Inflammatory Cytokine Concentrations in Saliva versus Plasma at Rest and in Response to Intense Exercise in Adolescent Athletes

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Abstract

Salivary and plasma concentrations of inflammatory cytokines at rest and following high-intensity interval exercise were compared in adolescent swimmers (21 male and 22 female, aged 13-17 years) to validate a non-invasive method of assessing inflammation in youth. Following provision of morning, fasted, resting blood and saliva samples, swimmers performed an intense swimming trial consisting of a maximal 200m swim, plus a high intensity interval swimming protocol (5x100m, 5x50m and 5x25m; 1:1 work-to-rest ratio), followed by post-exercise blood and saliva samples (~15 min). Salivary and plasma concentrations of interleukin-6 (IL-6), interleukin 10 (IL-10), and tumor necrosis factor alpha (TNF-α) were similar in males and females across time. Resting concentrations of IL-10 were significantly lower while IL-6 and TNF-α were significantly higher in saliva compared with plasma. IL-6 did not show a significant time effect or interaction, so although its relative decrease from pre- to post-swimming was higher in saliva than in plasma this difference was not significant (-21% vs. -3%, respectively; \( p = 0.06 \)). There was a significant time-by-media interaction for IL-10, which increased from pre- to post-swimming in plasma, but this response was attenuated in saliva (51% vs. 29%; \( p = 0.02 \)). TNF-α showed a significant time-by-media interaction, reflecting a decrease from pre- to post-swimming only in saliva (-27%, \( p = 0.01 \)). Intraclass correlation coefficients revealed no agreement between salivary and plasma cytokine levels, and low Pearson correlations revealed no association between these measures either at rest or in response to intense swimming. In conclusion, although the overall direction of the post-exercise response was similar between saliva and plasma, the magnitude of the response was consistently different in saliva compared with plasma, which combined with the different resting concentrations and the absence of correlation between measures, suggest that salivary cytokine measures are not representative of blood levels in young athletes.
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Chapter 1: Introduction

1.1 Rationale

It is well documented that circulating concentrations of both pro- and anti-inflammatory cytokines increase in response to physical stress, and that this response is influenced by the intensity and duration of exercise (Nielsen et al., 2016; Izquierdo et al., 2009; Pedersen, 2000). Specifically, studies in adults have consistently found an increase in interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α) and interleukin-10 (IL-10) concentrations after vigorous exercise (Nielsen et al., 2016; Izquierdo et al., 2009; Ostrowski et al., 1999; Windsor et al., 2018). In addition, the post-exercise increases in TNF-α and interleukin-1 beta (IL-1β) generally precede the dramatic rise of IL-6. This is supported by the release of cytokine inhibitors (IL-1ra and TNF receptors [TNF-R]) and the anti-inflammatory cytokine IL-10 (Pedersen, 2000; Petersen & Pedersen, 2005; Suzuki et al., 2002). In children, there are fewer studies examining the inflammatory response to exercise, reporting mixed results, showing both increases, or no changes in various cytokines post-exercise (Nemet et al., 2002; Rosa et al., 2007; Sanderson et al., 2020; Timmons et al., 2004). Although the intensity of the exercise in these studies varied from moderate to intense, the consensus is that the acute inflammatory response to exercise in both athletic and non-athletic youth is attenuated compared with the typical exercise-induced increases we see in adults (Sanderson et al., 2020; Timmons et al., 2004).

Methodologically, the cytokine levels in all these studies were measured in blood, using either plasma or serum. From a practical perspective, the invasive nature of blood sampling has negatively impacted the ability to conduct large exercise and training studies, especially in youth. Thus, as blood sampling is invasive, there is an increasing interest in the ability to identify inflammatory markers in response to stress using saliva, especially in children. Salivary sampling would be an advantage in conducting larger scale and more intensive studies, such as those involving large populations of children, and/or repeated measures over time (Slavish et al., 2015).
The literature regarding the measurement of salivary concentrations of cytokines following exercise is sparse. Indeed, two studies have found a significant change in salivary cytokine levels in response to exercise in adults, with effect sizes ranging from very small (0.11) to very large (>0.3) (Usui et al., 2011; Rahman et al., 2010). These studies report that a single bout of intense exercise leads to a transient increase in salivary levels of inflammatory cytokines, including IL-6 and TNF-α. To date, there is no study that has reported the salivary concentrations of anti-inflammatory cytokines, such as IL-10, either at rest or in response to exercise, or in children.

Furthermore, research examining the relationship of cytokine concentrations measured in saliva versus plasma is limited and contradictory. One study in healthy adolescent girls, found a significant correlation between saliva and plasma concentrations of IL-1β at rest (Riis et al., 2014). In adults, one study reported no correlation between IL-6 measured in plasma and passive drool saliva samples post-exercise (Minetto et al., 2005), while a more recent study found good correlation between saliva and blood concentrations of IL-1β, IL-6 and IL-18 in response to psychological stress (La Fratta et al., 2018). Thus, no consensus exists as to the association between salivary and blood (plasma or serum) concentrations of inflammatory markers in both children and adults.

In summary, because the use of saliva levels would decrease the burden of collecting blood samples in younger populations, research comparing the cytokine concentrations measured in saliva versus blood are needed to provide critical information to scientists and clinicians when designing pediatric studies.

1.2 Purpose

The present study compared the salivary to plasma concentrations of inflammatory cytokines (IL-6, IL-10, and TNF-α), at rest and following high-intensity interval exercise, in adolescent swimmers, to determine whether a similar response occurs. Potential sex differences in salivary and plasma concentrations, both at rest and in response to exercise, were also considered as a secondary objective.

It was hypothesized that: (a) there would be no difference in the concentrations of IL-6, IL-10, and TNF-α between saliva and plasma either at rest or following intense swimming; (b) there would be a significant correlation between the resting salivary and
plasma concentrations of IL-6, IL-10, and TNF-α; (c) there would be a significant correlation and agreement in terms of the relative pre- to post-exercise changes between saliva and plasma; and (c) there would be no sex related differences between salivary and plasma cytokine concentrations neither at rest or in response to exercise.
Chapter 2: Literature Review

2.1 Inflammation

Based on observation, the ancients defined inflammation by five cardinal signs, namely redness (rubor), swelling (tumor), heat (calor; only applicable to the body extremities), pain (dolor), and loss of function (functio laesa) (Punchard et al., 2004). More recently, inflammation was characterized as "the changes that happen when there is an injury in living tissue given the fact that the injury is not of such a degree as to at once destroy its structure and vitality" (Chatterjee et al., 2017; Punchard et al., 2004), and additionally, "the reaction to injury of the living microcirculation and related tissues" (Punchard et al., 2004; Spector & Willoughby, 1963).

In early times, inflammation was identified as part of the healing process, and up until the end of the 19th century, inflammation was seen as an unwanted reaction that was often fatal to the host. In the 19th century, Metchnikoff and colleagues described the benefits of inflammation to the body's defensive and healing process (Hurley, 1973). Later, inflammation was also considered as the base of pathology, in that the changes observed are indicative of injury and disease (Whelan, and Adcock, 2004).

Today, inflammation is well accepted as a significant response of the immune system to tissue damage and infection, and as a process far more complicated than it seems. However, inflammation is not only connected to infection (Punchard et al., 2004). Chronic inflammation can lead to atherosclerosis, changes in the bronchial wall in asthma and chronic bronchitis, and the debilitating destruction of the joints connected with rheumatoid arthritis. These deleterious actions involve the major cells of the immune system, including neutrophils, basophils, mast cells, T-cells, B-cells, etc. (Punchard et al., 2004). In response to acute tissue injury, the body starts a chemical signaling cascade that stimulates interventions directed to the healing of affected tissues. These signals activate leukocyte chemotaxis from the general circulation to the sites of tissue damage, where the activated leukocytes produce cytokines that induce inflammatory responses (Chen et al., 2018).
2.2 Cytokines

Cytokines are signaling molecules used widely in cellular communication. As such, they are released by specific immune system cells that carry signals between cells. Cytokines are classified as pro- or anti-inflammatory. Pro-inflammatory cytokines are those that increase systemic inflammation, such as interleukins 1, 2, 8, 12, 18 (IL-1, IL-2, IL8, IL-12, IL-18), interferon gamma (IFNγ), and tumor necrosis factor alpha (TNF-α) (Grindvik, 2013; Terra et al., 2012). Anti-inflammatory cytokines are those that decrease inflammation, such as IL-10. Depending on the situation, IL-6 can be both pro-inflammatory – if secreted by immune cells, or anti-inflammatory – if secreted by muscle fibres (Grindvik, 2013). Exercise triggers a rapid temporary increase in cytokine production, including IL-6, IL-10 and TNF-α (Grindvik, 2013), which will be examined in the proposed study.

IL-6 is produced by a wide variety of cell lineages, including monocytes/macrophages, T cells, fibroblasts, and endothelial cells. IL-6 has a central role in the neuronal reaction to nerve injury. There is evidence that IL-6 participates in the improvement of neuropathic pain behavior following a peripheral nerve injury; for example, sciatic cryoneurolysis, a sympathetically-independent model of neuropathic pain involving frequently freezing and thawing a section of the sciatic nerve, which results in enhanced IL-6 immunoreactivity in the spinal cord (Zhang & An, 2007). Moreover, primary immunodeficiency diseases, including those involving IL-6 or its signaling pathways, show that IL-6 is crucial in the defense against many types of pathogens (Tanaka et al., 2014; Terra et al., 2012).

IL-10 can attenuate the generation of inflammatory cytokines (Holdsworth & Can, 2015; Iyer & Cheng, 2012). Specifically, IL-10 is a cytokine with robust anti-inflammatory properties that play a primary part in limiting host immune response to pathogens, thereby decreasing damage to the host and maintaining healthy tissue homeostasis. Dysregulation of IL-10 correlates with enhanced immunopathology in response to infection as well as improved risk for the development of many autoimmune diseases (Holdsworth & Can, 2015; Iyer & Cheng, 2012).

Macrophages are the primary generators of TNF-α, which has two receptors, TNF-R1 and TNF-R2, and through them, TNF-α controls several critical cell functions, including
cell proliferation, survival, differentiation, and apoptosis (Parameswaran & Patial, 2010). Deviant TNF-α production and TNF receptor signaling have been associated with the pathogenesis of several diseases, including rheumatoid arthritis, atherosclerosis, diabetes, and obesity. TNF-α has a crucial role in organizing the cytokine cascade in many inflammatory diseases, and because of this role as a “master regulator” of inflammatory cytokine production, it has been proposed as a therapeutic target for many diseases (Parameswaran & Patial, 2010).

The five critical factors involved in the control of the macrophage response during inflammation consist of TNF-α, IL-1, granulocyte-monocyte colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and monocyte colony-stimulating factor (M-CSF). These factors are mainly produced by activated macrophage cells in the inflamed tissues and the rest by other inflamed tissue cells. This combination of TNF-α, IL-1, and colony-stimulating factors produce a robust feedback mechanism that form many defensive white blood cells, which in turn, help to eliminate the cause of the inflammation (Khonsary, 2017).

