BLOOD BIOMARKER MONITORING IN PROFESSIONAL SOCCER: LONGITUDINAL ANALYSIS OF INFLAMMATION IN ENGLISH PREMIER LEAGUE PLAYERS

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

GLOSSARY OF TERMS

AA, arachidonic acid

AA:EPA, Arachidonic acid:Eicosapentaenoic acid

AIFAI, Anti-Inflammatory Fatty Acid Index

ARH, alterations in redox homeostasis

AST, aspartate aminotransferase

AV, analytical variation

BMI, body mass index

BV, biological variation

CDV, critical difference value

CK, creatine kinase

COX, cyclooxygenase

CP, creatine phosphate

CRP, C-Reactive Protein

CV, coefficient of variation

CVa, analytical variation,

CVw, within subject variation

DGLA, Dihomo-γ-linolenic acid

DHA, docosahexaenoic acid

DOMS, delayed onset of muscle soreness

EIMD, Exercise-induced muscle damage

EM, Expectation-Maximisation

EMFA, erythrocyte membrane fatty acids

EPA, eicosapentaenoic acid

EPL, English Premier League

FS, fish oil supplementation

GSH: GSSG, reduced glutathione: oxidized glutathione

IL, Interleukin

LDH, lactate dehydrogenase

LIST, Loughborough Intermittent Shuttle Test

LME, linear mixed effect models

LOX, lipoxygenase

LTs, leukotrienes

m, metres

M1, classically activated pro-inflammatory monocytes

M2, alternatively activated anti-inflammatory monocytes

NADPH, nicotinamide adenine dinucleotide phosphate

NF-κB, nuclear factor-kappa B

NRF2, nuclear factor erythroid 2 related factor 2

OM3I, Omega-3 Index

OS, oxidative stress

PGs, prostaglandins

PMNs, polymorphonuclear cells

POC, point of care

PPAR-g, peroxisome proliferator activated receptor

RONS, reactive oxygen species and nitrogen species

RBE, repeated bout effect

RPE, rate of perceived exertion

SD, standard deviation

SPMs, specialized pro-resolving mediators

TAC, total anti-oxidant capacity

TD, total distance

THIR, Total high intensity running

TNF-α, Tumour Necrosis Factor alpha

TXs, thromboxanes

URS, upper respiratory symptoms

URTI, upper respiratory tract infection

WADA, World Anti-Doping Agency

DECLARATION

I declare that the present thesis is a record of my own work and has been completed by myself with full acknowledgement to the individual and institute in which assisted in the research. I have not obtained a degree in NUI Galway, or elsewhere, on the basis of the work described in the thesis.

Diarmuid Daniels

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ABSTRACT

The high intensity demands of elite soccer predispose players to recurrent episodes of acute inflammation, and during periods of frequent match play recovery time may not be adequate for the resolution of the inflammatory response. Given the link between chronic inflammation and a host of sport specific medical problems, the monitoring of blood biomarkers of inflammation in athletes may be a medical and performance objective for protecting player health, recovery and adaptation. However, longitudinal studies examining biomarkers of inflammation in the English Premier League are absent from the literature. Therefore, the primary aim of this thesis was to examine inflammation in English Premier League players. The secondary aim was to investigate strategies which may reduce inflammation during the competitive season when the ability to recover in sufficient time may be compromised.

The successful application of blood biomarker monitoring programs in elite sport is not only hindered by the need for more frequent testing but also by an understanding of meaningful changes in biomarker data. Therefore, the aim of the first study (Chapter 4) was to; Part 1: investigate the level of agreement between the standard laboratory method and a point of care test for C-reactive protein obtained from thirty five professional soccer players with physiological concentrations below 5 mg/L, over the course of 3 English Premier League seasons; Part 2 : calculate the analytical variation for the point of care measurement of C-reactive protein in well trained participants (duplicate samples) (n=10); Part 3: To calculate the biological variation and critical difference value for the point of care measurement of C-reactive protein in well trained participants who had capillary samples taken every morning for 5 consecutive days (n=8). The average difference between the two methods was 0.27 mg/L with the standard deviation of 0.115 mg/L and limits of agreement of -1.7 mg/L to 2.24 mg/L. Repeatability of the point of care assay was 5.26%, biological variation was 5.03% and the critical difference value was 20.1%. We report that the use of a point of care test for C-reactive protein in well-trained individuals is practical, but not interchangeable with the standard laboratory method, and the critical difference value reported here may be

used to enhance interpretation of meaningful changes in C-reactive protein in well trained individuals.

The aim of the second study (Chapter 5) was to examine blood biomarkers of inflammation and oxidative stress simultaneously with workload and wellness data in response to weekly match play in 22 players across the first half of an English Premier League season. We report that a competitive English Premier League game induces time-dependent changes in circulating markers of inflammation and oxidative stress, pre-match inflammation status predicts post-match inflammatory response, and pro-oxidant status significantly increase across the first half of an English Premier League season. The point of care measurement of inflammation was found to be highly sensitive in detecting subjective feelings of fatigue, muscle soreness and the presence of illness symptoms. No significant relationships were observed between match workload and biomarker responses. Our data suggests that the pre-game may be an important window for practitioners to reduce inflammation, since it is unlikely they can influence player match selection and that the POC measurement of C-reactive protein in conjunction with wellness data may offer an objective tool for identifying fatigue and illness risk in professional soccer.

The aim of the third study (Chapter 6) was to investigate the effect of curcumin supplementation on biomarkers of inflammation and oxidative stress in response to weekly match play during an EPL season. We used an interrupted time-series design to analyse the effect of curcumin supplementation on biomarkers of oxidative stress and inflammation in healthy players. Following a seven-game control period, participants completed a four-game supplementation block, followed by a second control period of two games and another supplementation block of five games. We report that curcumin ingestion had no significant effect on inflammation, and had borderline significant effects on pro-oxidant responses. However, the magnitude of this change does not appear to be physiologically relevant, suggesting that at a group level curcumin ingestion using the dose and protocol prescribed may not be effective for attenuating inflammation and pro-oxidant status within the real world, applied, field setting of elite

soccer. We therefore question the efficacy of curcumin supplementation in elite soccer players following their habitual diets.

The kinetics and resolution of inflammation may be influenced by the nutritional status of the athlete, and measuring the composition of the erythrocyte membrane fatty acids and examining its association with inflammation could clarify the role of fatty acids in the development of low-grade inflammation in soccer players. Therefore, the fourth aim of the thesis (Chapter 7) was to examine, for the first time, concurrent seasonal alterations in erythrocyte membrane fatty acid composition and inflammatory markers and determine whether inflammatory status is associated with certain fatty acid parameters in professional soccer players (n=35) over the course of 3 seasons. We report significantly higher mean inflammation values, in the winter period compared to the pre-season. Moreover, time-dependent changes were also observed for omega-6 and omega-3 fatty acid variables, with the former decreasing and the latter increasing from the pre-season. We report a significant negative association between C-reactive protein and the omega-3 Index, and a significant positive association between C-reactive protein and the omega-6:omega-3 ratio, suggesting that fatty acid status is a significant driver of low-grade inflammation in professional soccer players. Together, we present convincing evidence that the erythrocyte membrane fatty acid composition influences the changes in inflammation observed across time in the elite soccer player.

Large between subject variability in biomarkers mean that individualised approaches to biomarker monitoring may be of higher potential value in protecting player health or detecting an under-recovered state. Therefore, the fifth and final aim of the thesis (Chapter 8) was to explore the application of individualized adaptive reference ranges in professional soccer players competing in the English Premier League using point of care tests for C-reactive protein and hydroperoxides biomarkers. Secondly, we aimed to investigate whether the observed changes in blood as a result of curcumin supplementation are greater than the previously reported critical difference value for the aforementioned biomarkers and therefore of physiological significance for each player. Our study confirms that athletes deemed “healthy” or “non-healthy” present markedly

different within- and between-subject variations in biomarkers values, which has a significant impact on their respective individualized ranges. Physiologically relevant changes in C-Reactive Protein levels were observed for only 3 athletes in the study group in response to the intervention, with no athletes displaying substantial decreases in hydroperoxides. The individualized approach presented here may be used to identify athletes that require recovery or clinical review, however, the results of this study highlight that human interpretation of is still required in order to intuitively observe trends in biomarker data. Future work investigating this individualised approach to longitudinal biomarker monitoring are required in elite soccer.

The results of this thesis have shown that the point of care measurement of C-reactive protein provides a minimally invasive assessment that is sensitive to the changes in subjective feelings of wellness and illness and may assist in protecting player health and availability for team selection. We also show that the pre-match (i.e. game day -1) and the winter period of the English Premier League may be important windows to reduce inflammation. Furthermore, our findings provide rationale for augmenting the erythrocyte membrane fatty acid composition as a potential “pro-resolving” strategy during periods of limited recovery. We present an individualized approach to biomarker monitoring which may help guide the practitioner’s interpretation of individual biomarker data, however, future studies are warranted in order to transform this promising strategy into an efficient monitoring tool in a real world setting.

Keywords: Inflammation, C-reactive protein (CRP), oxidative stress (OS), hydroperoxides (HPX), English Premier League (EPL), workload, wellness, curcumin, omega-3, adaptive ranges.

CHAPTER 1: GENERAL INTRODUCTION

Chapter 1

1.1. Background

The English Premier League (EPL) is known as one of the most physically demanding leagues in soccer. Total high-intensity running (THIR) has been found to be 10–15% higher in the EPL (Bradley et al., 2009) than in the Danish (Mohr et al., 2003) and Swedish Premier Leagues (Andersson et al., 2008). In addition to the amount of distance covered at high intensity, the absolute number of explosive sprints and maximal running speeds have been found to be greater in the EPL than in previous studies conducted on players competing in the European Champions League and Spanish La Liga (Bush et al., 2015). Interestingly, the physical demands of match play for elite soccer players in the EPL have increased substantially in recent years. Bush et al. (2015) identified high-intensity running and sprint distances have increased by 30–50% across seven seasons in the EPL (2006-07 to 2012-13), however, notably, these substantial increases in high speed running do not appear to be attributed to improvements in the players' physical capacity (Barnes et al., 2014). Therefore, if the physical fitness of EPL players has remained stable across time then these findings suggest players are working at a higher proportion of their physical capacity during elite match play, which highlights the increasing importance of player recovery. The monitoring of the recovery status of the professional soccer player may therefore be an important objective of sports medicine and sports science staff.

The high intensity actions of elite soccer impose distinctive physiological and mechanical loading demands on players (Harper et al., 2019). The performance of concentric (e.g. accelerations/maximal sprinting), isometric (e.g. shielding the ball against offensive pressure) and particularly eccentric (e.g. jumping, landing and rapid deceleration movements) muscle contractions during the high velocity and technical actions of the game lead to various homeostatic perturbations including metabolic (i.e. muscle glycogen depletion), mechanical (i.e. muscle damage) and oxidative stress (i.e. production of reactive oxygen and nitrogen species) (Silva et al., 2018). These homeostatic perturbations stimulate the release of a number of inflammatory mediators, which act as "cellular messengers", activating signalling pathways and in turn regulating

the molecular machinery controlling inflammatory gene expression (Peake et al., 2015). Acute inflammation is a vital protective process, acting to clear damaged tissue and promote repair, however, overwhelming inflammation can result in secondary damage and promote maladaptive tissue remodelling (Markworth et al., 2016). Indeed, the rate of deterioration and recovery of performance and muscle soreness post-exercise is largely dependent on the magnitude of muscle damage and inflammation (Fatouros et al., 2016). Moreover, from a clinical perspective, chronic inflammation is a unifying feature of several disease states. Whilst these are not relevant to young healthy athletes, there is increasing attention on the long-term health of athletes as some sports are known to be strongly associated with disease continuums either during or post career (Lincoln et al, 2018). Inflammation has been described in the pathogenesis of heart disease in American Football players (McCarthy et al., 2016) and is a key mediator in the long term recovery from traumatic brain injury (Kokiko-Cochran et al., 2018), which has been suggested to be a potential mechanism for lifelong depression (Guskiewicz et al., 2007). This in itself provides rationale for future research to understand healthy vs. damaging levels of inflammation in elite athletes and long-term studies are warranted to define the relative contribution of inflammation in the pathogenesis of disease in athletes and the effects of repeated inflammatory episodes on career longevity and on post-career health.

Although, traditionally utilized for diagnostic or anti-doping purposes, blood biomarkers may provide an objective means of assessing recovery status (Hecksteden et al., 2017). The measurement of blood biomarkers of inflammation may be used to make inferences about the athletes underlying physiology and could provide the high performance practitioner with beneficial information to promote recovery and protect athlete health in the interval between successive matches. Little is known as to how inflammation fluctuates in response to match play and throughout a season in the EPL. Indeed, most studies investigating inflammatory disturbances induced by a competitive game have done so in response to a single match, which does not account for the overlap of acute and chronic changes in biomarker values (Carling et al., 2018), and there is little contextual information (i.e. workload and wellness data) surrounding these biomarker

responses in the literature. The challenges of frequently collecting and transporting samples to a laboratory mean that longitudinal studies examining biomarkers of inflammation in athletes are rare. In addition to the logistical burden of data collection and analysis, the challenges associated with player and staff buy-in, not to mention the schedule of the athlete including match preparation, travel, competition, media duties and often variable rest days may realistically reduce opportunities for monitoring in the professional setting (Carling et al., 2018). The point of care (POC) measurement of a capillary blood biomarker of inflammation may represent a practical solution to such problems for medical and performance staff. The simple application, small sample volume, and rapid result reporting mean that there are a number of practical advantages to POC testing which support its utility in the elite high performance environment. However, whilst convenience may be an important factor to the successful application of blood monitoring in elite athletes (Pedlar et al., 2019), considerations must be made regarding the validity and test-retest reliability of the POC device, the intra- and inter-individual variability between players, and the real-world meaningfulness of the biomarker data (i.e. does the biomarker provide actionable data or serve as a useful positive or negative outcome indicator) (Pedlar et al., 2019). Investigations into the practical application of a POC device for monitoring inflammation in the professional setting of the EPL are warranted.

