIgA 1. Derived from Woof & Russell (2011)

IgA is one of the most prevalent immune makers in the body and is synthesized more than all other immunoglobulins combined (de Sousa-Pereira & Woof, 2019). There are two subclasses of IgA, each comprising of two heavy chains and two light chains. IgA1 works to maintain homeostasis while IgA2 promotes inflammation (Steffen et al. 2020).

Out of the five classes of immunoglobulin, IgA is the one that primarily acts as the first line of defence against wide range of pathogens, including those that cause URTI (Rico-Gonzáles et al. 2021). It is especially present in the mucosa lining the respiratory tract, where it neutralizes bacterial toxins and prevents viral adhesion to epithelial cells (Lamm, 1997; Lamm et al. 1995; Rico-Gonzáles et al. 2021). IgA also provides protection just beneath the mucosal lining and acts as a 'net' inactivating any antigens that might have evaded detection within the mucosa (Lamm, 1997). A significant decrease in salivary SIgA, especially in the upper respiratory tract, is highly correlated with an increase in the opportunity for infection (Drummond et al. 2022; Tiernan, et al. 2020). Though it is dependent on the length and intensity, this decrease can often be brought on by strenuous exercise (Drummond et al. 2022).

2.3 Illness Among Athletes

Previous research within the sports industry has revealed that respiratory infections account for approximately half of all acute illnesses and infections reported in athletes (Gleeson, 2007; Rico-Gonzáles et al. 2021; Schwellnus et al. 2016). Though it would be sensible for athletes to abstain from exercise for the duration of their illness, many have traditionally ignored their symptoms in order focus on an important competition or support their fellow teammates (Orchard et al. 2021). However, training during an intense infection may further reduce musculoskeletal capacities and can potentially lead to infection complications (Friman

& Wesslén, 2000). While most athletes do not exhibit intense symptoms during a respiratory virus, many will experience mild symptoms such as sore throat, sneezing, nasal congestion, cough, and rhinitis (Ruuskanen et al. 2022).

Throughout competition season, the instance of respiratory infections, especially URTI, may become more ubiquitous in athletes. During the 13th Federation Internationale de Natation World Championships, 50.3% of all illnesses reported affected the respiratory tract (Mountjoy et al. 2010). Similarly, Alonso et al. (2010) found that URTI were the most common illness (comprising 30.4% of all reported cases) during the 2009 International Association of Athletics Federations World Championships in Athletics. Then, Schwellnus et al. (2011) found that during the 2010 Super 14 Rugby Tournament the majority (31%) of illnesses reported also occurred within respiratory tract.

Respiratory illnesses were even prevalent during the Olympics. Engebretsen et al. (2010) observed a high incidence (62.8%) of respiratory related illness at the 2010 Winter Olympics and Engebretsen et al. (2013) noted that 41% of illness reported affected the respiratory system at the 2012 Summer Olympics. During the 2018 Winter Olympics, 20 out of 44 athletes and 32% of staff members on the Finnish team experienced some degree of common cold symptoms (Valtonen et al. 2019). Many of the strains that impacted the Finnish team were viruses that specifically targeted the respiratory tract, such as coronavirus and human metapneumovirus.

While strenuous exercise may contribute to the development of URTI, consistent moderate exercise has been shown to have the opposite effect (Kostka et al. 2000; Spence et al. 2007). For example, Nieman et al. (1993) observed a significantly lower occurrence of the common cold in elderly women who exercised for approximately 1.5 hours per day (8%) compared to the incidence rate of the common cold in the sedentary control group (50%) during a 12 week follow up. Another observational study followed more than 500 healthy adults over 12-month period and demonstrated that high levels of moderate-intensity activity were associated with a 20-30% reduction in URTI risk (Matthews et al. 2002). And when recreational athletes are compared to their elite counterparts, they generally demonstrate a lower rate of both URTI and upper respiratory illnesses (Spence et al. 2007).

Exercising at a low to moderate frequency has also been associated with a reduced risk of illness related mortality (Wong et al. 2008). One study found that individuals who seldom or never exercised were associated with 5.8% to 8.5% excess risk of influenza-associated

mortality while individuals who engaged in low to moderate levels of exercise were associated with 4.2% to 6.4% excess risk of influenza associated mortality (Wong et al. 2008).

2.4 Immunity and Exercise

Physical exercise at either a moderate or light intensity generally has a beneficial effect on the immune system (da Silveira et al. 2021; Luzi & Radaelli 2020). Regular exercise may also strengthen the immune response to microbial antigens by enhancing toll-like receptor signalling pathways (Sameer & Nissar, 2021; Zheng et al. 2015). However, intense exercise may increase the risk of developing an URTI through exercise-related immunosuppression caused by tissue trauma acquired during an intensive exercise bout (Gleeson & Pyne, 2000; Lakier Smith, 2003). An up-regulation of humoral immunity, coupled with an altered focus of immune function and the suppression of cell-mediated immunity, may also contribute to an increased risk of infection (Lakier Smith, 2003).

As previously mentioned, IgA is integral to the immune system's initial response to foreign pathogens (Rico-Gonzáles et al. 2021). As such, the four following subsections will focus on the effect various exercise types have on IgA levels.

2.41 Intense Chronic Exercise and Immunity

Generally, intense chronic exercise has previously been shown to have a negative effect on salivary SIgA concentration. Gleeson et al. (1995) studied elite swimmers over a seven-month training period and analysed several immunoglobulins including IgG, IgM, and salivary SIgA. Although there were no remarkable changes in IgG and only one notable difference in IgM, salivary SIgA decreased significantly after individual training sessions and followed a downward trend throughout the study in both pre and post exercise samples. Gleeson et al. (2000) observed similar results during another elite 12-week training program for swimmers. Though there were significant decreases in salivary SIgA after individual training sessions, no significant patterns for salivary SIgA or any other measured immunoglobulin appeared for the duration of the program.

Throughout the course of a 50-week training period for elite yacht racers, Neville et al. (2008) noted that a decrease in salivary SIgA of more than 40% from baseline levels occurred roughly three weeks prior to an URTI. If the racer had not recently been infected with an URTI, this 40% decrease in salivary SIgA from baseline generally indicated a one in two

chance of contracting an URTI within the three weeks. These findings are supported by Perkins & Davison (2022), who studied football players during a 16-week training period and also discovered that a decrease in salivary SIgA of more than 40% from baseline levels was associated with a significantly increased likelihood of contracting an URTI within the subsequent weeks.

Yet, there have been discrepancies in the literature regarding the effect intense chronic exercise may have on salivary SIgA. Moreira et al. (2013) found no significant change in salivary SIgA concentration after a four-week intensive training program with futsal players. However, salivary SIgA concentrations were only assessed weekly, which prevented researchers from analysing any possible changes in salivary SIgA between training sessions. Filaire et al. (2003) also found that IgA levels remained relatively consistent in professional soccer players throughout a 12-month period. And, similar to Moreira et al. (2013), only four sets of samples were taken: before the start of training, at the end of the early season, at the end of the competitive sports season, and just before the commencement of a new season. Not collecting more saliva samples throughout the 12-month period also could have limited researchers from obtaining a more accurate overview of exercise-induced changes in salivary SIgA. The low frequency of sample collection could have also made it more difficult to detect the effects of periods of increased load and or stress that could have occurred during the training season. Collecting samples even weekly during data collection might have resulted in a different conclusion. While these studies demonstrated that intense chronic exercise appeared to have no long-term effect on salivary SIgA levels, they did not incorporate the short-term effects, which may contribute significantly more to exercise-induced changes in salivary SIgA.

2.42 Intense Acute Exercise and Immunity

There are numerous studies investigating intense acute exercise and salivary SIgA concentration that have observed a significant decrease in salivary SIgA post-exercise. During the 160- km Western States Endurance Run, Nieman et al. (2003) noted a significant decrease in salivary SIgA secretion rate 5-10 minutes post-race (254 ± 30 µg/min) compared to concentrations the morning of the race (508 ± 40 µg/min). Out of the forty-five runners included in the study, approximately one in four reported an URTI two weeks post-race. Analysis of salivary SIgA secretion rates revealed that many of the runners who reported an URTI also had low salivary SIgA secretion rates at the 90 km mark. Nieman et al. (2006)

were able to duplicate these results with a much larger sample size (n = 106). On average, saliva protein IgA concentration decreased 10% post-race compared to pre-race values. Libicz et al. (2006) observed significantly decreased levels the concentration of salivary SIgA following repeated triathlon races at the 2001 French Iron Tour. Saliva flow rate was also significantly decreased after each race (Libicz et al. 2006). During the 86.5 km Comrades Marathon in South Africa, Peters et al. (2010) found that mucosal IgA concentrations decreased immediately post-race but returned to baseline levels one day after the race. URTI symptoms were largely reported 7-14 days following the marathon.

Tauler et al. (2014) analysed the behaviour of salivary SIgA in response to the Ultra-trail Serra de Tramuntana, a 104 km ultra-marathon race, and the Cabrera Open Water Race, a 25 km swimming competition. Researchers collected saliva samples from participants before and after their respective race. Salivary SIgA concentration in both the Ultra-trail Serra de Tramuntana and the Cabrera Open Water Race was significantly reduced post-race compared to pre-race concentrations.

The decrease observed in salivary SIgA concentrations following intense acute exercise is not only limited to extreme marathons and competitions. Novas et al. (2003) measured salivary SIgA levels in elite female tennis players before and after one hour training sessions at 2 weekly intervals over a course of 12 weeks. Researchers found that salivary SIgA concentration was directly correlated to the amount of training athletes had undertaken in the days leading up to saliva sample collection.

Moreira et al. (2011) analysed saliva samples from professional Brazilian futsal players before and after 2 matches. There was a significant reduction in salivary SIgA concentration post-match (99 ± 17 μg/mL) compared to pre-match levels (175 ± 43 μg/mL). Pre-match salivary SIgA secretion rates and pre-match saliva flow rates were also significantly reduced post-match. There was no significant difference in the pattern of salivary SIgA concentration between matches. Murase et al. (2016) found a decrease in salivary SIgA after participants exercised on a bicycle ergometer at 50% VO2 max for one minute and then at 75% VO2 max for 59 minutes. Salivary SIgA concentrations post-exercise (18.5 ± 7.6 μg/mL) were significantly lower than pre-exercise levels (22.8 ± 8.5 μg/mL). Owen et al. (2016) compared the salivary SIgA concentrations of professional soccer players between four high intensity and low intensity training sessions. After the fourth training session, salivary SIgA

concentration in the high intensity session was significantly lower than salivary SIgA concentration in the low intensity session.

The majority of studies investigating the effect intense acute exercise has on salivary SIgA generally agree that this type of exercise has a negative impact on salivary SIgA levels post-exercise. When the decrease is large enough, it has been shown to increase the risk of URTIs in some studies. Presently, there has not been a reliable protocol in place for the prevention of exercise-induced changes in salivary SIgA. It is therefore imperative that the efficiency of possible preventative measures, such as caffeine supplementation, be fully investigated in order to combat an unfavourable decrease in salivary SIgA post-exercise.

2.43 Moderate Chronic Exercise and Immunity

Moderate exercise tends to improve the activity and levels of various immune markers (Nieman, 1997). For instance, it can increase anti-pathogenic macrophage activity and strengthen immune surveillance by increasing recirculation of immunoglobulins, neutrophils, anti-inflammatory cytokines, and natural killer cells (da Silveria et al. 2021; Nieman 2011). By enhancing the levels of immune markers in circulation, chronic moderate exercise can help reduce inflammatory damage to an infected organ by modulating the influx of inflammatory cells to the afflicted site (da Silveria et al. 2021).

In a study by Klentrou et al. (2002), sedentary participants took part in 45-minute exercise sessions consisting of aerobic activity and limb stretches for 12 weeks. At the end of the 12-week training period, salivary SIgA concentrations in the exercise group increased significantly compared to the control. Light URTI and influenza symptoms also decreased significantly in the exercise group. Akimoto et al. (2003) observed comparable results in elderly participants (64.9 ± 8.4 years). Participants completed a 60-minute resistance exercise and a 60-minute moderate endurance exercise once a week for 12 months. Saliva samples were collected before training began, four months into the program, and once more at the 12-month mark. Salivary SIgA concentrations had significantly increased at the end of the program (33.8 ± 18.5 μg/ml) compared to pre-program levels (24.7 ± 14.4 μg/ml). Lee et al. (2021) also observed a statistically significant increase in IgA levels in pre-frailty elderly women after a 12-week aquatic exercise program.

However, this increase in salivary SIgA following moderate exercise is not always guaranteed. Teixeira et al. (2008) assessed salivary SIgA concentration in elderly participants (aged 68-95) before and after a 19-week moderate aerobic exercise program. While the initial

concentration of salivary SIgA (92.92 ± 86.14 µg/ml) had markedly increased after the completion of the training program (133.87 ± 113.95 µg/ml), researchers determined that it was not statistically significant. As most physiological systems tend to decrease with age, the age range of participants in the study by Teixeira et al. (2008) might have contributed to the non-significant increase in the concentration of salivary SIgA. In order to prevent a similar outcome within this study, participants had to be under the age of forty-five.

2.44 Moderate Acute Exercise and Immunity

There are very few studies investigating the relationship between salivary SIgA and moderate acute exercise. Eda et al. (2018) found similar results as Akimoto et al. (2003) in elderly women (aged 60.4 ± 10.4 years) when far less vigorous exercise was performed. After 90 minutes of yoga stretching, secretory IgA concentration and secretion rates had significantly ($p < 0.05$) increased compared to baseline levels. In a study by Kunz et al. (2015), nine highly fit and experienced cyclists and eight recreational cyclists each completed three thirty-minute exercise workloads at increasing intensities (-5, +5, and +15% of individual blood lactate threshold). A significant increase in salivary SIgA post-exercise was only observed in the highly fit group, thus supporting the researcher's proposal that exercise induced changes in salivary antimicrobial proteins may be dependent on fitness level.

2.45 Intermittent, Continuous, and Other Exercise Effects on Immunity

While it has been established that the intensity of an exercise can influence salivary SIgA concentration, Sari-Sarraf et al. (2006) set out to determine if exercise type also had a similar effect. Participants completed either a soccer-specific intermittent exercise or a continuous exercise at the same overall work-rate on a motorized treadmill. Unstimulated saliva samples were taken pre-exercise, mid-exercise, post-exercise, and six, twenty-four, and forty-eight hours post-exercise. Researchers found no significant difference in the behaviour of salivary SIgA between the two exercise types.

With regards to intermittent exercise, salivary SIgA has previously been noted to decrease following an exercise bout. Fahlman et al. (2001) observed a decrease in secretory IgA following 'repeated bouts of brief, intermittent maximal effort exercise'. Participants completed three maximal 30 second Wingate leg cycling tests with three minutes of recovery in between each one. Both secretory IgA levels and saliva flow rate were significantly lower post-exercise compared to pre-exercise levels. Engels et al. (2003) repeated the study by Fahlman et al. (2001) and found similar results. Chidley & Davison (2018) analysed salivary

SIgA concentration in response to a 2-day exercise training program. The first day included a VO_2 max test and three 30 second maximal Wingate tests and the second day included a 90-minute cycle at approximately 60% VO_2 max followed by three 30 second maximal Wingate tests. There were significant decreases in the concentration of salivary SIgA following all four exercise sessions.

There have of course been deviations from general observations of salivary SIgA's response to intermittent exercise. For instance, Walsh et al. (1999) found that an acute bout of high-intensity intermittent exercise did not affect salivary SIgA concentration. However, in a study by Sari-Sarraf et al. (2007), the average concentration of salivary SIgA increased post-exercise after a single bout of football (soccer) related intermittent exercise.

Because both intermittent and intense acute exercise appear to have the most consistent and significant negative effect on salivary SIgA concentration, the exercise protocol utilized within this study incorporates both of these exercise types. In this manner, if caffeine supplementation is truly effective in influencing an exercise-induced decrease in salivary SIgA, its impact will be apparent.

Regardless of the type or duration, the vast majority of literature has shown that exercise can have a significant impact on salivary SIgA concentration. In cases of intense exercise where this impact can increase the risk of infection, it would be beneficial to investigate preventative measures that could combat adverse exercise induced changes in salivary SIgA. Given the gap in the literature within this particular area, a portion of this study is dedicated to analysing whether caffeine might serve as an effective tool in counteracting an exercise induced decrease in salivary SIgA concentration.

2.5 Caffeine and its use in Sports

Caffeine (1,3,7-trimethylxanthine) is a well-researched food component and is most commonly consumed in the form of a beverage (Heckman et al. 2010; Mitchell et al. 2014). It occurs naturally in the beans, leaves, and fruit of over 60 different plants in varying quantities (Heckman et al. 2010).

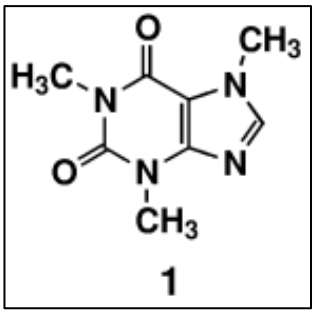

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Figure 3. Skeletal structure of caffeine. Derived from Waldvogel (2003)

Caffeine has deep roots in human history with reports of caffeine usage dating back to the Galla tribe in Ethiopia nearly one thousand years ago (Waldvogel, 2003). However, some historians suggest that caffeine was consumed as far back as 2737 BC in Ancient China (Arab & Blumberg, 2008). In today's modern world, approximately 80% of the population consumes one caffeinated product every day (Ogawa & Ueki, 2007).

Tea leaves and roasted coffee beans are the most common sources of dietary caffeine, but other sources include kola nuts, cacao beans, yerba mate, and guarana berries (Barone & Roberts, 1996). Outside of coffee and tea, caffeine can also be present in soft drinks, medications, dietary supplements, or products containing chocolate or cocoa (Andrews et al. 2007; Barone & Roberts, 1996). The caffeine concentration between these products varies drastically, with decaffeinated coffee containing very little caffeine (median concentration 5 μg/mL) and energy drinks containing very high levels of caffeine, ranging between 75 and 240 mg per container (Attipoe et al. 2016, Gilbert et al. 1976; Reissig et al. 2009). Variation in caffeine concentration can even occur within a particular source. For instance, brewed coffee will generally have higher levels of caffeine (107-151 mg) than instant coffee (55-65 mg) (Bunker & McWilliams, 1979).

Despite its widespread use, caffeine was a banned substance in sports for nearly twenty years between 1984 and 2004 (Diel, 2020). The World Anti-Doping Agency then moved it from the List of Prohibited Substances to the Monitoring Program effective January 1, 2004 (Aguilar-Navarro et al. 2019; Diel, 2020). Today, caffeine concentrations in athletes are monitored by the World Anti-Doping Agency as well as other entities but the cut off for acceptable concentrations may vary (Guest et al. 2021).

Since its legalization in 2004, caffeine usage has increased across a majority of athletic disciplines. Aguilar-Navarro et al. (2019) found caffeine concentrations in athletes to be significantly higher across several different sports including aquatics, boxing, judo, football, weightlifting, rowing, and cycling in 2015 compared to 2004. Despite the drastic difference in skill and technique required for the sports investigated during aforementioned study, they all demand a common ability: endurance.

Endurance sports involves repeated and prolonged isotonic contractions from large skeletal muscle groups (Morici et al. 2016). In order to enhance the performance of these muscle groups, it is common for endurance athletes to supplement their exercise with caffeine, often at higher doses than non-endurance athletes (Del Coso et al. 2011; Graham, 2001; Spriet, 2002). While there are some athletes that implement doses outside of this range, the vast majority of literature investigating the ergogenic effects of caffeine have administered doses between 3-6 mg/kg of body mass 60 minutes prior to exercise based on known pharmacokinetic interactions (Kreutzer et al. 2022). This range is also where caffeine's ergogenic effects are most commonly observed (Goldstein et al. 2010). Athletes that utilize caffeine supplementation below this range often include recreational athletes, who have reported consuming relatively small doses of caffeine prior to training sessions (1.6 ± 1.0 mg/kg of body mass) and races (2.0 ± 1.2 mg/kg of body mass) (Kreutzer et al. 2022). Because many studies have investigated the effects of high (5-6 mg/kg) or moderate to high (3-4 mg/kg) doses of caffeine, a 4 mg/kg dose was utilized during this study in order to align with previous literature. However, very few studies have examined the effects of lower caffeine doses (2 mg/kg or less). Therefore, the effects of a 2 mg/kg dose of caffeine were investigated in the present study with the aim of addressing the gap within the literature.

2.6 The Effects of Caffeine

As previously mentioned, caffeine is a well-established ergogenic aid within the athletic industry and has been shown improve performance across a broad range of exercise modalities such as aerobic endurance, muscle endurance, exercise speed, and muscle strength (Grgic et al. 2020). Although there have been several suggested physiological mechanisms by which caffeine's ergogenic effect might occur, improvement in performance can be largely attributed to caffeine's antagonistic, or opposing, effects on adenosine receptors (Graham, 2001; Spriet, 2002).

2.61 Physiological Effects of Caffeine

Adenosine is a neuroprotective modulator that minimizes neuron damage in noxious conditions and is incredibly similar in structure to caffeine (de Mendonça et al. 2000; Rodak et al. 2021).

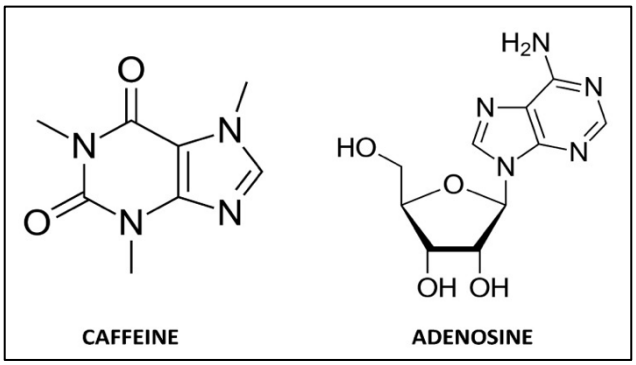

Figure 4. A visual representation of the structural similarities between caffeine and adenosine. Retrieved from Rodak et al. (2021)

Adenosine also hyperpolarizes neurons, controls the release of excitotoxic mediators, limits calcium influx, and acts as a modulator for glial cells in high concentrations (Rebola et al. 2005). Within the immune system, it is responsible for regulating immune and inflammatory responses (Antonioli et al. 2019).