2.3 Effects of Exercise on Cytokines (plasma/serum)

2.3.1 Adults

Exercise alters the normal functioning of the immune system, with transient immune responses being seen even in response to a single exercise bout (Hoffman-Goetz & Pedersen, 1994). Exercise dose is essential in determining the magnitude of the immune response. Thus, a single exercise bout causes a series of inflammatory responses, which mainly depend on the mode, intensity, duration, and training status. This cascade starts with a pro-inflammatory phase (1.5 - 24 h post-exercise), which is followed by an anti-inflammatory phase (24 - 72 h post-exercise) involving repair and skeletal muscle regeneration. The exercise-induced inflammatory response, and subsequent repair/regeneration, comes with an increase in the circulating levels of myokines (i.e., muscle derived cytokines), such as IL-6, and anti-inflammatory mediators, such as IL-10, and IL-1ra (Lombardi et al., 2019).
Recent studies in adults have also shown that plasma concentrations of several cytokines are elevated during and after vigorous exercise (Figure 1). This event is marked by an increase in TNF-α and IL-1β levels, which is followed by a significant rise in IL-6. This increase in pro-inflammatory cytokines is balanced by the release of cytokine inhibitors, such as IL-1ra and TNF receptors (TNF-R), as well as an increase in anti-inflammatory cytokines like IL-10 (Moldoveanu et al., 2001; K. Ostrowski et al., 1998; Pedersen, 2000; Pedersen et al., 2001). Indeed, it appears that an IL-6 response to endurance exercise is triggered by a decrease in cellular energy levels and an increase in heat stress, which is also related to the stress hormone responses. Moreover, IL-6 enhances the utilization of energy substrates, such as free fatty acids, which are helpful in endurance performance, and also promotes neutrophil mobilization and activation, together with the release of anti-inflammatory cytokines, such as IL-1ra and IL-10 (Suzuki, 2018). These findings imply that cytokine inhibitors and anti-inflammatory cytokines reduce the extent and duration of the inflammatory response to exercise. The finding of increased cytokine levels (TNF-α, IL-1β, IL-6, IL-2 receptors and INF-γ) in urine following exercise demonstrates the broad spectrum of cytokine responses to exercise (Pedersen, 2000).

*Figure 1:* Brief presentation of the changes in several cytokines in response to intense exercise over time. The enlarged double arrow indicates the period of strenuous exercise. The “relative increase” is the change in cytokine concentration at any time in comparison to time 0 levels. IL-1=interleukin-1; IL-1ra=interleukin-1 receptor antagonist; IL-6=interleukin-6; IL8=interleukin-8; MIP-1β=macrophage inflammatory protein 1 beta; TNF-α=tumor necrosis factor alpha; sTNFα R=soluble tumour necrosis factor alpha receptors. Adopted from Pedersen, 2000.)
Acute exercise results in an increased circulatory response to provide increased blood supply to the working tissues, which would facilitate the distribution of cytokines to areas of need. Some studies have reported that the acute effects of exercise include the mobilization of hematopoietic stem cells from bone marrow, and of senescent immune cells from external tissues, to the circulation for distribution. This might explain why in older subjects, where these mechanisms have declined with age, the response to acute exercise is different from that seen with younger individuals (Table 1). Note that the studies reported in Table 1, have been conducted in adults.

**Table 1: Summary of studies examining the immune responses to exercise (adopted from Sellami et al., 2018).**

<table>
<thead>
<tr>
<th>Study</th>
<th>Participants</th>
<th>Type of exercise</th>
<th>Time of sampling</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cannon et al., 1990</td>
<td>Sex: male (N=21) Age: 55-74 vs. 22-29 y Randomly assigned (double-blind placebo-controlled trial)</td>
<td>Running on an inclined treadmill (intense eccentric exercise) at 75% maximal heart rate</td>
<td>Samples collected 1 day before, the day of the test, and 1, 2, 5, and 12 days after the test</td>
<td>↑ neutrophils in young vs. old subjects ↑ Intramuscular CK in young vs. old subjects</td>
</tr>
<tr>
<td>Hamada et al., 2005</td>
<td>Sex: male (N=30) Age: 66-78 vs. 23-35 y</td>
<td>Acute eccentric exercise (45 min of downhill running, 16% descent, at 75% VO\textsubscript{2}max)</td>
<td>Samples collected 24h before and 72h after exercise</td>
<td>↑TNF-α mRNA ↑IL-1 mRNA ↔IL-6 mRNA ↑CD18 ↑TGF-β1</td>
</tr>
<tr>
<td>Bartlett et al., 2016</td>
<td>Sex: male and females (N=211 vs. 10 healthy young controls) Age: 67±5 y for male and 66±5 y for female vs. 23±4 y for controls</td>
<td>Different levels of activity</td>
<td>Accelerometry (one week)</td>
<td>↔IL-6 ↔IL-8 ↔MCP-1 ↔CRP ↔IL-10 ↔IL-13 ↑CD11b ↑Neutrophil ↔CXCR1 ↔CXCR2</td>
</tr>
<tr>
<td>Colbert et al., 2004</td>
<td>Sex: males and females (N=3075) Age: 70-79 y</td>
<td>Moderate activity; at least 180 min/week of walking and occupational activities</td>
<td>Self-administered questionnaire</td>
<td>↓CRP ↓IL-6 ↓TNF-α</td>
</tr>
</tbody>
</table>
2.3.2 Children and Adolescents

In children, exercise leads to a contemporary rise in antagonistic factors stimulating both the anabolic component of the growth hormone, i.e., the growth hormone → insulin-like growth factor-I axis (Eliakim & Nemet, 2013; Meckel et al., 2009) and the catabolic pro-inflammatory cytokines, namely IL-6, IL-1, and TNF-α (Nemet & Eliakim, 2010; Nemet et al., 2003). However, there are discrepancies among pediatric studies examining the changes in the systemic cytokine responses to exercise. For example, two previous studies have reported significant exercise-induced increases in serum IL-1β, IL-6 and TNF-α in both athletic and non-athletic adolescents (Nemet et al., 2002; Rosa et al., 2007). Others have reported no significant changes in plasma IL-6 and TNF-α from pre-to post-exercise in either pre-pubertal, non-athletic children or adolescent athletes, and they also reported a consistent increase in IL-10 (Sanderson et al., 2020; Timmons et al., 2004). According to these authors, the systemic cytokine response to intense exercise can be as much as 50% lower in children than in adults, suggesting a blunted inflammatory response to vigorous exercise in youth.

This attenuated cytokine response to exercise may be some type of ceiling effect because children have been found to have higher resting plasma concentrations of both IL-6 (Timmons et al., 2004) and TNF-α (Sanderson et al., 2020) than adults. In addition, a lower inflammatory response to exercise may reflect a growth-induced protective mechanism as increased levels of pro-inflammatory cytokines are known to restrain anabolic activity of the growth hormone → insulin-like growth factor-I axis directly (Nemet et al., 2002). In children with systemic inflammatory diseases, chronically high level of IL-6 leads to a decrease in the basal level of insulin-like growth factor-I and impairs somatic growth (Nemet et al., 2002). Prolonged dominance of the catabolic response, particularly if combined with poor nutrition, may eventually lead to over-reaching and over-training, and the dominance of the anabolic response will ultimately lead to enhanced muscle mass and improved fitness (Eliakim et al., 2015). Current thought is that a balance between the anabolic and catabolic responses to exercise would define effective training.
2.4 Salivary Measures of Inflammatory Cytokines

The majority of studies looking at cytokines and their response to exercise have done so through the collection of blood. Nevertheless, cytokines can also be measured in the saliva. However, there are inconsistent findings regarding salivary measures of inflammation. The variability seen in the published results is likely the result of a diverse collection of methodologies and protocols being used across the various studies (Slavish et al., 2015). A systematic review of the studies related to this area is needed; issues to be clarified include: (a) the degree to which inflammatory biomarkers can be reliably determined from saliva, (b) how, and with what time course, do the inflammatory biomarkers react to stress, and (c) what additional research is needed to develop reliable and reproducible methods (Slavish et al., 2015).

2.4.1 Response to Exercise and Other Stressors

Overall, there seems to be disagreement among adult studies about how well salivary cytokines reflect the inflammatory response to exercise in comparison to blood measures. Importantly, there are no studies examining the association between salivary and blood concentrations of cytokines in children, either at rest or in response to exercise. Specifically, only two studies in adults have evaluated the exercise induced effects of inflammation using salivary measures (Table 2). The first study by Usui et al. (2011) had 10 physically active, male participants (mean age 23 ± 3 years) assigned to a one-hour exercise on a stationary bicycle at 75% of VO$_{2}$max, or a control resting condition (random order) on seven different days. Saliva samples were taken immediately before, immediately after, as well as one hour and two hours after task completion. TNF-α levels were significantly raised during the exercise stress session compared to the resting control session, with levels returning to near baseline by 120 minutes post-stressor. The second study by Minetto et al. (2005) evaluated seven male and female “endurance-trained” athletes (mean age 29.5 ± 8.0 years) to a controlled cycling activity (“spinning” exercise task) for 3-hours (7:00-10:00 pm). Ten additional male athletes (two marathon runners, one triathlete, two rowers, two alpine skiers, two bodybuilders, one volleyball player; mean age 27.0 ± 6.9 years) followed a protocol involving a 15-minute cycling warm-up, followed by a 15-minute isokinetic exercise task consisting of 160 maximal knee
contractions on a Cybex 6000 device. Afterward, the responses of serum and salivary IL-6 were examined for the various groups of athletes. There was a significant increase in IL-6 concentration in both saliva and serum, but no correlation was found between the two cytokine sources, possibly reflecting a lack of ‘connection’ between the systemic/muscular and the salivary routes of IL-6 production (Minetto et al., 2005).

Beyond the limited exercise studies, salivary cytokines have been previously used to examine the response to other acute stressors that can influence inflammation (Table 2). Briefly, one study analyzed TNF-α responses of nine male and female university professors (mean age 40.9 ± 2.45 years) after having lectured for 2-hours (a presumptive stressor), compared to a control day without the lecture stressor. Eight unstimulated saliva samples were collected in total: four of them collected on a working day which included the lecture and four control samples on a day without a lecture. Lecturing resulted in notable increases in the concentration of TNF-α, IL-2, and IL-4, but did not affect the IL-10 values. These changes appeared to be related to changes in the concentrations of the stress markers, α-amylase, and cortisol (Filaire et al., 2011). Furthermore, Groer et al. studied 141 male and female police officers (mean age 34.5 years) with three or more years of experience. They examined salivary biomarkers during a critical incident fatal force scenario – baseline and 10- and 30-minutes post-scenario. Officers were randomly assigned to either a two-minute virtual reality motorcycle chase or a six-minute virtual reality workplace gunfight situation. Unfortunately, it was not stated whether the two scenarios were comparably stressful. Overall, the "workplace" scenario produced the most significant responses in biomarkers, such as a notable increase in IL-6 (Groer et al., 2010). In another study, saliva samples were collected from 34 first-year undergraduate students (94% female: aged 20–35 years) at baseline and during three exam periods. Saliva samples were taken two weeks before the first exam period (baseline), and one hour before each exam. The results revealed a gradual increase in IL-6 concentration from the first to the third exam (Lester et al., 2010).
Table 2: Summary of studies examining the immune responses to stressors.