Within a season, professional teams often compete up to three times a week, particularly during the winter period in the EPL, and the recovery time between successive games may not be adequate for the restoration of normal homeostasis and the resolution of the inflammatory response (Owen et al., 2019; Mohr et al., 2016). Indeed, although elite players are well adapted to their sport, the long season with consistent exposure to acute inflammation and limited periods of rest, places the soccer player at high risk of under recovery, potentially leading to the development of low grade inflammation. The long-term consequences of a lack of recovery may leave many players in an energy depleted state, both emotionally and physically, and could result in an increased risk for injury (Ekstrand et al., 2004). Reducing inflammation during periods of frequent match play

may therefore be an important objective for sports science and medicine staff in order to protect player health and recovery.

A recent UEFA consensus statement emphasised the importance of optimal nutrition and its role in optimising performance, facilitating post-exercise recovery and maintaining general health of the elite soccer player throughout the season (Collins et al., 2020). Moreover, there is increasing attention in the literature on nutritional supplementation strategies which aim to ameliorate the signs and symptoms related to exercise induced muscle damage and inflammation beyond the fundamentals of good hydration and macronutrient intake (Bongiovanni et al., 2020). Indeed, the targeted use of specific anti-inflammatory and antioxidant compounds (e.g. omega-3 fatty acids and polyphenols) may be advantageous during periods where facilitating recovery and hence, the ability to compete in subsequent competition is the priority (as opposed to adaptation) (Owens et al., 2019; Bell et al., 2014). Curcumin is a polyphenol found in the spice turmeric, and is often used to reduce exercise-induced inflammation (Sahin et al., 2016; Fernandez-Lazaro et al., 2020). Supplementation with oral curcumin may be beneficial for athletes participating in high-intensity exercise with a significant eccentric load (Heaton et al., 2017), however, data in elite soccer players are absent from the literature and warrant investigation. Moreover, the kinetics and resolution of inflammation may be influenced by the fatty acid status of the athlete (Calder, 2006; Calder, 2012). Measuring the composition of the erythrocyte membrane fatty acids (EMFA) and examining its association with inflammation could clarify the role of fatty acids in the development of low-grade inflammation in soccer players. The overall aim of this thesis is to longitudinally examine inflammation in professional soccer players competing in the EPL. A secondary aim is to investigate the efficacy of nutritional strategies which may be used to reduce inflammation and accelerate player recovery during the competitive season. These aims will be addressed by completion of the following objectives over the course of 5 sequential experimental chapters as described below:

1. To assess the precision of a POC test for C-Reactive Protein (CRP) against the gold standard method and quantify the biological variation (BV) and analytical variation (AV) in order to create an individual critical difference value (CDV) for CRP (Chapter 4).

2. To examine longitudinal alterations in blood biomarkers of inflammation and oxidative stress in conjunction with workload and wellness data in response to weekly match play during an EPL season (Chapter 5).

3. To investigate the efficacy of curcumin supplementation on biomarkers of inflammation and oxidative stress during an EPL season (Chapter 6).

4. To investigate the seasonal variation in blood biomarkers of inflammation and erythrocyte membrane fatty acids and to examine the strength of their association (Chapter 7).

5. To explore the application of individualized adaptive reference ranges and the individualized examination of redox and inflammation status in response to curcumin supplementation in professional soccer players competing in the EPL (Chapter 8).

CHAPTER 2: LITERATURE REVIEW

Chapter 2

2.1. Physiological Demands of Soccer

2.1.1. Physical Demands

The demands of elite football have increased in recent years (Bush et al,, 2015). Whilst, team success is multifactorial, technical indicators (e.g. ball possession, pass completion rates and number of shots) have been found to predict team success more accurately than physical indicators (Bush et al., 2015). However, physical performances can impact technical proficiency and may therefore be an important contributor to overall performance (Rampinini et al., 2008). Time–motion analysis is useful for examining the activity pattern and physical aspects of soccer (Mohr et al., 2003). The intermittent nature of soccer means that players are exposed to many episodes of high intensity running, interspersed with periods of low intensity running, over the course of a 90 minute game. It has been reported that THIR is a more sensitive indicator of performance than total distance (TD) covered as it correlates strongly with physical capacity (Bush et al, 2015). Indeed, top-class soccer players have been shown to perform more THIR during a game (28 and 58% more THIR and sprinting, respectively) and display greater performance during an intermittent recovery test than moderate professional players (Mohr et al, 2003). Furthermore, within elite soccer, different leagues display marked variations in workload data. The EPL is known as one of the most physically demanding leagues in soccer. THIR has been found to be 10–15% higher in the EPL (Bradley et al., 2009) than in the Danish (Mohr et al., 2003) and Swedish Premier Leagues (Andersson et al., 2008). In addition to the amount of distance covered at high intensity, the absolute number of explosive sprints and maximal running speeds have been found to be greater in the EPL than in previous studies conducted on players competing in the European Champions League and Spanish La Liga (Bush et al., 2015). Therefore, due to the intermittent nature of soccer, these findings indicate that soccer at the highest standard is characterized by the players' ability to perform high-intensity work repeatedly.

There are a number of factors which will influence the amount of THIR performed within a game. Findings suggest that activity profiles vary among playing positions. Bradley et al. (2009) analysed 28 EPL games during the 2005–2006 competitive season. Their results showed that high-intensity running and total distance covered were higher for wide (3138 and 11,535 metres (m)) and central midfielders (2825 and 11,450 m) than for full-backs (2605 and 10,710 m), attackers (2341 and 10,314 m), and central defenders (1834 and 9885 m). They also observed that wide midfielders, full-backs, and attackers cover a greater distance when sprinting (346, 287, and 264 m, respectively) than central midfielders (204 m) and central defenders (152 m). Given the evidence regarding positional differences, match activity profiles would appear to be influenced by the technical and tactical requirements of the game (Gregson et al., 2010). Indeed, changes in the tactical role of players are likely to lead to marked variability in high speed activity during competition (Di Salvo et al., 2007; Drust et al., 2007). Although it is difficult to discuss the impact of playing formations on physical performance as limited studies exist, the growing popularity of the EPL has brought with it an influx of new managers with unique tactical approaches, with some teams adopting an apparently less direct playing style (Barnes et al., 2014). For example, Tierney et al. (2016) reported that wide midfielders performed 20% more high-intensity decelerations when playing in a 3–4–3 compared to a 4–4–2 formation. Moreover, the compact 4-2-3-1 system, often requires wide midfielders to attract defenders inside and create space for full backs to move into, thus introducing more players into attacking positions, which in turn may result in greater high-intensity running demands for the modern full back (Bush et al., 2015). Furthermore, performance during match-play has been shown to be related to the physical activity completed by the opponent teams. Rampinini et al. (2007) found that the TD and THIR of the reference team was influenced by the activity profile of the opponent teams (the TD and THIR was higher against best opponent teams compared to worst opponent teams). Therefore, it appears that the physical output of elite soccer players may be influenced by changes in tactics/playing systems of opposing teams as well as their own.

Match-to-match variability in performance characteristics of elite soccer players is high (Gregson et al., 2010). Gregson et al. (2010) showed that match-to- match variability was generally high across all variables with a mean coefficient of variation (CV) of 16.2% and 30.8% reported for THIR and TD during a game. Furthermore, seasonal stage is another important consideration when analysing the physical demands of the elite soccer player. Mohr et al. (2003) found that match performance varied throughout the season and peaked at the end of the season (The CV in THIR was 24.8% between different stages of the season). Factors that explain this increase in match-related physical performance may be changes in fixture frequency, at the end of the season, rarely more than one match played a week at the end of the season, thus potentially increasing the amount of fitness training performed. Additionally, the cumulative effect of consistent competitive match play may have led to specific adaptations and improved fitness levels. Together, these findings highlight the limitations of using a single observation of high-speed activity within a game to characterize an individual's physical capacity and emphasize the importance of collecting repeated measurements when analysing the physiological demands of the elite soccer player.

2.1.2. Energy Demands

The activity profile of elite soccer means that there are extensive aerobic and anaerobic demands during periods of a game which lead to major metabolic changes (Bangsbo et al., 2007). The aerobic energy system is highly taxed during elite soccer, with average and peak heart rates of around 85 and 98% of maximal values, respectively (Krustrup et al., 2006), suggesting that blood flow and oxygen delivery to the exercising muscle is continuously high during a game (Bangsbo et al., 2006). The observation that elite soccer players perform 150-250 brief intense actions during a game, indicates that the rate of anaerobic energy turnover is high (Bangsbo et al., 2006). These intense actions lead to a high rate of creatine phosphate (CP) breakdown, which is supported by findings of reduced muscle CP levels (Krustrup et al., 2006). Furthermore, blood lactate concentrations of 2-14 mM have been observed during match play (Bangsbo et al., 1991; Reilly, 1997; Krustrup et al., 2006) suggesting that the rate of glycolysis is frequently high during a game. Muscle glycogen is the predominant carbohydrate source

during moderate to intense exercise (Egan et al., 2013), and is therefore an important substrate for the soccer player. However, evidence of a progressive depletion in muscle glycogen stores during match play, suggests that lipid oxidation is also important, particularly as exercise duration increases. Catecholamine concentrations are progressively elevated during a match (Bangsbo, 1994), stimulating a high rate of lipolysis and thus the release of free fatty acids into the blood (Bangsbo et al., 2006). Indeed, Krustrup et al. (2006) showed blood glucose was maintained at elevated levels throughout a match, whereas blood free-fatty acids increase progressively, particularly during the second half. These alterations in substrate utilization may cause higher uptake and oxidation of free-fatty acids by the contracting muscles and may partly compensate for the progressive lowering of muscle glycogen (MacLaren and Morton, 2011). The reduction in glycogenolysis with repeated sprints during match play suggests that aerobic metabolism becomes more important as the game progresses. Hence, it is essential for soccer players to have well developed aerobic systems in order to ensure high rates of oxygen uptake, delivery and utilization by the exercising muscles over the course of a game (MacLaren and Morton, 2011).

2.1.3. Recovery in Soccer

The physical demands of match play for elite soccer players in the EPL have increased substantially in recent years. Bush et al. (2015) identified THIR and sprint distances have increased by 30–50% across seven seasons in the EPL (2006-07 to 2012-13). The magnitude of these changes observed by Bush et al. (2015) were greater than the inherent match-to-match variability previously reported by Gregson et al. (2010), suggesting that the elevation in THIR is likely due to tactical modifications, as outlined above, as opposed to natural variability (Collins et al., 2020). Interestingly, these substantial increases in THIR do not appear to be attributed to improvements in the players' physical capacity. Indeed, Bush et al. (2015) indicate that the average distance covered by elite soccer players during the Yo-Yo Intermittent Endurance Level 2 Test increased minimally across a similar time period. Therefore, if the physical fitness of EPL players has remained stable across time then these findings suggest players are working at a higher proportion of their physical capacity during elite match play (Barnes

et al., 2014). Indeed, data suggests that players become progressively fatigued towards the end of a game and exhibit temporary fatigue after the most intense periods of a game (Mohr et al., 2003; Krustrup et al., 2006; Bradley et al., 2009; Harper et al., 2019), as evidenced by decrements in THIR and the frequency of high intensity accelerations and decelerations, which ultimately could prove decisive in critical match-play actions (Harper et al., 2019). The cause of the temporary and permanent states of fatigue are likely to be multifactorial, as both central and peripheral factors are suggested to act in unison to induce match related fatigue (Rampinini et al., 2011). Nevertheless, the high intensity actions of elite soccer impose distinctive physiological and mechanical loading demands on players, leaving players vulnerable to muscle damage (Harper et al., 2019), which in turn may exacerbate post-match residual fatigue (Silva et al., 2018).

Exercise-induced muscle damage (EIMD) can be briefly summarized into two phases; the initial phase or primary damage that occurs as a consequence of the mechanical work performed, and a secondary inflammatory response (Owens et al., 2019). The inflammatory response is characterized by the accumulation and infiltration of immune cells into the damaged tissue (Fatouros et al., 2016). Acute inflammation is a vital protective process, acting to clear damaged tissue and promote repair, however, overwhelming inflammation can result in secondary damage and promote maladaptive tissue remodelling (Markworth et al., 2016). The consequences of EIMD and inflammation for the elite soccer player is an increase in muscle swelling, stiffness, soreness and a temporary decrease in muscle function (Owens et al., 2019; Bongiovanni et al., 2020). Moreover, the rate of deterioration and recovery of performance and muscle soreness post-exercise is largely dependent on the magnitude of muscle damage and inflammation (Fatouros et al., 2016). Therefore, the inflammatory response to exercise appears to affect the time needed for optimal recovery between competition, and given the long season and intensive fixture schedule of the EPL, understanding inflammation in the EPL players may be of paramount importance for protecting player health and recovery.

The next section of the review will review the pertinent literature describing exercise induced inflammation, from its initiation to its resolution.