There are four types of adenosine receptors (A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R) and they can be found all across the body (Fredholm et al. 2000; Ribeiro & Sebastio et al. 2010). A_1 adenosine receptors are primarily located in the brain, spinal cord, adrenal gland, and heart while the A_{2A} adenosine receptors can be found in the spleen, thymus, and striatopallidal GABAergic neurons (Fredholm et al. 2000). A_1 receptors can also be found in skeletal muscle and adipose tissue, but their presence is far less extensive here. The same can be said for A_{2A} receptors located in the heart, lungs, and blood vessels. But no matter the type or location of the adenosine receptor, caffeine has the ability to antagonize them all, which can result in improved alertness and auditory vigilance (Ribeiro & Sebastio et al. 2010; Zwyghuizen-Doorenbos et al. 1990).

2.62 Caffeine's Effect on Sports Performance

Studies discussed within this section are summarized in the table below (Table 1). Caffeine supplementation has been shown to improve various components of exercise performance, especially time to fatigue, or time to exhaustion or task failure (Davis et al. 2003).

Table 1. Summary of studies discussed during Section 2.62; (+): improvement in performance; (-): no improvement in performance

Study	Exercise Protocol	Caffeine Dose	Performance Outcome with Caffeine Supplementation
Astorini et al. (2012)	Cycling Time-trial	5 mg/kg	Decrease in time to completion (+)
Bortolotti et al. (2014)	Cycling Time-trial	6 mg/kg	No improvement in performance (-)
Carr et al. (2011)	2000 m Rowing Ergometer Test	6 mg/kg	Increase in average power (+)
Christensen et al. (2014)	Maximal Performance Test	3 mg/kg	Increase in performance and mean power (+)
Church et al. (2015)	5 km Timed Run	Turkish Coffee	Decreased time to completion (+)
Graham-Paulson et al. (2016)	Cycling and Hand-cycling	4 mg/kg	Reduced time to compltion in cycing trials (+)
Hodgson et al. (2014)	Cycling Time-trial	5 mg/kg	Decrease in performance time (+)
Pitchford et al. (2014)	Cycling Time-trial	3 mg/kg	Decrease in time to completion (+)
Potgieter et al. (2018)	Triathalon	6 mg/kg	Improvement in overall performance (+)
Roelands et al. (2011)	Cycling Time-trial	6 mg/kg	No improvement in performance (-)
Santos et al. (2013)	4000 m Cycling Time-trial	5 mg/kg	Increase in performance (+)
Stadheim et al. (2021)	VO2 Max Test	4.5 mg/kg	Increase in VO2 max and time to exhaustion (+)
Stadheim et al. (2015)	Cross Country Double Poling (Skiing)	4.5 mg/kg	Increase in time to exhaustion (+)
Quinlivan et al. (2015)	Cycling Time-trial	3 mg/kg	Decrease in performance time (+)

Participants in a study by Astorino et al. (2012) completed a 10-kilometre timed cycling trial after consuming a drink containing 5 mg/kg of caffeine in two experimental sessions and a placebo drink in a third session. The caffeine treatment significantly ($P = 0.02$) increased performance in both of the visits it was used, demonstrating that caffeine's ergogenic effects could be repeated in participants across several days. Although the performance of some participants did not improve with caffeine supplementation, the majority of the study's sample size exhibited the effects of caffeine's ergogenic properties. Hodgson et al. (2013) also investigated the effects of a 5 mg/kg dose of caffeine on a timed cycling trial and found similar results as Astorino et al. (2012). Trained male cyclists and triathletes were given either a 5 mg/kg dose of caffeine or a placebo before completing 30 minutes of a steady state cycling at 55% VO_2 max followed by a timed trial. Performance times after caffeine ingestion (38.35 ± 1.35 minutes) improved significantly ($p < 0.05$) when compared to the placebo (40.23 ± 1.98 minutes). In a study by Santos et al. (2013), participants completed a 4000 m cycling timed trial after ingesting either a cellulose placebo or a 5 mg/kg dose of caffeine. Time to completion was notably reduced in the caffeine trial (409 ± 12 s) compared

to the placebo (419 ±13 s). Caffeine supplementation also resulted in a significantly higher power output (232.8 ±± 21.4 W) than the placebo (219.1 ± 18.6 W). Glaister et al. (2015) found that a 5 mg/kg dose of caffeine ingested one hour before a 20 km timed cycling trial resulted in a significant decrease in time to completion and a significant increase in power output. Researchers had also investigated dietary nitrate as a potential ergogenic aid for endurance exercise, but unlike caffeine, it did not produce any significant improvements in performance. Potgieter et al. (2018) also observed improvements in performance following caffeine supplementation. Researchers found that a 6 mg/kg dose of caffeine taken 60 minutes prior to a triathlon decreased both swim time and completion time across all participants.

Quinlivan et al. (2015) discovered that even if athletes are unable to obtain caffeine tablets with exact doses, commercially available caffeinated beverages will still be able to improve performance. Participants were given either a 9.4 mL/kg of Red Bull containing 3 mg/kg of caffeine, a 3 mg/kg dose of anhydrous caffeine in capsule form or a placebo 90 minutes before completing a cycling time trial equal to one hour at 75% peak power output. Performance improved with both the Red Bull and anhydrous caffeine treatments compared to the placebo. No statistical difference could be found in the performance time between the Red Bull and anhydrous caffeine treatments. Church et al. (2015) expanded on the efficiency of common caffeine products as exercise supplements by investigating the effect of Turkish Coffee on a 5 km timed trial run. Turkish coffee significantly decreased reaction time in participants and contributed to the reduction of completion time during the 5 km timed trial for caffeine responders (about 60% of participants). Although the majority of participants benefited from caffeine supplementation, a large portion experienced no improvement in performance. Genetic variability between individuals may have contributed to these results and is discussed further in a later section.

Caffeine's ergogenic effects can even be observed in extreme exercise conditions. Pitchford et al. (2014) examined nine well trained male participant's cycling performance in 35°C and 25% relative humidity after either a 3 mg/kg dose of caffeine or a placebo. Timed trial performance significantly improved after caffeine ingestion (3806 ± 359 seconds) compared to the placebo (4079 ± 333 seconds). A study done by Stadheim et al. (2015) examined athletes under different extreme conditions but discovered similar results as Pitchford et al. (2014). The performance of sub-elite male cross-country skiers was assessed via an 8-km cross country double poling (the technique and use of two poles while skiing) timed trial and

a timed test to exhaustion at a workload of approximately 90% of double poling VO_2 max. Testing took place in a hypobaric chamber at 800 mbar (equivalent to approximately 2000 m above sea level with inspired O_2 tension measuring roughly at 125 mmHg). A 4.5 mg/kg dose of caffeine significantly improved time to exhaustion (6.10 ± 1.40 min vs. 7.22 ± 1.30 min) as well as velocity for the first 4 km of the timed trial. Participants also reported a lower rating of perceived exertion as well as a lower incidence of pain in arms following caffeine ingestion.

Caffeine supplementation may also have the capability to increase VO_2 max. Stadheim et al. (2021) administered either a placebo or a 4.5 mg/kg dose of caffeine to elite runners before having them complete a VO_2 max test. Caffeine not only increased VO_2 max from 75.8 ± 5.6 mL/kg/min to 76.7 ± 6.0 mL/kg/min but also improved run time to fatigue from 355 ± 41 seconds to 375 ± 41 seconds. Their findings were supported by Pitchford et al. (2014), who had previously determined that the 3 mg/kg dose of caffeine utilized within their study was associated with moderate increases in VO_2 max.

Carr et al. (2011) found that mean power can also increase under caffeine supplementation. Well trained rowers performed four 2000 m rowing ergometer tests after ingesting either the 6 mg/kg caffeine dose or a placebo. There was a notable increase in average power during the caffeine trials and the largest power output observed (391 ± 72 W) was within the first 500 m of the ergometer test following caffeine supplementation. The power output with the placebo at this same time point was markedly lower (367 ± 65 W). Researchers also investigated whether a 0.3 mg/kg dose of sodium bicarbonate could impact performance, but found no substantial differences between the placebo and bicarbonate conditions. Christensen et al. (2014) also investigated the ergogenic effects of caffeine in rowers. Open-weight and light-weight elite rowers consumed either a 3 mg/kg dose of caffeine or a placebo before completing a 6-minute maximal performance test. Caffeine supplementation significantly increased the total distance covered (1878 ± 97 m) compared to the placebo (1865 ± 104 m). While participants' average performance improved during the caffeine trial, this improvement was far more pronounced in lightweight-rowers. Mean power under caffeine conditions (400 ± 58 W) was also significantly greater than the placebo (393 ± 61 W), especially during the final half of the maximal performance test.

While there is strong evidence for caffeine's ergogenic effects in endurance exercise, a handful of studies found no improvement in performance following caffeine

supplementation. Roelands et al. (2011) had participants consume either a 6 mg/kg dose of caffeine or a placebo one hour before cycling for 60 minutes at a constant workload at 55% VO_2 max followed by a timed trial in warm conditions (relative humidity maintained between 50-60% and temperature kept constant at 30°C). No significant difference could be found between the caffeine and placebo conditions for the timed trial ($p = 0.462$). Researchers admitted that caffeine supplementation raised participant's core temperature during exercise, which could have potentially counteracted caffeine's ergogenic effects. Utilizing a smaller caffeine dose such as one implemented by Pitchford et al. (2014) in future studies could prevent the ergogenic effects of caffeine from being masked. Roelands et al. (2011) also disclosed a number of other external factors that could have prevented an improvement in performance during caffeine trials including neurotransmitter concentrations and participant's individual responses to caffeine. Individual responses could be attributed to genetic differences and potential genetic influences on performance during caffeine supplemented exercise are discussed in a later section. Bortolotti et al. (2014) was also unable to observe an ergogenic effect of caffeine during their study. Male cyclists received either a 6 mg/kg dose of caffeine or a placebo one hour prior to completing a 20 km cycling timed trial. There was no significant difference between the placebo and caffeine treatments for power, speed, rotations per minute, rate of perceived exertion, or heart rate. However, researchers predominantly tested participants that could be classified as recreational athletes. As previously mentioned, recreational athletes benefit more from lower caffeine supplement doses during exercise. Lowering the caffeine dose could have allowed for caffeine's ergogenic effects to become more distinct and significant during analysis.

The cumulation of the studies discussed within this section make it clear as to why caffeine's use within the sports industry is not only common place, but somewhat justified. Caffeine can provide significant improvement in exercise performance and confer benefits on an exercising musculoskeletal system. But as with most supplements, caffeine's effects are generally systemic. As an adenosine receptor, caffeine has the capability to influence several physiological systems. However, when it comes to the immune system, there are only a few studies that examine the effects caffeine supplemented exercise has on immune markers, particularly salivary SIgA. Because of caffeine's widespread use and its apparent benefits on performance, it is vital to research any possible or lesser-known interactions it may have with other physiological systems for the betterment of the athlete.

2.7 Caffeine and Immunity

Due to the immune system's complex nature, caffeine's effect on it can also be quite complex. Adenosine receptors are present on a number of immune cells including monocytes, macrophages, Natural Killer cells, neutrophils, and dendritic cells, making them subject to caffeine modulation (Haskó et al. 2007). Under exercise conditions, caffeine ingestion can cause certain immune cells and immune markers to increase in circulation or expression. Studies that are discussed within this section that address the effect of caffeine supplemented exercise on immunity are summarized in the table below (Table 2).

Table 2. Summary of studies discussed during section 2.7; (+): increase in immune marker; (-): no change in immune marker; (\): decrease in immune marker

Study	Exercise Protocol	Caffeine Dose	The Effect of Caffeine Supplemented Exercise on Immunity
Bassini-Cameron et al. (2007)	simulated soccer match	5 mg/kg	Increase in lymphocyte counts, thrombocyltes, (+) neutrophils, and circulating monocytes
Bishop et al. (2005)	90 min cycle	6 mg/kg	Decrease in CD4+/CD8+ cells (+) Increase in CD69 expression (+)
Bishop et al. (2006)	90 min cycle	6 mg/kg	Increase in Salivary IgA concentration (+) Increase in salivary IgA flow rate and secretion rate (+)
Cheng et al. (2022)	40 min cycle	6 mg/kg sports drink	Salivary alpha-amylase activity increased (+) No effect on salivary lactoferrin (-)
Dulson et al. (2019)	70 min run	2, 4, 6, 8 mg/kg	No impact on salivary IgA (-)
Fletcher & Bishop (2011)	90 min cycle	2 and 6 mg/kg	Increase in CD3-CD56+ cells (+)
Fletcher & Bishop (2012)	90 min cycle	6 mg/kg	Increased CD69 expression in CD3- CD56+ cells (+) Decreased T-CD8+ and T-CD4+ cells (\)
Shirvani et al. (2020)	90 min cycle	6 mg/kg	Increase in neutrophils, monocyles, and interleuikin-6 levels (+)
Walker et al. (2006)	90 min cycle	6 mg/kg	Increase in number of circulating lymphocytes (+) No effect on neutrophil count (-)

Exercise alone has demonstrated an ability to enhance immune cell concentrations (Wang et al. 2003). However, previous studies have shown that when it is supplemented with caffeine, a synergistic effect may occur. Bassini-Cameron et al. (2007) administered a 5 mg/kg dose of caffeine to professional soccer players before they completed a 45-minute simulated soccer match. While non-caffeine supplemented exercise enhanced lymphocyte counts by 38%, caffeine supplemented exercise increased this count by an additional 35%. Concentrations of thrombocytes, segmented neutrophils, and circulating monocytes were also enhanced by

caffeine supplemented exercise, increasing by 24%, 58%, and 50% respectively. Bassini-Cameron et al. (2007) primarily attributed this increase to caffeine's exercise independent rise in monocytes. While increased cell counts may be beneficial to a certain point, it can also be associated with the stress response. An increase in the concentration of cortisol and other catecholamines can cause an increase in immune cell numbers while simultaneously decreasing the efficiency of cell function. The incorporation of catecholamine or cellular functional measurements could provide further clarification as to whether these increases in immune cell counts are beneficial.

Bassini-Cameron et al. (2007) is partially supported by Walker et al. (2006), who found that caffeine supplemented exercise also increased the number of circulating lymphocytes in their study. Nineteen endurance trained male participants cycled for 90 minutes at 70% VO_2 max for 60 minutes after consuming either a 6 mg/kg body mass dose of caffeine or a placebo. In addition to increasing lymphocyte count, caffeine also minimized the reduction of the f-MLP (N-formyl-methionyl-phenyl-alanine) stimulated response observed post-exercise in the placebo condition. However, caffeine was found to have no significant impact on neutrophil count or PMA (phorbol-12-myristate-13-acetate) stimulated oxidative burst responses (which directly activate protein kinase C, an essential step in signalling pathways) in blood neutrophils. Shirvani et al. (2020) also found that caffeine supplementation was associated with a post-exercise increase across several immune markers. Participants were given a 6 mg/kg dose of caffeine before cycling at 70% VO_2 max for 90 minutes. Compared to the placebo, the caffeine treatment significantly increased neutrophils post-exercise, monocytes one-hour post-exercise, and interleukin-6 levels post exercise and one hour post exercise. No significant changes were observed in lymphocytes. Within these studies, caffeine supplementation clearly has a positive impact on various immune markers post-exercise. It might therefore also have a positive impact on salivary SIgA levels post-exercise.

Fletcher & Bishop (2011) discovered similar patterns when investigating the effect of caffeine supplemented exercise on CD3-CD56+ Natural Killer cells. Endurance trained male athletes cycled at 70% VO_2 max for 90 minutes one hour after ingesting either a placebo, a low dose (2 mg/kg) or a high dose (6 mg/kg) of caffeine. The number of circulating CD3-CD56+ cells was significantly higher in the 6 mg/kg caffeine dose trial compared to the 2 mg/kg caffeine dose trial ($p < 0.05$) and placebo ($p < 0.01$). When cells were later exposed to a multi-antigen stimulant, a Pediacel 5 in 1 vaccine, the geometric mean fluorescence intensity (GMFI) of CD69 expression was found to be significantly higher in the 2 mg/kg

dose of caffeine compared to the placebo one-hour post-exercise. The GMFI of CD69 expression was also higher in the 6 mg/kg caffeine dose trial compared to the placebo, but this value did not reach statistical significance. Fletcher & Bishop (2012) conducted a similar study as Fletcher & Bishop (2011), but measured the response of several different lymphocytes. Participants cycled for 90 minutes at 70% VO_2 max one hour after consuming either a 6 mg/kg dose of caffeine or a placebo. The GMFI of CD69 expression in CD3(-) CD56(+) cells increased significantly after caffeine consumption compared to the placebo. However, the number of antigen-stimulated T-CD4(+) cells expressing CD69 and the GMFI of CD69 expression on T-CD8(+) cells significantly decreased one-hour post-exercise following caffeine supplementation compared to the placebo. Bishop et al. (2005) also noted a negative association between another immune marker and caffeine supplemented exercise. Participants ingested either a 6 mg/kg dose of caffeine or a placebo one hour before cycling for 90 minutes at 70% VO_{2max}. During the caffeine trial, the number of CD4+ cells and CD8+ cells decreased one-hour post-exercise by 54% and 55% respectively from pre-treatment levels. No changes to CD4+ cells or CD8+ cells could be identified under placebo conditions. Although caffeine had caused a decrease in the number of CD4+ and CD8+ cells, it had the opposite effect on CD69 expression levels. Compared to the placebo, CD69 expression levels in CD4+ cells were found to be significantly higher following caffeine supplementation at the pre-exercise, post-exercise, and one-hour post-exercise time points. The same was true for CD69 expression levels in CD8+ cells, but only at pre- and post-exercise.

Although Bishop et al. (2005) and Fletcher & Bishop (2012) examined different immune cells, a common theme between the two studies is an increase in CD69 expression following caffeine supplemented exercise (with the exception of T-CD8(+) cells). CD69 is one of the first surface cell proteins to be expressed following lymphocyte activation (Fletcher & Bishop, 2012; Ziegler et al. 1994). An induced increase in the number of activated lymphocytes could improve the quality of immune surveillance and contribute to a reduced rate of infection. Fletcher & Bishop (2012) speculated that under caffeine conditions, intracellular levels of cAMP (which typically regulate lymphocyte activation) are inhibited, which allowed for an increase in CD69 expression in CD3(-) CD56(+) cells. However, researchers also acknowledged that this increase could have occurred in order to compensate the reduction of T-CD8(+) cells, which were likely inhibited by the increase of epinephrine caused by caffeine ingestion. Further investigation could provide further insight into the extent caffeine supplementation impacts immune cell function.

Caffeine supplemented exercise has even been shown to have an effect on non-cellular elements of the immune system. Cheng et al. (2022) instructed twelve endurance trained male athletes to consume either a 6 mg/kg body weight sports drink or an equally weighted placebo drink prior to cycling for 40 minutes at 50% of maximum power output whilst maintaining a consistent pedal frequency of 60 rotations per minute. Researchers then analysed the response of two salivary antimicrobial proteins: salivary alpha-amylase and lactoferrin. Salivary alpha-amylase activity was significantly higher during the caffeine trial at the start of exercise, mid-exercise, and post-exercise compared to 60 minutes pre-exercise. Post-exercise activity was also seen to be significantly higher than mid-exercise activity. While concentrations of salivary lactoferrin were notably higher mid-exercise and post-exercise compared to levels 60 minutes pre-exercise, there were no differences between placebo and caffeine conditions.

The summation of results from aforementioned studies within this section clearly demonstrate a pattern between caffeine supplemented exercise and cellular immunity. However, no such pattern can yet be established between caffeine supplemented exercise and salivary SIgA. Bishop et al. (2006) is one of the few published studies that investigated caffeine's effect on salivary SIgA concentrations. Endurance trained male athletes cycled for 90 minutes at 70% VO_2 max after being given either a 6 mg/kg dose of caffeine or a placebo. An initial pilot study conducted prior to the main study found that the caffeine treatment had no effect on salivary SIgA while participants were at rest. Yet, when researchers incorporated exercise, salivary SIgA levels were significantly higher with caffeine supplementation post-exercise and one-hour post-exercise compared to placebo conditions. Saliva flow rate and salivary SIgA secretion rate was also higher during caffeine supplemented exercise. Dulson et al. (2019) conducted a similar study as Bishop et al. (2006) and observed results that contradicted those of Bishop et al. (2006). Over a course of five visits, participants received either a placebo, a 2 mg/kg, 4 mg/kg, 6 mg/kg or an 8 mg/kg dose of caffeine one hour before running on a treadmill at a work rate equivalent to 80% of their VO_2 max for 70 minutes. Saliva samples were taken pre-supplement, pre-exercise, post-exercise 35 minutes post-exercise, and one hour post exercise. Both salivary SIgA secretion rates and concentrations were unaffected by exercise or caffeine ingestion regardless of dose. This is a particularly interesting observation given previous literature has shown that concentrations of salivary SIgA tend to be at least temporarily influenced by exercise. Exercise induced physiological

changes unique to the participants involved could have caused salivary SIgA concentrations to remain consistent throughout the experimental trials.

The various elements that might have contributed to the stark contrast in results between Bishop et al. (2006) and Dulson et al. (2019) and their relation to the current study are discussed in Chapter V. However, one determinant that had not been previously considered is the CYP1A2 gene.

2.8 The Role of CYP1A2

CYP1A2 is an integral gene in the human body. It is a part of the CYP1 family, which is responsible for drug metabolism (Zhao, 2021). It is exclusively expressed in the human liver and metabolizes a broad range of xenobiotics ranging from theophylline, clozapine, and tacrine to ordinary caffeine (Faber et al. 2005; Yang et al. 2010; Zhao et al. 2021). CYP1A2 codes for the P-450 1A2 isoenzyme, the primary isoenzyme involved in the demethylation of caffeine into its primary dimethylxanthine metabolites: paraxanthine, theobromine, and theophylline (Lelo et al. 1986; Miners & Birkett 1996; Nehlig & Alexander, 2018). Paraxanthine accounts for the majority of the primary metabolites, as theobromine and theophylline represent only 7-8% of all caffeine metabolism and utilize a different pathway than paraxanthine (1-N-demethylation and 7-N-demethylation respectively) (Kot & Daniel, 2008; Nehlig & Alexander, 2018).

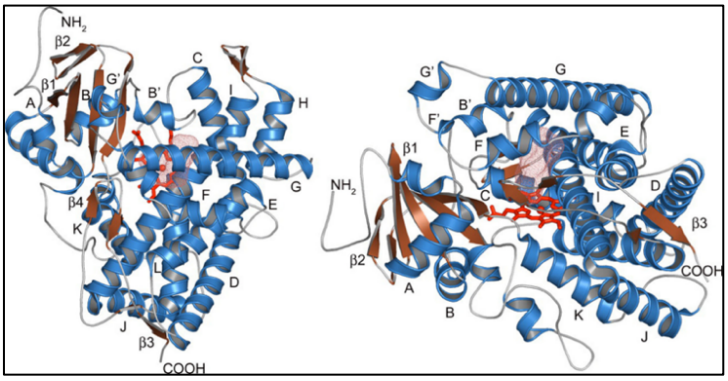

Figure 5. Secondary and tertiary structure of human P450 1A2 enzyme. Alpha helices in blue and beta strands in brown. Heme prosthetic group in red. Derived from Sansen

Other major metabolites of caffeine include 1-methylxanthine, 1-methyluric acid, 5-acetylamino-6-formylamino-3-methyluracil, and 1,7-dimethyl uric acid (Begas, et al. 2007). These metabolites are formed by the secondary metabolism of paraxanthine by the

cytochrome P450 1A2 enzyme and are present in urine along with xanthine oxidase and N-acetyltransferase 2 (Begas, et al. 2007).