<table>
<thead>
<tr>
<th>Study</th>
<th>Participants &amp; Activity</th>
<th>Sampling timeline</th>
<th>Collection method / assay</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minetto et al., 2005</td>
<td>$N = 7$ male endurance-trained athletes (9.5 ± 8.0 years); $N = 10$ male athletes of different disciplines (27.0±6.9 y) - spinning and isokinetic activity</td>
<td>Spinning: 15 and 5 min pre-, and 0 min post-exercise; Isokinetic: 15 and 5 min pre- and 0, 7, 15, 30, 45, 60, 90, 120 min post-exercise</td>
<td>IL-6: Salivettes; ELISA and immune-radiometric assay</td>
<td>IL-6 not detectable in IMRA method; for the isokinetic test, salivary levels only showed a non-significant increase immediately after the completion of the test; salivary and serum levels were not correlated at any time point</td>
</tr>
<tr>
<td>Groer et al., 2010</td>
<td>$N = 141$ police officers: 114 males, 27 females, aged 22-64 y in a “cycle scenario” (n = 49) vs. a “workplace scenario” (n = 92)</td>
<td>Pre-, and 10- and 30-min post-scenario</td>
<td>IL-6: passive drool; ELISA</td>
<td>IL-6: significant increase at 10 min post-scenario in the workplace group</td>
</tr>
<tr>
<td>Lester et al., 2010</td>
<td>$N = 36$ first year undergraduate students (2 males, 21-22 y; 34 females, 20-35 y)</td>
<td>Baseline between 1230 and 1300h - (two weeks prior to exams); and 60 min prior to each of the three exams, ~3 weeks apart</td>
<td>IL-6: passive drool; ELISA</td>
<td>IL-6: increase from the first to the third test ($p &lt; 0.05$)</td>
</tr>
<tr>
<td>Usui et al., 2011</td>
<td>$N = 10$ men (23±3 y); VO$_2$max = 49.7±4.8 ml/kg/min</td>
<td>Pre-exercise; 0, 60 and 120 min after completion of the exercise or control period</td>
<td>TNF-α: cotton swabs; ELISA kits</td>
<td>TNF-α: effects of time - baseline vs. immediately after ($p &lt; 0.01$) and 60 min after ($p &lt; 0.05$) the completion of the stressor; effect of condition - control vs. stress immediately after the completion of the stressor, ($p &lt; 0.01$); levels peak immediately after the completion of the stressor</td>
</tr>
<tr>
<td>Filaire et al., 2011</td>
<td>$N = 9$ university professors (7 males, 2 females), 12.1 years of experience in teaching</td>
<td>Pre-lecture (10 am), and 0 min, 120 min and 8h post-lecture</td>
<td>TNF-α, IL-10: cotton swab Salivettes; cytometric bead array kits</td>
<td>TNF-α: effect of sampling time - higher concentrations 120 min post-lecture compared with pre-lecture levels; IL-10: no effect for day or time of sampling</td>
</tr>
</tbody>
</table>
2.4.2 Association Between Plasma and Saliva

The correlation between saliva and blood measures of inflammatory cytokines in response to cognitive stress was determined by La Fratta al. (2018). Briefly, saliva and plasma samples were taken from 61 male undergraduate students (age: 22–26 y). The study was performed on two occasions: (a) on a day without an Academic Examination (AE), one week before the AE, and (b) the day of the exam. The correlation between plasma and saliva concentration compared the difference, i.e., Δ value, between the pre- and post-exam values, for each cytokine. There was a significant, positive association between saliva and plasma for IL-1β, IL-18, and IL-6 (Figure 2) for the exam day results, but no significant correlation was found between saliva and plasma for the resting day samples (La Fratta et al., 2018).

There is only one pediatric study (Riis et al.; 2014), which examined and compared the salivary with the serum levels of many cytokines at rest and across three years, in healthy adolescent girls (N = 114, 11-17 years at enrollment). Only resting salivary IL-1β levels were significantly associated with the serum concentrations, while no significant correlations were found between saliva and serum concentrations of IL-6, TNF-α, and other cytokines across the three annual assessments (Riis et al., 2014). In addition, salivary IL-10 levels were below the lower detection limit and was not included in the analysis (Riis et al., 2014). No other study has been found describing the cytokine levels at rest, or in response to stress in children and/or adolescents in terms of saliva and blood samples measurements.

There is significant difference in the way cytokines enter saliva compared to how they are released in blood. The components of saliva can be broadly categorized into two groups – those that enter saliva from plasma and serum, such as hormones, inflammatory markers, drug chemicals, and those that are locally secreted by the salivary glands (e.g., α-amylase, secretory IgA). Plasma components enter saliva though passive diffusion, active transport, ultrafiltration or leakage (Bosch, 2014). Plasma proteins, such as cytokines, protein hormones, or acute phase proteins, are too large to enter saliva via either diffusion or ultra-filtration; therefore, these molecules enter saliva via leaky patches, such as tissue damage sites and inflammation, as well as crevicular fluid. Another route by which such molecules may be moved to saliva is from the oral mucosa, through a form
of passive transport (largely) through osmotic pressure. Such proteins enter saliva via routes that are highly variable, both between and within participants. The efficiency in which they enter saliva is likely determined by local (as opposed to systemic) factors, such as local inflammation, tissue damage, and oral health. That is why data on immune proteins in saliva, like C-reactive protein and cytokines, have to be performed with some caution (Bosch, 2014; Kaufman & Lamster, 2002; Pfaff et al., 2011). In the systemic circulation, cytokines are released by immune cells in response to tissue damage or injury. When there is an injury in a tissue, multiple substances are released by the injured tissues, which causes both neutrophils and macrophages to move toward the chemical source. This phenomenon is known as chemotaxis. There are numerous substances that can lead to chemotaxis in the inflamed area, such as bacterial or viral toxins, degenerative products of the inflamed tissues themselves, complement complex, and some reaction products caused by plasma clotting in the inflamed area (Khonsary, 2017). In turn, activated macrophages produce cytokines, including tumor necrosis factor (TNF), interleukin-1 (IL-1), granulocyte-monocyte colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and monocyte colony-stimulating factor (M-CSF) in the inflamed tissues. This combination of TNF, IL-1, and colony-stimulating factors initiates a feedback mechanism that starts with tissue inflammation and continues to activate a large number of defensive white blood cells that help eliminate the cause of the inflammation (Khonsary, 2017).
Figure 2: Pearson (r) correlations between plasmatic and salivary levels of ΔIL-1β (A), ΔIL-18 (B) and ΔIL-6 (C) in male students (n = 61). Δcytokine = cytokine post-AE minus cytokine pre-AE. Adopted from (La Fratta et al., 2018).
2.5 Sex differences in inflammatory response

According to Crockett et al. (2006), both the innate and adaptive immune responses can be altered by sex hormones, and the response of females is greater than males (Crockett et al., 2006). However, no clear information exists that explains how sex hormones affect the immune system. Casimir et al. (2011) analyzed the expression of inflammatory markers, such as C-reactive protein, erythrocyte sedimentation rate, and neutrophil count underlying inflammatory processes in children under 10 years. They found a significant difference between girls and boys in all variables, with the girls having systematically higher levels than the boys. (Casimir & Duchateau, 2011). Klein & Flanagan (2016) suggested that both the innate and adaptive immune responses are affected by sex and age (Table 3 and 4), and that sex and age also influence cytokine and chemokine production by innate immune cells. Peripheral blood mononuclear cells (PBMCs) from human males were higher in IL-10 production compared with PBMCs from females and was correlated with androgen concentration in males. Also, PBMCs from human males produce more TNF than PBMCs from females. Moreover, male neutrophils express higher levels of Toll-like receptor 4 (TLR4) and produce more TNF than neutrophils from females, both constitutively and following activation with lipopolysaccharide (LPS) (Klein & Flanagan, 2016). The number and activity of cells associated with innate immunity also differ between the sexes. Females have lower natural killer (NK) cell frequencies than males (Abdullah et al., 2012); however, the phagocytic activity of neutrophils and macrophages is higher in females than males (Cannon & St. Pierre, 1997). On the other hand, given some enhanced inflammatory reactions in females (Table 3), cytokine production could be presumed to be higher in females than males. For example, Jenkins and colleagueages showed higher IL-6 in females, especially in the context of estrogen stimulation after a high-isoflavone soy diet, but no notable differences between sexes were observed in C-reactive protein (CRP), serum amyloid A (SAA), or TNF-α (Jenkins et al., 2002). In addition, although estrogen could affect cytokine levels, one study reported that IL-6 levels did not change during the menstrual cycle (Chaffin et al., 2011). In most cases, however, according to Casimir et al. (2013), the cytokine response to inflammation seems to be greater in males than in females across the lifespan (Casimir et al., 2013).
Finally, one pediatric study (Casimir et al., 2010) investigated the influence of sex on cytokines in healthy prepubescent males, females, and Turner syndrome patients with an XO genotype (X monosomy). They reported that the cytokine response to endotoxin and pokeweed mitogen stimulation of whole blood cells was greater in males than in females, suggesting a genetic-chromosomal influence (Casimir et al., 2010). However, in another study, there was no significant differences between boys and girls 3 to 17 years old (Sack et al., 1998).

Table 3: Changes in immune responses in human males and females over the lifespan.

<table>
<thead>
<tr>
<th></th>
<th>In utero</th>
<th>Childhood/Pre-puberty</th>
<th>Adulthood/Post-puberty</th>
<th>Old age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Innate immunity</td>
<td>Increased inflammatory responses in males</td>
<td>↑Inflammation in males</td>
<td>↑Inflammation in females</td>
<td>↑Inflammation in males</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑NK cells in males</td>
<td>↑NK cells in males</td>
<td>↑IL-10 in females</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↑NK cells in females</td>
</tr>
<tr>
<td>Adaptive immunity</td>
<td>Increased IgE levels in males</td>
<td>CD4/CD8 ratios and CD4+ T cell numbers equal</td>
<td>CD4/CD8 ratios and CD4+ T cell numbers ↑</td>
<td>CD4/CD8 ratios and CD4+ T cell numbers ↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD8+ T cell numbers equal</td>
<td>in females</td>
<td>in females</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IgA levels in males ≥ females</td>
<td>CD8+ T cell numbers ↑</td>
<td>CD8+ T cell numbers ↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B cell numbers equal</td>
<td>T cell activation/proliferation ↑</td>
<td>T cell activation/proliferation ↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T_{reg} cell numbers in males ≥ females</td>
<td>in females</td>
<td>in females</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T_{reg} cell numbers ↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>B cells ↑ in females</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>B cells ↑ in females</td>
</tr>
</tbody>
</table>
### Table 4: Summary of sex differences in innate and adaptive immune responses in adults.