2.2. Exercise Induced Inflammation

The precise stress mechanisms responsible for the initiation of muscle damage and inflammation during elite soccer match play are still unknown, however, these could be metabolic and mechanical in nature, and may occur separately or together (Pyne, 1994). In this regard, the physiological and mechanical demands of soccer fit within the metabolic and mechanical stress models of EIMD (Silva et al., 2018). The performance of concentric, isometric and particularly eccentric muscle contractions during the high velocity (e.g. accelerations/decelerations/maximal sprinting) and technical actions (e.g. control of the ball against offensive pressure) of the game, results in significant mechanical loading, leading to structural disturbances (Silva et al., 2018). Moreover, the high metabolic demand associated with match play leads to a very large decrement in muscle glycogen stores, which plays a role in sustaining high-intensity running performance and is implicated in the magnitude of post-exercise inflammatory disturbance (Fischer, 2006; Silva et al., 2018). The acute physiological changes associated with these metabolic and mechanical demands are sources of reactive oxygen species and nitrogen species (RONS) production that can favour increased oxidative stress (OS), and modulate key players involved in the inflammatory process such as pro-inflammatory cytokines (Ji, 2015).

The local response to muscle damage involves the production of cytokines (Ostrowski et al., 1999). Cytokines are small polypeptides which have immunoregulatory roles (Peterson and Pederson, 2005). Indeed, the term cytokine is derived from the Greek root meaning ‘‘to put cells into motion’’ (Peterson et al., 2005). The cytokine Interleukin (IL)-6 is produced in the largest quantity during exercise, by many cell types and has several roles, however, Pederson et al. (2011) found that contracting muscles contributes to most of the IL-6 present in the circulation in response to exercise, resulting in the naming of IL-6 as a “myokine”. Several mechanisms link muscle contractions to IL-6

synthesis. The homeostatic perturbations that occur during exercise, as described above, activate transcription factors which regulate IL-6 gene transcription, namely nuclear factor kappa B (NF-κB) (Fischer, 2006). Once synthesized, myokines work in a hormone-like fashion (Pederson et al., 2011). IL-6 may act locally via paracrine mechanisms, regulating carbohydrate metabolism in skeletal muscle, or IL-6 released into circulation may induce systemic effects (Fischer, 2006; Pederson et al., 2011; Egan et al., 2016).

The systemic response involves the production of acute phase proteins (Peterson et al., 2005). Increased circulating cytokines act on hepatocytes to stimulate the production of the acute phase reactant CRP, which is widely used as a systemic marker of inflammation (Ispirlidis et al., 2008). It is secreted by the liver in response to a variety of inflammatory cytokines, but predominantly under transcriptional control by IL-6 (Pepys et al., 2003). While in a resting or non-inflammatory state, CRP is released slowly from the endoplasmic reticulum of the hepatocytes, but following an increase in inflammatory cytokine levels, CRP is first synthesized as monomers and then assembled into its pentamer shape and secretion occurs more rapidly (Sproston and Ashworth, 2018). Upon secretion, several roles have been postulated for CRP. It is thought to act as a surveillance molecule, binding to phospholipids of damaged cells, and as an opsonin, thereby coating microbes and promoting the binding of phagocytes by activating and regulating the complement system (Du Clos, 2000).

Cytokines also act to stimulate the immune cells to infiltrate the damaged tissue and carry out specific roles (Owens et al., 2019). In the first 4-24 hours post injury, neutrophils are the dominant immune cell infiltrating the site of injury (Owens et al., 2019), after which macrophages predominate (Tidball, 2005; Butterfield et al., 2006). Localization of neutrophils to the damaged area is required for removal of damaged tissue and cellular debris through phagocytosis (Butterfield et al., 2006), however, neutrophils can release RONS during phagocytosis, in a process known as the ‘‘respiratory burst’’, which may exacerbate existing muscle damage (Toumi et al., 2003) (**Figure 2.1**). Importantly, studies suggest that a relationship exists between neutrophil

infiltration and degree of damage to stretch injured skeletal muscle (Toumi et al., 2003). On the other hand, Macrophages appear to perform multiple functions at various times in the inflammatory cascade dependent on the phenotype in question, however, it is generally thought that macrophages do not contribute to membrane disruption during the inflammatory process but may instead facilitate muscle recovery and adaptation (Butterfield et al., 2006; Owens et al., 2019).

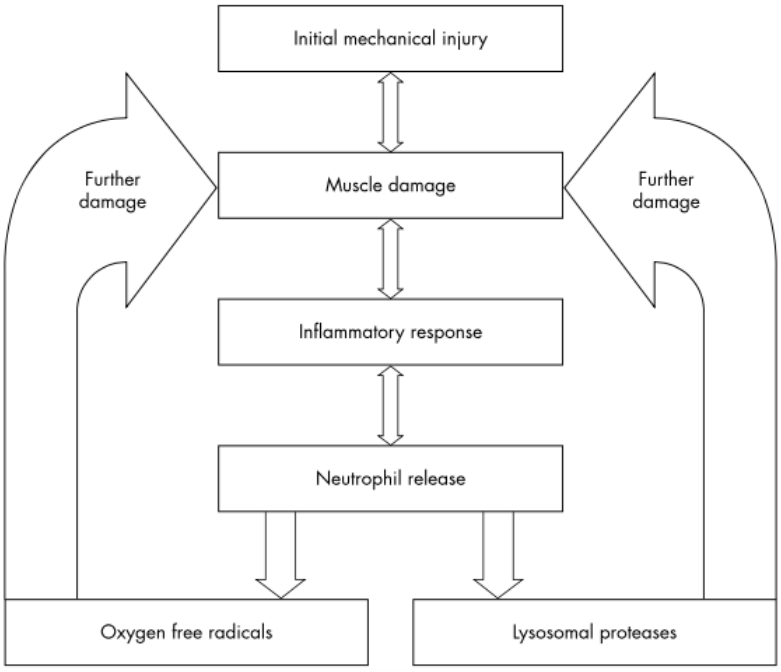

Figure 2.1. Proposed relationship between the inflammatory response to mechanical injury and further neutrophil mediated muscle damage (Toumi et al., 2003).

In the early stages following muscle injury, circulating classically activated pro-inflammatory monocytes (M1) infiltrate muscle and phagocytose polymorphonuclear cells (PMNs). This process triggers a switch to an alternatively activated anti-inflammatory phenotype (M2) (Markworth et al., 2016), which act as the predominant phenotype during the latter stages of inflammation and play an important role in facilitating recovery and regeneration. Indeed, systemic depletion of monocytes has been reported to delay resolution of muscle inflammation (Markworth et al., 2016). Resolution can be defined as the re-establishment of normal homeostasis and involves the termination of pro-inflammatory signalling pathways, the clearance of inflammatory

cells, cellular repair and the restoration of normal tissue function (Lawrence et al., 2010). If all of these pathways are followed then acute inflammation will resolve without causing excessive tissue damage (Lawrence et al., 2007), however, a failure of these mechanisms may lead to the development of chronic non-resolving inflammation (Lawrence et al., 2010).

The next section of the review will discuss the pertinent literature describing potential mediators of inflammation in elite soccer, with special emphasis given to oxidative stress and its role in the inflammatory response.

2.3. Mediators of Inflammation

2.3.1. Mechanical Stress

Soccer players perform a number of explosive types of movement that incorporate a strong eccentric component, such as sprinting, jumping, and rapid deceleration movements (whereby hamstrings act eccentrically to decelerate hip flexion and knee extension during running's landing phase) (Fatouros et al., 2010). Eccentric contractions involve fewer innervated motor units for a given work load when compared to concentric and isometric contractions, placing greater mechanical stress on a smaller number of muscle fibres (Enoka et al., 2016). Furthermore, the preferential recruitment of faster motor units during lengthening contractions suggests fast twitch fibres specifically are damaged (Owens et al., 2019), and damage may be exacerbated when eccentric exercise is performed at unaccustomed muscle length, force, velocity and overall volume (Fatouros et al., 2016; Bongiovanni et al., 2020).

The initial event after the eccentric contraction disrupts the contractile and non-contractile apparatus, which is followed by membrane damage and subsequent excitation-contraction coupling dysfunction (Owens et al., 2019). The popping sarcomere hypothesis states that stretch induced muscle damage results when active muscle is stretched beyond optimum length, at which point, some sarcomeres experience "popping", and increase tension on passive structures characterized by Z-line

streaming (evident for 1–3 days following eccentric exercise depending on the magnitude of the exercise stimulus) (Morgan et al., 2004; Fatouros et al., 2016). Moreover, it has been shown that stretch induced damage is also associated with deterioration of other structural (e.g. desmin and dystrophin) and myofibrillar proteins (e.g. myosin) (Fatouros et al., 2016). Repeated lengthening contractions further compromises weaker sarcomeres, and injury of muscle fibres is exacerbated causing a collapse of the membrane surrounding the sarcoplasmic reticulum, transverse tubules and the muscle fibres themselves (Fatouros et al., 2016). This in turn leads to an increase in membrane permeability and leaking of muscle proteins into circulation (Pyne, 1994). The increased concentration of cytosolic proteins (e.g. lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and creatine kinase(CK)), therefore reflects a significant change in the structure and permeability of the myofibrillar membrane (Pyne, 1994).

Increases in blood biomarkers of muscle damage have been shown to be related to exercise duration (Becatti et al., 2017; Devrnja et al., 2018), intensity (Devrnja et al., 2018), number of sprints performed during a game (Thorpe et al., 2012), and rate of perceived exertion (RPE) (Copalle et al., 2019) in elite soccer players. Ispirlidis et al. (2008) reported peak CK activity, occurred at 48 hours post-game, and was as high as 950 U/L, but notably within the reference ranges previously defined for male soccer players 83-1492 U/L (Mougios, 2007). Paulsen et al. (2012) describe that CK levels >1, 000 U/L may be associated with moderate performance decline and an intense inflammatory response. Indeed, the cascade of events which compromises the process of excitation–contraction coupling described above results in the release of calcium ions from the sarcoplasmic reticulum into the cytoplasm, known as the calcium overload phase (Pyne, 1994), and represents a key step responsible for the initiation of the subsequent inflammatory cascade.

2.3.2. Metabolic Stress

The inability to perform high intensity actions during the second half of a game may be explained by depletion of muscle glycogen, especially in type II fibres (MacLaren and

Morton, 2011). Some investigations have observed that muscle glycogen decreases to levels below the required value to maintain maximal glycolytic rate (Krustrup et al., 2006). Krustrup et al. (2006) observed that when pre-match muscle glycogen levels were low, muscle glycogen stores were almost depleted at half time, whereas when players started the game with normal muscle glycogen levels, values remained high at half time, but depleted at the end of the game. Indeed, analysis of individual fibres in soccer players revealed that 50% of individual fibres were classified as empty at the end of a game (Krustrup et al., 2006), suggesting that depletion of glycogen in some fibres may influence a players ability to produce maximal effort in single and repeated sprints. This is supported by evidence that elevating muscle glycogen stores before prolonged intermittent exercise using a carbohydrate diet elevates performance during such exercise (Balsom et al., 1999), which together emphasises the importance of effective fuelling strategies by the high performance staff in order to limit the magnitude of muscle glycogen depletion during match play.

Evidence suggests that exercising with low carbohydrate availability will increase inflammatory cytokine release (Robson, 2003). The greater duration of the soccer game leads to a greater depletion of muscle glycogen and it has been shown that the transcription rate of the IL-6 gene and the rate of IL-6 secretion from skeletal muscle may be exacerbated when exercising in a glycogen depleted state (Keller et al., 2001). Interestingly, the rapid increase of IL-6 towards the end of exercise, may be partly explained by IL-6 enhancing its own transcription (Keller et al., 2001) which may contribute to the peak in IL-6 found immediately post game (Ispirlidis et al., 2008). Carbohydrate loading has been shown to diminish the IL-6 response (Nehlson-Cannarella et al., 1997). However, to the authors knowledge, only one study has investigated the effect of carbohydrate supplementation on the inflammatory response from soccer specific activity, albeit in amateur athletes (Naclerio et al., 2015). The authors found no significant differences between treatment groups on inflammatory markers, which they hypothesize was due to the intermittent sprint protocol not being challenging enough to significantly deplete muscle glycogen. Nonetheless, it is noteworthy that the recovery of muscle glycogen after a game has been shown to display

a slow recovery phase. Indeed, glycogen synthesis is impaired in the presence of muscle damage (Costill et al., 1990), and recent findings suggest that muscle glycogen content in type II fibres may not be completely restored 48 hours after a match (Gunnarsson et al., 2013). Together, these findings are likely problematic for the recovery of the elite soccer player during congested fixture schedules as a combination of limited recovery time and suboptimal glycogen re-synthesis patterns as a result of muscle damaging exercise, may result in excessive cytokine release and contribute to the development of an inflammatory state.

2.3.3. Oxidative Stress

Oxidative stress has been defined as "a disturbance in the pro-oxidant anti-oxidant balance in favour of the former"(Sies, 1985), and the acute physiological changes associated with match-related activity patterns may favour pro-oxidant alterations and increase markers of oxidative stress (OS). The performance of eccentric contractions inducing muscle damage, inflammation and superoxide production via neutrophil oxidative burst generated by the enzyme nicotinamide adenine dinucleotide phosphate (NADPH)- oxidase; Ischemia-reperfusion events associated with power-related actions (e.g. accelerations and isometric contractions to shield the ball), augmenting xanthine oxidase free- radical-generation system activity; The excessive trauma that occurs during impacts with the ground (e.g. foot strike haemolysis) and opponents, causing the autoxidation of iron-containing proteins; and increased oxygen consumption during the game, leading to superoxide production via the mitochondrial electron transport chain; are all sources of RONS production related to the metabolic and mechanical demands of elite match play that can favour increased OS (Bloomer et al., 2009; Silva et al., 2018). Other sources of reactive species production during exercise include peroxynitrate formation through the reaction of nitric oxide with superoxide and the metabolic changes that occur during exercise such as the increased release of catecholamines, increased hydrogen ions and elevations in core temperature (Powers et al., 2008; Lewis et al., 2015a).