The P450 1A2 enzyme is particularly important as it accounts for more than 95% of all caffeine metabolism in the human body (Kalow & Tang, 1993). The predominant pathway used to break down caffeine is through 3-N-demethylation, which is the removal of a methyl (CH_3) group from the third atom of caffeine's cyclic structure (nitrogen) to form paraxanthine (Kot & Daniel, 2008). Compared to other pathways in the CYP family, the intrinsic clearance, or the ability for the liver to remove drugs or other toxins, of the 3-N-demethylation pathway utilized by CYP1A2 is significantly more efficient, so much so that it accounts for roughly 80% of all caffeine metabolism in humans (Benowitz et al. 1995; Thorn et al. 2012).

2.9 The Role of CYP1A2 in Exercise

Although the CYP1A2 gene is primarily responsible for caffeine metabolism, it may also be responsible for the variability of performance seen during caffeine supplemented exercise (Grgic et al. 2021).

The CYP1A2 gene has several variations that can affect the rate of caffeine metabolism (Yang, et al. 2010). These variations often appear as single nucleotide polymorphisms (SNPs), which are a single base change in a DNA sequence (Vignal et al. 2002). In order for this single base change to be considered a SNP, the frequency of the resulting allele should be at least 1% or greater (Vignal et al. 2002). There are more than 150 identified SNPs for CYP1A2, with some resulting in significantly distinguishable phenotypes (Yang et al. 2010). The most extensively studied SNP is rs762551, which substitutes adenine (A) to cytosine (C) at position 163 (Nikrandt et al. 2022). Individuals with the homozygous AA allele tend to metabolize caffeine at a faster rate than their CC and CA genotypic counterparts (Gunes & Dahl, 2008). There have been several studies exploring as to whether this SNP might also influence the effectiveness of caffeine as an ergogenic aid because of its control over caffeine metabolism. A summary of the studies discussed within this section is listed below (Table 3).

Table 3. Summary of studies discussed during Section 2.9; AA genotype is fast metabolizer of caffeine; AC and CC are slow metabolizers of caffeine; (+): improvement in performance; (-): no improvement in performance; (/): decrease in performance

Study	Exercise Protocol	Caffeine Dose	Performance Outcomes of Gentoypes under Caffeine Supplementation
Carswell et al. (2020)	20 min cycle at 70% VO2 max and a Cycling Timed Trial	3 mg/kg	No genetic influence (-)
Davenport et al. (2020)	Cycling Timed Trial	200 g	No genetic influence (-)
Glaister et al. (2021)	Cycling Timed Trial	5 mg/kg	No genetic influence (-)
Guest et al. (2018)	10 kilometer cycle	2 and 4 mg/kg	Performance increase for AA (+) Performance decrease for CC (/) No effect on AC (-)
Klein et al. (2013)	Tennis skill test	6 mg/kg	No genetic influence (-)
Pataky et al. (2016)	Four Cycling Timed Trials	Caffeine rinse 6 mg/kg	Performance likely improved for AC (+)
Puente et al. (2018)	Basketball game	3 mg/kg	No genetic influence (-)
Spineli et al. (2020)	Various exercise tests	6 mg/kg	No genetic influence (-)
Womack et al. (2012)	40 kilometere cycle	6 mg/kg	Performance increase for AA (+)

Womack et al. (2012) found that participants with the homozygous AA genotype performed significantly better compared to other genotypes during caffeine supplemented exercise. Participants consumed either a placebo or a 6 mg/kg dose of caffeine before completing a 40-kilometre computer simulated cycle ergometer time trial. Caffeine supplementation decreased cycling time by 4.9% in participants with the AA allele and only 1.8% in C allele carriers. Guest et al. (2018) also found that caffeine improved the performance of participants with the homozygous AA allele. Following either a 4 mg/kg dose of caffeine, a 2 mg/kg dose of caffeine, or a placebo, participants cycled for 10 kilometres. Cycling times among the AA genotype were reduced by 4.8% with a 2 mg/kg caffeine treatment and 6.8% with a 4 mg/kg dose of caffeine. The 4 mg/kg caffeine treatment had the opposite effect on the CC genotype, increasing cycling times by 13.7%. No significant effect was observed in the AC genotype. While Pataky et al. (2016) also observed a caffeine x genotype interaction during their study, but their findings contradicted the results of their previous work, Womack et al. (2012). This time, they were not able to find a significant difference in performance between the caffeine and placebo conditions for participants with the homozygous AA allele. However, Pataky et al. (2016) utilized a different exercise protocol and athlete type than Womack et al. (2012). Instead of having elite trained athletes complete one timed trial, recreational cyclists

performed four separate 3-kilometre timed trials on a cycle ergometer after either rinsing with a 25 mL mouth wash with a 1.4% of caffeine, consuming a 6 mg/kg dose of caffeine, being administered a combination of these treatments, or ingesting a placebo. The power output for participants with the heterozygous AC allele likely improved by 5.1 ± 6.1% more than participants with the homozygous AA allele with the 6 mg/kg caffeine dose condition (P = 0.12). Although performance for the AA genotype likely improved under the caffeine and rinse condition, it was unclear if performance improved under caffeine ingestion alone. Researchers speculated that their contradictory results could stem from the duration of the physical activity as well as the activity levels of the participants. As previously mentioned, Womack et al. (2012) utilized elite trained athletes, who may naturally have a larger density of adenosine receptors than their untrained counterparts (Mizuno et al. 2005).

There have also been quite a few studies that were not able to find evidence of genetic influence over the performance outcome of caffeine supplemented exercise. Carswell et al. (2020) had participants ingest either a 3 mg/kg dose of caffeine or a placebo 70 minutes prior to cycling for 20 minutes at 70% VO_2 max while performing a psychomotor vigilance test. After a five-minute rest period, participants then performed a 15-minute cycling timed trial. While caffeine enhanced exercise performance, researchers determined that this improvement was not influenced by the CYP1A2 gene. There was, however, a significant difference between genotypes for the psychomotor vigilance test. Participants with the homozygous AA allele were found to have the greatest improvement in reaction times compared to C allele carriers. It is possible that the effects of caffeine's metabolites could have been more apparent in the central nervous system than in peripheral tissues. These metabolites could be directly influenced by a CYP genotype, which could explain the differences in reaction times between genotypes. Davenport et al. (2020) observed similar results regarding exercise performance during their study. Participants consumed a commercially available supplement containing 200 mg of caffeine at one of three time points: 35 minutes before a 30-minute steady state exercise on a cycle ergometer at 80% VO_2 max, at the onset of the steady state exercise, or immediately before a 15-minute timed cycling trial. Compared to the placebo, average work during the 15-minute timed cycling trial was 5% higher when the caffeine was ingested 35 minutes prior to the steady state exercise. When comparing genotypes, researchers were unable to find a distinct difference in performance under any of the experimental conditions. While researchers used a relatively high dose of caffeine (200 mg), this amount cannot be efficiently standardized across participants. Two-hundred mg of caffeine might equate to a 4

mg/kg dose for one participant and a 2 mg/kg dose for another. Standardizing doses could have also revealed a potential caffeine x genotype interaction, such as the one observed by Guest et al. (2018), or produced different results in general. However, there are studies that have implemented this design that did not find a caffeine x genotype interaction. Glaister et al. (2021) instructed sixty-six cyclists to consume a 5 mg/kg dose of caffeine or a placebo 60 minutes prior to completing a submaximal increment cycling test followed by a thirty-minute timed trial. Caffeine supplementation significantly reduced time to completion (29.7 ± 1.8 min) compared to the placebo (30.8 ± 2.3 min) for the timed cycling trial. Caffeine also reduced average perceived exertion by 0.5 ± 0.8. However, no caffeine x genotype interaction observed within the study. Klein et al. (2013) were also unable to find a caffeine x genotype interaction when they analysed the performance of collegiate tennis players. Sixteen male and female athletes ingested either a placebo or a 6 mg/kg dose of caffeine prior to completing a 45-minute intermittent treadmill exercise and a tennis skill test which assessed stroke accuracy. Caffeine supplementation significantly improved stroke accuracy (295 ± 11 shots) compared to the placebo (289 ± 10 shots). The only caffeine x genotype interaction observed within the study was with participants' heart rate during the tennis skill test. C allele carriers on average had a lower heart rate under caffeinated conditions (150 ± 16 beats per minute) than participants with the homozygous AA allele (155 ± 17 beats per minute). Puente et al. (2018) investigated as to whether the CYP1A2 gene influenced caffeine supplemented exercise in elite basketball players and were unable to find any correlation. Participants consumed either a 3 mg/kg dose of caffeine or a placebo one-hour before completing several exercises: an Abalakov jump test, a Change-of-Direction and Acceleration test, and a 20-minute simulated basketball game. The Abalakov jump test and the Change-of-Direction and Acceleration test were each repeated ten times before the simulated basketball game. Caffeine supplementation improved performance during the Abalakov jump test for both C allele carriers and homozygous AA participants. No significant difference in performance could be established between the two genotypes during the caffeine trial. A similar trend was observed in self-perceived muscle power but not in the Change-of Direction and Acceleration test. A study by Spineli et al. (2020) also found no caffeine x genotype interaction when examining the effects of caffeine on muscular endurance and performance in adolescent athletes. Participants (aged 15 ± 2 years) ingested either a 6 mg/kg dose of caffeine or a placebo before being tested on handgrip strength, vertical jump height, agility, sit-ups, push-ups, and the Yo-Yo intermittent recovery test level one. Caffeine supplementation increased the total distance covered in the Yo-Yo intermittent recovery test and the number of push-up

repetitions completed. Researchers could not find a statistically significant difference in performance between homozygous AA allele and C allele carriers for either the Yo-Yo intermittent recovery test or the push-up test. No improvement was observed during the caffeine trial for any other physical test. The lack of caffeine x genotype interaction within these studies could be contributed to a number of differences, including differences between methodology and participant type. The general consensus as to whether the CYP1A2 gene influences caffeine supplemented exercise is divided in the current literature. However, the CYP1A2 gene might impact other physiological systems during exercise, such as the immune system.

Although there have been quite a few studies analysing CYP1A2's effect on exercise performance, to my knowledge no study has yet investigated whether this gene may account for the variability in salivary SIgA concentrations observed in response to caffeine supplemented exercise (Bishop et al. 2006; Dulson et al. 2019). As such, this study aims to investigate whether CYP1A2 has any influence over exercise-induced change in salivary SIgA concentration. Due to the limited number of studies focusing on the impact of caffeine supplementation on salivary SIgA levels during exercise, this study also aims to determine whether a high dose (4 mg/kg) or a low dose (2 mg/kg) of anhydrous caffeine has an influence on the exercise-induced change in salivary SIgA.

Chapter III: Methodology

3.1 Participants

This study was performed on eleven (nine males and two females) healthy, active volunteers 32 ± 9.2 years old who engaged in regular exercise. Originally, twelve participants volunteered and enrolled, but one participant withdrew due to scheduling conflicts. Participants had no historically adverse reaction to caffeine and were free from any autoimmune, cardiovascular, pulmonary, or other disease that would prevent them from safely engaging in the study. Participants also reported no upper respiratory infections within the 1 month prior to or during the study. This study was carried out according to the Declaration of Helsinki and was fully approved by the University of Kent School of Sport and Exercise Sciences Research Ethics and Advisory Group (REAG) (Ethics Reference: 16_20_22). Participants received a verbal and complete written description of the protocol. The written explanation outlined potential risks, benefits, and why the study was occurring. If participants were still comfortable taking part, they provided written informed consent and completed the Physical Activity Readiness Questionnaire to ensure they were physiologically fit to volunteer. Participants were given a food diary and were asked to write down everything they ate 24 hours prior to any laboratory visit and keep diets consistent before each visit. Food diaries were collected after the end of the fourth visit. Female participants were given a One Step® Ovulation Test to determine the phase of their menstrual cycle during each laboratory visit. Female participants were given at least 20 One Step® Ovulation Tests during their first laboratory visit along with written guidelines on how to use it. Female participants were instructed to urinate into a cup and lower the ovulation test strip into the urine and hold it for 10-15 seconds. Participants were told not to allow the ovulation test strip to submerge further than the black line marked 'MAX.' Once the 10-15 seconds was up, the ovulation test strip was to be laid flat on a clean surface and results were to be read in 5 minutes' time. If the test line on the ovulation test strip had a stronger pigmentation than the control strip, the result was positive. Female participants were instructed to begin testing on the sixth day of their menstrual cycle and stop when they had received a positive result. From this positive result, the phase of the menstrual cycle each female participant was in during testing was determined. While this was originally meant to account for caffeine's effects on the menstrual cycle, this data was unable to be included. Details are discussed in a later section.

3.2 Laboratory Visit Preparation

Prior to the familiarization visit, participants were asked to abstain from physical exercise for at least 24 hours. Participants received a reminder email 72 hours prior to each experimental visit reminding them to abstain from physical exercise and all forms of caffeine for 48 hours and complete a 12 hour fast the night before their next experimental visit. Participants were also given a food diary and asked to write what they ate the day before all testing laboratory visits. These food diaries were collected at the end of the last laboratory visit.

3.3 Participant Characteristics

Participant's body mass and stature were measured with their respective exercise apparel on but without shoes. Body mass (kg) was measured using the SECA 760 mechanical flat scale and is presented with a precision of 1 kg. Height (cm) was measured using the SECA 213 Portable Stadiometer and was recorded with a precision of 1 cm. Participant characteristics are displayed in Table Four.

Table 4. Participant Characteristics

Sex	Participant ID	Height (cm)	Weight (kg)	VO2 Max (ml/min/kg) 5 s rolling average
Female	1	161	52	49.6
Male	2	171	58	63.5
Male	3	182	78	72.0
Male	4	196	79	67.8
Male	5	177	81	53.0
Female	6	167	68	43.0
Male	7	183	84	33.1
Male	8	165	65	49.0
Male	9	195	125	45.0
Male	10	171	73	50.0
Male	11	177	68	48.0
	Mean ± Standard Deviation	176.8 ± 10.9	75.5 ± 18.2	52.2 ± 10.9

3.4 Treatment Preparation

A 2 mg/kg and a 4 mg/kg dose of caffeine was prepared for each participant by placing 99% Anhydrous caffeine (Sigma-Aldrich food grade W222402, Merck Life Science UK, Dorset, UK) in Bulk size 0 vegetarian capsules. A Kern ADB Analytical Weighing Scale was first tared for the capsule. Caffeine was then scooped into the capsule and weighed until the desired amount was reached. Caffeine tablet weight (grams) was measured at least twice for accuracy. Placebo treatments contained an unmeasured amount of Blackburn Distributions microcrystalline cellulose powder 102 in the same capsules as the caffeine treatments. Both caffeine doses and placebos for all participants were made up by myself. Treatments for all

participants were double blinded by laboratory technicians utilizing a colour coding system. The colour coding key was not revealed until after data analysis was complete.

3.5 Saliva Sample Label Organization

Four separate saliva samples were taken at different time points during each experimental visit. Each of these tubes was marked with the participant's unique ID number, which of the four saliva samples it contained (1-4), and the experimental visit number (1-3). Saliva samples collected during the first, second, and third experimental visits were stored in Yellow, Red and Blue 1.5 mL Eppendorf Safe-Lock Tubes respectively. After initial processing, saliva samples were stored in a Haier Biomedical Freezer at -79°C.

3.6 Aerobic Capacity and Familiarization Procedures

During the first laboratory visit, participant's maximal oxygen consumption (VO_2 max) was determined by use of an h/p/cosmos quasar® treadmill and a Cortex Metalyzer 3B, which was calibrated before each familiarization visit. Participants waited in the University of Kent Chipperfield Building main lobby upon their arrival and were escorted to the physiology research lab by a researcher at their scheduled visit time. Following height and weight measurements, volunteers completed a Caffeine Frequency Questionnaire to determine the level of their daily caffeine use. A capillary sample was then taken for genetic testing. DNA prepared from whole blood has been shown to perform significantly better than that prepared from saliva with higher yields and rates of genotyping (Philibert et al. 2008). Either the index, middle or ring finger was pricked to obtain the bloods sample using an Owen Mumford Unistik 3 Normal Lancet (23g x 1.8 mm needle). Blood was collected using a Microvette CB 300. Capillary samples were immediately centrifuged in a Cole-Parmer Stuart SCF3 microcentrifuge at 1531 relative centrifugal force (RCF) for ten minutes. Plasma was transferred to a 1.5 mL Fisherbrand Premium Microcentrifuge Tube (Natural) with a 20 µL SciPette Micropipette before being placed inside of a VWR Cryobox (with dividers) and frozen inside a Haier Biomedical Freezer at -79 °C until data analysis. All cells were properly disposed of in a biomedical waste bin. A basal saliva sample was also taken during the familiarization visit. Participants were asked to passively drool into a SciQuip 50 mL PP Conical Centrifuge Tube and fill it to just below the 5 mL mark. Participants were instructed allow saliva to pool in a closed mouth. Once they felt sufficient saliva pool had been formed (i.e. mouth felt full), participants were instructed to open their mouths and then lean forward to allow for the saliva to drip into the SciQuip 50 mL PP Conical Centrifuge Tube. A portion

of the saliva sample was then transferred to a 1.5 mL Fisherbrand Premium Microcentrifuge Tube (Natural) and centrifuged at the same speed and length of time as the capillary sample. Any surplus of saliva was properly disposed of in a biohazard waste bin. If the precipitant had not properly accumulated at the bottom of the tube at the end of the ten minutes, the sample would undergo additional bouts of centrifuging until the precipitant and the supernatant were clearly separated. The supernatant was then transferred to another 1.5 mL Fisherbrand Premium Microcentrifuge Tube (Natural) with a SciPette micropipette. As much of the supernatant was transferred into the second tube as possible, but no exact amount was extracted from each participant due to the variable nature of each sample. The white saliva precipitant was properly disposed of in a biomedical waste bin. The basal saliva sample was then placed inside of the same cyrobox and freezer the capillary sample. Samples within the cryobox were grouped together based on participant. The basal saliva samples were kept as a back-up in case capillary samples did not produce conclusive results during genotype analysis. Basal saliva samples did not end up being used.

Participants were then fitted with a Hans Rudolph V2 mask and Hans Rudolph headgear that corresponded to their mask size. Once participants' information and final calibrations had been entered into the Cortex Metalyzer 3B, the turbine and O_2 sensor were attached to the participant's mask and secured with 3M Transpore Surgical Tape. The emergency stop buttons on the treadmill were made apparent to participants and the protocol for the submaximal test was briefly described before it began. Participants were also reminded that they could stop the test at any time for any reason. Participants were also offered the choice of a wall mounted Panasonic Air Conditioner set at 18 °C and or an AIRCONCO Tank – 3076 Fan set on the 'one' intensity level directed towards the treadmill to be on during their submaximal run. The treadmill was first increased manually to 6 km/hr at which point data collection would begin. The speed of the treadmill would increase 1 km/hr every minute until the participant indicated with a 'thumbs up' that they had reached a rating (here and throughout) of perceived exertion of 11. Participants would then complete three 'stages' three minutes in duration. They would remain at their rate of perceived exertion of 11 speed for three minutes, following which the treadmill would be increased by 1 km/hr every three minutes for six additional minutes. Participants were then given the option of either immediately coming off the treadmill after the third stage or cool down on the treadmill at a slow walking pace. Once the participant came off the treadmill, they were given a 17-minute rest before the VO_2 max test. The Hans Rudolph V2 mask was taken off during the break and

participants were encouraged to stretch and drink water. Following the 17-minute break, participants returned to the treadmill and the Hans Rudolph V2 mask and Cortex Metalyzer 3B were set up in an identical fashion as the submaximal test. Participants were again offered the choice of the wall mounted Panasonic Air Conditioner set at 18 °C and or an AIRCONCO Tank – 3076 Fan set on the 'one' intensity level directed towards the treadmill to be on during their VO_2 max test. The treadmill speed would be increased to each participant's rate of perceived exertion of 11 at which point data collection would begin. The speed would then be increased by 1 km/hr every minute until either exhaustion or 16 km/hr. If the participant had still not reached exhaustion by 16 km/hr, the incline of the treadmill was increased by 1% every minute until exhaustion. Once participants had reached exhaustion, they would indicate as such by a 'thumbs' up to the researcher. Data collection would also stop once exhaustion was reached and the mask would be taken off. The treadmill speed would then decrease to approximately 6 km/hr and participants would be allotted a cool down. Once participants had stepped off the treadmill, the speed of the treadmill at 70% of their VO_2 max and at 40% of their VO_2 max was calculated. After a 30-minute break period, participants would then complete one interval of the exercise to be performed during the experimental trial. Participants would warm up on the treadmill for 2.5 minutes at a moderate walking pace. The treadmill would then be sped up gradually to the participant's speed at 70% VO_2 max. The participant would remain at this speed for four complete minutes before the treadmill was gradually brought down to the participant's speed at 40% VO_2 max. The participant would then remain at this speed for one whole minute before the treadmill was decreased once until the participant was at a moderate walk for a cool-down. After 2.5 minutes, the treadmill was stopped and the participant safely stepped off the machine.

At the end of the familiarization visit, participants were given a list of common caffeinated foods and beverages to avoid for their next visit. Participants were then reminded of their next laboratory visit and were given the opportunity to ask any final questions. Participants were also encouraged to email the researcher at any time with questions. All participant queries were addressed within 24 hours. At the end of the visit, they were then directed back towards the University of Kent Chipperfield Building main lobby.