<table>
<thead>
<tr>
<th>Immune component</th>
<th>Characteristic</th>
<th>Sex difference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex difference in the innate immune system</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macrophages</td>
<td>TLR4 expression</td>
<td>Higher in males</td>
</tr>
<tr>
<td></td>
<td>Activation</td>
<td>Higher in females</td>
</tr>
<tr>
<td></td>
<td>Phagocytic capacity</td>
<td>Higher in females</td>
</tr>
<tr>
<td></td>
<td>Pro-inflammatory cytokine production</td>
<td>Higher in males</td>
</tr>
<tr>
<td></td>
<td>IL-10 production</td>
<td>Higher in females</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>Phagocytic capacity</td>
<td>Higher in females</td>
</tr>
<tr>
<td></td>
<td>TLR expression</td>
<td>Higher in males</td>
</tr>
<tr>
<td>NK cells</td>
<td>NK cell numbers</td>
<td>Higher in males</td>
</tr>
<tr>
<td><strong>Sex difference in the adaptive immune system</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T cells</td>
<td>CD4 T cell counts</td>
<td>Higher in females</td>
</tr>
<tr>
<td></td>
<td>CD4/CD8 T cell ratio</td>
<td>Higher in females</td>
</tr>
<tr>
<td></td>
<td>CD8 T cell counts</td>
<td>Higher in males</td>
</tr>
<tr>
<td></td>
<td>Number of activated T cells</td>
<td>Higher in females</td>
</tr>
<tr>
<td></td>
<td>T cell proliferation</td>
<td>Greater in females</td>
</tr>
<tr>
<td>B cells</td>
<td>B cell numbers</td>
<td>Higher in females</td>
</tr>
</tbody>
</table>
Chapter 3: Methods

3.1 Participants

Participants in the current study were recruited for a larger study, which was designed to examine the effect of whey protein supplementation during recovery from high-intensity swimming exercise on subsequent swimming performance and muscle damage in adolescent swimmers (McKinlay et al., 2020). Specifically, blood samples from 43 swimmers (21 male and 22 female, 13-17 years of age), which were taken before the protein/carbohydrate supplementation protocol and solely focus on acute responses to intense swimming, were available to be analysed for the purpose of this study (Table 5).

All participants were competitive swimmers training and competing for at least four years before the testing date and were free of injuries, medications, and any medical condition that prevented them from participating. Prior to completing any questionnaires or tests, participants and their parents/legal guardians were provided with oral and written information and a consent/assent form to be completed. The original study was cleared by the Research Ethics Boards of Brock University (REB# 16-279) and the Canadian Sports Institute of Ontario (REB# 2017-01) and has been registered with ClinicalTrials.gov (PRS- NCT04114045). The supplementation protocol was approved by Health Canada’s - Natural and Non-prescription Health Products Directorate (NOA- 229774). The secondary analysis of data was also approved by the Research Ethics Boards of Brock University (REB# 18-296).

Table 5: Participant characteristics (mean ± standard deviation).

<table>
<thead>
<tr>
<th></th>
<th>Male (n=21)</th>
<th>Female (n=22)</th>
<th>Total Cohort (n=43)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>14.1 ± 1.6</td>
<td>13.8 ± 1.7</td>
<td>13.9 ± 1.6</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>167.1 ± 13.1†</td>
<td>160.3 ± 7.7†</td>
<td>163.4 ± 11.0</td>
</tr>
<tr>
<td>Mass (kg)</td>
<td>56.4 ± 15.6</td>
<td>52.9 ± 10.7</td>
<td>54.5 ± 13.1</td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>12.3 ± 5.1†</td>
<td>19.7 ± 5.7†</td>
<td>16.3 ± 6.5</td>
</tr>
<tr>
<td>Somatic Maturity</td>
<td>+0.2 ± 1.6</td>
<td>-0.1 ± 1.4</td>
<td>+0.02 ± 1.5</td>
</tr>
<tr>
<td>(years from age of peak height velocity)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Training Experience (years)</td>
<td>4.6 ± 1.7</td>
<td>4.9 ± 2.5</td>
<td>4.8 ± 1.9</td>
</tr>
<tr>
<td>Training Volume (hours/week)</td>
<td>11.0 ± 2.5</td>
<td>9.7 ± 3.2</td>
<td>10.3 ± 2.9</td>
</tr>
</tbody>
</table>

† denotes significant difference between sexes (p<0.05).
3.2 Study Design

The first visit was a familiarization session, during which participants and their parents/guardians received detailed explanation on the purpose and procedures of the study and signed the informed consent/assent form. Subsequently, participants completed a screening questionnaire to report any injuries, allergies, and/or health related conditions, and had their anthropometric and maturity measurements taken.

The second visit was the testing session. Participants were asked to avoid any physical activity for at least 24 h prior to the testing session. During this session, both blood and saliva samples were collected before, and ~15 min following, an intense multi-task swimming trial as described below. After the first blood and saliva sample, before swimming, participants were provided with a standardized breakfast that included a banana, granola bar, and a juice box (fruit punch or apple flavored).

Figure 3: Timeline of procedures relevant to the purpose of the present study.

3.3 Intense Swimming Trial

The swimming tasks were performed by all participants, in the same order, and followed immediately after the initial blood and saliva sampling and breakfast as illustrated in Figure 3. After a 1000 m warm-up, participants performed a maximal 200m freestyle swim (25 m pool). This test was performed in groups to simulate a swim competition. Following the maximum swim, all swimmers performed a high-intensity interval freestyle
swimming protocol consisting of 5 swims of 100 m, 5 swims of 50 m, and 5 swims of 25 m. All swims in the interval portion of the trial were required to be at 90% of maximal effort, as determined by their 100 m split times, during the 200 m swim performance test. The interval swimming protocol involved a swim to rest ratio of 1:1.

### 3.4 Measurements

#### 3.4.1 Physical Characteristics

Body mass and percent body fat were measured via bioelectric impedance analysis using the InBody 520™ analyzer (InBody520; BioSpace Co, Ltd, Madison, MI, USA) during the familiarization session. Participants were self-hydrated prior to the session and were asked to void their bladder prior to measurement. Participants were required to wipe the bottom of their feet as well as their hands on a clean wet towel before stepping onto the scale to ensure proper conductivity. Seated and standing height were recorded with a stadiometer (SECA-217; CAN), and were used to assess somatic maturity, expressed as years from age of peak height velocity (Mirwald et al., 2002).

#### 3.4.2 Sample Collection and Analysis

Sample collection occurred at two-time points: rest (i.e., pre-exercise, fasted), as well as immediately post-exercise. All the samples were taken in winter and in the early morning hours in order to control for any effect of circadian rhythm. Specifically, saliva and blood samples were collected between 0600 and 0800 hours. Participants were asked to provide a one-milliliter sample of unstimulated whole saliva using oral cotton swabs (Sarstedt salivette cotton swab, Sarstedt Inc, Nümbrecht, Germany). The samples were provided by participants gently chewing on the swab for 60 seconds. After sampling, the swabs were placed directly into storage tubes. This method of saliva collection was chosen based on the study of Hiremath et al. (2015), which compared the difference between passive drool and oral swabs, and found that the oral swab method is more appropriate for analyzing cytokines (Hiremath et al., 2015). After the saliva collection, a total of 10 ml of blood was obtained from an antecubital vein using a standard venipuncture technique. Both blood and saliva samples were immediately centrifuged at 1,405g at 24 °C using a benchtop centrifuge for 15 min and 2 min, respectively. Following
centrifugation, all samples were aliquoted for their given media (saliva or plasma) into Eppendorf tubes and stored at -80 °C until analysis.

Plasma levels of inflammatory cytokines (IL-6, IL-10, and TNF-α) were measured in duplicate, using a total of 8 high-sensitivity Multiplex MAGPIX kits (EMD Millipore Corporation, Billerica, MA, USA - Cat. #HSTCMAG-28SK). However, these kits are designed for serum and have a serum matrix that mimics serum to dilute the standard for the standard curve. Thus, we replaced serum matrix as the diluent for the standard curve with assay diluent from a saliva enzyme linked immunosorbent assay for IL-6 (Salimetrics, State College, PA, USA - Cat. #1-3602) to mimic saliva. The in-house, average inter-assay coefficients of variation for plasma IL-6, IL-10, and TNF-α were 9.2%, 5.7%, and 6.4%, respectively, and the average intra-assay coefficients of variation were 5.8%, 5.4%, and 6.4%, respectively. Saliva samples were analysed using the same Multiplex kits. The in-house average inter-assay coefficients of variation for salivary IL-6, IL-10, and TNF-α were 12.8%, 11.2%, and 6.4%, respectively, and the average intra-assay coefficients of variation were 8.8%, 6.0%, and 8.0%, respectively.

Hematocrit was measured in triplicate by the same investigator using microhematocrit capillary tubes treated with heparin (VWR International, Mississauga, ON). The relative change in plasma volume (%ΔPV) was estimated using the following formula by (Van Beaumont, 1972):

\[
% \Delta PV = \left[ \frac{100}{(100 - HCT_{pre})} \right] \times \frac{100 (HCT_{pre} - HCT_{post})}{HCT_{post}}
\]

where HCT_{pre} is the hematocrit at baseline, and HCT_{post} is the hematocrit post-exercise. The %ΔPV was used to adjust the post-exercise plasma values of the various cytokines prior to statistical analysis. The mean %ΔPV in plasma volume was 2.41 ± 3.2 %.

3.5 Statistical Analysis

All variables were checked for normality using the Kolmogorov-Smirnov test, z-scores for skewness and kurtosis of ±3 and visual screening of histograms for symmetry. The screening showed that IL-6 and TNF-α concentrations in both saliva and plasma were not normally distributed, and were Log transformed for further analysis. Differences between
male and female swimmers in physical characteristics, training history, and baseline cytokine concentrations were analyzed using independent t-tests. The intra-individual variability in the salivary and plasma concentrations of all cytokines, was then determined by the percent coefficients of variation ($\%CV = \frac{\text{standard deviation}}{\text{mean}} \times 100$).

A three-way (group x time x media) repeated measures analysis of variance (RM-ANOVA) was used to examine changes from pre- to post-exercise in the salivary and plasma levels in both male and female swimmers. For this analysis, 3 missing values of IL-6 (2 out of 43 pre-exercise saliva samples and 1 out of 43 post-exercise saliva samples) were replaced with the corresponding group mean value. Specifically, from a total of 172 saliva and plasma samples (43 participants x 2 media x 2 sampling times), three salivary IL-6 values from different participants were not detectable in the analysis. In the event of a significant time-by-media interaction, in absence of a sex effect (i.e., no differences between males and females), further post-hoc pairwise comparisons were performed with male and female data combined using paired t-tests for each media. However, due to the high interindividual variability, especially in salivary concentrations, there was potential for non-uniformity bias when comparing individuals who differ in absolute concentrations at baseline. Thus, we calculated the relative changes ($\%\Delta$) from pre- to post-exercise for each cytokine and used one-way ANOVA to examine differences in the $\%\Delta$ between saliva and plasma in the combined cohort. Finally, two-way random model intraclass correlation coefficient (ICC) analysis was used to assess agreement, and Pearson correlation analysis was used to assess association between salivary and plasma concentrations. Statistical significance was accepted at $p \leq 0.05$. All analyses were conducted using SPSS version 26 for windows (SPSS Inc., USA).
Chapter 4: Results

4.1 Resting Cytokine Concentrations

There were no differences in resting cytokine levels between male and female adolescents, for both saliva and plasma (Table 6). Thus, the resting concentrations of IL-10 were significantly lower and TNF-α were significantly higher in saliva compared with plasma, independent of sex, and there was a much greater variability in the salivary concentrations of all three cytokines as determined by the percent coefficients of variation (Table 6).