Direct assessment of reactive species is possible via electron spin resonance spectroscopy involving spin traps (Fisher-Wellman and Bloomer, 2009). However, whilst the short half-life of these species makes them excellent signalling molecules (Ho et al., 2013), it also means direct measurement is difficult to employ, expensive and laboratory intensive (Fisher-Wellman and Bloomer, 2009). Direct measurements are key in greater understanding the mechanisms underpinning how reactive species exert their effects in response to exercise, however, they are not practical in the elite sport setting. Instead, much of the focus in the applied literature has been on measuring indirect markers in the circulation that may reflect systemic OS or as Lewis et al. (2015a) aptly described "biological footprints of oxidative damage downstream of the site of RONS production." Blood-based OS biomarkers can be classified as molecules that are modified by interactions with RONS such as DNA, lipids, and proteins (Ho et al., 2013). These indirect markers may be used to make inferences about OS before and after the exercise insult and by examining these markers it may be possible to draw some conclusion about extent of OS as a result of the exercise insult, or perhaps provide evidence of an adaptation process (Margaritelis et al., 2018), ultimately, informing athlete management decisions.

The acute response of redox biomarkers to match-play has been investigated in elite male soccer players measuring the concentration of markers of lipid peroxidation, protein oxidation, plasma total anti-oxidant status, plasma concentration of specific endogenous non-enzymatic anti-oxidant molecules and endogenous enzymatic anti-oxidant molecules (Ispirlidis et al., 2008; Ascensao et al., 2008; Fatouros et al., 2010; Silva et al., 2013; Silva et al., 2018; Souglis et al., 2018). The evidence to date suggests that match-play leads to alterations in redox biomarkers and promotes oxidative damage, probably as a part of the exercise-induced inflammatory response. Indeed, a competitive game results in a substantial pro-oxidant insult (Ispirlidis et al., 2008; Ascensao et al., 2008; Fatouros et al., 2010; Souglis et al., 2018), an upregulation of the antioxidant defence as evidenced by increases in endogenous anti-oxidant enzymes (Fatouros et al., 2010; Silva et al., 2013) and total anti-oxidant capacity (TAC) (Ascensao et al., 2008; Fatouros et al., 2010), which may indicate compensation in response to intense exercise

(Ascensao et al., 2008). Moreover, alterations in redox biomarkers have been found to be associated with a short lived performance deterioration in some (Ispirlidis et al., 2008; Ascensao et al., 2008; Fatouros et al., 2010) but not all studies (Silva et al., 2013). Research suggests that redox markers are prominent in the blood until game day (GD)+48 hours, with biomarkers generally returning to near baseline levels at GD+72 hours (Silva et al., 2018), although there are some exceptions (Souglis et al., 2018). Furthermore, extensive muscle damage and associated inflammation in response to match play may lead to the depletion of endogenous antioxidant resources, as evidenced by the decline in the reduced glutathione: oxidized glutathione (GSH: GSSG) ratio during recovery from a single match (Ispirlidis et al. 2008), and during periods of frequent match play with very short recuperation time (Silva et al., 2014; Le Moal et al., 2015; Mohr et al., 2016). Together, research suggests that match-play shifts the blood to a more oxidizing environment. Currently, there is limited information regarding OS responses following a soccer game in the EPL. Indeed, knowledge of OS manifestations during recovery from an EPL game would inform sport scientists regarding the time-course of post-game inflammation as production of RONS represent an integral part of the post-exercise inflammatory response (Fatouros et al., 2010).

2.4. Monitoring Inflammation in Professional Soccer

2.4.1. Biomarkers of Inflammation

The main pro-inflammatory cytokines are Tumour Necrosis Factor alpha (TNF-α), and members of the IL family IL-1, IL-2, IL-8 and IL-6, which are directly inhibited by anti-inflammatory cytokines, most notably IL-4, IL-10, and IL-1ra (Elenkov et al., 2000). The most commonly assessed cytokines in the literature include IL-1, IL-6 and TNF-α (Bruunsgaard et al., 1997; Ostrowski et al., 1999; Suzuki et al., 2003; Peterson and Pederson, 2005). Exercise induced increases in the plasma concentration of IL-6 has been a consistent finding in the literature (Pedersen and Febbraio, 2008) and research suggests its appearance precedes that of the other cytokines (Pederson et al., 2011). Plasma-IL-6 increases in an exponential manner during exercise (Ostrowski et al., 1999), peaking at the end of exercise followed by a rapid decrease towards pre-exercise

levels (Fischer, 2006). Together, evidence suggests that the cytokine cascade induced by exercise involves an increase primarily in IL-6, followed by increases in anti-inflammatory cytokines IL-1ra and IL-10 (Pederson et al., 2011).

The measurement of CRP is also widely used to monitor various inflammatory states (Du Clos, 2000). CRP remains stable over prolonged time periods and has a half-life of nineteen hours (Pepys et al., 2003) and because this half-life remains constant under conditions of health and disease, the sole determinant of circulating CRP is its synthesis rate (Pepys et al., 2003). The synthesis rate will directly reflect the intensity of the pathological process(es) stimulating CRP production (Pepys et al., 2003), meaning that CRP levels increase very rapidly in response to trauma and infection, and decrease just as rapidly with the resolution of the condition (Du Clos, 2000). The recent emphasis on "high-sensitivity" CRP refers simply to the lower detection limit of the assay procedures being used, the protein being measured is the same regardless of the assay range (Pepys et al., 2003). IL-6 is often used as a global measure of inflammation in response to match play (Silva et al., 2018), however, the IL-6 half-life has been shown to range between 5 to 11 minutes (Morettini et al., 2017), and unlike baseline CRP concentrations (Meier-Ewert et al., 2001), are subject to time-of-day variation and thus may be a less convenient measure of inflammation status than CRP. Moreover, numerous authors have reported CRP to be a more sensitive marker of inflammation after a soccer match (Ispirlidis et al., 2008; Fatouros et al., 2010; Mohr et al., 2016; Thorpe et al., 2017) with CRP showing a marked but transient rise within 24 hours post-game (Ispirlidis et al., 2008). When we consider how these biomarkers are deployed in a field setting, data collection is likely to occur in the days following the game as opposed to immediately after, thus the measurement of CRP may be considered to have higher ecological validity.

In elite athletes, who are well adapted to training, it can be expected that inflammation will be negligible at rest when compared to non-trained individuals, due to the protective effect of regular exercise on the pro/anti-inflammatory cytokine balance (Capo et al., 2016). Indeed, a low plasma IL-6 concentration at rest as well as in response to exercise

(Fischer, 2006) and low baseline CRP levels (Kasapis et al., 2005; Puglisi et al., 2008), appears to characterize the inflammatory response after training adaptation. Recent findings have highlighted the differences in inflammatory responses between those who are untrained, trained and elite (Gokhale et al., 2007; Handzlik et al., 2013). Furthermore, the time interval required for recreational athletes to achieve recovery and resolution of the inflammatory response might not reflect responses in professional soccer players. Together, this highlights the limitations associated with extrapolating findings from non-athletes to the elite soccer player, and therefore, the following section of the review will focus on the information derived only from studies of elite soccer players.

2.4.2. Evidence following a game

A competitive soccer match results in significant time dependent changes in inflammatory markers and is accompanied by an acute performance deterioration in elite soccer players (Ispirlidis et al., 2008). Inflammatory responses to soccer-specific activity in elite male athletes have been investigated by CRP, IL-6 and TNF-α biomarkers (Ispirlidis et al., 2008; Silva et al., 2013; Souglis et al., 2015; Naclerio et al., 2015; Romagnoli et al., 2016; Souglis et al., 2018). Souglis et al. (2018) report significant increases in IL-6 immediately post-game (before returning to baseline at GD+1), and CRP at 13 hours post-game (before returning to baseline at GD+2) in professional soccer players competing in the Greek Super League. Souglis et al. (2015) observed significant increases in inflammatory cytokines immediately after a game (440% increase in IL-6 and a 240% increase in TNF-α), as well as a 290% delta change in CRP at 13 hours post game in players competing in the Greek first division. Moreover, Ispirlidis et al. (2008) also found significant inflammatory responses to a soccer game, consisting of a post-game peak of leukocyte count, cytokines, and cortisol, a 24-hour peak of CRP (150% delta change), as well as significant changes in OS and muscle damage markers for up to 48-72 hours post game. However, whilst the authors define the players as elite, they do not report the playing standard or league. Silva et al. (2013) observed a significant increase in plasma CRP content at 24 h post-match (with levels ranging from 1.1 - 4 mg/L) as compared to baseline, before declining thereafter and

returning near baseline levels at 72 hours post game, in a group of high-level male soccer players from a team competing in the Portuguese Professional Soccer League. A similar trend was observed in a group of young professional soccer players competing in an Italian Serie A team in response to an official soccer match, with IL-6 and CRP peaking at 30 mins and 24 hours post-game respectively, the latter of which returning near baseline levels at 48 hours post game (Romagnoli et al., 2016). Additionally, Romagnoli et al. (2016) found that the TD covered during the match was positively correlated to the increases in IL-6 post-game. Overall, the evidence suggests there are substantial elevations in inflammatory markers throughout the recovery period from a game before biomarker values return to baseline before or at 72 hours post game. Currently, the inflammatory disturbances induced by a competitive game in the EPL are unknown. Whether or not players competing in the EPL display faster recovery patterns than what has been observed to date and thus possess a higher capability to cope with the demands of the game warrants further exploration. Moreover, understanding the time course of biomarker responses to EPL match play has implications for the prescription of training volume and intensity after a soccer match, as practitioners may seek to adjust exercise training programs in order to reduce excessive systemic inflammation and promote recovery.

Importantly, the majority of studies investigating recovery from match-play have only followed the response to a single game, however, in a real-world setting, there are multiple games often separated by limited recovery time. Pedlar et al. (2019) report that the insights gained from the assessment of blood biomarkers are narrow if only a single data point is available and therefore, render interpretation of one-off changes in values reported across the literature difficult (Carling et al., 2018). Indeed, caution is necessary when interpreting findings from single match studies, as they do not account for the recognised large match-to-match variation in physical demands (Gregson et al., 2010), or the cumulative effects of prior exercise exposure (Carling et al., 2018), which may in turn lead to inaccurate benchmark profiling of inflammatory responses to competition for the elite soccer player.

With the exception of the findings presented by Romagnoli et al. (2016), it is noteworthy, that studies examining the relationship between measures of game workload and inflammatory biomarkers in elite soccer are absent from the literature. As such, contextual information used to interpret the inflammatory response to competition need to be extrapolated from laboratory protocols. Indeed, protocols such as the Loughborough Intermittent Shuttle Test (LIST) have been validated to replicate the internal and external load recorded during match play, and allow the researcher to investigate recovery patterns in a more controlled environment. Bell et al. (2016) demonstrated significantly elevated levels of IL-6, IL-8 , TNF-α and CRP in semi-professional, male soccer players in the 72 hours following an adapted version of the LIST. However, research suggests that these laboratory protocols do not accurately recreate the biomarker responses and perception of muscle soreness that is associated with real world 11 vs. 11 match play. In a recent systematic review, Silva et al. (2018) concluded that whilst on-field and treadmill-based simulation protocols resulted in similar impairments in muscle function and performance, 11 vs. 11 match play induced a greater magnitude of change in muscle damage, OS and inflammatory markers and delayed onset of muscle soreness (DOMS). This discrepancy may be due to a combination of factors related to the unique physical and cognitive demands of elite match play that cannot be captured during on-field (e.g. LIST) and treadmill conditions. The performance of high-velocity soccer-related tasks (e.g. kicking and jumping) and lateral movements may result in a greater mechanical strain inducing more pronounced inflammatory responses, and it is reasonable to assume that competitive match play involves higher cognitive demands than simulation protocols, potentially resulting in higher levels of mental fatigue, and in turn higher perceptual responses (i.e. DOMS).

In conclusion, there is little research studying inflammation levels specific to elite soccer, with data in the EPL absent from the literature, and the laboratory models that have been developed to simulate match play may not replicate the demands of competition. Furthermore, the need for contextualization is a common feature of many biomarkers (Lee et al., 2017), and ideally blood biomarkers should be analysed longitudinally alongside physiological and physical data. There is potential for research

merging biomarker data with external workload information derived from time–motion analyses (Carling et al., 2018; Pedlar et al., 2019), which could potentially optimize the utility of blood monitoring in elite soccer and help contextualize the inflammatory response to competition.