3.7 Experimental Visit Procedures

Similar to the familiarization visit, participants waited in the lobby of the Chipperfield Building at the University of Kent and were escorted to the physiology research lab at their scheduled visit time by a researcher. Each experimental visit was conducted under the exact

same protocol with the exception of the final experimental visit in which participants filled out a blinding questionnaire near the end of the visit. Participants were first asked to complete the following questionnaires: The University of Kent School of Sport & Exercise Sciences Pre-test/Re-Test Questionnaire, a Competitive State Anxiety Inventory-2, and The Groningen Sleep Quality Scale. The first of four saliva samples were collected once questionnaires were complete. Participants were instructed to passively drool into a SciQuip 50 mL PP Conical Centrifuge Tube until just below the 5 mL mark. The saliva sample was then transferred into a 1.5 mL Fisherbrand Premium Microcentrifuge Tube (Natural) via a SciPette Micropipette and centrifuged in a Cole-Parmer Stuart SCF3 microcentrifuge at 1531 relative centrifugal force (RCF) for ten minutes. Additional bouts of centrifuging (at 1531 RCF) were completed if the supernatant and precipitant were not notably distinguishable from one another. Once the saliva supernatant could be extracted without disturbing the precipitant, it was transferred from the 1.5 mL Fisherbrand Premium Microcentrifuge Tube (Natural) into either a yellow, red, or blue 1.5 mL Eppendorf Safe-Lock Tubes depending on the experimental visit number. All saliva samples taken during the experimental visits were collected in this manner. All saliva samples were prepared for and centrifuged immediately after participants had completed drooling into the SciQuip 50 mL PP Conical Centrifuge Tube.

Participants then received either a 2 mg/kg dose of caffeine, a 4 mg/kg dose of caffeine, or a placebo. Although it was common for participants to bring their own water bottle, each participant was offered a cup of water to swallow the capsule with. This cup was thoroughly washed after each use. Participants sat quietly in the physiology research lab during the one hour waiting period between the treatment ingestion and the exercise portion of the visit. However, participants were allowed to use the bathroom and drink water, but not within 10 minutes of sample collection. A second saliva sample was taken after the one hour waiting period and before the exercise portion of the visit. Before the exercise portion of the visit began, participants were reminded of the procedures as well as the speed (km/hr) they would be running at and for how long. They were also re-familiarized with the safety mechanisms of the treadmill and the exercise would not commence until the participant had voiced that they were comfortable with the protocol. For the experimental visits, participants ran without the use of the Hans Rudolph V2 mask or the Cortex Metalyzer 3B. Before the exercise portion began, participants were offered the choice of a wall mounted Panasonic Air Conditioner set at 18 °C and or an AIRCONCO Tank – 3076 Fan set on the ‘one’ intensity level directed towards the treadmill to be on during their run. Participants were also given the option to

listen to music while they ran or be strapped into the treadmill's safety harness. These choices were repeated for all participants during all laboratory visits. Participants began the exercise portion with a warm up at 6 km/hr on the treadmill for 2.5 minutes. The treadmill's speed was then increased to the speed at 70% of the participant's VO_2 max. This speed was maintained for four minutes. The speed was then decreased to 40% of the participant's VO_2 max for one minute. The speed of the treadmill alternated between 70 and 40% of a participant's VO_2 max for five complete cycles. Participants then cooled down by walking moderately on the treadmill for 2.5 minutes. The speed of the treadmill did not change until the time intended to spend at that speed was complete. During the exercise, participants would occasionally be encouraged with verbal praise. A third saliva sample was collected immediately following the completion of the 2.5-minute warm down. The fourth and final saliva sample was then taken 30 minutes post-exercise. At the end of the third experimental visit, participants completed a blinding questionnaire following the collection of the fourth saliva sample. Participants were encouraged to drink water during this time, but were still not permitted to consume other forms of food or beverage. Participants sat quietly in the physiology laboratory during the time between the third and fourth saliva sample. The participant was then reminded of the date of their next laboratory visit and were given the opportunity to ask any final questions. Participants were encouraged to contact the researcher via email at any time should they have additional questions or concerns. All participant queries were addressed within 24 hours. At the end of the session, the participant was directed back to the University of Kent Chipperfield main lobby. Once the supernatant from the fourth saliva sample had been transferred to its appropriately labelled and coloured 1.5 mL Eppendorf Safe-Lock Tube, it along with the other three saliva samples were frozen in a Haier Biomedical Freezer at -79 °C until data analysis. All saliva samples were grouped together based on participant.

3.8 Saliva Sample Analysis

Saliva samples were analysed for osmolality and the concentration of salivary SIgA. Both absolute salivary SIgA and salivary SIgA relative to osmolality were considered during analysis because absolute salivary SIgA represents the working concentration available for immune function and defence in vivo while salivary SIgA relative to osmolality is able to account for dehydration and reflect the concentration of salivary SIgA per µL of saliva (Bishop & Gleeson, 2009). Saliva IgA concentration was determined using two separate methods: The FineTest® Human sIgA (Secretory IgA) ELISA Kit and an 'in house' ELISA

procedure modified from Leicht et al. 2011. The modification involved altering the capture antibody used to give specificity for human secretory IgA. Although all samples had initially been measured with the FineTest® ELISA Kit, we had some concerns regarding the validity of results for some of the samples. We therefore reverted to the 'in-house' method that has previously been used within the University of Kent School of Sports and Exercise Science labs. Results from The FineTest® ELISA Kit were analysed from participants 1-3 as we were satisfied that these results were valid and acceptable. Samples from all other participants were analysed using the 'in house' ELISA procedure. Osmolality was measured using a Gonotec® Osmomat™ 3000. All sample analysis took place at the Chipperfield Building at the University of Kent, Canterbury.

3.81 FineTest® ELISA Kit Saliva Sample Analysis

The FineTest® Human sIgA (Secretory IgA) ELISA Kit was used to measure concentration of salivary SIgA. The assay was performed in accordance with manufacture instructions. Once samples had completely thawed, they were centrifuged for 30 seconds at the highest speed within a Fisher Scientific Refrigerated accuSpin Micro 17R m/n. This allowed for any remaining mucins within the sample to precipitate at the bottom of the tube. Reagents within The FineTest® Human sIgA (Secretory IgA) ELISA Kit were brought to room temperature at least 20 minutes before use. All reagents except for deionised water and phosphate buffered saline (PBS) were from The FineTest® Human sIgA (Secretory IgA) ELISA Kit. All samples were placed on the assay in duplicate and all samples for any given subject were on the same assay plate.

3.82 Sample Preparation for FineTest® ELISA Kit

A wash buffer was first prepared in accordance with the manufacturer instructions. During the 10-minute waiting period, the standard sample tubes were prepared for six serial dilutions. 1.5 mL Fisherbrand™ Premium Microcentrifuge Natural Tubes were labelled according to the decreasing concentration of the original 200 mg in the Human sIgA Standard Tube: 200, 100, 50, 25, 12.5, 6.25, and 3.125 respectively. A Gilson™ PIPETMAN Classic™ Pipets micropipette along with 1000 µL filtered low-retention pipette was used to add 0.3 mL of PBS into the 100, 50, 25, 12.5, 6.25, and 3.125 tube. This micropipette and pipette combination was used throughout the creation of the standard solutions. An equal amount of PBS was also added to a tube labelled 'BLANK,' which would serve as the control for the diluent. Once the Sample Solution Buffer and Human sIgA Standard Tube mixture had stood for 10 minutes at room temperature, the entirety of the vial was added to the standard sample

tube labelled 200. 0.3 mL of solution was taken from the tube labelled 200 and placed into the tube labelled 100 and was mixed well by pipetting rapidly 8 times just below the surface and blowing out the remaining contents of the pipette above the surface. This process was repeated for the transfer of solution between the tubes labelled 100 and 50, 50 and 25, 25 and 12.5, 12.5 and 6.25, and 6.25 and 3.125. A new pipette was used for each transfer between tubes. The standard samples were used within 2 hours.

Saliva samples were diluted 2500-fold using two sets of 1.5 mL Fisherbrand Premium Microcentrifuge Tube (Natural). 500 µL of PBS was first placed into each set of tubes using an Eppendorf™ Explorer™ Multi-Channel Electronic Pipette and Eppendorf™ epT.I.P.S.™ Reloads Pipette Tips (Eppendorf Quality™, 50-1250 µL). 10 µL of the saliva sample was placed in the corresponding first set dilution tube, employing the same micropipette used to create the standard solutions along with Fisherbrand™ SureOne™ Aerosol Barrier Pipette Tips. This solution was mixed well by pipetting rapidly below the surface 10 times followed by a blow-out above the surface. The tube was then vortexed manually for 1-2 seconds using the Fisher Scientific TopMix FB 15024 Vortex Mixer at 15 Hz. 10 µL from the first set dilution tube was then placed into the corresponding second set dilution tube and mixed in the same manner as the first set dilution tube.

3.83 Assay Procedure for FineTest® ELISA Kit

In the first two columns of the ELISA assay, 100 µL of the standard solution were arranged in duplicates using an Eppendorf™ Explorer™ Multi-Channel Electronic Pipette and Eppendorf™ epT.I.P.S.™ Reloads Pipette Tips (Eppendorf Quality™, 50-1250 µL). 100 µL of the diluted saliva samples were placed in the remain columns as side-by-side duplicates using the Sartorius Picus™ NxT Single Channel Electronic Pipette and Fisherbrand™ SureOne™ Aerosol Barrier Pipette Tips.

Once the standards and diluted saliva samples were placed in their proper wells, the plate was sealed with a plastic cover and incubated in a BMG LABTECH FLUOstar OPTIMA Reader at 37 °C for 90 minutes.

When there was approximately 30 minutes of incubation remaining, the HRP-detection Antibody and Antibody Dilution Buffer were combined at a 1:100 ratio and mixed by gently shaking the bottle. This solution was used within one hour of preparation. After the incubation period was completed, the plastic cover was removed and any remaining liquid within the wells was disposed of in the sink. 200 µL of the wash buffer was allocated into

each well using an Eppendorf™ Explorer™ Multi-Channel Electronic Pipette and Eppendorf™ epT.I.P.S.™ Reloads Pipette Tips (Eppendorf Quality™, 50-1250 µL). The wash buffer was allowed to sit in the well for approximately 1.2 minutes before being poured out. The assay was dried slightly by tapping it well-side down on a paper towel on the lab workbench. This process was repeated two more times for a total of three washes. 100 µL of the HRP-labelled antibody and Antibody Dilution Buffer solution was then added into each well using Eppendorf™ Explorer™ Multi-Channel Electronic Pipette and Eppendorf™ epT.I.P.S.™ Reloads Pipette Tips (Eppendorf Quality™, 50-1250 µL). The assay was sealed with a new cover and incubated a second time at 37 °C for 30 minutes along with the TMB substrate bottle in a BMG LABTECH FLUOstar OPTIMA Reader. Although both the assay and the TMB substrate bottle were incubated within the same space, both components were kept separate during the incubation period.

After the 30-minute incubation, the assay was rinsed with the wash buffer with a procedure nearly identical to the wash after the first incubation. The single differentiating factor being that the assay was washed a total of five times instead of three. Due to the light sensitive nature of TMB Substrate, the lab's florescent lights were turned off before the bottle was opened. However, natural light was allowed to enter the lab from windows. 90 µL of TMB Substrate was placed into each well using Eppendorf™ Explorer™ Multi-Channel Electronic Pipette and Eppendorf™ epT.I.P.S.™ Reloads Pipette Tips (Eppendorf Quality™, 50-1250 µL). The plate was covered with a new plastic cover and was incubated for a final time at 37 °C for 16 minutes in the BMG LABTECH FLUOstar OPTIMA Reader. When the incubation time was complete, the seal was taken off and 50 µL of Stop Solution was added into each well.

3.84 'In House' Modified ELISA Procedure from Leicht et al. (2011)

The measurement of human s-IgA took place over a three-day time period. On the first day, a 0.05 M carbonate and bicarbonate coating buffer was prepared for a sterile Costar 96-Well Microplate. Each Costar 96-Well Microplate could hold the complete sample set of up to three participants at a time. 0.208 g of Sigma Adrich S2127-1KG Sodium Carbonate and 0.030 g Sigma Life Science S5761-1KG Sodium Bicarbonate were individually placed inside of a plastic weigh boat using separate sterile stainless steel measuring spoon and measured inside of an A&D BM Series Micro Balance. 55 mL of dH_2O was measured inside of a Fisherbrand™ Class B Graduated Cylinder (100 mL) before being poured into a Pyrex® Narrow Mouth Conical Flask (250 mL). The carbonate and bicarbonate powders were poured

into the Erlenmeyer flask and the weigh boats that held them were disposed of inside a biohazard waste bin. The Erlenmeyer flask was gently hand shaken until the bicarbonate and carbonate were completely dissolved. A Fisherbrand™ Class B Graduated Cylinder (100 mL)and a µL 1000 Gilson™ PIPETMAN Classic™ Pipets micropipette fitted with a 1000 µL filtered low-retention pipette was used to measure out exactly 52.2 mL of this solution and transfer it into a Thermo Scientific™ Matrix™ Reagent Reservoirs. A 30 µL of a neat sample from a Sigma-Adrich I6635 Monoclonal Anti-Secretory Component (IgA) antibody produced in mouse sample to the Thermo Scientific™ Matrix™ Reagent Reservoirs using a 20 µL Gilson™ PIPETMAN Classic™ Pipets equipped with a Molecular BioProducts ART 20P 20 µL 2149P-05-R Barrier Pipette Tips. To ensure proper incorporation of the capture antibody into the coating buffer solution, the pipette tip on the 20 µL Gilson™ PIPETMAN Classic™ Pipets containing the capture antibody was pipetted rapidly 8 times just below the surface of the coating buffer solution before blowing out the remaining contents of the pipette above the surface. 100 µL of the coating buffer and antibody mixture were placed into each well of the Costar 96-Well Microplate using an Eppendorf™ Explorer™ Electronic Pipettes, Multi-Channel micropipette fitted with Eppendorf™ epT.I.P.S.™ Reloads Pipette Tips (Eppendorf Quality™, 50-1250 µL). The Costar 96-Well Microplate was then sealed with a clear plastic sticker cover and incubated overnight in a Fridgemaster Refrigerator at 4°C.

3.85 Sample Preparation for ‘In House’ Modified ELISA Procedure

On the second day, the saliva samples scheduled to be analysed were removed from the Haier Biomedical Freezer and allowed to thaw at room temperature. While the samples defrosted, a 1 L phosphate buffered saline (PBS) solution a made up in by dissolving five Sigma P4417-100TAB Phosphate Buffered Saline Tablets in 1 L of deionised water inside of a 1000 mL Fisherbrand™ Reusable Glass Media Bottle with Cap. A second 1 L PBS solution was created in an identical manner in order to provide the main component of the washing buffer. 20.75 g of Sodium Chloride, Extra Pure, SLR, Fisher Chemical was placed inside of a plastic weigh boat using a sterile stainless steel measuring spoon and measured inside of an A&D BM Series Micro Balance. The sodium chloride, along with 1 mL of Sigma-Adrich TWEEN®20 measured out using a 1000 µL Gilson™ PIPETMAN Classic™ Pipets equipped with a 1000 µL low retention sterile pipette tip with a filter, were added to the Fisherbrand™ Reusable Glass Media Bottle designated for the washing buffer. The micropipette containing the Sigma-Adrich TWEEN®20 was pipetted rapidly 8 times just below the surface of the PBS solution before blowing out the remaining contents of the pipette above the surface to

ensure total transfer of the Sigma-Adrich TWEEN®20 into the washing buffer solution. The bottle was then capped and shaken vigorously several times to incorporate the ingredients of the wash buffer.

A 2% Sigma-Adrich A2153 Bovine Serum Albumin lyophilized powder, ≥96% (agarose gel electrophoresis) 10 gr (BSA) in PBS was then prepared as the blocking solution.

The appropriate amount of BSA was placed into a plastic weigh boat using a sterile stainless steel measuring spoon and weighed inside of an A&D BM Series Micro Balance. The corresponding amount of PBS was measured inside of a Fisherbrand™ Class B Graduated Cylinder (100 mL) and poured into a clean sterile glass jar. The BSA was transferred from the plastic weigh boat into the same sterile glass jar and the lid was tightly secured. The jar was then gently shaken until all of the BSA had completely dissolved within the PBS.

Once the blocking solution had been prepared, the ELISA assays were removed from Fridgemaster Refrigerator. The sticker covers were then peeled off and properly disposed of and the contents of the assay were poured down the sink. The washing buffer prepared earlier was poured into Thermo Scientific™ Matrix™ Reagent Reservoirs for easy access. 200 µL of the washing buffer was then placed into each assay well utilizing the Eppendorf™ Explorer™ Electronic Pipettes, Multi-Channel micropipette equipped with the Eppendorf™ epT.I.P.S.™ Reloads Pipette Tips (Eppendorf Quality™, 50-1250 µL). The assay was gently shaken by hand before its contents were once again decanted. The assay was dried by aggressively banging it upside down on a paper towel on the lab bench. This process was repeated three more times for a total of four washes.

Following the final wash, the blocking solution was then poured into Thermo Scientific™ Matrix™ Reagent Reservoirs for easy access. Each dry well was then filled with 100 µL of the blocking solution utilizing the Eppendorf™ Explorer™ Electronic Pipettes, Multi-Channel micropipette and the Eppendorf™ epT.I.P.S.™ Reloads Pipette Tips (Eppendorf Quality™, 50-1250 µL). The assay was re-covered with a new clear plastic sticker cover and incubated at room temperature for 60 minutes.

During the 60-minute incubation period, the standard solutions and saliva samples were prepared to be plated onto the assay. For the standard solutions, nine Fisherbrand Premium Microcentrifuge Tubes (Natural) were labelled X, A, B, C, D, E, F, G, and H respectively. Tube X was filled with 800 and tube A was filled with 990 µL of PBS utilizing a 1000 µL Gilson™ PIPETMAN Classic™ Pipets and a 1000 µL low retention sterile pipette tip with a

filter. The seven remaining tubes were filled with 500 µL of PBS using the same micropipette and tip combination. A 1:1700 dilution of Sigma-Adrich I2636 IgA from human colostrum was prepared by first diluting 50 µL of the neat IgA from human colostrum sample in tube X using a Sartorius Picus™ NxT Electronic Pipette, single channel equipped with Fisherbrand™ SureOne™ 300 µL Aerosol Barrier Pipette Tips. To ensure as much of the sample as possible had exited the pipette, the solution was pipetted just below the surface 10 times followed by a blow-out above the surface. 10 µL from tube X was then transferred to tube A utilizing a 20 µL Gilson™ PIPETMAN Classic™ Pipets and a Molecular BioProducts ART 20P 20 µL 2149P-05-R Barrier Pipette Tips. An identical pipetting technique was applied tube A in order to ensure this solution was mixed thoroughly. Tube A then underwent six serial dilutions utilizing a 1000 µL Gilson™ PIPETMAN Classic™ Pipets and a 1000 µL filtered low-retention pipette. 500 µL was taken from tube A and placed into tube B and was mixed well by pipetting rapidly 8 times just below the surface followed by a blow-out of the remaining contents of the pipette above the surface. This process was repeated for the transfer of solution between tube B and tube C, tube C and tube D, tube D and tube E, tube E and tube F, and tube F and tube G. Tube H served as the 'blank' containing only PBS. A new pipette was used for each transfer between tubes. Used pipettes were discarded of properly in a biohazard waste bin.

All samples were prepared for analysis with a 900-fold dilution. In order to achieve this, two sets of 1.5 mL Fisherbrand Premium Microcentrifuge Tubes (Natural) were identically labelled as to correspond to each individual sample. 290 µL of the PBS solution was then dispensed into each set utilizing the Eppendorf™ Explorer™ Electronic Pipettes, Multi-Channel micropipette and Eppendorf™ epT.I.P.S.™ Reloads Pipette Tips (Eppendorf Quality™, 50-1250 µL).

Once the samples had thawed completely, they were spun in a for 30 seconds at the highest speed within a Fisher Scientific Refrigerated accuSpin Micro 17R m/n. This allowed for any remaining mucus within the sample to precipitate at the bottom of the tube. 10 µL of a saliva sample was placed in the corresponding first set dilution tube using a 20 µL Gilson™ PIPETMAN Classic™ Pipets equipped with a Molecular BioProducts ART 20P 20 µL 2149P-05-R Barrier Pipette Tips. To ensure as much of the sample as possible had exited the pipette, the solution was rapidly pipetted just below the surface 10 times followed by a blow-out above the surface. The tube was then vortexed manually for 1-2 seconds using the Fisher Scientific TopMix FB 15024 Vortex Mixer at 15 Hz. 10 µL from the first set dilution tube

was then placed into the corresponding second set dilution tube. An identical pipetting technique was applied to the transfer of solution from the first set dilution tube into the second set dilution tube. The second set dilution tube was also vortexed in an identical manner as the first set dilution tube. This process was repeated until all saliva samples scheduled to be analysed within that particular ELISA assay had been diluted 900-fold.

Once the 60-minute incubation period was complete, the assay was washed for a second time following the same washing procedure that had taken place after it was first removed from the Fridgemaster Refrigerator After the final wash in the first two columns of the ELISA assay, 50 µL of the standard solution were arranged in duplicates using a Sartorius Picus™ NxT Single Channel Electronic Pipette and Fisherbrand™ SureOne™ Aerosol Barrier Pipette Tips. 50 µL of the diluted saliva samples were placed in the remaining wells as side-by-side duplicates using the same micropipette and pipette tip combination as the standard solutions.

Once the standards and diluted saliva samples were placed in their proper wells, the plate was sealed with a new clear plastic sticker cover and incubated overnight in a Fridgemaster Refrigerator at 4°C.

3.86 Preparation of Assay for Analysis for 'In House' Modified ELISA Procedure

The third day began with the preparation of the detection antibody. A BioLegend HRP Goat anti-human IgA Antibody sample was diluted 1:2000 in PBS.

The appropriate amount of HRP Goat anti-human IgA antibody was measured using a 20 µL Gilson™ PIPETMAN Classic™ Pipets and a Molecular BioProducts ART 20P 20 µL 2149P-05-R Barrier Pipette Tips and the corresponding amount of PBS was measured in a Fisherbrand™ Class B Graduated Cylinder (100 mL) and poured into a clean Thermo Scientific™ Matrix™ Reagent Reservoirs. To ensure proper incorporation of the detection antibody into the PBS solution, the pipette tip on the 20 µL Gilson™ PIPETMAN Classic™ Pipets containing the detection antibody was pipetted rapidly 8 times just below the surface of the PBS before blowing out the remaining contents of the pipette above the surface.

The assay was removed from the Fridgemaster Refrigerator and the sticker cover as well as the contents of the well were properly disposed of. The assay was then washed for a third time following an identical procedure as the two previous washes. Once the plate had been dried following the final wash, 50 µL of the HRP Goat anti-human IgA Antibody and PBS solution was added to each well utilizing the Eppendorf™ Explorer™ Electronic Pipettes,

Multi-Channel micropipette and Eppendorf™ epT.I.P.S.™ Reloads Pipette Tips (Eppendorf Quality™, 50-1250 µL). The assay was then re-covered with a new clear plastic sticker cover and left to incubate at room temperature on the lab bench for 60 minutes.