Table 6: Resting (pre-exercise) circulating levels of inflammatory cytokines in male (N=21) and female (N=22) adolescent swimmers.
Values presented as mean ± standard error [% coefficient of variation]

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Saliva</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-6 (pg/ml)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>6.05 ± 2.09 [151%]</td>
<td>3.09 ± 0.50 [75%]</td>
</tr>
<tr>
<td>Females</td>
<td>4.52 ± 1.43 [149%]</td>
<td>2.75 ± 0.34 [58%]</td>
</tr>
<tr>
<td>Total Cohort</td>
<td>5.27 ± 1.17# [146%]</td>
<td>2.92 ± 0.30# [67%]</td>
</tr>
<tr>
<td><strong>IL-10 (pg/ml)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>2.46 ± 0.48# [89%]</td>
<td>11.69 ± 1.36# [53%]</td>
</tr>
<tr>
<td>Females</td>
<td>2.73 ± 0.47# [80%]</td>
<td>11.39 ± 1.20# [49%]</td>
</tr>
<tr>
<td>Total Cohort</td>
<td>2.60 ± 0.33# [83%]</td>
<td>11.53 ± 0.89# [51%]</td>
</tr>
<tr>
<td><strong>TNF-α (pg/ml)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>8.63 ± 1.63# [87%]</td>
<td>4.49 ± 0.32# [33%]</td>
</tr>
<tr>
<td>Females</td>
<td>12.55 ± 2.38# [89%]</td>
<td>4.09 ± 0.27# [31%]</td>
</tr>
<tr>
<td>Total Cohort</td>
<td>10.64 ± 1.47# [91%]</td>
<td>4.28 ± 0.21# [32%]</td>
</tr>
</tbody>
</table>

# denotes significant differences between media. IL-6=interleukin 6; IL-10=interleukin 10; TNF-α=tumor necrosis factor alpha.
4.2 Exercise-Induced Changes in Absolute Cytokine Concentrations

There was no significant main effect for sex in response to high intensity swimming for any of the cytokines.

IL-6 showed a significant main effect for media, reflecting higher salivary levels compared to plasma ($F = 4.17$, $p = 0.044$, partial $\eta^2 = 0.05$), but no significant main effect for time, and no significant interaction. Thus, there were no significant changes from pre- to post-exercise in neither saliva nor plasma concentrations (Figure 4).

![Figure 4. Plasma concentrations (mean ± standard error) at rest (pre-exercise) and in response to high intensity exercise (post-exercise) of interleukin 6 (IL-6) in male and female adolescent swimmers (N=43). No main effects of interactions were observed. #denotes significant difference between media ($p \leq 0.05$). *denotes significant change from pre- to post-exercise ($p \leq 0.05$).]
For IL-10, significant main effects were observed for both media ($F = 115.87, p < 0.001$, $partial \eta^2 = 0.59$) and time ($F = 8.98, p = 0.004$, $partial \eta^2 = 0.10$), and a significant time-by-media interaction ($F = 5.48, p < 0.022$, $partial \eta^2 = 0.06$), reflecting significantly lower concentrations in saliva compared with plasma, and an overall increase in plasma concentration of IL-10 from pre- to post-exercise, which was not observed in saliva (Figure 5).

**Figure 5.** Plasma concentrations (mean ± standard error) at rest (pre-exercise) and in response to high intensity exercise (post-exercise) of interleukin 10 (IL-10) in male and female adolescent swimmers (N=43). #denotes significant difference between media ($p \leq 0.05$). *denotes significant change from pre- to post-exercise ($p \leq 0.05$).
There were no significant main effects for media in TNF-α, but there was a significant effect for time ($F=23.65$, $p<0.0001$, partial $\eta^2=0.224$), and a significant time-by-media interaction ($F=21.61$, $p<0.0001$, partial $\eta^2=0.209$), reflecting a significant decrease from pre- to post-exercise in saliva, which was not observed in plasma (Figure 6).

![Graph](image)

**Figure 6.** Plasma concentrations (mean ± standard error) at rest (pre-exercise) and in response to high intensity exercise (post-exercise) of tumor necrosis factor alpha (TNF-α) in male and female adolescent swimmers (N=43). #denotes significant difference between media ($p \leq 0.05$). *denotes significant change from pre- to post-exercise ($p \leq 0.05$).

### 4.3 Relative (%Δ) Changes in Response to Exercise

Since there were no differences between male and female swimmers at rest and no sex effect in response to exercise, differences in the %Δ between saliva and plasma were examined in the combined cohort and presented in **Figure 7**. Specifically, the relative increase (%Δ) from pre- to post-exercise for IL-10 was less pronounced in saliva.
compared with plasma (29% versus 51%, respectively, $p = 0.02$). In contrast, the relative decrease for IL-6 was greater in saliva than in plasma, but this difference was not statistically significant (-21% versus -3%, respectively, $p = 0.06$) while for TNF-α this difference in the exercise-induced response between saliva and plasma was significant (-27% versus -1%, respectively, $p = 0.01$).

**Figure 7.** Percent changes from pre to post-exercise in plasma concentrations of interleukin 6 (IL-6), interleukin 10 (IL-10) and tumor necrosis factor alpha (TNF-α) in male and female adolescent swimmers (N=43). # denotes significant difference between media ($p \leq 0.05$). * denotes significant change from pre- to post-exercise ($p \leq 0.05$).

### 4.4 Correlations between Saliva and Plasma

The intraclass correlation coefficients showed weak or no agreement between resting salivary and plasma cytokine levels (**Figure 8**). IL-10 was the only cytokine with a borderline significant, yet moderate, ICC between saliva and plasma resting levels (**Figure 8B**). However, when all values (i.e., n=86) were considered (pre- and post-swimming combined), the intraclass correlation between salivary and plasma IL-10 was
no longer significant (ICC=0.12, $p=0.27$). The ICC between salivary and plasma IL-6 were not significant at rest (Figure 8A) and did not change considerably with pre- and post-swimming values combined (ICC=0.26, $p=0.09$). Likewise, a weak ICC was found between salivary and plasma concentrations of TNF-α at rest (Figure 8C), which worsened with pre- and post-swimming values combined (ICC=0.05, $p=0.60$).

Figure 8. Relationship between salivary and plasma resting concentrations of: A) interleukin 6 (IL-6), B) interleukin 10 (IL-10) and C) tumor necrosis factor alpha (TNF-α) in male and female adolescent swimmers (N=43).
Finally, Pearson correlation analysis showed no significant association between the resting salivary and plasma concentrations of IL-6 ($r=0.24$, $p=0.13$), IL-10 ($r=0.09$, $p=0.38$) and TNF-α ($r=0.11$, $p=0.47$). These associations did not improve when post-swimming values were included in the analysis, apart from a significant correlation in the relative pre- to post-swimming change of IL-10 ($r=0.33$, $p=0.03$).
Chapter 5: Discussion

5.1 Most Significant Results

To our knowledge, this is the first study to compare the salivary concentrations of pro- (IL-6, TNF-α) and anti-inflammatory (IL-10) cytokines to simultaneously sampled plasma concentrations, at rest and following high-intensity interval exercise, in adolescent athletes, in order to validate a non-invasive method of assessing indices of post-exercise inflammation in youth. Our first hypothesis was that there would be no difference in the concentrations of IL-6, IL-10, and TNF-α between saliva and plasma either, at rest or following intense swimming. However, IL-10 was significantly lower in saliva, while both IL-6 and TNF-α concentrations were significantly higher in saliva compared with plasma. Furthermore, IL-6 did not show a significant time effect or interaction, and although its relative decrease from pre- to post-swimming was more pronounced in saliva than in plasma (-21% versus -3%, respectively), this difference was not statistically significant. In contrast, a significant time-by-media interaction was found in IL-10, which increased significantly from pre- to post-swimming in plasma (+51%), but the magnitude of this response was lower in saliva (29%). A significant time-by-media interaction was also found for TNF-α, which decreased significantly from pre- to post-swimming in saliva, but this response was not evident in plasma (-27% versus -1%). We also hypothesized that the salivary and plasma concentrations of all three cytokines would be significantly correlated, both at rest and in response to exercise. However, this hypothesis was also rejected. Furthermore, the correlation analyses showed weak or no agreement, and no association, between salivary and plasma pro-inflammatory cytokine levels, both at rest and in response to exercise. IL-10 was the only cytokine with a weak, but significant ICC at rest and a significant association between the relative exercise-induced changes measured in saliva versus plasma. Finally, our secondary hypothesis that there would be no sex differences between salivary and plasma cytokine concentrations, either at rest or in response to exercise, was accepted. Indeed, resting concentrations of all cytokines were not different between males and females, nor there was a sex effect in the cytokine responses to exercise.
5.2 Cytokine Concentrations at Rest

One of the primary findings of the present study is that the resting salivary cytokine concentrations were all different than in plasma; IL-6 and TNF-α concentrations were higher in saliva than in plasma, while IL-10 concentrations were lower in saliva than in plasma. There are two possible explanations why the pro-inflammatory cytokine concentrations, such as IL-6 and TNF-α, might be higher in saliva compared with plasma. One explanation relates to the direct influence of central factors on the blood levels, in that both the hypothalamus-pituitary-adrenal (HPA) axis and sympathetic nervous system (SNS) activation lead to elevation in systemic inflammation (Rohleder, 2012). Plasma proteins, such as cytokines, enter saliva via leaky patches. Thus, the saliva concentrations of plasma proteins would be difficult to manage, and would be sensitive to intraindividual variations related to local factors, such as local inflammation, tissue damage and oral health (Bosch, 2014; Byrne et al., 2013; Slavish et al., 2015). The other explanation relates to acute stress directly influencing oral inflammation. Many cell types in the mouth, especially in the salivary glands, are controlled by sympathetic innervation and release inflammatory cytokines locally (Cox et al., 2008; Schapher et al., 2011). It is possible, therefore, that the action of chewing on the cotton swab may have increased the local secretion of pro-inflammatory cytokines. In adults, IL-6 has been also found to be higher in saliva compared with plasma, supporting the suggestion that this cytokine may have been secreted locally into saliva in addition to entering saliva by way of blood (Cox et al., 2008). However, none of these explanations apply to the anti-inflammatory cytokine IL-10, which was significantly lower in saliva compared with plasma. Either IL-10 is not secreted locally, or, since saliva was collected prior to blood, the differences observed may be due to a deference in the timing of the local versus the systemic secretion between pro- and anti-inflammatory cytokines. In any case, salivary cytokine concentrations do not seem to reflect their plasma concentrations.