2.4.3. Evidence across a season

Data describing the variations in inflammation levels in elite soccer players throughout a season are scarce. To the authors knowledge, only two studies exist investigating seasonal variation in elite soccer players. Meyer et al. (2011) conducted biomarker testing at 4 distinct time points across a soccer season in a large sample of professional soccer players competing in the 2 highest German leagues and reported no statistically significant changes detected for CRP. In contrast, Silva et al. (2014) investigated seasonal variation in biomarkers of physiological strain in a group of professional soccer players competing in the Portuguese professional soccer league across four time points in a season, and found CRP content to be significantly higher at the middle of the season when compared to measurements taken at the beginning of the pre-season period (+130%). Clearly, the research conducted to date are limited by the number of observational periods. The challenges of frequently collecting and transporting samples to a laboratory may explain why studies examining inflammation during recovery from multiple match play in the EPL absent from the literature. Indeed, the delayed retrospective analysis associated with venous blood sampling mean that results are unable to inform athlete management decisions at the time of sampling, and analysis in the interval between successive matches logistically difficult, particularly during the in-season period. The POC measurement of a capillary blood biomarker of inflammation may represent a practical solution to such problems for medical and performance staff. To the authors knowledge, only one study has investigated the application of a POC test for monitoring inflammatory status in elite soccer players. Becker et al. (2020) investigated intra- and inter- individual variation of CRP using a POC test in a group of 11 youth soccer players during the German under-19 Bundesliga season. The authors data demonstrate considerable inter-individual variations and intra-individual variations (demonstrated by large intra-individual CV of 45% and inter-individual CV of 15-

136%) for CRP. These findings highlight an important point regarding the interpretation of biomarker results. Population-based reference ranges are typically used to interpret biomarker results (Pedlar et al., 2019). However, given the recognised differences in the inflammatory responses of trained vs untrained individuals, and the wide inter-individual variability reported by Becker et al. (2020), the utilisation of such ranges may be of limited value to the elite soccer player. Therefore, individualised approaches used to identify atypical measures in the context of the athletes own historic data may be of higher potential value to the sports scientist (Pedlar et al., 2019). Indeed, the elimination of the inter-individual component by deriving reference ranges from repeated measurements in a given athlete may lead to a significant improvement of the biomarker signal and assist the practitioner in understanding what is 'normal' for each individual athlete and whether variations in biomarker data are adaptive or pathological in nature. This individualisation is used in the Athlete Biological Passport, as implemented by the World Anti-Doping Agency (WADA), and a recent report has demonstrated its potential utility for the purpose of individualizing athlete recovery (Hecksteden et al., 2017). However, data exploring the application of this approach in elite soccer players are absent from the literature.

2.5. Chronic Inflammation: Development & Implications

A critical balance exists between the beneficial and detrimental effects of inflammation. The favourable effect of inflammation may be explained by the theory of hormesis, whereby low levels of stress can be beneficial through induction of adaptive mechanisms increasing the tolerance against future stress incidents (e.g. enhanced resistance to fatigue during a future exercise bout) (Margaritelis et al., 2015). For example, the microdamage experienced by athletes during a game may act as the mechanical trigger for positive physiological adaptations. The eccentric actions associated with the high intensity actions of the game acutely upregulate pro-inflammatory/pro-oxidant agents, chronically, this may be counteracted by an upregulation of anti-inflammatory/anti-oxidant agents (Margaritelis et al., 2015). Indeed, Coppalle et al. (2019) observed a significant and strong negative association between

THIR and CRP after a pre-season period in a group of professional soccer players and match accumulated time has been shown to positively influence redox parameters in professional soccer players competing in the Portuguese league (Silva et al., 2014). However, prolonged production of inflammatory mediators can activate proteolytic pathways, exert negative effects on muscle mass, and overwhelm endogenous defence mechanisms (Peake et al., 2015), resulting in chronic inflammation, and it appears that recovery time between repeated bouts of exercise plays an important role in the regulation and resolution of the inflammatory response (Ronsen et al., 2002).

2.5.1. Recovery Time

A soccer season includes weekly micro-cycles consisting of training, taper, competition, and recovery that occur repetitively throughout 38–40 successive cycles (Ispirlidis et al., 2008). However, given the volume of games required to complete a season in most top leagues and the fixtures associated with cup competitions, it is normal for elite soccer players be exposed to periods of fixture congestion comprising of two to three games within the same week. Indeed, fixture congestion is a frequent in the EPL due in part to the increased commercialisation of the sport (Julian et al., 2020), and it has been described that recovery time between successive games during periods of frequent match play may not be adequate for the restoration of normal homeostasis (Mohr et al., 2016; Owen et al., 2019).

It has been shown that post-match performance recovery and inflammatory adaptations in response to a three game weekly micro-cycle (Sunday, Wednesday and Sunday) reveal different response patterns, with the largest physiological stress evident after the middle game, which was preceded by the fewest days allocated to recovery (Mohr et al., 2016). This is also supported by findings in elite level players competing in the European Champions League (Owen et al., 2019). Mohr et al. (2016) found repeated sprint performance to be markedly impaired, with the largest impairment after game 2. The authors observed a 7–14% decrement in THIR covered in game 2 compared to the game 1 and 3. However, it is noteworthy, that this difference in THIR is lower than the CV previously reported for this measure (Gregson et al. 2010), and therefore the

decrements in THIR observed by Mohr et al. (2016) may be reflective of match-to-match variability as opposed to residual fatigue from game 1 (Julian et al., 2020). A review of the literature on the effect of fixture congestion on performance showed that competitive performance is generally unaffected in professional players, although the authors do report a negative effect on distances covered at low- and moderate-intensity, suggesting that players may employ pacing strategies to maintain high-intensity actions during periods of frequent match play (Julian et al., 2020). Consideration must also be given to the calibre of opposition, as players cover more THIR when playing superior opposition (Rampinini et al., 2007). Therefore, if a team is to compete against better-ranked teams during these congested periods, players may display a higher physical output, and in turn result in an exacerbated fatigue response in the recovery phase (Julian et al., 2020).

Although the evidence suggests physical performance remains constant during periods of fixture congestion, Mohr et al. (2016) demonstrate that DOMS and post-game inflammation were more pronounced after game 2, with changes in CRP from baseline of 169%, 192% and 184% for game 1, 2 and 3 respectively. The authors suggest that a recovery time of less than 3 days may not be adequate for resolution of the inflammatory response. Similarly, Leeder et al. (2019) reported that a simulated tournament (participants completed the LIST on three occasions in five days) resulted in a gradual increase across time for CRP in a group of team sport athletes, albeit at a collegiate level. This is particularly pertinent to the EPL player, as the competitive demands of the EPL is characterized by an intensive fixture schedule with very limited recovery time, particularly during the winter period (Morgans et al., 2014). Indeed, although elite players are well adapted to the sport, the long season with consistent exposure to acute inflammatory episodes and limited periods of rest, places the soccer player at high risk of under recovery. This could potentially lead to the development of low grade inflammation, which is reflected by increased CRP concentrations and systemic levels of some cytokines (Petersen et al., 2015). Moreover, the professional player is also subject to various forms of stress (physical, psychological, lifestyle) over the course of a season, which may occur as a result of or contribute to the development

of chronic inflammation, which once developed, has both practical and clinical implications for the health and recovery of the player.

2.5.2. Practical Implications

Studies suggest that muscle soreness is markedly upregulated by a competitive game and is associated with losses in muscle function (Ispirlidis et al., 2008; Mohr et al., 2016). The post-game changes in inflammatory markers observed by Ispirlidis et al. (2008) were accompanied by a 730% increase in DOMS at 24 hours post game, as well as a reduction in anaerobic performance for 24 to 72 hours after the game, suggesting that soccer players may not be able to perform anaerobic activities at maximal level for at least 3 days after their most recent competition. Research suggests that DOMS usually peaks 1–2 days post-exercise, returning to baseline levels after 4–7 days (Fatorous et al., 2016), and it has been described that DOMS and strength loss could be related to the sensitization of nocioceptors from inflammatory cell infiltrates (Fatouros et al., 2016; Owens et al., 2019). Fatouros et al. (2016) describe that proinflammatory cells synthesise and release inflammatory agents post-exercise which may trigger pain receptors and in turn produce soreness. However, research corroborating this relationship in EPL players are absent from the literature.

Data suggests that fatigue manifests over the course of a game (see section 2.1.3), and inadequate rest and regeneration may expose players to residual fatigue post-game (Silva et al., 2018; Carling et al., 2018). Indeed, Thorpe et al. (2017) found perceived ratings of fatigue were sensitive to daily fluctuations in THIR distance in a sample of elite soccer players. Specifically, the authors reported that every 400m increase in THIR distance led to a one unit decrease in fatigue (using a seven-point Likert scale with 1 and 7 representing very, very poor and very, very good respectively). IL-6 has previously been referred to as a ‘fatigueogen’, and increases in muscle glycogen depleted states (Lewis et al., 2015b). Indeed, it has been proposed that excessive cytokine release may be implicated in the pathophysiology of chronic fatigue and overtraining syndrome (Robson, 2003). Given the evidence presented in section 2.3.2, elite soccer players may be at risk of excessive cytokine release during periods of limited recovery, which may in

turn lead to augmented sensations of fatigue and reductions in physical performance during subsequent games. However, longitudinal data examining the relationship between perceptions of fatigue and biomarkers of inflammation in elite soccer are lacking.

Muscle fatigue and soreness may also adversely affect sleep, and sub-optimal sleeping patterns have been noted in elite soccer players (Leeder et al., 2012; Nedelec et al., 2015). There are a number of factors which may predispose the elite soccer player to inadequate sleep hygiene. In addition to the demanding fixture schedule of the EPL inducing muscle fatigue and soreness, the scheduling of later kick-off times to accommodate television schedules invariably results in inconsistent wake up and bedtimes and may affect the sleep patterns of the elite athlete. Indeed, elite players are often required to perform high intensity exercise involving high levels of concentration at a time that is incongruent to their circadian rhythm (Nedelec et al., 2015). The consumption of products containing caffeine and the exposure to bright lights from the stadium and from the use of electronic devices may further compromise the sleep hygiene of the athlete (Nedelec et al., 2015). Acute sleep deprivation is known to increase background levels of inflammation (Irwin et al., 2016). Indeed, disruption of normal sleep is described to have proximal effects on IL-6, which induces CRP, and therefore, persistent or severe sleep disturbance may increase CRP levels (Irwin et al., 2016).

2.5.2. Clinical Implications

Being ranked among the elite is related to increased susceptibility to depression, particularly in relation to failed performance and recovery from injury (Hammond et al., 2013). Emerging research suggests that inflammation may play a role in the progression of depressive symptoms. The strenuous schedule of the elite athletes can lead to disturbances in the balance of inflammatory mediator levels which may act as potential triggers of depression (Ostapiuk-Karolczuk et al. 2015). Albeit not in an athletic population, patients with depression show mildly elevated (>1 mg/L) CRP levels (Osimo et al., 2019). However studies linking psychological stress and inflammation in

elite soccer players are absent from the literature. In addition to the psychological demands of elite performance, the physical demands during intensive periods, such as the increase in workload during the winter period of the EPL, may be particularly problematic for player health and well-being given that susceptibility to illness and infection is exacerbated during the winter months (Morgans et al., 2014). Furthermore, it has been shown that cytokine responses to treadmill running differ between healthy and illness-prone endurance athletes (Cox et al., 2007). Illness-prone distance runners showed evidence suggestive of impaired inflammatory regulation in the hours after exercise that may account for the greater frequency of upper- respiratory symptoms (URS) and upper respiratory tract infections (URTI). This appears to be the result of inadequate anti- inflammatory capacity, resulting in a dysregulated inflammatory cytokine balance (Cox et al., 2007). In contrast, the same authors found that CRP response to exercise was not useful in distinguishing between healthy and illness-prone runners (Cox et al., 2009). However, they did show a relationship between resting CRP concentrations and the peak pro- and anti- inflammatory cytokine responses which supports the likely contribution of CRP in the regulation of post-exercise inflammatory disturbance. Reducing illness susceptibility is clearly an important issue for medical and performance staff in protecting player health and availability for match selection. Research is warranted to establish whether monitoring biomarkers of inflammation longitudinally may prove beneficial to the elite practitioner in establishing illness risk and identifying illness prone athletes.

Negative adaptations to training load are dose related, with the highest incidence of illness and injuries occurring when training loads are high (Foster, 1999; Owen et al., 2015). In addition to the higher game demands observed in comparison to other leagues, another differentiating factor is the absence of a winter break, which has been associated with a higher injury burden in elite soccer players (Ekstrand et al., 2019). Ekstrand et al. (2019) report that teams without a winter break lost on average 303 days more per season due to injuries than teams with a winter break. Furthermore, Brockett et al. (2004) suggested that the microtears induced by eccentric actions can increase the susceptibility of the muscle to a more serious tear, and playing soccer matches with

extremely high levels of CK could increase the risk of injury during competition (Lazarim et al., 2009). Indeed, exposure to competition whilst players are still 'recovering and regenerating' and experiencing the symptoms associated with EIMD and inflammation, may increase the risk of injury (Howatson et al., 2008; Howatson et al., 2016; Silva et al., 2018). It is plausible therefore that athletes with chronic non-resolving inflammation may be at greater risk of injury. However, at present no research group has investigated this hypothesis across a season in the EPL and therefore this remains purely speculative.

Objectively capturing these environmental stressors (e.g. measuring sleep using wrist actigraphy) may represent a challenge in the applied environment given the size of first team squads and the potential reluctance of players to engage in such practices outside the team facility. Many team sports often adopt short, customized questionnaires which can be administered on daily basis, and provide a means of capturing athlete well-being. Indeed, research in the EPL has shown custom psychometric scales (fatigue, sleep quality, stress, mood, and muscle soreness) to be significantly correlated with daily training load in EPL players (Thorpe et al., 2017). The application of blood monitoring, in conjunction with wellness data routinely collected in the high performance environment, has received little attention in the literature to date (Pedlar et al., 2019), and may assist the practitioner in understanding whether the biomarker serves as a useful positive or negative outcome indicator, gain context into the cause of disturbance in the athlete's physiological state, and be used to better inform the subsequent course of action to protect player health and promote timely recovery.

2.6. Reducing Inflammation

It appears that reducing inflammation may be an important objective for protecting player health during periods of frequent match play. However, the dichotomy between the effects of acute and chronic exposure to inflammatory stimuli is important to consider when investigating strategies to accelerate recovery in the elite soccer player.