Once the 60 minutes had passed, the sticker cover was removed and the contents of the assay were properly disposed of. The assay was washed for a final time following an identical procedure as the three previous washes. An ample number of R&D Systems™ Stop Solution 2N Sulfuric Acid was poured into a clean Thermo Scientific™ Matrix™ Reagent Reservoirs to be used following the development of the assay.

Plate development involved the use of Salimetrics Tetramethylbenzidine (TMB) Substrate Solution. Due to the light sensitive nature of this reagent, all of the lab's florescent lights were turned off and the mesh shades on the lab windows were closed before the TMB bottle was opened. The TMB was poured into a Thermo Scientific™ Matrix™ Reagent Reservoirs and 50 µL of the TMB Substrate Solution was placed into each well utilizing the Eppendorf™ Explorer™ Electronic Pipettes, Multi-Channel micropipette and Eppendorf™ epT.I.P.S.™ Reloads Pipette Tips (Eppendorf Quality™, 50-1250 µL). The assay was covered with a paper towel and was allowed to develop for approximately 30-45 seconds. After which, 75 µL of the Stop Solution was added to each well using the same micropipette and pipette tip combination that was used with the TMB Substrate Solution.

3.9 Measurement of Saliva Osmolality

The Gonotec® Osmomat™ 3000 was first calibrated with 50 µL of distilled water followed by 50 µL of a 300 and 50 µL of an 850 mOsmol/kg $NaCl/H_2O$ solution. Each solution was measured twice during the calibration of The Gonotec® Osmomat™ 3000. Once the machine had been calibrated successfully, a 50 µL saliva sample was then placed in the measuring vessel included in the Gonotec® Osmomat™ 3000 kit. The osmolality of the sample was recorded and included in the calculation of the sample's salivary SIgA concentration. Measuring vessels containing both calibration liquids and salivary samples were properly disposed of in biohazardous waste bins after use. Salivary SIgA data analyzed during this study has been expressed relative to salivary osmolality, largely because saliva osmolality is proportionate to saliva flow rate (Bishop & Gleeson, 2009; Blannin et al. 1998). Saliva osmolality also primarily indicates inorganic electrolyte concentration and protein found within saliva encompasses less than 1% of saliva osmolality (Bishop & Gleeson, 2009; Blannin et al. 1998).

3.10 Absorbance Measurement

For both The FineTest® Human sIgA (Secretory IgA) ELISA Kit and the 'in house' ELISA procedure modified from Leicht et al. 2011, A BMG LABTECH FLUOstar OPTIMA Reader with its companion OPTIMA Control program was used to measure the absorbance of the ELISA assay. Plates were read at 450 nm with background correction at 610 nm. The OPTIMA software was then used to export the data into an Excel Version 2304 spreadsheet where a standard curve would be manually created for each assay. Excel Version 2304 was also utilized to create the graphs representing the means and standard deviation for absolute salivary SIgA concentration, salivary osmolality, and salivary SIgA normalised with osmolality.

For samples analysed using the modified ELISA protocol, the absorbance determined at 610 nm was subtracted from the absorbance measured at 450 nm for each well. The average of each standard solution duplicate from the first two columns were calculated and graphed against the known concentration of the Sigma-Adrich I2636 IgA from human colostrum sample within the serial dilutions. A polynomial trendline with an order of four was added to the graph along with the graph's equation and R^2 value. The R^2 value was used solely to determine if the polynomial trendline created the best fit for the standard curve. The absorbance values were then entered into the graph's equation to ensure the correct concentration of Sigma-Adrich I2636 IgA from human colostrum was unaltered during the ELISA.

Once the standard curve was created, the average of the saliva sample duplicates was calculated. This mean was then entered into the standard curve's equation and then multiplied by the dilution factor of 900 and then divided by 1000 to achieve the saliva sample's absolute concentration. The absolute concentration was then corrected by dividing it by its corresponding osmolality value. This was done manually within the same excel spreadsheet where the standard curve was created. Absolute salivary SIgA concentration is expressed as mg/L and salivary SIgA concentration relative to osmolality is expressed as mL/mOsmol.

For samples analysed using The FineTest® Human sIgA (Secretory IgA) ELISA Kit, the absorbance determined at 610 nm was subtracted from the absorbance measured at 450 nm for each well. The average of the 'BLANK' duplicate would then be subsequently subtracted from the values corresponding to each well. From this point, salivary SIgA concentrations were determined using a similar protocol as the 'in house' modified ELISA procedure.

However, there were two distinct variations in calculation procedure. The standard curve was created against the known concentration of Human sIgA Standard Tube included in The FineTest® Human sIgA (Secretory IgA) ELISA Kit and values were instead multiplied by a dilution factor of 2500.

3.11 Genotyping Procedures

Capillary plasma samples were analysed for the CYP1A2 SNP rs762551. All sample analysis took place at the Chipperfield Building at the University of Kent, Canterbury. The Zymo Research *Quick*-DNA Miniprep Kit was used to extract DNA from the plasma samples and the Roche LightCycler® 96 Instrument was used to analyse ELISA absorbance. Reagents used during DNA extraction were all taken from The Zymo Research *Quick*-DNA Miniprep Kit. All eleven participants were genotyped at the same time. Aurelia Sonic Nitrile Powder-Free Examination Gloves (Non-Sterile) and MSAFE Model 5057 Blue Disposable Medical Masks were worn when handling the capillary samples or reagents.

Capillary samples were allowed to thaw on the lab countertop while all lab equipment utilized for the DNA extraction was exposed to UV light (253 nm) for at least 15 minutes in a Grant Bio Life Science Benchtop PCV Ultra Violet Cabinet in order to create a sterile environment and destroy any potential contaminating DNA or DNases. The following procedures took place inside the same Grant Bio Life Science Benchtop PCV Ultra Violet Cabinet to prevent the possibility of cross contamination. Only the cabinet's fluorescent light was on during sample preparation. 200 µL of Genomic Lysis buffer was added to 50 µL of the capillary sample with a Fisherbrand Single Channel 20-200 µL Pipette and Fisherbrand™ SureOne™ Aerosol Barrier Pipette Tips. This pipette and micropipette combination was used throughout the DNA extraction process. The solution was vortexed manually for approximately 5 seconds in a Velp Scientifica Vortex at 10 Hz. The solution then sat in the covered Grant Bio Life Science Benchtop PCV Ultra Violet Cabinet for 5 minutes at room temperature.

The entirety of the solution was transferred to a Zymo-Spin II CR Column in a collection tube and was centrifuged in a Sigma Centrifuge 1-14 CW 24place Rotor at 1669 relative centrifugal force (RCF) for 1 minute. The collection tube was discarded and the Zymo-Spin II CR Column was placed inside a new collection tube. 200 µL of DNA Pre-wash Buffer was added to the spin column and centrifuged in a Sigma Centrifuge 1-14 CW 24place Rotor at 1669 RCF for 1 minute. 500 µL of g-DNA Wash Buffer was added to the Zymo-Spin II CR

Column before the column was transferred to a 1.5 mL Fisherbrand Premium Microcentrifuge Tube (Natural). 50 µL of DNA Elution Buffer was added to the Zymo-Spin II CR Column. The column was then incubated for 5 minutes at room temperature. Following the incubation period, the Zymo-Spin II CR Column was centrifuged at the highest speed possible on the Sigma Centrifuge 1-14 CW 24place Rotor for 30 seconds in order to elute the DNA. rhAmp™ SNP Genotyping reagent mixes were utilized in order to prepare the eluted DNA to be analysed with PCR in accordance with manufacturer instructions. rhAMP Genotyping Master Mix and rhAmp Reporter Mix were combined in a 20:1 volume ratio in a 1.5 mL Eppendorf tube. A SNP genotyping reaction mix was then created by combining 5.3 µL of the rhAMP Genotyping Master Mix and rhAmp Reporter Mix with 2.2 µL of nuclease free water and 0.5 µL of the rhAmp SNP Assay for every 10 µL of liquid sample. 8 µL of the SNP genotyping reaction mix was added to each appropriate Roche® LightCycler® PCR assay wells along with 2 µL of the liquid sample. The Roche® LightCycler® PCR assay was sealed and placed into the Roche LightCycler® 96 Instrument for absorbance measurement. Thermal cycles were set up as follows: enzyme activation for 1 cycle at 95°C for 10 minutes, denaturation, annealing, and extension at 95°C for 10 minutes, 60°C for 30 minutes, and 68°C for 20 minutes respectively for 40 cycles. An allelic determination plate read was done with FAM assigned to the reference allele (C) and VIC™ assigned to the alternative allele (A). The LightCycler® 96, Roche Diagnostics software was used to determine participant genotype. Participants who had requested so during their laboratory visits were given their CYP1A2 SNP rs762551 genotype.

3.12 Statistical Analysis

Summarized quantitative data is presented with the mean value and standard deviation. Saliva osmolality, absolute salivary SIgA concentration, and salivary SIgA concentration relative to saliva osmolality were statistically analysed utilizing IBM SPSS Statistics Version 27.0 (Armonk, NY: IBM Corp). Data was first checked for normal distribution prior to analysis using Shapiro-Wilk statistics and Z-scores for skewness and kurtosis. If the data was not normally distributed, values were transformed using natural log. These values were then used during statistical analysis instead of the raw values. Both absolute salivary SIgA concentration and salivary SIgA concentration normalised with osmolality were transformed with natural log. Raw data was utilized when analysing osmolality. Data was analysed using a mixed measures for an analysis of variance (ANOVA) for three different conditions measured across four time point measurements. Data was also analysed using a mixed

measures ANOVA for three different conditions measured across four time points for two genotype groups. Mauchly's Test of Sphericity was checked for significance across subject effects. Appropriate adjustments were made using the Greenhouse-Geisser method where required. Statistical significance was only assigned if $p < 0.05$. Significant main effects were followed up with post hoc paired t-tests with a 95% Confidence Interval and Least Significant Difference for multiple comparisons where necessary. Bonferroni corrections were applied manually where necessary.

Percent coefficient variability was performed intra and inter assay to determine variability among samples. Excel Version 2304 was utilized for the calculations. The statistical procedure for intra-assay variability was the same for all assay plates analysed. The standard deviation of duplicate values for each sample was divided by the average of the duplicate values for each sample. This value was then multiplied by 100 to achieve the percent coefficient variability between the two duplicates. The average percent coefficient variability for all sample duplicates were taken to achieve intra-assay percent coefficient variability. Inter-assay percent coefficient variability was achieved by dividing the summation of all intra-assay percent coefficient variabilities for all assay plates analysed by the total number of assay plates. See Appendix K for percent coefficient variability values.

Chapter IV: Results

4.1 CYP1A2 rs762551 SNP Genotype of Participants

All elven participants were genotyped for the rs762551 CYP1A2 SNP. Capillary samples collected during the familiarization visit all produced conclusive results. The divide between allele type among participants was roughly even, with approximately 55% of participants carrying the homozygous AA allele and 45% of participants being heterozygous AC. The homozygous CC genotype was not present within this study.

Participant ID	CYP1A2 rs762551 SNP Genotype
1	AA
2	AC
3	AA
4	AC
5	AA
6	AA
7	AC
8	AA
9	AA
10	AC
11	AC

Table 5. CYP1A2 Genotype of individual participants; AA homozygous indicates fast a metabolizer of caffeine and C allele carriers indicates slow a metabolizer of caffeine

4.2 Salivary SIgA Concentration, Osmolality, and Salivary SIgA Relative to Osmolality

Absolute salivary SIgA concentration data is included as it represents the working concentrations available for immune function and defence in vivo (Bishop & Gleeson, 2009). There was no significant effect of condition ($p = 0.565$) and no significant condition x timepoint interaction ($p = 0.306$). However, a main effect of time was observed. Post hoc analysis revealed that absolute salivary SIgA concentration was significantly higher post-exercise compared to initial ($p = 0.027$) and pre-exercise levels ($p = 0.003$). Salivary SIgA concentration at 30-minutes post-exercise not different from initial levels ($p = 0.138$, see figure 6).

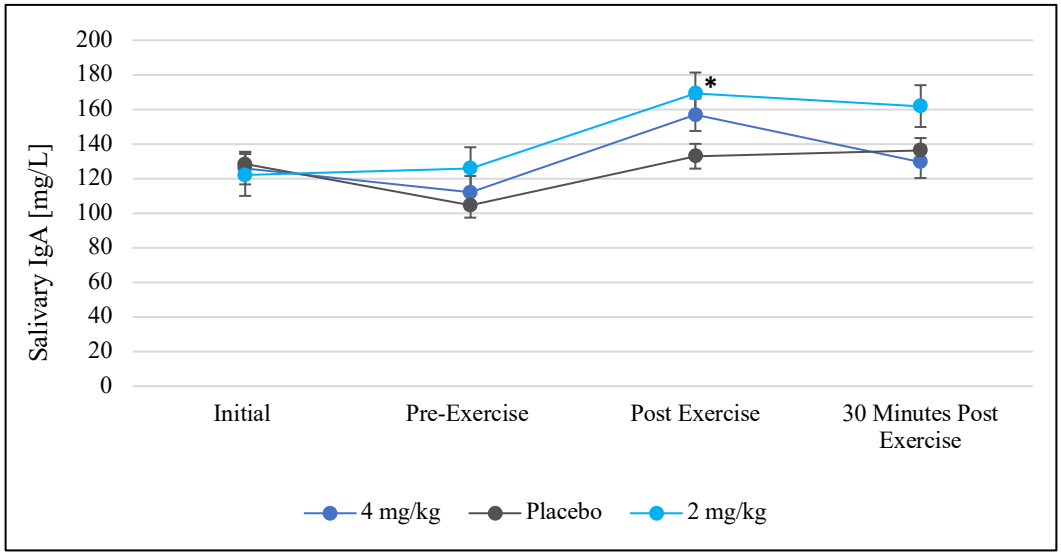

Figure 6. Absolute Salivary SIgA concentration across four measured time points. Data is presented as means with standard error bars. *p < 0.05 for Bonferroni Corrected

For osmolality, there was no significant main effect for condition (p = 0.85) or condition x time point interaction (p = 0.759). However, significant main effect of time was observed. Post hoc analysis revealed saliva osmolality to be significantly higher post-exercise compared to initial levels, pre-exercise and 30 minutes post-exercise (p < 0.01 for all).

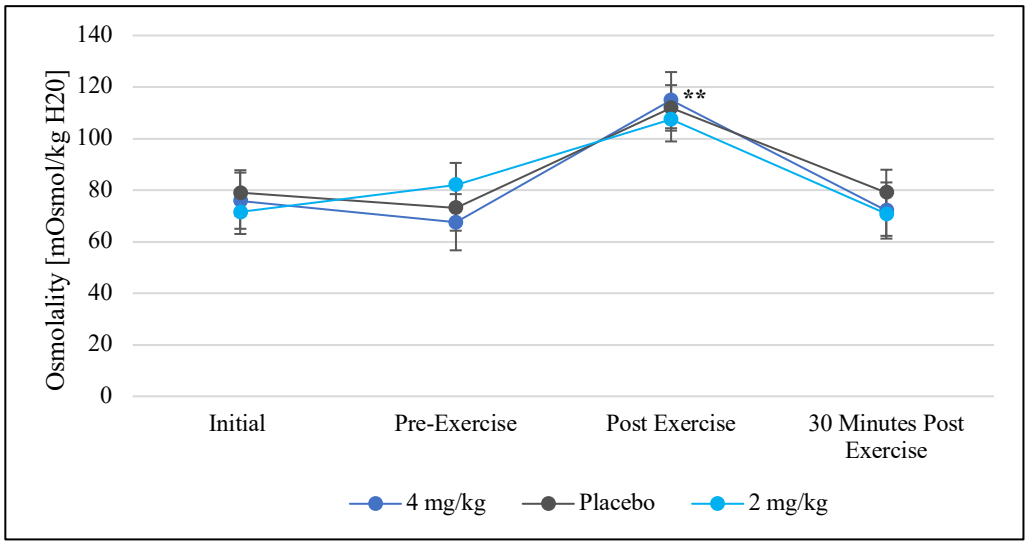

Figure 7. Change in saliva osmolality over the four measured time points. Values are presented as means with standard error bars. **p < 0.01

For salivary SIgA concentration relative to osmolality, there was no significant effect of condition (p = 0.403) and no significant condition x time point interaction (p= 0.481). However, there was a main effect for time between saliva collection points. Post hoc analysis

showed that salivary SIgA concentration relative to osmolality post-exercise was significantly different than 30 minutes post-exercise (p = 0.006).

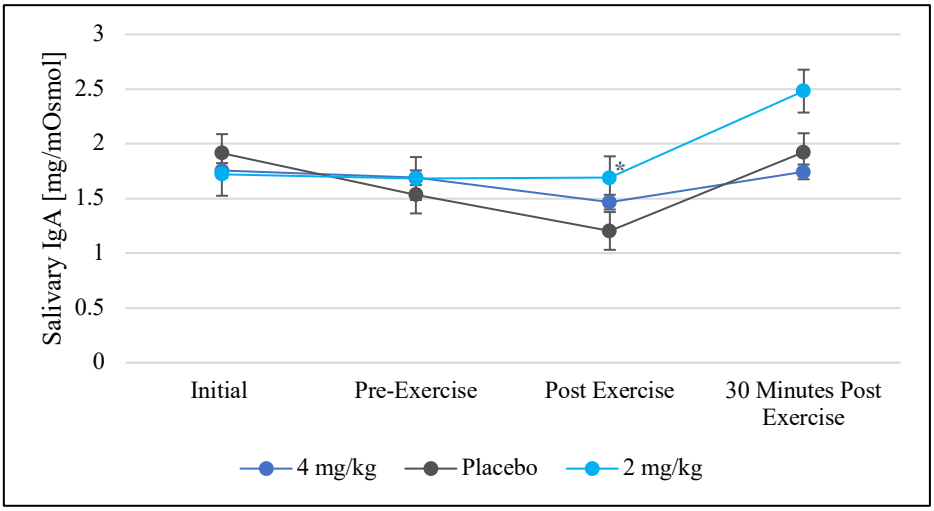

Figure 8. Concentration of salivary SIgA relative to osmolality. Data is presented as participant means with standard error bars. *p < 0.05

4.3 CYP1A2 rs762551 SNP, Salivary SIgA Concentration, and Caffeine Ingestion

For absolute salivary SIgA concentration, there was no significant main effect for condition (p = 0.605), no significant condition x genotype interaction (p = 0.665), no significant timepoint x genotype interaction (p = 0.585), no significant condition x timepoint interaction (p = 0.352), and no significant condition x timepoint x genotype interaction (p =0.36).

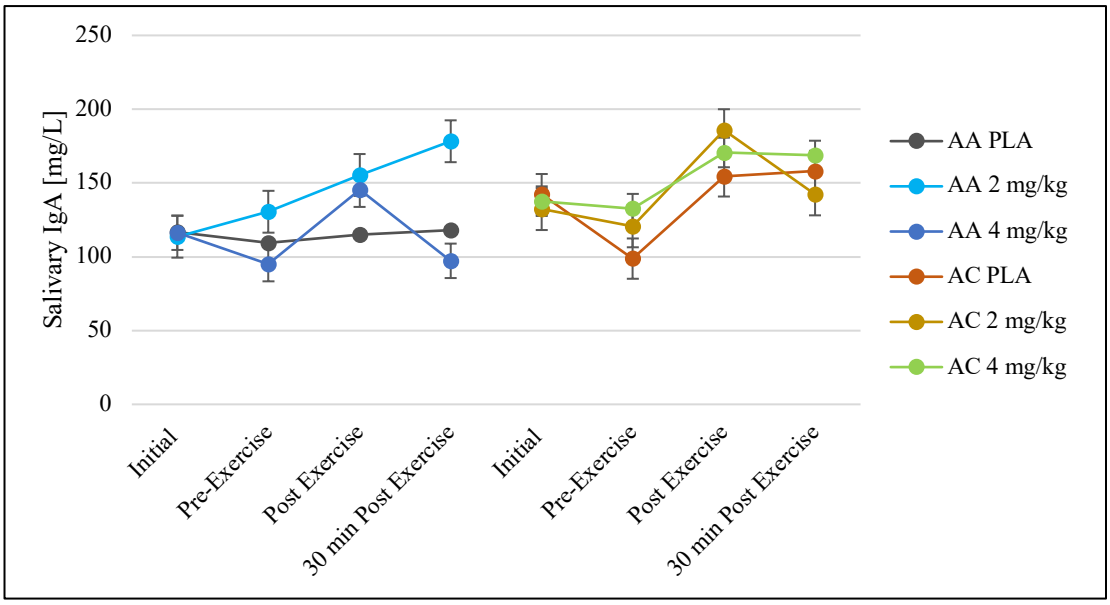

Figure 9. Absolute salivary SIgA concentration compared between homozygous AA participants and heterozygous AC individuals across four measured time points in three different conditions. 2m/kg = 2 mg/kg body mass dose caffeine, 4 mg/kg = 4 mg/kg body mass dose caffeine, PLA = placebo. Values are expressed as means with standard error bars.

For salivary SIgA relative to osmolality, there was no significant main effect for condition ($p = 0.421$), no significant condition x genotype interaction ($p = 0.526$), no significant timepoint x genotype interaction ($p = 0.196$), no significant condition x timepoint interaction ($p = 0.577$), and no significant condition x timepoint x genotype interaction ($p = 0.648$).

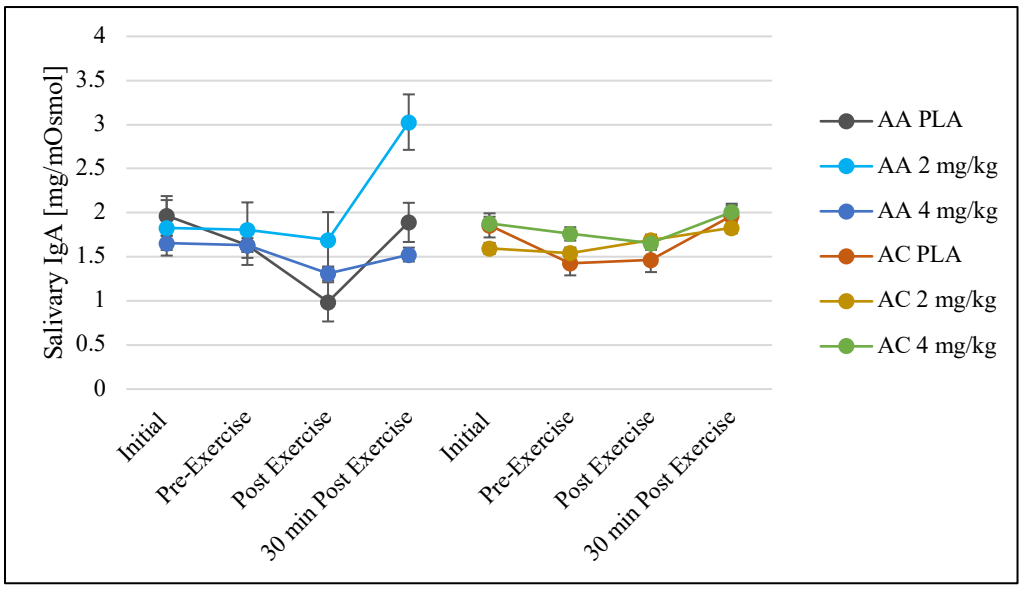

Figure 10. Salivary SIgA (relative with osmolality) concentration compared between homozygous AA participants and heterozygous AC individuals across four measured time points in three different conditions. 2m/kg = 2 mg/kg body mass dose caffeine, 4 mg/kg = 4 mg/kg body mass dose caffeine, PLA = placebo. Values are expressed as means with standard error bars.