Furthermore, in agreement with previous studies in adults (Cox et al., 2008; Cullen et al., 2015; Minetto et al., 2007), there were no significant correlations between the salivary and plasma cytokine concentrations, and our ICC showed weak or no agreement between resting salivary and plasma cytokine levels. The only significant ICC was observed in IL-10, but even this was no longer significant when post-swimming
concentrations were considered. Riis et al. (11) also reported no significant correlations between saliva and serum concentrations of IL-6, TNF-α and other cytokines in adolescent girls across three annual assessments. Only one other adult study reported a significant correlation between serum and salivary IL-6 levels in response to stress (La Fratta et al., 2018). This discrepancy in the results could be attributed to the different collection times when it comes to saliva. In our study, we collected saliva by chewing on cotton oral swabs for 1 min, while the adult study had participants chewing on the cotton swab for 4 min. When using cotton oral swabs one must consider the potential ‘cotton-interference’ effect on the salivary IL-6 assay analysis (Minetto et al., 2007), which could explain the lower salivary IL-6 concentrations found with longer chewing time in adults (La Fratta et al., 2018) compared with our salivary IL-6 levels in adolescents. On the other hand, in spite of the potential ‘cotton-interference’, saliva collection using oral swabs appears to offer methodological advantages over the passive drool method and more precise detection and analysis of cytokines related to allergic inflammation in children (Hiremath et al., 2015). Finally, another factor to consider in comparing the exercise-induced cytokine response in saliva versus plasma is the post-exercise decrease in plasma volume, for which we did adjust our post-swimming plasma concentrations, but not the salivary concentrations.

In terms of sex differences, our study agrees with a previous adult study showing no difference in resting, morning, plasma concentrations of IL-6, IL-10 and TNF-α between males and females (Snipe & Costa, 2018). In addition, the resting levels of both plasma IL-6 and TNF-α measured in our group of adolescent swimmers were comparable to those previously measured in non-athletic pre-pubertal boys (Timmons et al., 2004), but higher than those previously reported in adolescent girls (Riis et al. 2014). Furthermore, the IL-10 salivary levels in the Riis et al. (11) study were below the lower limit of detection, so to date, there is no previous data on salivary levels of IL-10 to compare our results.

5.3 Cytokine Concentrations in Response to Exercise

In this study, we found no change in plasma concentrations and a decrease in the salivary concentrations of IL-6 and TNF-α post-swimming, which is in line with a few previous
studies in adults, which also reported a post-exercise decrease in TNF-α (Koh & Park, 2017; Starkie et al., 2005). However, studies in adults typically have found an increase in both the salivary and plasma concentrations of these cytokines in response to acute stress (Minetto et al., 2007; Usui et al., 2011; Cabral-Santos et al., 2016; Nieman et al., 2015; Peake et al., 2008; Rivier et al., 1994; Pereira et al., 2013; Starkie et al., 2001). Likewise, plasma concentrations of IL-6 and IL-10 increased significantly after a marathon race (26-fold and 28-fold, respectively), while there was no change in TNF-α (Nielsen et al., 2016). Although the increase in IL-10 is consistent with our results, there is a clear discrepancy regarding IL-6 and TNF-α. In addition, Minetto et al. (2005) also reported a significant increase in salivary IL-6 concentration immediately after strenuous exercise in endurance-trained male and female athletes, however, they found no association between salivary and serum IL-6 responses to exercise, suggesting a discrepancy between the systemic/muscular and the salivary routes of IL-6 production.

Discrepancies also exist among pediatric studies examining the cytokine responses to exercise, although this commentary relates only to blood measures as there are no studies using saliva samples. For example, two previous studies have reported significant exercise-induced increases in serum IL-1β, IL-6 and TNF-α in both athletic and non-athletic adolescents (Nemet et al., 2002; Rosa et al., 2007). Others, in agreement with our study, found no significant changes in plasma IL-6 and TNF-α from pre- to post-exercise in either pre-pubertal, non-athletic children or adolescent athletes, and they also reported a consistent increase in IL-10 (Sanderson et al., 2020; Timmons et al., 2004). According to these authors, the systemic cytokine response to intense exercise can be as much as 50% lower in children than in adults, suggesting a blunted inflammatory response to vigorous exercise in youth, possibly due to a growth-induced protective mechanism or some type of ceiling effect. Indeed, children have been found to have higher resting plasma concentrations of both IL-6 (Timmons et al., 2004) and TNF-α (Sanderson et al., 2020) than adults. On the other hand, as seen with adults, it is possible that the differences among pediatric studies in reported values and responses to exercise could be associated with variations in exercise mode, intensity and/or duration (Sanderson et al., 2020), or perhaps the differences in values are the result of
methodological variances, such as the process used to adjust for changes in plasma volume (i.e., hemoconcentration effect), or the timing of sample collection.

In this study, the samples were collected at ~15 min following the bout of high-intensity interval swimming, so it is possible that we may have missed the time when the inflammatory markers reach a peak in the systemic response to physical stress. Although systemic levels of inflammatory biomarkers seem to peak around 60-120 min post-stressor and take 120-180 min to return to baseline (Steptoe et al., 2007), the temporal behavior of the salivary response is unclear. Since in our adolescent swimmers, the IL-6 and TNF-α response from pre- to 15 min post-exercise was different between saliva and plasma, it is reasonable to suspect that initial kinetics of the salivary versus the systemic response of these cytokines may differ. This is supported by the fact that the relative pre-to post-swimming changes in the salivary concentrations of both pro-inflammatory cytokines, were not associated with those in plasma. Thus, the post-exercise time of collection should be considered in future research examining post-exercise cytokine levels, especially those of IL-6 and TNF-α, depending on the choice of tissue collected. Ideally, post-stressor samples should include multiple time points (until ~2h after) in order to examine peak response and full recovery to baseline (Filaire et al., 2011; Minetto et al., 2005; Slavish et al., 2015). On the other hand, the relative changes in salivary IL-10 concentrations were significantly correlated with its plasma concentrations. Therefore, salivary IL-10, although lower in absolute levels found in plasma, was the only cytokine that, within this short-time frame, may be representative of post-exercise systemic responses.

5.4 Strengths and Limitations

A strength of the current study is the adjustment of post-exercise plasma volume for changes that occur with exercise, which adds rigour to the experimental design. Another strength is the use of the same assay panels to analyse the salivary and plasma cytokine levels. Often studies have used differing methods to assess biomarker concentrations in saliva and plasma. Other important strengths of this study lie on the strict control of physical activity and nutrition before and during the study (e.g., standardized breakfast), as these factors have been shown to significantly impact cytokine concentrations, at least
in adults (Abedelmalek et al., 2015; Hennigar et al., 2017). Similarly, all samples were obtained during the same time of day (morning) for all participants in the study to avoid any influences of circadian rhythm on our results. However, a significant limitation in the current study is the relatively small sample size, which, although larger than that used in previous studies, still presents a limitation in that there is a limited power to detect significant change.

5.5 Conclusions

Early morning high-intensity exercise resulted in no change in plasma concentrations, a decrease in the saliva concentrations of pro-inflammatory IL-6 and TNF-α, and an increase in the plasma concentrations of the anti-inflammatory IL-10, which was not significant in saliva, in both male and female adolescent swimmers. Consequently, although the direction of the post-exercise response seemed similar between saliva and plasma samples, the differences in the resting concentrations, and in the magnitude of the exercise-induced responses, combined with the weak correlations, suggest that the salivary cytokine measures are not representative of blood levels in young athletes.
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**Appendices**

**Appendix A:** Brock REB #16-279 clearance of original study

**Appendix B:** CSIO #16-279 clearance of original study

**Appendix C:** Brock REB #18-296 clearance of the secondary analysis (latest modification)

**Appendix D:** Invitation letter to participants and parents

**Appendix E:** Information and consent to participate in research – parent

**Appendix F:** Information and assent to participate in research
Bioscience Research Ethics Board

Certificate of Ethics Clearance for Human Participant Research

DATE: 4/11/2017

PRINCIPAL INVESTIGATOR: FALK/KLENTROU, Bareket/Nota - Kinesiology

CO-INVESTIGATOR(S): Heather Sprenger (hsprenger@csiontario.ca)

FILE: 16-279 - FALK/KLENTROU

TYPE: Faculty Research

STUDENT: Brandon/Tony McKinlay/Adeboro

SUPERVISOR:

TITLE: Protein supplementation and exercise recovery following intense exercise in young athletes

ETHICS CLEARANCE GRANTED

Type of Clearance: NEW

Expiry Date: 4/30/2018

The Brock University Bioscience Research Ethics Board has reviewed the above named research proposal and considers the procedures, as described by the applicant, to conform to the University’s ethical standards and the Tri-Council Policy Statement. Clearance granted from 4/11/2017 to 4/30/2018.

The Tri-Council Policy Statement requires that ongoing research be monitored by, at a minimum, an annual report. Should your project extend beyond the expiry date, you are required to submit a Renewal form before 4/30/2018. Continued clearance is contingent on timely submission of reports.

To comply with the Tri-Council Policy Statement, you must also submit a final report upon completion of your project. All report forms can be found on the Research Ethics web page at http://www.brocku.ca/research/policies-and-forms/research-forms

In addition, throughout your research, you must report promptly to the REB:

a) Changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
b) All adverse and/or unanticipated experiences or events that may have real or potential unfavourable implications for participants;
c) New information that may adversely affect the safety of the participants or the conduct of the study;
d) Any changes in your source of funding or new funding to a previously unfunded project.

We wish you success with your research.

Approved:

Sandra Peters, Chair
Bioscience Research Ethics Board

Note: Brock University is accountable for the research carried out in its own jurisdiction or under its auspices and may refuse certain research even though the REB has found it ethically acceptable.

If research participants are in the care of a health facility, at a school, or other institution or community organization, it is the responsibility of the Principal Investigator to ensure that the ethical guidelines and clearance of those facilities or institutions are obtained and filed with the REB prior to the initiation of research at that site.
MEMORANDUM

DATE: March 7th, 2017
TO: Dr. Bareket Falk, Principle Investigator
     Dr. Nota Klentrou, Principle Investigator

REB #: 2016-04 converted to 2017-01
Title: Protein supplementation and exercise recovery following intense exercise in young athletes

The Canadian Sport Institute Research Ethics Board (REB) has reviewed the revisions made to project submission REB #2016-04 and has granted your project approval. Your project has been assigned a new REB #2017-01. Please refer to your project REB number for future correspondence.

If this project changes in any way, you have the explicit responsibility to notify the Lead of Research & Innovation at that time in writing.

Research records must be retained for a minimum of 3 years after completion of the research; if the study involves medical treatment, it is recommended that the results are retained for 5 years.

You are responsible for notifying all parties about the approval of this project, including your co-investigators, PSO/NSO coaches, and management. Please be advised that you will need to submit a progress report every 6 months until the study is completed and a final report outlining the key findings of the study.