Indeed, there is increasing attention on strategies to enhance exercise recovery in athletes within the context of hormesis in skeletal muscle, and readers are directed to comprehensive reviews on such interventions by Peake et al. (2015) and Howatson et al. (2008). Whilst recovery interventions are widely adopted in the professional setting, it is important to note that any potential gains from such strategies remain futile in the absence of getting the basic principles of recovery correct, such as nutrition, hydration, sleep hygiene and appropriate rest (Howatson et al., 2016). However, the purpose of this section of the review is to discuss how nutritional interventions, beyond the fundamentals of good hydration and macronutrient intake, may be used to reduce inflammation and enhance the recovery of the elite soccer player.

Interestingly, Leeder et al. (2014) showed that well trained male team sport collegiate athletes did not receive a protective effect after repeated exposure to the LIST (known as the repeated bout effect (RBE)) (Leeder et al., 2014), suggesting that interventions which target EIMD may be justified in trained soccer players. Nonetheless, caution should be used when the elite soccer player is training to improve aerobic capacity or maximize strength gains because of the role the acute production of inflammatory mediators plays in the adaptive response to exercise. Consideration of anti-inflammatory and anti-oxidant use therefore rests within the timing of the season. In the professional setting, athletes may seek to increase their adaptive response to training during the pre-season, while their focus may shift to maintenance during the season (Heaton et al., 2017). Hence, supplementation may be advantageous during times when adaptation is inconsequential and recovery is paramount, such as during the in-season period when an athlete has multiple training bouts or competitions in a short period of time and the player may be at risk of under-recovery (Owens et al., 2019) (**Figure 2.2**).

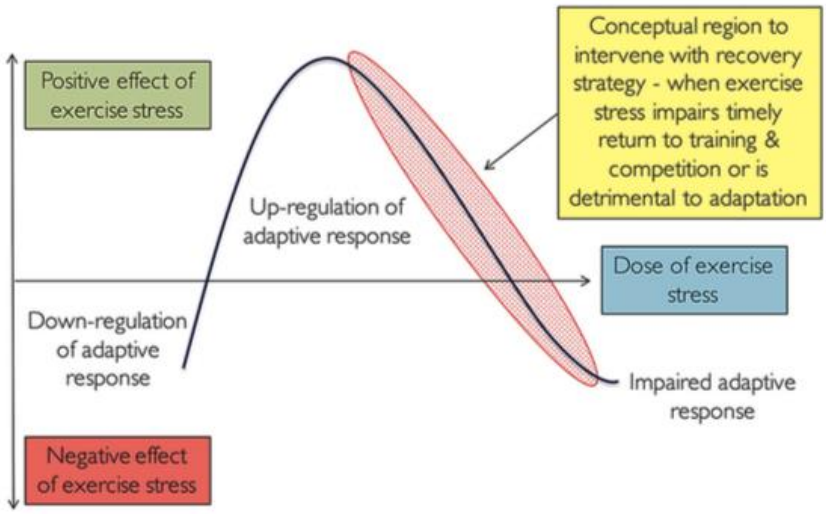

Figure 2.2. Theoretical framework for the hormesis theory in the context of nutritional interventions for the management of muscle damage and inflammation. The authors suggest a conceptual region (i.e. yellow box) for intervention where the exercise stress impairs timely return to training & competition or is detrimental to adaptation (Owens et al., 2019).

In addition to timing, consideration must also be given to the *means* of intervening. A nutritional intervention is unlikely to interact with the primary phase of the exercise induced muscle damage but is more likely to interact with the secondary damage process (i.e. the production of RONS and inflammation), and consequently, further exacerbation of damage may be modulated (Howatson et al., 2008; Bell et al., 2014; Owens et al., 2019). Importantly, studies suggest that chronic anti-oxidant supplementation may have a harmful effect on performance (Cobley et al., 2011), and mega-dosing with anti-oxidant supplements has been described to be detrimental to health (Powers et al., 2004). However, the targeted use of specific anti-inflammatory and antioxidant compounds from so-called “functional foods” (e.g. omega-3 fatty acids and dietary polyphenols) may be used to manage the negative effects of inflammation (Owens et al., 2019). The next section of the review will focus on the evidence derived from studies on curcumin supplementation and omega-3 fatty acids.

2.6.1. Curcumin

Curcumin is a polyphenol found in the spice turmeric and reports suggest that curcumin can reduce inflammation (Suhett et al., 2020; Hewlings et al., 2020; Fernández-Lázaro et al. 2020). Furthermore, it has the potential to attenuate DOMS, suggested by its effects on pain intensity (Drobnic et al., 2014). Consuming adequate quantities of curcumin via the spice turmeric in the diet in an effort to decrease inflammation is unrealistic, however, oral curcumin supplementation may prove effective (Heaton et al., 2017; Fernandez-Lazaro et al., 2020). Its mechanism of action is likely related to the inhibition of the cyclooxygenase (COX) enzymes (Heaton et al., 2017) and its effect on inflammatory signalling cascades (Sahin et al., 2016). Indeed, studies suggest that curcumin plays a role in the regulation of NF-κB and nuclear factor erythroid 2 related factor 2 (NRF2) pathways (Sahin et al., 2016). The latter of which has been described as the master regulator of endogenous antioxidant enzymes (Done et al., 2016), and as such, curcumin may act as a booster of the body's endogenous antioxidant response (Drobnic et al., 2014). Therefore, by simultaneously blocking pro-inflammatory pathways and upregulating endogenous anti-oxidant defences, curcumin supplementation could in principle be useful to increase tissue resistance to the harmful effects of the secondary damage cascade and the progress of its associated symptoms during periods of high demand in the EPL.

Of the research conducted to date, conflicting evidence exists. Nicol et al. (2015) investigated the effects of oral curcumin supplementation (2.5 g twice daily) versus placebo on single-leg jump performance and DOMS following unaccustomed heavy eccentric exercise in untrained individuals. Curcumin or placebo was taken 2 days before to 3 days after the exercise protocol, and was found to reduce DOMS at 24 and 48 h post-exercise. Despite observing a small reduction in CK concentration, levels of inflammatory biomarkers did not change significantly. McFarlin et al. (2016) investigated the effect of curcumin supplementation (400 mg/day) following muscle damaging exercise (eccentric-only dual-leg press exercise) in a group of healthy participants supplementing 2 days prior to 4 days after the protocol. The authors reported no reduction in serum levels of IL-6, however, a 48% reduction in CK and a

25% decrease in circulating levels of the inflammatory cytokines TNF-α and IL-8 were observed. Similarly, Tanabe et al. (2018) examined the effect of 180 mg/day of curcumin for 7 days prior to a muscle damaging protocol in ten healthy men and reported significantly lower IL-8 in the curcumin group, suggesting that curcumin ingestion before eccentric exercise attenuates early inflammation.

In contrast, endurance exercise trials have not produced significant reductions in DOMS or muscle damage and inflammatory markers (Heaton et al., 2017). Sciberras et al. (2015) investigated the effect of curcumin supplementation in male recreational athletes consuming 500 mg/day of highly bioavailable curcumin for 3 days and an additional 500 mg dose immediately prior to 2 hours of endurance cycling exercise. The authors deliberately adopted a low carbohydrate diet in an attempt to exacerbate the cytokine response, however, there were no significant differences in inflammatory biomarker levels after curcumin supplementation. Participants did report less DOMS after supplementation, however, the absence of an eccentric component will have limited the magnitude of muscle damage induced by the exercise protocol. Drobnic et al. (2014) prescribed a 200 mg/day dose 48 hours prior to a downhill running test and continued the supplementation regimen for 24 hours after the test (4 days in total) in a group of male healthy, moderately active volunteers. Similarly, subjects in the curcumin group reported less pain compared with subjects in the placebo group, and had significantly less evidence of muscle injury in the lower limb, as indicated by magnetic resonance imaging. However, whilst increases in markers of muscle damage and inflammation tended to be lower in the curcumin group, significant differences were only observed for the cytokine IL-8 at 2 h post-exercise. Given the data demonstrating that a bioactive dose of at least 400 mg/day is needed to alter biological indices of inflammation (McFarlin et al., 2016), the dose used in the study by Drobnic et al. (2014) likely did not supply enough free curcumin to elicit changes in a greater number of inflammatory biomarkers.

In summary, it appears that curcumin supplementation taken prior to exercise may be beneficial for athletes participating in high-intensity exercise with a significant eccentric

load (McFarlin et al., 2016; Heaton et al., 2017; Tanabe et al., 2018). However, research investigating its utility in elite soccer players are absent from the literature. Moreover, it is noteworthy, that the level of training status investigated varied from non-athlete to recreationally active individuals. Therefore, investigations are warranted to determine if the magnitude of inflammatory biomarker reduction is of physiological relevance in a cohort of elite soccer players. Anecdotally speaking, we are aware that curcumin supplements are garnering attention from players in the EPL, and scientific investigation into its efficacy in a cohort of EPL players are warranted.

2.6.2. Omega-3 Polyunsaturated Fatty Acids

In response to muscle damage and the subsequent calcium overload phase (see section 2.3.1), the omega-6 polyunsaturated fatty acid, arachidonic acid (AA) is rapidly released from the cell membrane phospholipids by the action of phospholipase A2 (Markworth et al., 2016). Arachidonic acid (AA) is the precursor for lipid mediators involved in the initiation of inflammation (Calder, 2009). The mobilised AA acts as a substrate for various cyclooxygenase (COX) and lipoxygenase (LOX) enzymes, giving rise to lipid mediators, such as prostaglandins (PGs), thromboxanes (TXs) and leukotrienes (LTs), collectively known as eicosanoids (Calder, 2009). Eicosanoids are key mediators and regulators of inflammation involved in modulating the intensity and duration of inflammatory responses (Calder, 2006), and during the early stages of muscle damage, act to drive and elicit the cardinal signs of inflammation such as redness, heat, swelling and pain (Markworth et al., 2016). Later in the inflammatory response to muscle damage, there is a shift to biosynthesis of lipid mediators with the anti-inflammatory and pro-resolving properties. The omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), act as precursors for specialized pro-resolving mediators (SPMs), such as resolvins, protectins and maresins (Kohli and Levy, 2009), which have been described to act in both an anti-inflammatory and pro-resolving manner, by simultaneously limiting PMN trafficking and actively promoting monocyte/macrophage recruitment and function (Markworth et al., 2016) (**Figure 2.4**). Therefore, there is biological rationale behind the notion that increased consumption of omega-3 fatty acids, which are most abundant in marine sources such as oily fish and fish oils, may

accelerate the recovery process (Bongiovanni et al., 2020), through its effects on inflammation resolution.

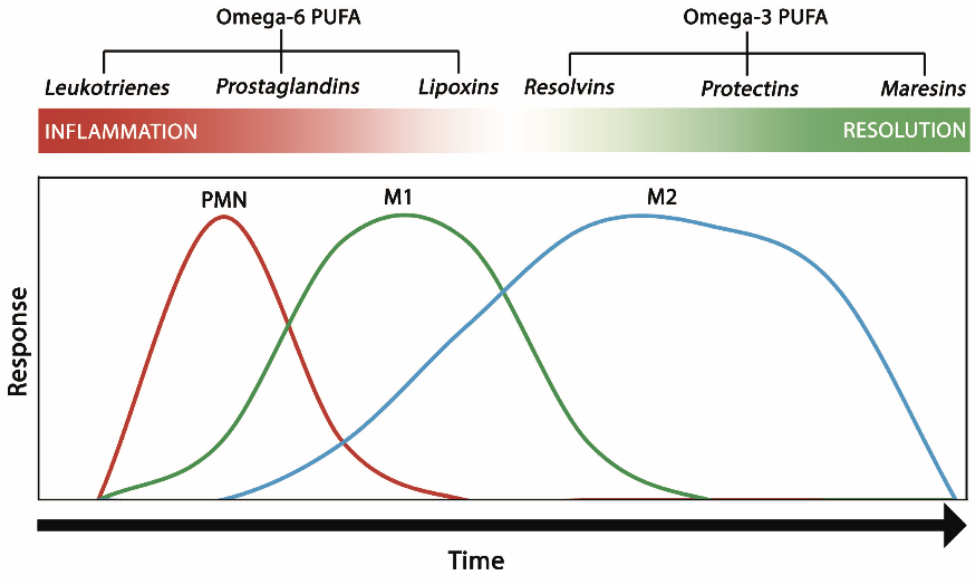

Figure 2.3. Time-course of leukocyte populations and lipid mediators in the inflammatory response to muscle injury (Markworth et al., 2016). Abbreviations: polymorphonuclear cells (PMNs), classically activated pro-inflammatory monocytes (M1), alternatively activated anti-inflammatory (M2) phenotype. Polyunsaturated fatty acids (PUFA).