4.4 Participant Caffeine Consumption

Rate of caffeine consumption was determined by a Caffeine Frequency Questionnaire. The questionnaire covered a wide range of caffeine sources including various types of coffee, tea, energy drinks, soft drinks, chocolate, and miscellaneous sources which consisted of supplements or medications containing caffeine. A comprehensive list of the specific type of caffeine sources included in the questionnaire can be found in Appendix E.

Participants were sorted into three different categories based on how often they consumed caffeine from any source. Infrequent users consumed caffeine either never, less than once per month, or 1-3 times per month. Moderate users consumed caffeine at a rate of once per week, 2-4 times per week, or 5-6 times per week. Habitual users of consumed caffeine once per day, 2-3 times per day, 4-5 times per day, or more than 6 times per day. Only one participant reported being an infrequent user of caffeine while the remainder of participants were evenly split between moderate (5) and habitual (5) users. Average daily caffeine intake for participants was calculated to be approximately 130.4 ± 140.4 mg/day using caffeine content values from Mitchell et al. (2015).

Tea and coffee were the most common sources of caffeine for participants; eight participants reported either a moderate or habitual intake of caffeinated tea and seven participants reported either a moderate or habitual intake of coffee. Very few participants within this study consumed energy drinks, soft drinks, chocolate, or miscellaneous sources of caffeine regularly with nine, nine, eight, and ten participants respectively reporting that they either never consumed these sources or consumed them at a low frequency.

4.5 Competitive State Anxiety Inventory-2 Questionnaire Results

The Competitive State Inventory-2 Questionnaire was primarily used as a precautionary measure to ensure participants were mentally ready to perform the required exercise. Previous studies have shown that pre-exercise anxiety can have a significant effect on the immune response to exercise (Edwards et al. 2018). Results of the questionnaire were collected prior to each experimental visit to ensure that scores were roughly the same before each trial for each participant. The questionnaire assessed cognitive anxiety, somatic anxiety, and self-confidence. The scores for each category range from 9 to 36, with a low score indicating low somatic or cognitive anxiety and a high score indicating high cognitive or somatic anxiety. The scoring model is applied in reverse for the self-confidence category. There was no significant difference in scores between visits for any category (Appendix K).

Please refer to the table below for information regarding participant's scores for all three categories across the three experimental visits.

Experimental Visit Number	Cognitive State Anxiety Score (Mean ± SD)
1	12.54 ± 3.60
2	11.36 ± 2.77
3	10.64 ± 2.35
Experimental Visit Number	Somatic State Anxiety Score (Mean ± SD)
1	11.18 ± 0.83
2	11.27 ± 1.86
3	10.45 ± 0.66
Experimental Visit Number	Self-Confidence Score (Mean ± SD)
1	25.27 ± 3.57
2	26.72 ± 6.15
3	28.90 ± 5.10

Table 6. Scores from the Competitive State Anxiety Inventory-2 Questionnaire given across all three experimental visits.

4.6 Groningen Sleep Quality Scale Questionnaire Results

The Groningen Sleep Quality Scale was used as an additional measure to ensure participants were well-rested enough in order to complete the required exercise. Previous studies have shown that poor sleep quality can negatively impact the immune system (Prather et al. 2015). Results of the questionnaire were collected prior to each experimental visit to ensure that scores were roughly the same before each trial for each participant. A maximum score of 14 indicated a substandard level of sleep the night before and a score of 0 indicated good quality of sleep the night before. On average, participant sleep quality scores remained consistent across all three experimental visits. There was no significant difference in sleep scores between visits (Appendix K). Please refer to the table below for these sleep scores.

Experimental Visit Number	Groningen Sleep Quality Score (Mean ± SD)
1	2.82 ± 3.40
2	4.18 ± 3.40
3	3.64 ± 3.23

Table 7. Groningen Sleep Quality scores presented as Mean ± SD for all participants across the three experimental visits.

4.7 Results of the Blinding Questionnaire

A blinding questionnaire was included in this study to gain the volunteer's subjective perspective as to which treatment they think they were given during each experimental visit.

Due to treatments being randomized and double-blind, results are represented as participants' guess as to which treatment corresponded to which colour within the double-blind colour code. A large number of participants believed the placebo, high dose of caffeine, and low dose of caffeine to correspond to the blue, yellow, and red coded treatments respectively. Success of the blinding questionnaire is discussed in a later section.

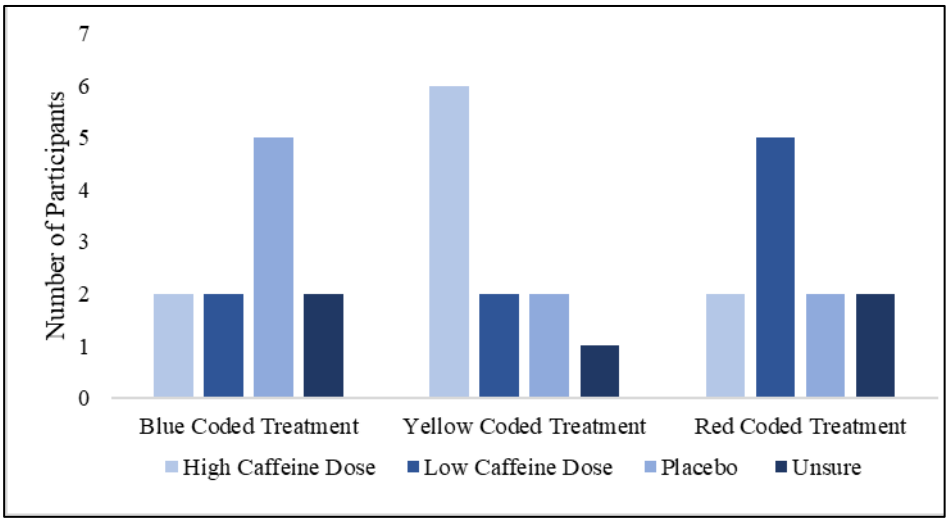

Figure 11. Number of participants that believed a certain treatment to correspond to a certain treatment colour code. Study remained double blind until after analysis was complete.

Chapter V: Discussion

5.1 Caffeine, Salivary SIgA, and the CYP1A2 SNP rs762551

While there was a significant main effect of time for both absolute salivary SIgA and salivary SIgA relative to osmolality, the primary results of the present study indicate that the CYP1A2 rs762551 SNP has no significant influence over the salivary SIgA concentration during caffeine supplemented exercise (Figure 9, Figure 10). Salivary SIgA concentration in both the homozygous AA allele and C allele carriers experienced a main effect of time, but was not influenced by any other independent variable. Additionally, neither the high caffeine (4 mg/kg body mass) or low caffeine (2 mg/kg body mass) dose significantly affected salivary SIgA concentration pre-exercise, post-exercise or 30-minutes post-exercise (Figure 6, Figure 8).

Although caffeine is a known adenosine receptor antagonist and can potentially reduce antibody production and lymphocyte circulation in the absence of exercise, previous studies have shown that certain adenosine receptor carrying immune cells may improve with caffeine supplemented exercise (Bassini-Cameron et al. 2007; Fletcher & Bishop 2011; Graham, 2001; Horrigan et al. 2006; Shirvani et al. 2020; Walker et al. 2006). A recent study has even shown that caffeine supplemented exercise may improve IL-4 and IL-10 levels, both of which have important anti-inflammatory and immunoregulator properties (Rahimi & Beaven, 2023). However, given the number of physiological systems caffeine, and therefore the CYP1A2 gene, influences both inside and outside of the immune system (central nervous system, digestive system, respiratory system, urinary tract, etc.) (Rodak et al. 2021), it is possible that caffeine does not interact with the adenosine receptors of plasma cells enough to elicit a caffeine-induced change in salivary SIgA in response to exercise. Bishop et al. (2006) conducted an initial pilot study where they found caffeine had no impact on salivary SIgA during rest, suggesting adenosine receptors on plasma cells may not interact with caffeine to the extent other cells might. However, additional research would be required in order to test this assumption.

Furthermore, caffeine supplemented exercise might not have had an influence over salivary SIgA concentration because of the possibility that its effects were masked by an exercise induced increase in catecholamines. Generally, the concentration of circulating catecholamines increase as exercise intensity increases (Allgrove et al. 2008; McMurray et al. 1987). But when high doses of caffeine (above 4 mg/kg of body mass) are utilized during

exercise, previous studies have shown that catecholamine levels will be significantly higher than non-caffeine supplemented exercise (Jackman et al. 1985; Anderson & Hickey, 1994). The caffeine doses used within this study might not have been large enough to significantly alter the levels of catecholamines during exercise, but the expected increase in catecholamines brought on by exercise might have been large enough to mask the effect of caffeine.

While the present study demonstrates that caffeine and the CYP1A2 rs762551 SNP do not play a role in exercise-induced changes in salivary SIgA, they might influence other aspects of the immune system. The potential impact of the CYP1A2 gene should especially be considered when analysing immune markers that have consistently responded to caffeine supplemented exercise (see Table 2).

5.2 Comparisons to Previous Literature

In regards to previous literature, and to the best of my knowledge, there have only been two other studies investigating the interaction between caffeine supplemented exercise and salivary SIgA. One of these studies, published by Dulson et al. (2019) is partially supported by the results of the present study. Dulson et al. (2019) examined the possible effect that a wide range of caffeine doses (2 mg/kg, 4 mg/kg, 6 mg/kg, and 8 mg/kg) might have on salivary SIgA and, similar to this study, found no significant difference between doses and the placebo. Unlike this study, Dulson et al. (2019) found no significant main effect for time even though such an effect has been previously observed (Gonzalo et al. 2019; Tharp & Barnes 1990). Researchers speculated that the ‘surprising’ lack of change in salivary SIgA levels could possibly be attributed to a stimulus sufficient enough to activate both immunoinhibitory and immunostimulatory pathways, thus producing counteractive effects on salivary SIgA levels. Dulson et al. (2019) also hypothesized that an insufficient stimulus could have prevented a change in salivary SIgA levels. However, it is difficult to confirm either theory with the current data.

The second of these studies, published by Bishop et al. (2006), is not supported by the results of the present study. Bishop et al. (2006) found that caffeine supplementation increased salivary SIgA concentration mid and post exercise. Researchers speculated that this increase in salivary SIgA concentration could be attributed to an increase in adrenergic stimuli and an increase in the response of epinephrine. While it can be argued that the smaller caffeine doses utilized during this study (2 mg/kg of body mass and 4 mg/kg of body mass) might not have

been large enough to induce a strong autonomic response in order to influence salivary SIgA concentration, Dulson et al. (2019) utilized an exercise with a similar intensity as Bishop et al (2006) and still found caffeine had no influence on salivary SIgA concentration. Future renditions of the present study could include measurements of alpha-amylase, a marker of epinephrine activity, in order to further investigate a potential correlation between the adrenergic response and salivary SIgA as present results are indeterminate.

Within the present study, salivary SIgA concentration was found to have returned to initial levels 30-minutes post-exercise. These observations are supported by a systematic review by Drummond et al. (2022). Data pooled from nine trials (mean effect size of 0.16) showed acute bouts of exercise to have a non-significant effect on the salivary SIgA levels of untrained participants. The lack of significant change in the initial concentration of salivary SIgA across the three experimental trials along with the recreationally active fitness profile of the participants involved within this study are consistent with the patterns observed by Drummond et al. (2022).

Unsurprisingly, there have been frequent inconsistencies in previous literature concerning the levels of salivary SIgA observed in the period of time following an acute bout of high intensity exercise (Bishop & Gleeson, 2009). Variation in methodology for analysing salivary SIgA data may be a driving factor in these inconsistencies (Bishop & Gleeson, 2009; Lindsay & Costello, 2017). This also makes it complicated to make direct comparisons between some studies (Bishop & Gleeson, 2009). For instance, participants in both the present study and Bishop et al. (2006) were relatively fit but VO_2 max means and standard deviation differed between studies: 52.2 ± 10.9 mL/kg/min (mean ± standard deviation) and 61.6 ± 1.4 ml/kg/min, (mean ± standard deviation) for the present study and Bishop et al. (2006) respectively. Bishop et al. (2006) also tested much younger participants (age: 23 ± 1 year, mean ± standard deviation) compared to the current study (32 ± 9.2 years, mean ± standard deviation) and utilized a different form of acute exercise (90-minute cycling at 70% VO_2 max). This distinction in exercise duration may be the most significant differentiating factor between the present study and Bishop et al. (2006). While the exercise implemented during the present study falls within the nadir of the J-shaped curve, the exercise utilized by Bishop et al. (2006) falls further to the right under strenuous exercise. Because the present study and Bishop et al. (2006) investigate very different exercise bouts, there are likely different mechanisms by which exercise influences salivary SIgA within each study. There were similar differences in participant characteristics and experimental procedures between the

study completed by Dulson et al. (2019) and the present study. Regardless of methodological differences, because of the similar conclusions made by this study and Dulson et al. (2019), there is slightly stronger evidence to suggest that caffeine supplemented exercise does not influence salivary SIgA concentrations. However, further research would be required in order to further prove any lack of correlation.

5.3 Potential Mechanisms for Exercise-Induced Changes in Salivary SIgA

As previously mentioned, a main effect of time was observed for both absolute salivary SIgA concentration and salivary SIgA concentration relative to osmolality. While the mechanisms behind exercise-induced changes in salivary SIgA are still not well-known, there are a few physiological systems impacted by exercise that might influence this change.

While previous literature has shown that salivary SIgA tends to significantly decrease post-exercise compared to initial levels, the present study found that the concentration of salivary SIgA relative to osmolality post-exercise was only significantly lower than the concentration 30 minutes post-exercise. It has been suggested that a significant decrease in salivary SIgA post-exercise can be partially attributed to the stimulation of the autonomous nervous system (Trochimiak & Hübner-Woźniak, 2012). Similar to Dulson et al. (2019), it is possible that an insignificant stimulus within this study caused the difference in the concentration of salivary SIgA relative to osmolality between initial and post-exercise time points to approach significance, but not achieve it. However, the significant increase in salivary SIgA relative to osmolality 30 minutes post-exercise could indicate that the mechanisms that promote a return to homeostasis were notably active following exercise.

In order to begin assessing these possible mechanisms, it would be beneficial to evaluate the potential causes of an exercise induced decrease in salivary IgA levels. There may be a likelihood that the two pathways are related and investigating potential theories could help explain patterns observed by previous literature. One possibility behind an exercise-induced decrease in salivary SIgA concentration relative to osmolality could be a temporary inhibition of local salivary SIgA production due to the mobilization of immune cell subpopulations in the blood (Pedersen & Toft, 2000). Eccentric contractions, or a lengthening contraction that occurs when the force applied to the muscle exceeds the force produced by the muscle, play a critical role in muscle action (Bijker et al. 2002; Hody et al. 2019). Some sarcomeres may stretch beyond the myofilament overlap during eccentric contraction and thus become disrupted during muscle relaxation (Brockett et al. 2001). The number of disrupted

sarcomeres grow until membrane damage to the muscle or a 'micro-tear' occurs (Armstrong, et al. 1983; Armstrong, 1990; Brockett et al. 2001). Signalling molecules released by the muscle will then recruit immune cells to the damaged site (Tidball, 2017). B cells responsible for producing salivary SIgA could be amongst the immune cells called to repair the sarcomere membrane damage (Turner et al. 2016).

The behaviour of plasma cells following acute exercise in a study by Turner et al. (2016) somewhat mirrors the pattern of salivary SIgA relative to osmolality observed within the present study. Plasma cells significantly increased post-exercise and returned to baseline levels +30 minutes post exercise. Triplett-McBride et al. (1998) also found that antibody producing B cells increased in circulation in fit participants following acute exercise. Given the opposing behaviour of circulating B cells in the aforementioned studies and salivary SIgA concentration relative to osmolality in this study, it is possible that "micro-tears" could be responsible for exercise-induced changes in salivary SIgA. If this theory holds true, then the mode of exercise implemented could provide an additional explanation for different outcomes between studies. Certain exercises may contribute to more "micro-tears" than others, thus influencing salivary SIgA concentration. However, B cell levels nor physiological markers of muscle damage were analysed during the present study, thus making this correlation purely theoretical. To the best of my knowledge, no literature currently exists investigating as to whether there is a cause-effect relationship between "micro-tears," and exercise-induced changes in salivary SIgA.

Glucocorticoids can also influence the redistribution of polymeric IgA, thus indirectly decreasing salivary SIgA levels (He et al. 2010; Wira & Rossoll, 1991). Previous studies have shown that following in vivo administration of synthetic glucocorticoids, polymeric IgA levels in serum increased while salivary SIgA levels were notably inhibited (Wira and Rossol, 1991). Glucocorticoids may also contribute to diminished B cell responsiveness, which would directly affect salivary SIgA production (Saxon, et al. 1978). Cortisol, the main type of glucocorticoid, can increase due to stimulation of the hypothalamic-pituitary-adrenal axis and contribute to a post-exercise decrease in salivary SIgA (Thau, et al. 2022; Hill et al. 2008; Usui et al. 2011). A correlation between cortisol and salivary SIgA has been previously observed by Naughton et al. (2006). Salivary SIgA and cortisol had significantly decreased and increased respectively in response to a 90-minute cycle ergometer trial. However, research investigating the mechanism between salivary SIgA and cortisol is currently minimal and inconclusive (Rutherfurd-Markwick, et al. 2022). Previous literature has shown

either a positive correlation or no relation between salivary SIgA and cortisol under exercise conditions (Cieslak, et al. 2003; Leicht, et al. 2018). He et al. (2010) speculated that these differences may be caused by either the intensity or duration of the exercise or both. Cortisol levels were not measured within the present study and thus the influence of cortisol is conjectural.

Any change in salivary SIgA concentration relative to osmolality within this study is not likely to contribute to the development of an URTI. Prior research has demonstrated that a 40% or more post-exercise decrease from initial salivary SIgA concentrations is a relatively reliable indication that a URTI risk is increased (Perkins & Davison 2022; Neville et al. 2008). The average concentration of salivary SIgA relative to osmolality in this study did not decrease below 40% of the initial concentration thus making the risk of an URTI relatively low.

5.4 Blinding Questionnaire

The double blinding and randomization procedure employed within this study was successful. Across each condition, only 45% of participants correctly guessed the treatment they received. Out of the eleven participants involved, six correctly guessed the dosage of only one condition and two were unable to guess the dosage of any condition. The three other participants correctly guessed the dosage across all three experimental conditions.

5.5 Limitations

As it was not the main focus of the study, performance measures were not taken during experimental laboratory visits. Relative exercise intensity was standardized among participants based on individual VO_2 max as determined during the initial study visit. Taking performance measures might have compromised the success of blinding (i.e., if participants perceived their performance and 'better' or easier) and since the ergogenic effects of caffeine on physiology and performance have been well documented, so performance and perceptual measures were not included to minimise the risk to blinding and any potential influence on the primary outcomes. Although collecting physiological measures such as heart rate and perceptual measures such as RPE may provide valuable information in certain instances, they did not contribute the primary investigation of this study and as such were not included.

Previous literature exploring the response of salivary SIgA to caffeine supplemented exercise primarily utilized trained endurance athletes. However, the present study largely recruited

recreationally active healthy adults. While this may make comparisons difficult, this study further supports conclusions established by previous research by replicating results in a different population. However, it is premature to determine whether salivary SIgA concentrations in either trained or recreationally active athletes are not influenced by caffeine supplemented exercise.

Saliva caffeine concentration was not taken into consideration during this study because participants had been genotyped for CYP1A2, the gene largely in control of caffeine metabolism. Because participants' genotype, and therefore the relative speed at which they metabolized caffeine, would be revealed post-analysis, it was decided that saliva caffeine concentration would not be taken into consideration. Furthermore, the kinetics behind caffeine's appearance in circulation has been well established. The rate at which caffeine appears in saliva reflects caffeine absorption more than caffeine metabolism. As such, it would not contribute to the primary research question. In order to determine a more accurate rate of caffeine metabolism, an analysis of caffeine's metabolites would have been required, but was not feasible for the current study.

Participants were asked to keep a detailed food diary of their diet 24 hours before each visit to the lab. This measure was to ensure individual diets were kept relatively consistent prior to each visit, but did not serve as a disqualifying measure in the Pre-Test Health Questionnaire. General information regarding participant food diary entries from ten participants is included in Appendix A. The remaining participant failed to submit their food diary to researchers following the completion of their fourth visit.

While the phase of the menstrual cycle was originally intended to be analysed as a covariate , this measure was unable to be effectively included. Each female participant was in a different phase of the menstrual cycle for each analogous visit, which complicated the possibility of including the phase of the menstrual cycle as a covariate.

Competitive State Anxiety Inventory-2 questionnaires and Groningen Sleep Quality questionnaires were only included at the beginning of each experimental visit in order to ensure participants were well rested and mentally ready to perform the exercise. Although participant scores appeared to indicate that all participants were relatively rested and mentally ready to exercise, there was not a set score for either of the two questionnaires that would disqualify a participant from exercise. The only true disqualifiers during experimental visits were those taken into account by the Pre-Test Health Questionnaire. Participant scores for the

Competitive State Anxiety Inventory-2 and Groningen Sleep Quality Questionnaires were not significantly different across visits.

Most samples were analysed by an in-house ELISA that was prepared from a modified procedure by Leicht et al. (2011). However due to a shortage of supplies, samples from 3 participants were analysed using The FineTest® ELISA Kit. All samples for any given subject were measured with the same method. Although two different methods were employed for saliva sample analysis, the percentage of coefficient variability inter-assay and intra-assay was less than 7% and 12% respectively across all assays (Appendix K). This suggests both The FineTest® Human sIgA (Secretory IgA) ELISA Kit and the modified in-house ELISA procedure produced reliable results. However, utilizing one analysis method for all participants could have produced a lower variability percentage and reduced overall time spent analysing saliva samples. Both ELISA procedures were specific only to secretory IgA. Kits and or procedures that could test for any IgA type might have introduced a source of variability and were therefore not utilized.