Good luck with your research pursuits,

Dr. Samantha Yuristy, PhD
CSIO Research Ethics Board Co-Chair
Certificate of Ethics Clearance for Human Participant Research

DATE: November 11, 2020

PRINCIPAL INVESTIGATOR: FALK/KLENTROU, Bareket/Nota - Kinesiology

FILE: 18-296 - FALK/KLENTROU

TYPE: Faculty Research

STUDENT: Brandon McKinlay

SUPERVISOR: Bareket Falk & Nota Klentrou

TITLE: Protein supplementation and bone markers during exercise recovery following intense exercise in young athletes

ETHICS CLEARANCE GRANTED

Type of Clearance: MODIFICATION  Expiry Date: 3/1/2021

The Brock University Health Science Research Ethics Board has reviewed the above named research proposal and considers the procedures, as described by the applicant, to conform to the University’s ethical standards and the Tri-Council Policy Statement.

Modification: Addition of Malcolm Sanderson, Abrisham Beigpoor, Debbie O’Leary, and Mike Plyle; comparison of pre- and post-exercise saliva and plasma samples of inflammatory cytokines; comparison of inflammatory cytokines and irisin between the adolescent and the young adult swimmers (both related to the original study REB #16-279).

The Tri-Council Policy Statement requires that ongoing research be monitored by, at a minimum, an annual report. Should your project extend beyond the expiry date, you are required to submit a Renewal form before 3/1/2021. Continued clearance is contingent on timely submission of reports.

To comply with the Tri-Council Policy Statement, you must also submit a final report upon completion of your project. All report forms can be found on the Office of Research Ethics web page at https://brocku.ca/research-at-brock/office-of-research-services/research-ethics-office/#application-forms

In addition, throughout your research, you must report promptly to the REB:
   a) Changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
   b) All adverse and/or unanticipated experiences or events that may have real or potential unfavourable implications for participants;
   c) New information that may adversely affect the safety of the participants or the conduct of the study;
   d) Any changes in your source of funding or new funding to a previously unfunded project.

We wish you success with your research.

Approved:

Craig Tokunoo, Chair
Health Science Research Ethics Board

Note: Brock University is accountable for the research carried out in its own jurisdiction or under its auspices and may refuse certain research even though the REB has found it ethically acceptable. If research participants are in the care of a health facility, at a school, or other institution or community organization, it is the responsibility of the Principal Investigator to ensure that the ethical guidelines and clearance of those facilities or institutions are obtained and filed with the REB prior to the initiation of research at that site.
Invitation Letter: Protein supplementation and exercise performance

Principal Investigators: Dr. Bareket Falk and Dr. Nota Klentrou Department of Kinesiology, Brock University and Dr. Heather Sprenger, Department of Sports Physiology, Canadian Sport Institute Ontario

Co-Investigators: Tony Adebero and Alexandros Theocharidis MSc Student, Department of Kinesiology, Brock University

Principal Student Investigator: Brandon McKinlay, PhD Student, Department of Kinesiology, Brock University

Your son or daughter is invited to participate in a research study, investigating the effects of protein supplementation on recovery and performance in young athletes.

The purpose of this research project is to determine whether protein consumption following an intensive exercise session improves recovery, as indicated by exercise performance as well as indicators of inflammation.

All testing and measurements will take place at Brock University unless specified otherwise. Testing will occur over four testing sessions, for a total time commitment of 9-10 hours (day 1 – 30-45 min, day 2 – 7 hrs, day 3 30-45min and day 4 2hr 30min) At the end of the study, your child will be given a summary of the findings, upon request.

What will happen to you if your son or daughter is in the study?

If you agree to volunteer for this study, you will:

1. Visit Brock University unless specified otherwise, where we will do some tests and take some measurements which are described below. The tests will require a total of three visits to Brock University.
2. Your child will be asked complete several questionnaires, outlining your medical and training history, as well as what you eat on a regular basis. In all questionnaires, you or your child may choose not to answer any question without penalty.
3. We will measure your child’s height, weight and body fat percentage.
4. Your child’s muscular power will be tested using several maximal pulls on a swim bench. It will also involve sport specific performance testing in the form of a 200m free-swim sprint.
5. Your child will be asked to provide us with some saliva (spit) and a blood sample taken from your arm. We will use this information to determine exactly how hard your child worked during the exercise session, as well examine the genomic and epigenetic response. More specifically, white blood cells (leukocytes) will be examined for changes in gene expression resulting from exercise and supplementation. The saliva (spit) sample will be collected using specifically designed swabs (cotton-balls). Your child will be asked to chew these swabs and then deposit them back into a collection tube. The blood will be collected using specially designed needles. Once the arm has been
‘pricked’, a small amount of blood will be collected and placed into the special tubes. Your child may experience some discomfort during the blood sample but not for long. If you want, you may ask for the special cream (e.g. EMLA) that takes the pain away.

Participation in this project will allow your child to have personal information on his/her muscular power, as well as other information, such as height, weight, current nutritional status, and body composition. All this information will be provided to you in the form of an individualized participant feedback sheet.

If you are interested in finding out more about this study, please contact us by email (brandon.mckinlay@brocku.ca, bfalk@brocku.ca or nklentrou@brocku.ca) or by phone (905-688-5550 ext: 4979,5623 or 4538).

The study has been reviewed and received ethics clearance though the REB (file #16-279). If you have any questions about your rights as a research participant, please contact the Brock University Research Ethics Officer (905 688-5550 ext 3035, reb@brocku.ca).

Thank you,

Investigators:

Dr. Bareket Falk, Dr. Nota Klentrou, Dr. Heather Sprenger, Brandon McKinlay, Tony Adebero and Alexandros Theocharidis

Faculty of Applied Health Science, Brock University and CSIO

Tel: 905-688-5550 ext: 4979, 5623 or 4538 Email: bfalk@brocku.ca, nklentrou@brocku.ca or brandon.mckinlay@brocku.ca
INFORMATION AND CONSENT TO PARTICIPATE IN RESEARCH - PARENT

Protein supplementation and exercise performance

You and your child are invited to participate in a research study being conducted by the investigators listed below. Prior to participating in this study please read this form to find out about the purpose and the tests of this study. This study is part of the Faculty of Applied Health Sciences of Brock University as well as the Canadian Sport Institute Ontario.

INVESTIGATORS: DEPARTMENT: CONTACT:
Dr. Bareket Falk FAHS, Brock University bfalk@brocku.ca
Dr. Nota Klentrou FAHS, Brock University nklentrou@brocku.ca
Dr. Heather Sprenger Canadian Sports Institute hsprenger@csiontario.ca
Brandon McKinlay FAHS, Brock University brandon.mckinlay@brocku.ca
Tony Adebero FAHS, Brock University tony.adebero@brocku.ca
Alexandros Theocharidis FAHS, Brock University Alexandros.theocharidis@brocku.ca

PURPOSE:
The purpose of the study is to investigate the effects of protein supplementation on recovery and performance in young athletes. Specifically, we are interested in the effect of supplemental whey protein following an intensive exercise session, to determine if protein improves recovery as indicated by performance as well as indicators of hormonal changes or inflammation.

DESCRIPTION OF TESTING PROCEDURES:
If your child agrees to volunteer for this study, he/she will visit the pool at which he/she trains, over four testing sessions, for a total time commitment of up to 9-10 hours (day 1 – 30-45 min, day 2 – 7 hrs, day 3 – 30-45min and day 4 – 2.5hrs). At the end of the study, he/she will be given a summary of the findings, upon request. Your child should come prepared to exercise in athletic shoes, shorts/a short sleeved shirt, as well as swimsuit apparel.

Please note that participation in this study will not affect the status of your child within the swim team/academy.

Your child will undergo the measurements and procedures listed below; please note that in all questionnaires, your child may choose not to answer any question, EXCEPT in the medical history questionnaire, where all questions MUST be answered to ensure participant eligibility and safety. Your child may also choose not to participate in any procedure listed below.

A. Assessments:
1. Your child will complete several questionnaires, outlining his/her training history and current/on-going nutritional status. In all questionnaires, your child may choose not to answer any question without penalty.

2. Your child **MUST** complete a medical history questionnaire to assess your child’s safety and eligibility for this study.

3. Body Composition: we will measure your child’s height, weight and body fat percentage. Body fat percentage will be estimated using skinfold thicknesses.

4. Heart rate monitors will be used during exercise. Your child will be connected to a radio-transmitter monitor (chest strap). The procedure involves no discomfort.

5. The measurement of your child’s muscular power will be tested using several maximal pulls on a swim bench. It will also involve sport specific performance testing in the form of a 200m free-swim sprint. Performance testing will be repeated before, immediately following, 2, 6 and 24hrs after the fatigue protocol.

6. A saliva and a venous blood sample will be collected to determine hormonal and inflammatory changes, as well genomic and epigenetic response. More specifically, white blood cells (leukocytes) will be examined for changes in gene expression resulting from exercise and supplementation. A total of seven samples will be collected to assess hormonal and inflammation response, as follows: Five samples will be collected in one of the visits: pre-exercise, 5 min post exercise, 2, 6, and 24 hours post-exercise. On a separate visit, two samples will be collected, two hours apart. The saliva samples will be collected using specifically designed swabs, these swabs will be chewed and deposited into a collection tube. Venous blood will be drawn using a standard venipuncture technique by a certified Life Lab technician. Up to 20 ml of blood will be withdrawn at each time. It should be noted that the venous blood drawing procedure is a routine procedure performed by a certified technician and offers minimal risk to participants. In rare instances, participants may experience slight pain and/or tingling in the area and/or a minor bruise from the needle. However, with the use of anaesthetic creams (e.g., EMLA), which we use in the laboratory, any sensation of pain is minimal. Additionally, blood draws will be performed by a trained phlebotomist, using standardized operative procedures to ensure sanitary cleanliness during all blood collections, thereby minimizing the likelihood of any adverse infections.

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**B. Exercise Protocol:**

Your child will undergo a swim-specific exercise session. This session will be administered and supervised by the principal student investigator (a registered kinesiologist) and other members of the research team. Each child will perform a series of high-intensity swimming intervals (5 x 100m, 5 x 50m, 5 x 25m). This procedure may result in some muscle soreness within 24 hours of the test. If this occurs, it will only be temporary.

**C. Beverage Supplementation:**
Your child will be assigned to a group which will be required to consume either a protein supplement or placebo immediately following the exercise protocol (33% chance of being in either protein, carbohydrate, or water group). The protein is NSF certified and the quantities administered fall within safe guidelines for protein ingestion in children. The protein supplement or placebo will be mixed with water before consumption. The current study will be performed utilizing double blind procedures meaning both the administering investigator and participant will be unaware of their drink’s contents (protein or placebo).

**List of Ingredients:**

**Protein** – Whey protein isolate (milk), Cocoa processed with alkali, Natural flavors, Sunflower lecithin, Stevia extract.