The fatty acid composition of the membrane may influence aspects of inflammatory responses and hence, it is important that the fatty acid membrane composition of the athlete is known. The fatty acid membrane composition can be assessed through the analysis of the EMFA, via a dried blood spot technique (Pedlar et al., 2019). EMFA are strongly correlated to skeletal muscle phospholipid fatty acids (Fenton et al., 2016), which are the result of dietary supply, the efficiency of fatty acid metabolism in the body, and are also affected by training load (Davinelli et al., 2019; Helge et al., 2001; Andersson et al., 2000). The omega-3 index (OM3I) represents the percentage of EPA and DHA as a proportion (%) of the total EMFA composition, and has been shown to be a validated and reliable biomarker for the assessment of omega-3 status (Harris et al., 2010). Measuring and augmenting the OM3I in athletes may be a useful endeavour to

enhance recovery (Pedlar et al., 2019). A study investigating the influence of the OM3I on performance and well-being in healthy college students found that participants with an OM3I of <4% experienced significantly higher CRP concentrations compared to participants with an OM3I of >4% (Lembke et al., 2014). However, it is noteworthy, that the model employed to elicit eccentric exercise-induced muscle damage in the study consisted of non-athletes performing repeated muscle contractions using an isokinetic dynamometer, and therefore the external validity of their findings to the professional soccer player must be considered with caution. In contrast, using a similar exercise protocol on a isokinetic dynamometer, Philpott et al. (2018) found no significant differences in CRP concentrations between groups following a 6 week fish oil supplementation (FS) regimen in competitive soccer players, despite having observed a 58% increase in blood omega-3 status from baseline and significant decrease in CK concentrations and perceived ratings of muscle soreness. Complicating these findings further, Capo et al. (2014) showed that 8 weeks of DHA supplementation resulted in a 36% increase in RBC DHA concentration and significantly reduced the rate of TNF-α and IL-6 production post-exercise (consisting of a 2 hour habitual physical training session including small sided games), but had no effect on basal plasma cytokines, in a group of professional soccer players. Studies investigating the role of omega-3 fatty acid status on the inflammatory response to repeated match play in the professional soccer player are absent from the literature and could provide rationale for augmenting the EMFA composition of the professional soccer player during the competitive season.

2.7. Perspectives

In summary, this section highlights the importance of player recovery given the growing physicality of the EPL. The inflammatory response to exercise appears to affect the time needed for optimal recovery between competition, thus understanding inflammation in the EPL players may be of paramount importance for protecting player health and recovery. However, data describing the variations in inflammation levels in elite soccer players throughout a season are scarce. The POC measurement of a biomarker of inflammation would allow for more routine testing in elite soccer, however, accounting

for the analytical and biological variation of the biomarker so that meaningful changes can be distinguished from natural variations in the biomarker is a key issue. Therefore, the initial investigation in the current thesis will attempt to evaluate the validity of a POC device for monitoring inflammation in elite soccer players and report the AV and BV of the biomarker. Furthermore, there is little contextual information (i.e. workload and wellness data) surrounding these biomarker responses in the literature, subsequent investigations will attempt to quantify the sensitivity of the biomarker in response to repeated match play and examine the intra- and inter-individual variability between players during the competitive season. Finally, it appears that reducing inflammation may be an important objective for protecting player health during periods of frequent match play. However, the efficacy of such interventions in elite soccer players in response to real world muscle damaging exercise (i.e. match play) in the EPL are absent from the literature and warrant investigation.

CHAPTER 3: GENERAL METHODS

Chapter 3

3 General Methodology

3.1. Participants

Participants for Chapter 4 (Part 2) were recruited from the surrounding area (Galway, Ireland) by word of mouth, all other participants were chosen from a full-time professional soccer team competing in the EPL. Testing for each experimental chapter was conducted at the same venue throughout (i.e. within the clubs' training facility and lab). The procedures were approved by the research ethics committee of the National University of Ireland Galway (see appendix 1 for ethics approval letter). Written informed consent was obtained from every participant before data collection began. Participants were provided with a written information sheet outlining the procedures of the study (see appendix 2 for the information sheet and consent forms). Prior to the commencement of the study participants were provided with a verbal outline of the study procedure, and were given opportunities to ask any questions. Participation in each study was voluntary and participants were free to withdraw at any time. Individual participants are not identifiable from any of the data presented.

3.2. Procedures

3.2.1. Match Load Assessment

Games were analysed during the 2019-2020 season, using a multi-camera computerised tracking system (Second Spectrum Inc., CA, USA). Game minutes, total distance (km) and high speed running distance (defined as distance covered at >21km/h) were recorded each game via cameras positioned in each of the stadiums and were analysed using match-analysis software (Second Spectrum Inc., CA, USA) to produce a single data set of each player's activity during a match.

3.2.2. Perceived Ratings of Wellness and Illness Symptoms

Subjective assessments of well-being were recorded prior to each blood sampling. This questionnaire contained 5 questions: Fasted? (Yes/No); Rested? (Yes/No); Rate your

energy levels (Very Low/Low/OK/Good/High); Rate your muscle soreness (Very Sore/Sore/Average/Not Sore/Feel Great); Symptoms: Fever, Cold/Sore Throat, Headache, Joint/Muscle Ache, Diarrhoea/Sickness (Options: Today/In the last week/None).

3.2.3. Blood Sampling

Disposable latex gloves were worn for all procedures involving contact with bodily fluids and clinical waste. Consumables used in the collection and analysis of sampling were disposed of appropriately in yellow biohazard sharps containers. Participants were tested in a fasted, rested and hydrated state and blood sampling was conducted at the same time of day for each measurement (8.30-10.30am).

3.2.4. Blood Sampling: C-Reactive Protein

POC Test: Whole blood capillary samples (20 μL) were taken from the participant's ear lobe, and immediately analysed at room temperature in line with the manufacturer's instructions. Inflammation was measured with an immunoturbidimetric high sensitivity CRP assay on the Cube-S POC analyzer (Eurolyser Diagnostica GmbH, Salzburg, Austria). This photometric measurement of CRP is based on an antigen-antibody reaction between antibodies to human CRP bound to polystyrene particles and CRP present in the sample. All the biochemistry is contained within the test cartridges supplied by the manufacturer. The manufacturer reports a coefficient of variation for the assay of 2.83% for whole blood with a correlation coefficient R^2 of 0.951 against a clinical laboratory gold standard. The measurement range of the analyzer for whole blood is 0.5-20.0 mg/L.

Laboratory Test: Blood draws via the antecubital vein were conducted with 1 × 5 mL venous blood samples collected in a serum separator tube for the analysis of CRP. Samples were immediately mixed and capped and transported to the laboratory on the morning of testing, for analysis on the same day. Serum CRP was measured using the CRP Vario test (ultra-sensitive method), a latex immunoassay (Architect cSystem,

Abbott laboratories, USA). Manufacturer's report an intra-sample CV for the analyser of <1.47% for CRP.

3.2.5. Blood Sampling: Plasma Hydroperoxides

Pro-oxidant status was assessed by measuring plasma hydroperoxides (HPX) using the Free Oxygen Radical Test calorimetric assay (FORT; Callegari, Parma, Italy). Whole-blood capillary samples (20 L for FORT) were taken from the earlobe in heparinized capillary tubes. These were mixed immediately with reagent, centrifuged at 5000 $r \cdot min^{-1}$ (2000 G) for 1 min, and analyzed according to the manufacturer's instructions, using a Callegari analyzer (Callegari SpA, Catellani Group, Parma, Italy) at 37°C, with absorbance set at a wavelength of 505 nm for the calculation of FORT. Lewis et al. (2016a) previously published in detail the methodology for the FORT assay. The manufacturer of the assays reports a coefficient of variation <5% for FORT, and Lewis et al. (2016a) have reported a coefficient of variation of 3.9%.

3.2.6. Blood Sampling: Erythrocyte Membrane Fatty Acids

EMFAs were measured from a 100 μL drop of blood pipetted from a tube containing lithium heparin anti-coagulant on to filter paper before being left to dry and shipped immediately to the Omega Metrix laboratory for analysis. Measurement methodologies have been previously defined by Harris et al. (2010). The OM3I is the erythrocyte membrane content of the two marine long-chain omega-3 fatty acids, and is expressed as EPA+DHA as a percent of total identified EMFAs. The manufacturer reports an analytical coefficient of variation for the OM3I as 3.9%.

CHAPTER 4: THE EVALUATION OF A POINT OF CARE DEVICE FOR ASSESSING INFLAMMATION IN HEALTHY ATHLETES

Chapter 4

4.1. Abstract

Consistent exposure to acute inflammatory episodes and limited periods of rest throughout a season, places the soccer player at high risk of under recovery. The monitoring of inflammation in athletes may therefore be a medical and performance objective for protecting player health, recovery and adaptation. Evaluating the precision of a POC test for CRP against the gold standard method and quantifying the BV and AV in order to create an individual CDV for CRP, would aid the interpretation of an individuals' biomarker results and assists practitioners in assessing individual tolerance of training. PURPOSE: Part 1: To investigate the level of agreement between the standard laboratory method and a POC test for CRP; Part 2: To calculate the AV for the POC measurement of CRP in well trained participants; Part 3: To calculate the BV and CDV for the POC measurement of CRP in well trained participants. METHODS: Part 1: Values for CRP (Laboratory and POC test: 126 total samples) were obtained from professional soccer players (n=35) over the course of 3 EPL seasons. Part 2: Well-trained participants had duplicate capillary samples for the analysis of AV (n=10). Part 3: Well-trained participants had capillary samples taken every morning for 5 consecutive days (n=8) for the analysis of BV. RESULTS: The average difference between the two methods is 0.27 mg/L with the standard deviation of 0.115 mg/L and limits of agreement of -1.7 mg/L to 2.24 mg/L. Repeatability of POC assay was 5.26%, BV was 5.03% and CDV was 20.1%. CONCLUSION: We report that the use of a POC CRP test in well trained individuals is practical, but not interchangeable with the standard laboratory method, and the CDV reported here may be used to enhance interpretation of meaningful changes in CRP in well trained individuals.

Chapter 4

4.2. Introduction

Soccer match play is characterized by many high intensity episodes of physical stress, inducing skeletal muscle fibre damage, and as a result, a game produces significant increases in inflammatory response markers (see section 2.4.2). During the inflammatory process, increased circulating cytokines act on hepatocytes to stimulate the production of acute phase proteins (Ispirlidis et al., 2008). CRP is an acute phase reactant, widely used as a marker of systemic inflammation. It is secreted by the liver in response to a variety of inflammatory cytokines, but predominantly under transcriptional control by the cytokine IL-6 (Pepys et al., 2003). Upon secretion, several roles have been postulated for CRP (Du Clos, 2000). It has been described as a surveillance molecule, binding to phospholipids of damaged cells, and as an opsonin, thereby coating microbes and promoting the binding of phagocytes by activating and regulating the complement system (Du Clos, 2000).

There are many factors that can alter baseline CRP levels including the cumulative effect of recent training sessions (Kasapis et al., 2005), as well as alterations in lifestyle and wellness factors such as sleep quality (Irwin et al., 2016), dietary patterns (Puglisi et al., 2008) and mood state (Ostapiuk-Karolczuk et al., 2015). Long-term physical activity and cardiorespiratory fitness are inversely associated with inflammation (Kasapis et al., 2005). Indeed, in elite athletes, it can be expected that CRP levels will be negligible at rest when compared to non-trained individuals. However, prolonged increases in inflammation beyond the transient increases associated with competition indicate the presence of systemic inflammation and imply that the athletes recovery may be compromised. Where this becomes chronic or excessive, irreparable damage to host tissues and disease can occur (Calder, 2009). Indeed, given the link between inflammation and a host of sport specific medical problems (Cook et al., 2004; Kokiko-Cochran et al., 2018), the monitoring of CRP in athletes may be a valuable medical and performance objective in order to assess adaptation/recovery and protect player health. The challenges of frequently collecting and transporting samples to a laboratory mean that longitudinal studies examining biomarkers of inflammation in athletes are rare.

Furthermore, the invasive nature of repeated venous sampling (Pedlar et al., 2019; Garvican et al., 2011), mean that venous blood draws should be kept to a minimum with a well justified purpose. The POC measurement of capillary blood biomarkers may represent both an ethical and practical solution to such problems for medical staff. Moreover, Lewis et al. (2016a) outline that the successful application of biomarker monitoring programs in elite sport is not only hindered by the need for a rapid turnaround of results but also by an understanding of meaningful changes in biomarker data. Population based reference ranges are often used when interpreting changes in biomarker data, however, such an approach has limited value because of BV (i.e. each analyte in an individual varies around the homeostatic setting point of that individual) in each biomarker (Lewis et al., 2016a). Quantifying the BV and AV (i.e. the test-retest reliability of the biomarker) in order to create an individual CDV for CRP, aids the interpretation of an individuals' biomarker results and assists practitioners in assessing individual tolerance of training (Pedlar et al., 2019). Numerous studies have quantified the BV of CRP in healthy individuals (Clark et al., 1993; Macy et al., 1997; Melzi d'Eril et al., 2001; Sennels et al., 2007; Rudez et al., 2009; Nunes et al., 2010; Franzini, 2011; Braga et al., 2012). To our knowledge, only one study has reported BV and CDV data for CRP in well trained individuals (Nunes et al., 2010), however, to date, no studies have reported the BV for the POC measurement of CRP in well trained individuals.

Ultimately, the POC measurement of CRP coupled with knowledge of the BV (Lewis et al., 2016a) may help sport scientists support the adaptation and recovery needs of elite athletes. Therefore, we aimed to: (1) To investigate the level of agreement between the standard laboratory method and a POC test for CRP, (2) calculate the AV for the POC measurement of CRP in well-trained participants, (3) calculate the BV and CDV for the POC measurement of CRP in well-trained participants. We aimed to focus our study on healthy, well trained individuals and professional soccer players with physiological CRP concentrations below 5.1 mg/L (clinical range for soccer players previously defined by Meyer et al. (2011)) in line with the target of monitoring athletes during normal training micro- and macro-cycles.

4.3. Methods

4.3.1. Part 1: Method Agreement

Values for CRP (Laboratory and POC test: 126 total samples) were obtained from professional soccer players (n=35 males; age (mean (standard deviation(SD)) 25.3 (3.1) y; weight 75.2 (7.2) kg; height 182 (7.2) cm, body mass index (BMI) 22.7(1.6)), who were participating in a biomarker monitoring program over the course of 3 English Premier League (EPL) seasons. Players were tested in a fasted, rested and hydrated state, with no game or heavy training undertaken the day prior to testing.