5.6 Conclusion

The CYP1A2 gene and caffeine supplementation in 2 and 4 mg/kg of body mass doses does not appear to influence salivary SIgA concentration during exercise. No significant difference could be established between AA and AC genotype groups. However, a main effect of time was observed in both absolute salivary SIgA concentration and salivary SIgA concentration relative to osmolality. Further investigation into a possible correlation between salivary SIgA, glucocorticoid, or B cell levels during exercise may provide a more complete understanding of underlying mechanisms behind exercise induced changes in salivary SIgA.

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Appendix A: Summation of Participant Food Diary Entries

Table 8. Food groups consumed by each participant 24 hours prior to the familiarization visit (DAY 1), Experimental Visit 1 (DAY 2), Experimental Visit 2 (DAY 3), and Experimental Visit 3 (DAY 4). FR = Fruits, V = Vegetables, S = Starchy Foods, P = Protein, FA = Fats. No available data for Participant 9.

Participant ID	DAY 1	DAY 2	DAY 3	DAY 4
1	S, P, FA, V	FA, S, P, FR, V	P, FA, FR, V	P, FA, FR, S, V
2	S, FR, P, V, FA	FA, FR, S, P, V	S, FR, P, V, FA	S, FR, P, V, FA
3	FR, P, V, S, FA	S, FA, FR, P, V	FA, FR, S, P, V	P, V, FA, FR, S
4	FA, P, V, S, FR	V, FR, P, S, FA	S, FA, FR, V, P	S, FA, P, FR, V
5	M, FR, S, FA	FA, S, P, V, FR	FA, S, FR	FA, S, FR, P, V
6	FA, P, FR, S, V	V, FA, S, FR	S, FA, P, V, FR	FA, S, P, V
7	P, S,V, FR	P, V, S, FR	S, FR, P, V	S, P, FR
8	S, P	S, FA, P	P, S	FA, P, FR, S
9	n/a	n/a	n/a	n/a
10	S, P, FA, V, P	S, FA, FR, P, V	S, FA, FR, V, P	S, FA, FR, P
11	V, FR, FA, S, P	P, V, S	FR, V, FA, P, S	FR, FA, P, V, S

Appendix B: Participant Consent Form

Project Title: Genetic influence of caffeine's ergogenic effects on the immune system

Name of Investigators: Lauren "Ren" D'Empaire

Participant Identification Number for this Project:

Initial Box

1. I confirm that I have read and understand the Information sheet (version ___, dated _____ 202__ for the study above. I have had the opportunity to consider the information, ask questions, and have had those questions answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving a reason. If I wish to withdraw, I will contact Lauren "Ren" D'Empaire via email (ld498@kent.ac.uk). ☐

3. I give permission for samples to be used for the purposes described for this study, and for members of the research team to have access to my anonymized data. ☐

4. I understand that I must read through all questionnaires carefully and answer the questions to the best of my ability, and that the researchers will use my answers during data analysis and to assess my suitability for participation. ☐

5. I agree to take part in the research project listed above. ☐

Name of Participant	Date	Signature
Name of person taking consent – to be signed and dated in presence of participant	Date	Signature
Lead researcher	Date	Signature

When completed, one copy is to given to participant, one for researcher's site file and original to be kept in main file

Appendix C: Health Questionnaire

HEALTH QUESTIONNAIRE

Participant ID……

Please answer these questions truthfully and completely. The sole purpose of this questionnaire is to ensure that you are in a fit and healthy state to complete the exercise test.

ANY INFORMATION CONTAINED HEREIN WILL BE TREATED AS CONFIDENTIAL.

SECTION 1: GENERAL HEALTH QUESTIONS

Please read the 12 questions below carefully and answer each one honestly: check YES or NO.

	YES	NO
1. Has your doctor ever said that you have a heart condition or high blood pressure?	□	□
2. Do you feel pain in your chest at rest, during your daily activities of living, or when you do physical activity?	□	□
3. Do you lose balance because of dizziness or have you lost consciousness in the last 12 months? (Please answer NO if your dizziness was associated with over-breathing including vigorous exercise).	□	□
4. Have you ever been diagnosed with another chronic medical condition (other than heart disease or high blood pressure)?	□	□
If yes, please list condition(s) here:		
5. Are you currently taking prescribed medications for a chronic medical condition?	□	□
If yes, please list condition(s) and medications here:		
6. Do you currently have (or have you had within the past 12 months) a bone, joint or soft tissue (muscle, ligament, or tendon) problem that could be made worse by exercise? Please answer NO if you had a problem in the past but it *does not limit your ability* to be physically active.	□	□
If yes, please list condition(s) here:		
7. Has your doctor ever said that you should only do medically supervised physical activity?	□	□
8. Have you had a viral infection in the last 2 weeks (cough, cold, sore throat, etc.)? If YES please provide details below:	□	□

	YES	**NO**
9. Do you have an allergy or intolerance to any foods or food components? If YES, please provide details here:	□	□
10. Have you ever had an adverse reaction to caffeine consumption?	□	□
11. Are you particularly sensitive to the effects of caffeine? If yes, please give further details here:	□	□
12. Have you ever been advised to avoid caffeine?	□	□
10. Please provide brief details of your current weekly levels of physical activity (sport, physical fitness or conditioning activities): Activity Duration (mins.) Monday Tuesday Wednesday Thursday Friday Saturday Sunday		
11. Are you a regular caffeine consumer? If yes, please provide details below of what you normally consume (this could be tea, coffee, energy drinks, chocolate/cocoa products, or any other caffeine-containing foods or drinks… please list all items), and how many you typically consume per day or per week:	□	□
12. Are there any reasons why you would not be able to consume caffeine? If YES, please provide details here:	□	□

You are approved to take part. Please sign the declaration on the consent form. You do not need to complete section 2.

If you answered YES to one or more of the questions in Section 1 - PLEASE GO TO SECTION 2.

SECTION 2: CHRONIC MEDICAL CONDITIONS

Please read the questions below carefully and answer each one honestly: check YES or NO.

		YES	NO
1.	**Do you have arthritis, osteoporosis, or back problems?** If YES answer questions 1a-1c. If NO go to Question 2.	□	□
1a.	Do you have difficulty controlling your condition with medications or other physician-prescribed therapies? (Answer NO if you are not currently taking any medications or other treatments).	□	□
1b.	Do you have joint problems causing pain, a recent fracture or fracture caused by osteoporosis or cancer, displaced vertebrae (e.g. spondylolisthesis), and/or spondyloysis/pars defect (a crack in the bony ring on the back of the spinal column)?	□	□
1c.	Have you had steroid injections or taken steroid tablets regularly for more than 3 months?	□	□
2.	**Do you have cancer of any kind?** If YES answer questions 2a-2b. If NO, go to Question 3.	□	□
2a.	Does your cancer diagnosis include any of the following types: lung/bronchogenic, multiple myeloma (cancer of plasma cells), head and neck?	□	□
2b.	Are you currently receiving cancer therapy (such as chemotherapy or radiotherapy)?	□	□
3.	**Do you have heart disease or cardiovascular disease? This includes coronary artery disease, high blood pressure, heart failure, diagnosed abnormality or heart rhythm.** If YES answer questions 3a-3e. If NO go to Question 4.	□	□
3a.	Do you have difficulty controlling your condition with medications or other physician-prescribed therapies? (Answer NO if you are not currently taking any medications or other treatments).	□	□
3b.	Do you have an irregular heartbeat that requires medical management? (e.g. atrial fibrillation, premature ventricular contraction)	□	□
3c.	Do you have chronic heart failure?	□	□
3d.	Do you have a resting blood pressure equal to or greater than 160/90mmHg with or without medication? Answer YES if you do not know your resting blood pressure.	□	□
3e.	Do you have diagnosed coronary artery (cardiovascular) disease and have not participated in regular physical activity in the last 2 months?	□	□

		YES	NO
4.	**Do you have any metabolic conditions? This includes Type 1 Diabetes, Type 2 Diabetes and Pre-Diabetes.** If YES answer questions 4a-4c. If NO, go to Question 5.	□	□
4a.	Is your blood sugar often above 13mmol/L? (Answer YES if you are not sure).	□	□
4b.	Do you have any signs or symptoms of diabetes complications such as heart or vascular disease and/or complications affecting your eyes, kidneys, OR the sensation in your toes and feet?	□	□
4c.	Do you have other metabolic conditions (such as thyroid disorders, current pregnancy related diabetes, chronic kidney disease, or liver problems)?	□	□
5.	**Do you have any mental health problems or learning difficulties?** This includes Alzheimer's, dementia, depression, anxiety disorder, eating disorder, psychotic disorder, intellectual disability and down syndrome. If YES answer questions 5a-5b. If NO go to Question 6.	□	□
5a.	Do you have difficulty controlling your condition with medications or other physician-prescribed therapies? (Answer NO if you are not currently taking any medications or other treatments).	□	□
5b.	Do you also have back problems affecting nerves or muscles?	□	□
6.	**Do you have a respiratory disease?** This includes chronic obstructive pulmonary disease, asthma, pulmonary high blood pressure. If YES answer questions 6a-6d. If NO, go to Question 7.	□	□
6a.	Do you have difficulty controlling your condition with medications or other physician-prescribed therapies? (Answer NO if you are not currently taking any medications or other treatments).	□	□
6b.	Has your doctor ever said you blood oxygen level is low at rest or during exercise and/or that you require supplemental oxygen therapy?	□	□
6c.	If asthmatic, do you currently have symptoms of chest tightness, wheezing, laboured breathing, consistent cough (more than 2 days/week), or have you used your rescue medication more than twice in the last week?	□	□
6d.	Has your doctor ever said you have high blood pressure in the blood vessels of your lungs?	□	□
7.	**Do you have a spinal cord injury?** This includes tetraplegia and paraplegia. If YES answer questions 7a-7c. If NO, go to Question 8.	□	□
7a.	Do you have difficulty controlling your condition with medications or other physician-prescribed therapies? (Answer NO if you are not currently taking any medications or other treatments).	□	□
7b.	Do you commonly exhibit low resting blood pressure significant enough to cause dizziness, light-headedness, and/or fainting?	□	□
7c.	Has your physician indicated that you exhibit sudden bouts of high blood pressure (known as autonomic dysreflexia)?	□	□

		YES	NO
8.	**Have you had a stroke?** This includes transient ischemic attack (TIA) or cerebrovascular event. If YES answer questions 8a-8c. If NO go to Question 9.	□	□
8a.	Do you have difficulty controlling your condition with medications or other physician-prescribed therapies? (Answer NO if you are not currently taking any medications or other treatments).	□	□
8b.	Do you have any impairment in walking or mobility?	□	□
8c.	Have you experienced a stroke or impairment in nerves or muscles in the past 6 months?	□	□
9.	**Do you have any other medical condition which is not listed above or do you have two or more medical conditions?** If you have other medical conditions, answer questions 9a-9c. If NO go to Question 10.	□	□
9a.	Have you experienced a blackout, fainted, or lost consciousness as a result of a head injury within the last 12 months OR have you had a diagnosed concussion within the last 12 months?	□	□
9b.	Do you have a medical condition that is not listed (such as epilepsy, neurological conditions, and kidney problems)?	□	□
9c.	Do you currently live with two or more medical conditions?	□	□
	Please list your medical condition(s) and any related medications here:		
10.	**Have you had a viral infection in the last 2 weeks (cough, cold, sore throat, etc.)?** If YES please provide details below:	□	□
11.	**Is there any other reason why you cannot take part in this exercise test?** If YES please provide details below:	□	□

If you answered NO to all of the follow-up questions about your medical condition, you are cleared to take part in the exercise test.

If you answered YES to one or more of the follow-up questions about your medical condition it is strongly advised that you should seek further advice from a medical professional before taking part in the exercise test.

This health questionnaire is based around the PAR-Q+, which was developed by the Canadian Society for Exercise Physiology www.csep.ca

Appendix D: Food and Activity Diary

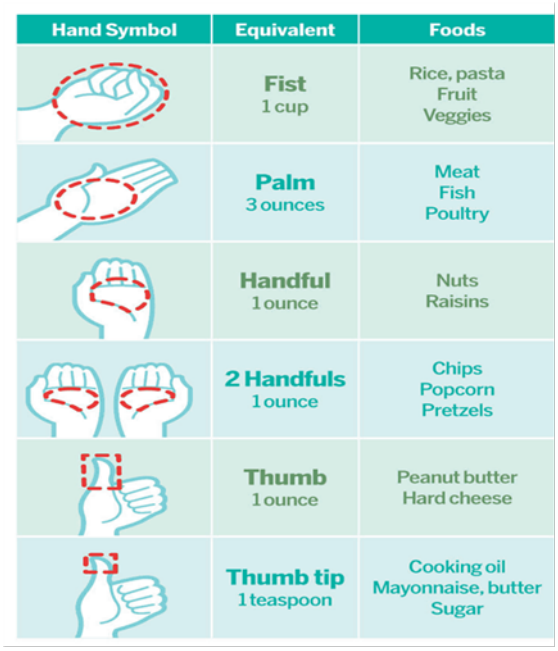
Participant Food and Activity Diary

Additional notes:

Please use kitchen scales to weigh the food consumed (i.e. weigh the amount served and subtract the weight of the amount leftover if not all is eaten). However, if it is impossible to weigh foods (e.g. when eating out) you may use the following estimation methods: Please try and stick to the following codes, weights and measures when completing the food diary

Grams	g
Ounces	oz
Kilograms	kg
Pounds	lbs
Pints	pts
Millilitres	mls
*Portions	P

1 pint =	480 grams
1 cup =	240 grams
½ cup =	120 grams
1 glass =	240 grams
½ glass =	120 grams



**Portion sizes are taken as an average helping of a particular food and therefore will give you a less accurate analysis than if exact weights are used, however, if a precise weight is not known, portions give an idea of the quantity eaten. A portion is for example 1 apple or 1 slice of bread.*

DAY 1 (Before Familiarization Visit). Date: _____

Time	**Detailed food description**	**Weighed amount or estimated portion weight** (note weight of any leftovers also)	**Activity** What exercise, training or other physical activity have you done today?

DAY 2 (Before experimental visit 1) Date:_____

Time	**Detailed food/ activity description**	**Weighed amount or estimated portion weight**

DAY 3 (Before experimental visit 2) Date: _____

Time	**Detailed food/ activity description**	**Weighed amount or estimated portion weight**

DAY 4. (Before experimental visit 3) Date: _____

Time	Detailed food/ activity description	Weighed amount or estimated portion weight

Appendix E: Caffeine Frequency Questionnaire

Caffeine Frequency Questionnaire. ID_____

Please complete as comprehensively and as honestly as possible. The results that are obtained can only be as accurate as the information put in. The purpose of this questionnaire is to get some indication of your general dietary habits of caffeine intake.

For each of the items listed below, please indicate your **typical** intake (i.e. what would be normal for you) using the following.

	Never	Less 1/month	1-3/month	1/week	2-4/week	5-6/wk	1/day	2-3/day	4-5/day	6+/day
Instant coffee										
Filter/cafetiere coffee										
Sachet coffee										
Single espresso shot										
Double espresso shot										
Iced coffee										
Decaf coffee										
Black tea										
Green tea										
Iced tea										
Decaf tea										
Energy drink small (250 mL)										
Energy drink large (500 mL)										
Energy shot										
Cola										
Dr Pepper										
Irn-Bru										
Mountain Dew										
Cream soda										
Lucozade energy										
Milk choc 50g										
Milk choc 200g										
Dark choc 50g										
Dark choc 200g										
Hot choc										
Choc milk										
Coffee/choc ice-cream										
Coffee choc frozen yoghurt										
Pre-workout supplement w/ caf										
Caf gels										
Caf energy bars										

Caf chewing gum										
Pro-plus caf tablets										
Paracetamol tablets w/ caf										

Appendix F: Menstrual Diary

Please remember to take the urine dipstick test on day 6 of your menstrual cycle and mark whether the test was positive or negative in the appropriate boxes. If you know you have a short cycle, you can begin taking the test earlier. Once you receive a positive result, you can stop taking the test until day 6 of your next cycle. If you forget to take the urine dipstick test, mark an "X" through the date you forgot to take the test.

	Check One Only	
DATE:	Positive Test	Negative Test
Date		
Date		
Date		
Date		
Date		
Date		
Date		
Date		
Date		
Date		
Date		
Date		
Date		
Date		

Appendix G: Competitive State Anxiety Inventory-2

The following are several statements that performers use to describe their feelings before a performance or competition. Read each statement and circle the appropriate number to indicate how you feel right now—at this moment. There are no right or wrong answers. Do not spend too much time on any one statement.

	Not at all	Somewhat	Moderately So	Very Much So
am concerned about this performance	1	2	3	4
feel nervous	1	2	3	4
feel at ease	1	2	3	4
have self-doubts	1	2	3	4
feel jittery	1	2	3	4
feel comfortable	1	2	3	4
am concerned I may not do as well in this performance as I could	1	2	3	4
My body feels tense	1	2	3	4
feel self-confident	1	2	3	4
am concerned about losing or doing poorly	1	2	3	4
feel tense in my stomach	1	2	3	4
feel secure	1	2	3	4
am worried about performing well	1	2	3	4
My body feels relaxed	1	2	3	4
'm confident I can meet this challenge	1	2	3	4
'm concerned about performing poorly	1	2	3	4
My heart is racing	1	2	3	4
'm confident about performing well	1	2	3	4
'm worried about reaching my goal	1	2	3	4
feel my stomach sinking	1	2	3	4
feel mentally relaxed	1	2	3	4
'm concerned that others will be disappointed with my performance	1	2	3	4
My hands are clammy	1	2	3	4
'm confident because I mentally picture myself reaching my goal	1	2	3	4
'm concerned I won't be able to focus	1	2	3	4
My body feels tight	1	2	3	4
'm confident of coming through under pressure	1	2	3	4

Scoring

This scale divides anxiety into three components: cognitive anxiety, somatic anxiety, and a related component—self-confidence. Self-confidence tends to be the opposite of cognitive anxiety and is another important factor in managing stress.

To score the CSAI-2, take the scores for each item at face value with the exception of item 14, where you "reverse" the score. For example, if you circled 3, count that as 2 points.

When totaling your rankings, you will arrive at the following three scores:

_____ **Cognitive State Anxiety:** (Sum of items 1, 4, 7, 10, 13, 16, 19, 22, & 25)

_____ **Somatic State Anxiety:** (Sum of items 2, 5, 8, 11, 14, 17, 20, 23, & 26)

_____ **Self-Confidence:** (Sum of items 3, 6, 9, 12, 15, 18, 21, 24, & 27)

Your scores for each will range from 9 to 36: 9 indicating low state anxiety and 36 indicated high state anxiety.

Appendix H: Groningen Sleep Quality Scale

Circle True or False for each question.

I had a deep sleep last night.	TRUE	FALSE
I feel like I slept poorly last night.	TRUE	FALSE
It took me more than half an hour to fall asleep last night.	TRUE	FALSE
I felt tired after waking up this morning.	TRUE	FALSE
I woke up several times last night.	TRUE	FALSE
I feel like I didn't get enough sleep last night.	TRUE	FALSE
I got up in the middle of the night.	TRUE	FALSE
I felt rested after waking up this morning.	TRUE	FALSE
I feel like I only had a couple hours of sleep last night.	TRUE	FALSE
I feel l slept well last night.	TRUE	FALSE
I didn't sleep a wink last night.	TRUE	FALSE
I didn't have any trouble falling asleep last night.	TRUE	FALSE
After I woke up last night, I had trouble falling asleep again.	TRUE	FALSE
I tossed and turned all last night	TRUE	FALSE
I didn't get more than 5 hours sleep last night.	TRUE	FALSE

Scoring:

The first question doesn't count toward the total score.

One point if answer is "TRUE" for questions 2, 3, 4, 5, 6, 7, 9, 11, 13, 14, 15

One point if answer is "FALSE" for questions 8, 10, 12

Maximum score of 14 points indicates poor sleep the night before.

NOTE: The Groningen Sleep Quality Scale is a **tool** that can be used to understand your patters in overall sleep quality. Answer these 15 questions for at least 14 days in a row to help understand your individual sleep pattern.

Approximately what time did you go to bed last night? _____

Approximately what time did you wake up this morning? _____

SCHOOL OF SPORT & EXERCISE SCIENCES

PRE-TEST / RE-TEST QUESTIONNAIRE

PARTICIPANT ID:

RESEARCHER:

	YES/NO
1. HAS THERE BEEN ANY CHANGE IN YOUR HEALTH STATUS?	☐
2. HAVE YOU HAD ANY KIND OF ILLNESS OR INFECTION IN THE LAST 2 WEEKS?	☐
3. ARE YOU TAKING ANY MEDICATION OR NUTRITIONAL SUPPLEMENTS, OR INVOLVED IN ANY OTHER RESEARCH PROJECTS? IF YES, PROVIDE DETAILS: …….	☐
4. DO YOU HAVE ANY FORM OF INJURY? IF YES, PLEASE PROVIDE DETAILS: ……...	☐
5. HAVE YOU EATEN IN THE LAST 12 HOURS?	☐
6. HAVE YOU CONSUMED ANY ALCOHOL IN THE LAST 24 HOURS?	☐
7. HAVE YOU CONSUMED ANY CAFFEINE (TEA, COFFEE, CAFFEINATED SOFT DRINKS (E.G. RED BULL, COKE, ETC.) IN THE LAST 48 HOURS?	☐
8. HAVE YOU PERFORMED EXHAUSTIVE EXERCISE WITHIN THE LAST 48 HOURS?	☐
9. ARE YOU SUFFICIENTLY HYDRATED?	☐

IF THE ANSWER TO ANY OF THE ABOVE QUESTIONS IS YES (APART FROM Q9), THEN YOU MUST CONSULT WITH YOUR SUPERVISOR BEFORE UNDERGOING AN EXERCISE TEST, OR RE-SCHEDULE THE TEST.