**Carbohydrate** – Maltodextrin, Chocolate flavoring

**Water** – Water, Chocolate flavoring

**Visit Summary:**

<table>
<thead>
<tr>
<th>Visits</th>
<th>Events</th>
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| 1- Familiarization Session (60 – 90 mins) | -Informed consent form  
- Medical history and screening questionnaire  
- Emergency contact information form  
- Training questionnaire  
- Anthropometry  
- Familiarization with tests and procedures  
**PERFORMED AT SWIM TEAMS FACILITY** |
| 2- Exercise protocol and protein supplementation (8-hrs) | -Saliva/blood collection (baseline)  
- Breakfast  
- Performance tests: maximal strength and 200m free (before, after, 2hr and 6hr following the fatiguing protocol) and Swim-bench  
- Fatiguing protocol (5x100m, 5x50m, 5x 25m / 1:1 work to rest ratio)  
- Protein, Carbohydrate or water ingestion (immediately following and 2hrs min post fatigue protocol)  
- Food frequency questionnaire (FFQ)  
- Lunch  
- Tour and presentations at Brock university  
- Saliva/blood collection (pre, post, 2hr, 6hr following fatigue protocol) |
| 3- 24 hrs post fatiguing protocol and protein supplementation (30-45 mins) | -Saliva/blood sampling  
- Muscle soreness questionnaire  
- Performance Testing- 200m free and swim bench |
| 4- 0 and 2hr resting sample collection | -Saliva/blood sampling |

**CONFIDENTIALITY:**
All data collected during this study will remain confidential and will be stored in offices and on secured computers to which the principal and co-investigators have access. Furthermore, auditor(s), regulatory ethics board (REB), Health Canada and other regulatory authority(ies) will be granted direct access to the study participant’s original screening questionnaire for verification of clinical trial procedures and/or data. You should also be aware that the results of this study will be made available to scientists, through publication in a scientific journal but your child’s name and any personal data will not appear in compiling or publishing these results. The name of the swimming organization may appear in the report. Data will be kept for 25 years after the date of publication, at which time all information will be destroyed. Additionally, you will have access to your child’s data, as well as group data when it becomes available and if you are interested. This can be provided to you by simply contacting the principal investigator. The records identifying the study participants will be kept confidential for 25 years. If the results of the study are published, study participant’s identity will not be revealed. Study participants will not be provided with any legal rights by participating in the trial and they do not release the investigators from liability for negligence.

**PARTICIPATION AND WITHDRAWAL/DISCONTINUATION:**
You and your child can choose whether to participate in this study or not and may withdraw or remove your child’s data from the study, by simply telling one of the investigators. In case you or your child chooses to withdraw from the study by telling the investigator, you will be asked whether his/her data can still be used for analysis. In case your child withdraws by not showing up, partial data will be used. Your child may also refuse to answer any questions posed to him during the study and remain as a participant in the study. The investigators reserve the right to withdraw your child from the study if they believe that it is necessary.

You child should not feel obligated to participate in the study and his/her decision to participate or not participate or withdraw from participation will in no way impact the standing of your child within the swimming team/academy.

**REASONS FOR PARTICIPANT WITHDRAWL:**
- Clinical reasons – A participant becomes ill during study duration inhibiting their ability to perform tasks maximally.
- Protocol violation – A participant violates clearly outlined guidelines by obtaining any supplements/medication and non-pharmacological therapies (physiotherapy, acupuncture, massage) that may affect performance prior to or during the study.
- Poor compliance – Failure of participant to attend all protocol required sessions.
- Serious or adverse events – Circumstances beyond control of participants resulting in poor compliance.
- Voluntary withdrawal – Participant or parent guardian no longer wishes to participate within study.

**COMPENSATION:**
You and your child will be compensated $20 total (gift card to a local store, pro-rated in case participants do not complete the study) for travel expenses, parking and time specific to the testing sessions. High school students will also be receiving 20 volunteer hours for their time spend in the study.

**RISKS AND BENEFITS:**
Participation will allow you and your child to become exposed to a research protocol, potentially improve your child’s physical performance, contribute to the advancement of science, and gain personal and general knowledge about your child’s fitness and nutrition.

The only foreseeable risks involved in participation include:

a) Possible muscle soreness within 24 hours of training or testing. If this occurs, it will only be temporary.

b) With any participation in physical activity there is a risk of injury. Therefore, at each testing session there will be one or more team members who have certified first aid training, as well as a registered kinesiologist.

c) In rare instances, participants may experience slight pain and/or tingling in the area and/or a minor bruise from the venous blood draw. Children and youth are also at a risk of fainting at the sight of or introduction of a needle. However, for the younger participants a butterfly needle will be used that does not look like a typical needle. Both the nurse and researcher will show the younger participants the butterfly needle and explain the procedure. Participants who are not allergic to medications may choose to use an aesthetic cream that usually has no side effects. However, there is a small risk for minor effects such as burning, swelling, itching, or skin rash at application site.

d) Like all clinical trials, there may be unknown risk with taking the investigational natural health product (whey protein).

**NOTE:** In the unlikely event that participants should experience an adverse event because of the procedures outlined above, they should **IMMEDIATELY** contact one of the members of the research team to report adverse event(s). If the adverse event is life-threatening, participant should attend the closet emergency room.

**FEEDBACK and STUDY RESULTS:**
Your child’s and group results will be provided to you upon request. If any results outside the norm appear during data collection, you and your child will be informed within one month.

**FUTURE RESEARCH**
At the completion of this study, we may wish to use the data for a future study that might or might not be directly related to the current research. All records identifying your child will remain confidential and information about your child will not be released. If the results of such a study are published, your child’s identity will remain confidential. An appropriate research ethics board would clear any future research with the current data.

**Check this box if you consent for us to retain your data for use in future work** □
RIGHTS OF RESEARCH PARTICIPANTS:
Study participants will receive a signed copy of this consent form. The study has been reviewed and received ethics clearance though the REB (file #2017-01). If you have any pertinent questions about your rights as a research participant, please contact the CSIO Research Ethics Board or the Brock University Research Ethics Officer REB (file #16-279) (905 688-5550 ext 3035, reb@brocku.ca). Study participants will not be giving any legal rights by participating in the trial, and they do not release the investigators from liability or negligence.

INFORMATION:
Please contact Dr. Bareket Falk at 905 688-5550 (ext. 4979), bfalk@brocku.ca, Dr. Nota Klentrou at 905 688-5550 (ext. 4538), nklentrou@brocku.ca or Brandon McKinlay at 905 688-5550 (ext. 5623), brandon.mckinlay@brocku.ca if you have any questions about the study.

I HAVE READ AND UNDERSTAND THE ABOVE EXPLANATION OF THE PURPOSE AND PROCEDURES OF THE PROJECT. I HAVE ALSO RECEIVED A SIGNED COPY OF THE INFORMATION AND CONSENT FORM. MY QUESTIONS HAVE BEEN ANSWERED TO MY SATISFACTION AND I AGREE TO PARTICIPATE IN THIS STUDY.

SIGNATURE OF PARENT/GUARDIAN

______________________________  ______________________________

DATE

PRINTED NAME OF PARTICIPANT

______________________________

In my judgment the participant is voluntarily and knowingly giving informed consent and possesses the legal capacity to give informed consent and participate in this research study.

SIGNATURE OF INVESTIGATOR

______________________________  ______________________________

DATE

______________________________
INFORMATION AND ASSENT TO PARTICIPATE IN RESEARCH

Protein supplementation and exercise performance

Hello,

You are being invited to take part in a research study by Brock University, in partnership with the Canadian Sports Institute Ontario (CSIO). Before deciding if you want to take part please read this form to find out about the study.

Why are we doing this study?

We are doing this study to investigate how protein supplementation affects recovery after a tiring session of exercise in young athletes. Specifically, we are interested in how protein immediately following the exercise session, will affect performance and inflammation.

What will happen to you if you are in the study?

If you agree to volunteer for this study, you will:

1. Visit the pool in which you train, where we will do some tests and take some measurements which are described below. The tests will require a total of four visits to the pool.
2. You will be asked complete several questionnaires, outlining your medical and training history, as well as what you eat on a regular basis. In all questionnaires, you may choose not to answer any question without penalty.
3. We will measure your height, weight and body fat percentage. Body fat percentage will be estimated using skinfold thicknesses.
4. We will measure how powerful your muscles are by asking you to perform maximal (all out) arm pulls on a swim bench and ask you to show us how fast you are during a timed 200m free-swim race.
5. You will be asked to provide us with some saliva (spit) and a blood sample taken from your arm. We will use this information to determine exactly how hard you worked during the exercise session. The saliva (spit) sample will be collected using specifically designed swabs (cotton-balls), you will be asked to chew these swabs and then deposit them back into a collection tube. The blood will be collected using specially designed needles. Once the arm has been ‘pricked’, a small amount of blood (20 ml) will be collected and placed into the special tubes. You may experience some discomfort during the blood sample but not for long. If you want, you may ask for the special cream (e.g. EMLA) that takes the pain away.

What kind of exercise will you do?

The exercise session will be approximately 45-60 min in length. Within this session you will perform swimming intervals. You may (or may not) experience some muscle soreness in the days following the exercise, but this is nothing to worry about and is only temporary (24-72hrs).
**Do you have to participate?**

No. It will help our study if you volunteer but you don’t have to participate. Whether you participate in this study or not, your decision will not affect your position or how people treat you within your swim team/academy.

If you decide to participate now and then later change your mind, you can tell the researchers that you don’t want to be in the study anymore. Again, this decision will not affect your position or how people treat you within your swim team/academy.

If there are any tests that you do not want to do or questions that you do not want to answer, you can tell the researcher and you won’t have to do them.

We want you to feel comfortable so remember that you can ask the researchers questions at any time.

**Who will know that you are in the study?**

We will write papers about what we find and share the information with other researchers but when we talk about your measurements and how you did, we will not use your name.

**Will you get paid for participating?**

You and your parents will be given a $20 gift card to a local store (or less if you do not finish the entire study). If you are a high school student, you will also be receiving 20 volunteer hours for their time spend in the study.

**Are there good things and bad things about being in the study?**

By taking part in this study, you will get to see how we do research and contribute to science. You may learn new exercises to use and learn about your current fitness and nutrition status.

There are some possible risks that we want you to know about:

a) Possible muscle soreness within 24-72 hours of training or testing. If this occurs, it will only be temporary.

b) With any participation in physical activity there is a risk of injury. Therefore, at each testing session there will be one or more team members who have certified first aid training, as well as a registered kinesiologist.

c) Some point tenderness in the blood draw site, if this occurs, it will only be temporary.

**What if you have questions about the study?**
Please contact Dr. Bareket Falk at 905 688-5550 (ext. 4979), bfalk@brocku.ca, Dr. Nota Klentrou at 905 688-5550 (ext. 4538), nklentrou@brocku.ca or Brandon McKinlay at 905 688-5550 (ext. 5623), brandon.mckinlay@brocku.ca if you have any questions about the study.

**FUTURE RESEARCH**

After this study is completed, we may wish to use the data for a future study that might or might not be directly related to this study. Personal information about you will not be released.

Check this box if you consent for us to keep your results for use in future work  □

If you want to part of the study, please print your name below.

__________________________________________  __________________________
SIGNATURE OF PARTICIPANT                  DATE

__________________________________________
PRINTED NAME OF PARTICIPANT

In my judgment the participant is voluntarily and knowingly giving informed consent to participate in this research study.

__________________________________________  __________________________
SIGNATURE OF INVESTIGATOR                  DATE