SIGNATURE OF PARTICIPANT: ……

DATE: ……

Appendix J: Blinding Questionnaire

Blinding questionnaire Date _____

Participant ID # _____

During experimental **trial 1** I think I was given:

	CHECK OFF ONE BOX ONLY
High Caffeine Dose	
Low Caffeine Dose	
Placebo	
Do Not Know/Unsure	

During experimental **Trial 2** I think I was given:

	CHECK OFF ONE BOX ONLY
High Caffeine Dose	
Low Caffeine Dose	
Placebo	
Do Not Know/Unsure	

During experimental **trial 3** I think I was given:

	CHECK OFF ONE BOX ONLY
High Caffeine Dose	
Low Caffeine Dose	
Placebo	
Do Not Know/Unsure	

Appendix K: Statistical Results

Statistical Values for Concentration of Salivary SIgA Normalised with Osmolality

Table 9. Mauchly's Test of Sphericity: Tests the null hypothesis that the error covariance matrix of the orthonormalized transformed dependent variables is proportional to an identity matrix
a. Design: Intercept; Within Subjects Design: Condition + TimePoint + Condition * TimePoint
b. May be used to adjust the degrees of freedom for the averaged tests of significance. Corrected tests are displayed in the Tests of Within-Subjects Effects table.

Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilonb Greenhouse-Geisser	Huynh-Feldt	Lower-bound
Condition	0.512	6.03	2	0.049	0.672	0.738	0.5
TimePoint	0.62	4.166	5	0.528	0.782	1	0.333
Condition * TimePoint	0.016	32.036	20	0.057	0.504	0.748	0.167

Table 10. Tests of Within Subject Effects for Salivary SIgA Concentration Normalised with Osmolality

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Condition	Sphericity Assumed	0.463	2	0.232	0.859	0.439
	Greenhouse-Geisser	0.463	1.344	0.345	0.859	0.403
	Huynh-Feldt	0.463	1.477	0.314	0.859	0.412
	Lower-bound	0.463	1	0.463	0.859	0.376
Error(Condition)	Sphericity Assumed	5.397	20	0.27		
	Greenhouse-Geisser	5.397	13.438	0.402		
	Huynh-Feldt	5.397	14.766	0.365		
	Lower-bound	5.397	10	0.54		
TimePoint	Sphericity Assumed	1.401	3	0.467	4.788	0.008
	Greenhouse-Geisser	1.401	2.346	0.597	4.788	0.014
	Huynh-Feldt	1.401	3	0.467	4.788	0.008
	Lower-bound	1.401	1	1.401	4.788	0.053
Error(TimePoint)	Sphericity Assumed	2.927	30	0.098		
	Greenhouse-Geisser	2.927	23.459	0.125		
	Huynh-Feldt	2.927	30	0.098		
	Lower-bound	2.927	10	0.293		
Condition * TimePoint	Sphericity Assumed	0.482	6	0.08	0.929	0.481
	Greenhouse-Geisser	0.482	3.026	0.159	0.929	0.44
	Huynh-Feldt	0.482	4.485	0.108	0.929	0.464
	Lower-bound	0.482	1	0.482	0.929	0.358
Error(Condition*TimePoint)	Sphericity Assumed	5.195	60	0.087		
	Greenhouse-Geisser	5.195	30.256	0.172		
	Huynh-Feldt	5.195	44.852	0.116		
	Lower-bound	5.195	10	0.519		

Table 11. Pairwise Comparisons**.** Based on estimated marginal means; * = The mean difference is significant at the .05 level; ** = Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

(I) TimePoint	(J) TimePoint	Mean Difference (I-J)	Std. Error	Sig.b	95% Confidence Interval for Difference** Lower Bound	Upper Bound
1	2	0.067	0.054	0.239	-0.052	0.187
	3	.200*	0.079	0.03	0.023	0.376
	4	-0.082	0.077	0.316	-0.254	0.091
2	1	-0.067	0.054	0.239	-0.187	0.052
	3	0.133	0.094	0.187	-0.076	0.341
	4	-0.149	0.079	0.09	-0.325	0.028
3	1	-.200*	0.079	0.03	-0.376	-0.023
	2	-0.133	0.094	0.187	-0.341	0.076
	4	-.281*	0.073	0.003	-0.444	-0.119
4	1	0.082	0.077	0.316	-0.091	0.254
	2	0.149	0.079	0.09	-0.028	0.325
	3	.281*	0.073	0.003	0.119	0.444

Statistical Values for Absolute Salivary SIgA Concentration

Table 12. Mauchly's Test of Sphericity: Tests the null hypothesis that the error covariance matrix of the orthonormalized transformed dependent variables is proportional to an identity matrix

a. Design: Intercept; Within Subjects Design: Condition + TimePoint + Condition * TimePoint

b. May be used to adjust the degrees of freedom for the averaged tests of significance. Corrected tests are displayed in the Tests of Within-Subjects Effects table.

Mauchly's Test of Sphericity

Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Greenhouse-Geisser	Epsilonb Huynh-Feldt	Lower-bound
condition	0.737	2.745	2	0.254	0.792	0.916	0.5
timepoint	0.732	2.727	5	0.743	0.816	1	0.333
condition * timepoint	0.042	24.665	20	0.25	0.55	0.853	0.167

Table 13. Tests of Within Subject Effects for Absolute Salivary SIgA Concentration

Tests of Within-Subjects Effects

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
condition	Sphericity Assumed	0.334	2	0.167	0.588	0.565
	Greenhouse-Geisser	0.334	1.584	0.211	0.588	0.529
	Huynh-Feldt	0.334	1.832	0.182	0.588	0.551
	Lower-bound	0.334	1	0.334	0.588	0.461
Error(condition)	Sphericity Assumed	5.672	20	0.284		
	Greenhouse-Geisser	5.672	15.837	0.358		
	Huynh-Feldt	5.672	18.323	0.31		
	Lower-bound	5.672	10	0.567		
timepoint	Sphericity Assumed	1.338	3	0.446	5.971	0.003
	Greenhouse-Geisser	1.338	2.449	0.546	5.971	0.005
	Huynh-Feldt	1.338	3	0.446	5.971	0.003
	Lower-bound	1.338	1	1.338	5.971	0.035
Error(timepoint)	Sphericity Assumed	2.241	30	0.075		
	Greenhouse-Geisser	2.241	24.494	0.091		
	Huynh-Feldt	2.241	30	0.075		
	Lower-bound	2.241	10	0.224		
condition * timepoint	Sphericity Assumed	0.638	6	0.106	1.226	0.306
	Greenhouse-Geisser	0.638	3.3	0.193	1.226	0.317
	Huynh-Feldt	0.638	5.12	0.125	1.226	0.31
	Lower-bound	0.638	1	0.638	1.226	0.294
Error(condition*timepoint)	Sphericity Assumed	5.207	60	0.087		
	Greenhouse-Geisser	5.207	33.003	0.158		
	Huynh-Feldt	5.207	51.202	0.102		
	Lower-bound	5.207	10	0.521		

Table 14. Based on estimated marginal means; * = The mean difference is significant at the .05 level; ** = Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Pairwise Comparisons

(I) timepoint	(J) timepoint	Mean Difference (I-J)	Std. Error	Sig.b	95% Confidence Interval for Difference** Lower Bound	Upper Bound
1	2	0.078	0.058	0.212	-0.052	0.208
	3	-.196*	0.061	0.009	-0.333	-0.06
	4	-0.068	0.067	0.332	-0.218	0.081
2	1	-0.078	0.058	0.212	-0.208	0.052
	3	-.274*	0.062	0.001	-0.413	-0.135
	4	-.146*	0.064	0.046	-0.289	-0.003
3	1	.196*	0.061	0.009	0.06	0.333
	2	.274*	0.062	0.001	0.135	0.413
	4	0.128	0.087	0.17	-0.065	0.321
4	1	0.068	0.067	0.332	-0.081	0.218
	2	.146*	0.064	0.046	0.003	0.289
	3	-0.128	0.087	0.17	-0.321	0.065

Statistical Values for Comparisons Between Salivary SIgA Concentration Normalised with Osmolality, Condition, and CYP1A2 Genotype

Table 15. Mauchly's Test of Sphericity: Tests the null hypothesis that the error covariance matrix of the orthonormalized transformed dependent variables is proportional to an identity matrix
a. Design: Intercept + Genotype; Within Subjects Design: Condition + TimePoint + Condition * TimePoint
b. May be used to adjust the degrees of freedom for the averaged tests of significance. Corrected tests are displayed in the Tests of Within-Subjects Effects table.

Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilonb Greenhouse-Geisser	Huynh-Feldt	Lower-bound
Condition	0.377	7.808	2	0.02	0.616	0.744	0.5
TimePoint	0.663	3.178	5	0.675	0.802	1	0.333
Condition * TimePoint	0.015	28.677	20	0.125	0.5	0.86	0.167

Table 16. Tests of Within Subject Effects

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Condition	Sphericity Assumed	0.437	2	0.219	0.784	0.472
	Greenhouse-Geisser	0.437	1.232	0.355	0.784	0.421
	Huynh-Feldt	0.437	1.487	0.294	0.784	0.441
	Lower-bound	0.437	1	0.437	0.784	0.399
Condition * Genotype	Sphericity Assumed	0.372	2	0.186	0.667	0.526
	Greenhouse-Geisser	0.372	1.232	0.302	0.667	0.462
	Huynh-Feldt	0.372	1.487	0.25	0.667	0.487
	Lower-bound	0.372	1	0.372	0.667	0.435
Error(Condition)	Sphericity Assumed	5.025	18	0.279		
	Greenhouse-Geisser	5.025	11.089	0.453		
	Huynh-Feldt	5.025	13.386	0.375		
	Lower-bound	5.025	9	0.558		
TimePoint	Sphericity Assumed	1.288	3	0.429	4.699	0.009
	Greenhouse-Geisser	1.288	2.405	0.536	4.699	0.016
	Huynh-Feldt	1.288	3	0.429	4.699	0.009
	Lower-bound	1.288	1	1.288	4.699	0.058
TimePoint * Genotype	Sphericity Assumed	0.459	3	0.153	1.672	0.196
	Greenhouse-Geisser	0.459	2.405	0.191	1.672	0.208
	Huynh-Feldt	0.459	3	0.153	1.672	0.196
	Lower-bound	0.459	1	0.459	1.672	0.228
Error(TimePoint)	Sphericity Assumed	2.468	27	0.091		
	Greenhouse-Geisser	2.468	21.644	0.114		
	Huynh-Feldt	2.468	27	0.091		
	Lower-bound	2.468	9	0.274		
Condition * TimePoint	Sphericity Assumed	0.426	6	0.071	0.796	0.577
	Greenhouse-Geisser	0.426	2.998	0.142	0.796	0.507
	Huynh-Feldt	0.426	5.161	0.083	0.796	0.562
	Lower-bound	0.426	1	0.426	0.796	0.396
Condition * TimePoint * Genotype	Sphericity Assumed	0.376	6	0.063	0.703	0.648
	Greenhouse-Geisser	0.376	2.998	0.126	0.703	0.558
	Huynh-Feldt	0.376	5.161	0.073	0.703	0.628
	Lower-bound	0.376	1	0.376	0.703	0.424
Error(Condition*TimePoint)	Sphericity Assumed	4.818	54	0.089		
	Greenhouse-Geisser	4.818	26.982	0.179		
	Huynh-Feldt	4.818	46.451	0.104		
	Lower-bound	4.818	9	0.535		

Statistical Values for Comparisons Between Absolute Salivary SIgA Concentration Condition, and CYP1A2 Genotype

Table 17. Mauchly's Test of Sphericity: Tests the null hypothesis that the error covariance matrix of the orthonormalized transformed dependent variables is proportional to an identity matrix

a. Design: Intercept + Genotype; Within Subjects Design: Condition + TimePoint + Condition * TimePoint

b. May be used to adjust the degrees of freedom for the averaged tests of significance. Corrected tests are displayed in the Tests of Within-Subjects Effects table.

Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilonb Greenhouse-Geisser	Huynh-Feldt	Lower-bound
Condition	0.682	3.061	2	0.216	0.759	0.982	0.5
TimePoint	0.601	3.93	5	0.562	0.752	1	0.333
Condition * TimePoint	0.026	24.829	20	0.255	0.535	0.958	0.167

Table 18. Tests of Within Subject Effects

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Condition	Sphericity Assumed	0.312	2	0.156	0.517	0.605
	Greenhouse-Geisser	0.312	1.518	0.205	0.517	0.558
	Huynh-Feldt	0.312	1.964	0.159	0.517	0.602
	Lower-bound	0.312	1	0.312	0.517	0.49
Condition * Genotype	Sphericity Assumed	0.252	2	0.126	0.418	0.665
	Greenhouse-Geisser	0.252	1.518	0.166	0.418	0.612
	Huynh-Feldt	0.252	1.964	0.128	0.418	0.661
	Lower-bound	0.252	1	0.252	0.418	0.534
Error(Condition)	Sphericity Assumed	5.42	18	0.301		
	Greenhouse-Geisser	5.42	13.658	0.397		
	Huynh-Feldt	5.42	17.672	0.307		
	Lower-bound	5.42	9	0.602		
TimePoint	Sphericity Assumed	1.404	3	0.468	6.051	0.003
	Greenhouse-Geisser	1.404	2.257	0.622	6.051	0.007
	Huynh-Feldt	1.404	3	0.468	6.051	0.003
	Lower-bound	1.404	1	1.404	6.051	0.036
TimePoint * Genotype	Sphericity Assumed	0.153	3	0.051	0.658	0.585
	Greenhouse-Geisser	0.153	2.257	0.068	0.658	0.546
	Huynh-Feldt	0.153	3	0.051	0.658	0.585
	Lower-bound	0.153	1	0.153	0.658	0.438
Error(TimePoint)	Sphericity Assumed	2.088	27	0.077		
	Greenhouse-Geisser	2.088	20.313	0.103		
	Huynh-Feldt	2.088	27	0.077		
	Lower-bound	2.088	9	0.232		
Condition * TimePoint	Sphericity Assumed	0.587	6	0.098	1.141	0.352
	Greenhouse-Geisser	0.587	3.209	0.183	1.141	0.351
	Huynh-Feldt	0.587	5.75	0.102	1.141	0.352
	Lower-bound	0.587	1	0.587	1.141	0.313
Condition * TimePoint * Genotype	Sphericity Assumed	0.579	6	0.096	1.125	0.36
	Greenhouse-Geisser	0.579	3.209	0.18	1.125	0.357
	Huynh-Feldt	0.579	5.75	0.101	1.125	0.361
	Lower-bound	0.579	1	0.579	1.125	0.316
Error(Condition*TimePoint)	Sphericity Assumed	4.629	54	0.086		
	Greenhouse-Geisser	4.629	28.879	0.16		
	Huynh-Feldt	4.629	51.746	0.089		
	Lower-bound	4.629	9	0.514		

Salivary Osmolality

Table 19. Mauchly's Test of Sphericity: Tests the null hypothesis that the error covariance matrix of the orthonormalized transformed dependent variables is proportional to an identity matrix
a. Design: Intercept; Within Subjects Design: Condition + TimePoint + Condition * TimePoint
b. May be used to adjust the degrees of freedom for the averaged tests of significance. Corrected tests are displayed in the Tests of Within-Subjects Effects table.

Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilonb Greenhouse-Geisser	Huynh-Feldt	Lower-bound
Condition	0.608	4.483	2	0.106	0.718	0.806	0.5
TimePoint	0.335	9.543	5	0.091	0.671	0.84	0.333
Condition * TimePoint	0.07	20.643	20	0.46	0.537	0.822	0.167

Table 20. Tests of Within Subject Effects

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Condition	Sphericity Assumed	260.591	2	130.295	0.163	0.85
	Greenhouse-Geisser	260.591	1.436	181.415	0.163	0.78
	Huynh-Feldt	260.591	1.612	161.7	0.163	0.805
	Lower-bound	260.591	1	260.591	0.163	0.695
Error(Condition)	Sphericity Assumed	15942.409	20	797.12		
	Greenhouse-Geisser	15942.409	14.364	1109.859		
	Huynh-Feldt	15942.409	16.116	989.246		
	Lower-bound	15942.409	10	1594.241		
TimePoint	Sphericity Assumed	33683.424	3	11227.808	24.252	0
	Greenhouse-Geisser	33683.424	2.012	16741.448	24.252	0
	Huynh-Feldt	33683.424	2.52	13365.149	24.252	0
	Lower-bound	33683.424	1	33683.424	24.252	0.001
Error(TimePoint)	Sphericity Assumed	13889.076	30	462.969		
	Greenhouse-Geisser	13889.076	20.12	690.319		
	Huynh-Feldt	13889.076	25.202	551.101		
	Lower-bound	13889.076	10	1388.908		
Condition * TimePoint	Sphericity Assumed	1956.985	6	326.164	0.561	0.759
	Greenhouse-Geisser	1956.985	3.221	607.621	0.561	0.656
	Huynh-Feldt	1956.985	4.931	396.88	0.561	0.727
	Lower-bound	1956.985	1	1956.985	0.561	0.471
Error(Condition*TimePoint)	Sphericity Assumed	34868.015	60	581.134		
	Greenhouse-Geisser	34868.015	32.207	1082.611		
	Huynh-Feldt	34868.015	49.309	707.129		
	Lower-bound	34868.015	10	3486.802		

Table 21. Pairwise Comparisons: Based on estimated marginal means; * = The mean difference is significant at the .05 level; ** = Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).
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					95% Confidence Interval for Difference**	
(I) TimePoint	(J) TimePoint	Mean Difference (I-J)	Std. Error	Sig.b	Lower Bound	Upper Bound
1	2	1.242	2.833	0.67	-5.069	7.554
	3	-35.970*	5.936	0	-49.196	-22.743
	4	1.455	3.945	0.72	-7.335	10.244
2	1	-1.242	2.833	0.67	-7.554	5.069
	3	-37.212*	5.635	0	-49.768	-24.656
	4	0.212	5.291	0.969	-11.578	12.002
3	1	35.970*	5.936	0	22.743	49.196
	2	37.212*	5.635	0	24.656	49.768
	4	37.424*	7.055	0	21.704	53.144
4	1	-1.455	3.945	0.72	-10.244	7.335
	2	-0.212	5.291	0.969	-12.002	11.578
	3	-37.424*	7.055	0	-53.144	-21.704

Cognitive State Anxiety Inventory-2 Statistics: Cognitive Anxiety

Table 22. Mauchly's Test of Sphericity: Tests the null hypothesis that the error covariance matrix of the orthonormalized transformed dependent variables is proportional to an identity matrix
a. Design: Intercept; Within Subjects Design: Visit
b. May be used to adjust the degrees of freedom for the averaged tests of significance. Corrected tests are displayed in the Tests of Within-Subjects Effects table.

Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Greenhouse-Geisser	Epsilonb Huynh-Feldt	Lower-bound
Visit	0.549	5.398	2	0.067	0.689	0.763	0.5

Table 23. Tests of Within Subject Effects

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Visit	Sphericity Assumed	4.424	2	2.212	1.604	0.226
	Greenhouse-Geisser	4.424	1.519	2.912	1.604	0.232
	Huynh-Feldt	4.424	1.735	2.55	1.604	0.23
	Lower-bound	4.424	1	4.424	1.604	0.234
Error(Visit)	Sphericity Assumed	27.576	20	1.379		
	Greenhouse-Geisser	27.576	15.194	1.815		
	Huynh-Feldt	27.576	17.349	1.589		
	Lower-bound	27.576	10	2.758		

Cognitive State Anxiety Inventory-2 Statistics: Somatic Anxiety

Table 24. Mauchly's Test of Sphericity: Tests the null hypothesis that the error covariance matrix of the orthonormalized transformed dependent variables is proportional to an identity matrix
a. Design: Intercept; Within Subjects Design: Visit
b. May be used to adjust the degrees of freedom for the averaged tests of significance. Corrected tests are displayed in the Tests of Within-Subjects Effects table.

Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Greenhouse-Geisser	Epsilonb Huynh-Feldt	Lower-bound
Visit	0.684	3.423	2	0.181	0.76	0.867	0.5

Table 25. Tests of Within Subject Effects

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Visit	Sphericity Assumed	20.424	2	10.212	1.737	0.202
	Greenhouse-Geisser	20.424	1.378	14.818	1.737	0.213
	Huynh-Feldt	20.424	1.527	13.379	1.737	0.211
	Lower-bound	20.424	1	20.424	1.737	0.217
Error(Visit)	Sphericity Assumed	117.576	20	5.879		
	Greenhouse-Geisser	117.576	13.783	8.53		
	Huynh-Feldt	117.576	15.266	7.702		
	Lower-bound	117.576	10	11.758		

Cognitive State Anxiety Inventory-2 Statistics: Self-Confidence

Table 26. Mauchly's Test of Sphericity: Tests the null hypothesis that the error covariance matrix of the orthonormalized transformed dependent variables is proportional to an identity matrix
a. Design: Intercept; Within Subjects Design: Visit
b. May be used to adjust the degrees of freedom for the averaged tests of significance. Corrected tests are displayed in the Tests of Within-Subjects Effects table.

Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilonb Greenhouse-Geisser	Huynh-Feldt	Lower-bound
Visit	0.732	2.804	2	0.246	0.789	0.912	0.5

Table 27. Tests of Within Subject Effects

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Visit	Sphericity Assumed	73.697	2	36.848	2.153	0.142
	Greenhouse-Geisser	73.697	1.578	46.712	2.153	0.156
	Huynh-Feldt	73.697	1.823	40.424	2.153	0.148
	Lower-bound	73.697	1	73.697	2.153	0.173
Error(Visit)	Sphericity Assumed	342.303	20	17.115		
	Greenhouse-Geisser	342.303	15.777	21.696		
	Huynh-Feldt	342.303	18.231	18.776		
	Lower-bound	342.303	10	34.23		

Groningen Sleep Quality Statistics

Table 28. Mauchly's Test of Sphericity: Tests the null hypothesis that the error covariance matrix of the orthonormalized transformed dependent variables is proportional to an identity matrix
a. Design: Intercept; Within Subjects Design: Visit
b. May be used to adjust the degrees of freedom for the averaged tests of significance. Corrected tests are displayed in the Tests of Within-Subjects Effects table.

Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilonb Greenhouse-Geisser	Huynh-Feldt	Lower-bound
Visit	0.985	0.138	2	0.933	0.985	1	0.5

Table 29. Tests of Within Subject Effects

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Visit	Sphericity Assumed	10.364	2	5.182	0.852	0.441
	Greenhouse-Geisser	10.364	1.97	5.261	0.852	0.44
	Huynh-Feldt	10.364	2	5.182	0.852	0.441
	Lower-bound	10.364	1	10.364	0.852	0.378
Error(Visit)	Sphericity Assumed	121.636	20	6.082		
	Greenhouse-Geisser	121.636	19.7	6.174		
	Huynh-Feldt	121.636	20	6.082		
	Lower-bound	121.636	10	12.164		

Table 30. Coefficient Variability inter and intra assay. Four assay plates in total were analysed.

Plate Type	% Coefficient Variability
Intra-Assay Plate 1	3.103455565
Intra-Assay Plate 2	7.393108815
Intra-Assay Plate 3	2.037917319
Intra-Assay Plate 4	11.90304309
Inter-assay, n = 4	6.109381198