4.3.2. Part 2: Analytical and Biological Variation

Ten well-trained participants were recruited (n=6 males and 4 females; age (mean (SD) 33.7 (6.2) y; weight 71.6 (12.5) kg; height 175.2 (8.4) cm, BMI 23.2(2.8)). Participants were selected based on recommendations for performing studies on BV (apparently healthy, and undertaking and maintaining their usual lifestyles) (Fraser, 2001). In line with other studies investigating the biological variability of CRP, we included both male and female participants (Clark et al., 1993; Macy et al., 1997; Melzi d'Eril et al., 2001; Sennels et al., 2007; Rudez et al., 2009; Franzini, 2011; Braga et al., 2012). Rifai et al. (2003) demonstrated similar distribution of CRP concentrations among apparently healthy men and women and Orri et al. (2010) found CRP levels in physically active college students and intercollegiate athletes did not differ significantly between sexes, indicating that sex-partitioned reference values are not required for this protein. Strict control of conditions was undertaken to reduce variability and to control for factors known to influence inflammatory status e.g. vigorous or unaccustomed exercise (Kasapis et al., 2005), infection (Sproston et al., 2018), anti-inflammatory supplements (Bloomer et al., 2009), anti-inflammatory medication (Wigmore et al., 1995), recent competitive activity (Ispirlidis et al., 2008). All participants abstained from vigorous physical exercise for 48 hours prior to the commencement of the study period, and maintained this throughout the study. Participants maintained their normal dietary habits throughout the study. Participants arrived at the laboratory at the same allotted time, between 8-10 am, each morning for 5 consecutive days. Participants were required to

attend testing sessions in a fasted (overnight) and rested (abstain from vigorous physical exercise for duration of study) rest. To minimise sources of variation participants were required to remain supine on an examination chair for a minimum of 20 minutes prior to each blood draw. Duplicate capillary blood samples were collected on the first morning for the analysis of analytical variation.

4.3.3. Blood Sampling: C-Reactive Protein

Capillary blood samples were obtained as described in Chapter 3. Biomarkers of inflammation were measured as described in Chapter 3.

4.3.4. Data Analysis

Numerical and graphical summaries were generated in order to identify potential anomalies and visually assess the level of agreement within athlete and between methods. The level of agreement between the two methods was assessed using the Bland and Altman approach (Bland and Altman, 1999). In the Bland–Altman method the mean difference between two methods of measurement (the 'bias') is estimated with the corresponding 95% limits of agreement, the limits that are likely to contain 95% of differences between the two measurement methods in the population of athletes under consideration. Bland and Altman's approach was originally devised for a single measurement taken on each subject by each of the methods. Extensions to account for repeated measures were presented in their 2007 paper (Bland and Altman, 2007), where 'replicates' was defined as two or more measurements on the same individual taken in identical conditions and in quick succession. In order to account for within subject variability due to multiple measurements per player, linear mixed models (LME) were used to calculate the limits of agreement using the approach proposed by Carstensen et al. (2008) where method was specified as a fixed effect and replicate as a random effect to account for the correlation (modelled as a temporal process) within athlete and over time and a B-spline to model the functional relationship between the difference and the mean to account for the proportional bias evident. These assumptions were assessed by visual inspection of the Bland-Altman plots and plots of the (standardised) residuals from the fitted models. The resulting Limits of Agreement represent the likely

differences between the POC and Gold Standard in the population of footballers of interest where wide limits of agreement indicate large discrepancies between the two methods and therefore a lack of agreement. All analyses were carried out using R (version 4.0.1).

The analytical coefficient of variation (CVA), the intra-assay CVA (%) were calculated for the Eurolyser CRP test using methodology of Fraser and Harris (1989).
CVA is calculated using the following formula:

$$\mathrm{CVA} = \frac{\mathrm{SD}}{\mathrm{X}} \times 100(\%)$$

Where X = mean and SD = standard deviation.
The CVA for Eurolyser CRP were calculated using the formula above, and derived from duplicate samples. The within subject biological variation (CVw), and CDV were calculated according to methods of Fraser and Harris (1989). CDV was calculated using the following formula: $CDV = 2^{\frac{1}{2}} \times Z \times (CVa^2 + CVw^2)^{\frac{1}{2}}$

4.4. Results

A scatterplot of CRP by method with the line of equality as a reference is given in **Figure 4.1**. It is apparent from **Figure 4.1** that the methods do not agree perfectly. There is evidence that, in general, at lower values (<1 mg/L), CRP concentrations returned by the Eurolyser method tends to be higher than that returned by the laboratory method. The mean is offset in **Figure 4.2**, is above zero, suggesting a mean positive mean bias for Eurolyser CRP. The data cluster in the upper left quadrant, suggests that the mean bias is greater for smaller CRP values. This may be explained by the smaller limits of detection for laboratory based CRP vs Eurolyser CRP. The lower limit of detection for the Eurolyser CUBE analyser is 0.5 mg/L compared to the 0.1 mg/L reported by the laboratory for CRP measurement. The average difference (i.e. the estimated bias) between the two methods is 0.27 mg/L with a standard deviation of 0.115 mg/L and the limits of agreement for the difference between Eurolyser CRP and Laboratory CRP are -

1.7 mg/L to 2.24 mg/L (n=35). As this interval contains zero, the data are consistent with there being no bias (on average) between the methods in the population.

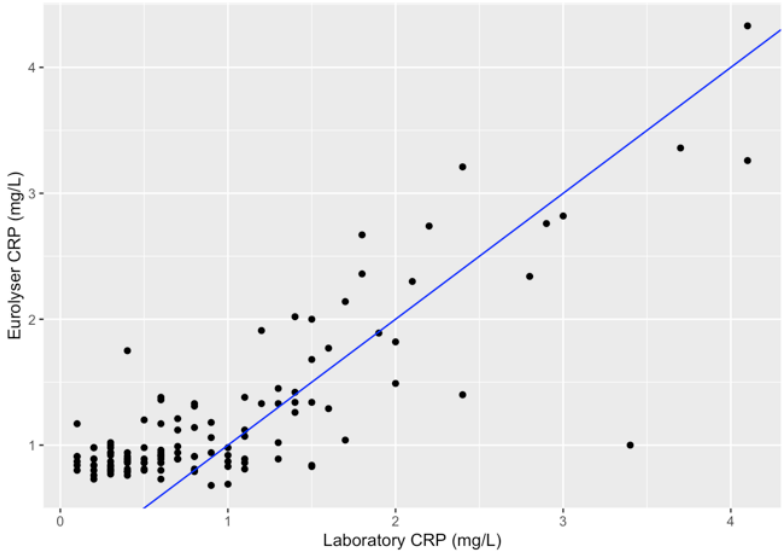

Figure 4.1: Scatter plot of Eurolyser measured C-reactive protein (CRP) (mg/L) and laboratory measured CRP (mg/L) (with line of equality superimposed).

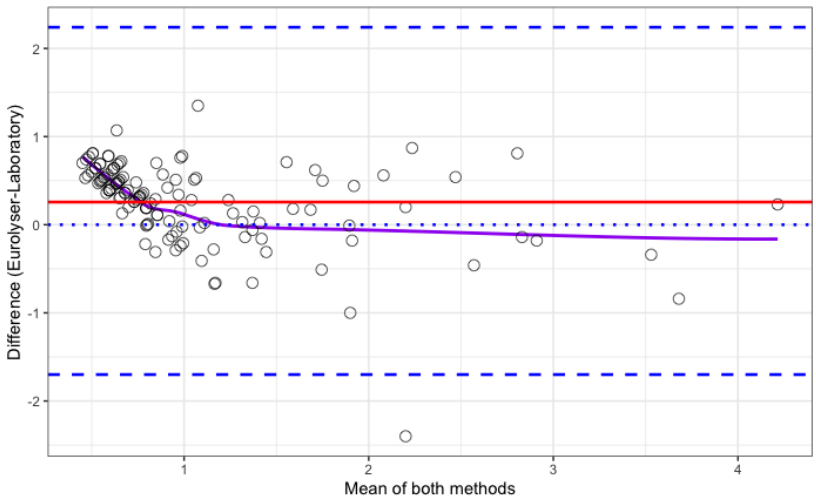

Figure 4.2: Bland and Altman plot: Comparison of pairs of C-reactive protein (CRP) measurements determined separately from Eurolyser measured CRP (mg/L) and Laboratory measured CRP (mg/L) with smoother. *Solid line* (red) mean difference, *dashed line (blue)* 95% limits of agreement adjusting for replicates.

The magnitude of the bias does not appear to be additive across the range of CRP however as evidenced by the smoother in the Bland Altman plot (**Figure 4.2**). Our analysis shows that the level of agreement is different at low and high values, where the bias is positive for low values and negative by a consistently small amount for values larger than 2 mg/L. This suggests that a different calibration is needed at low values compared to high values, and that the limits of agreement are dependent on the magnitude of CRP, as presented in **Figure 4.3**. However, it is important to note, that the CRP values obtained in this study were less than 5.1 mg/L and consequently the statistical analysis of the data is only applicable to that limit.

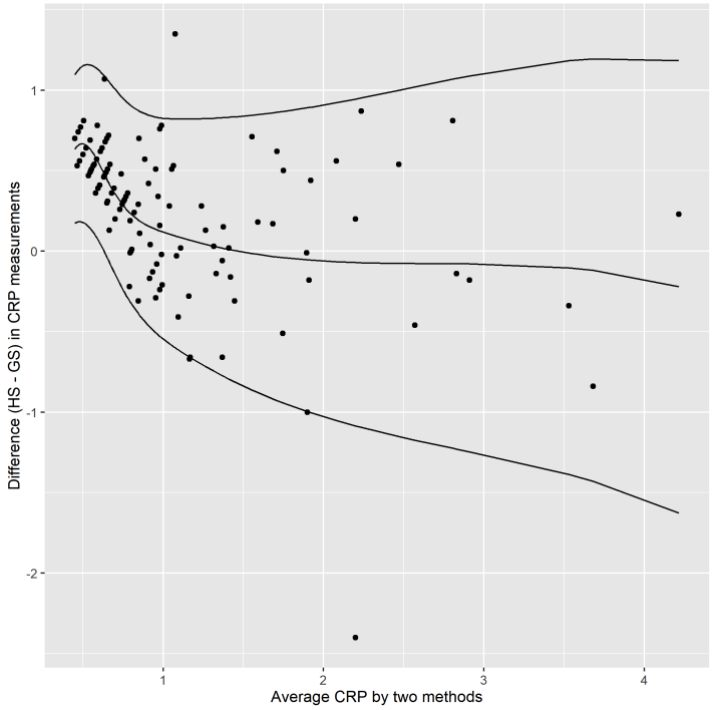

Figure 4.3: Bland and Altman plot: Comparison of pairs of C-reactive protein (CRP) measurements determined separately from Eurolyser measured CRP (mg/L) and laboratory measured CRP (mg/L) with smoother and new limits of agreement dependent on CRP level. *HS denotes Eurolyser measured CRP and GS denotes laboratory measured CRP.*

The repeatability of the Eurolyser CRP assay was 5.26% (n=10) **(Figure 4.4).** Abstaining from vigorous physical exercise is an important inclusion criteria for BV studies. The data from two male subjects were excluded from the statistical analysis because of a sudden increase in their physical activity during the study resulting in transitory but consistent increases in their CRP concentrations. The BV observed in the current study was 5.03% (n=8) (**Figure 4.5**). The mean (standard deviation) results for the participants were 0.95 (0.20) mg/L. **Table 4.1** summarises the analytical (n=10) and biological variability (n=8) and CDV for Eurolyser CRP.

Table 4.1: Analytical variation, biological variation and critical difference values for Eurolyser C-reactive protein (CRP (mg/L))

	CVa (%)	CVw (%)	CDV (%)
Eurolyser CRP	5.26	5.03	20.1

CVa % = analytical variation, CVw % = within subject variation, CDV % = critical difference value.

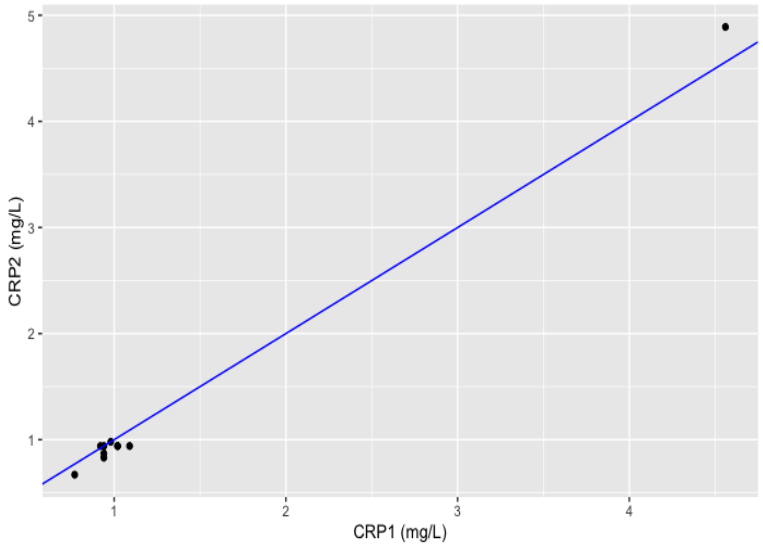

Figure 4.4: Scatterplot of the test-retest reliability of Eurolyser measured C-reactive protein (CRP) (mg/L) (with line of equality superimposed). *CRP1 and CRP2 denote first and second measurements respectively.*

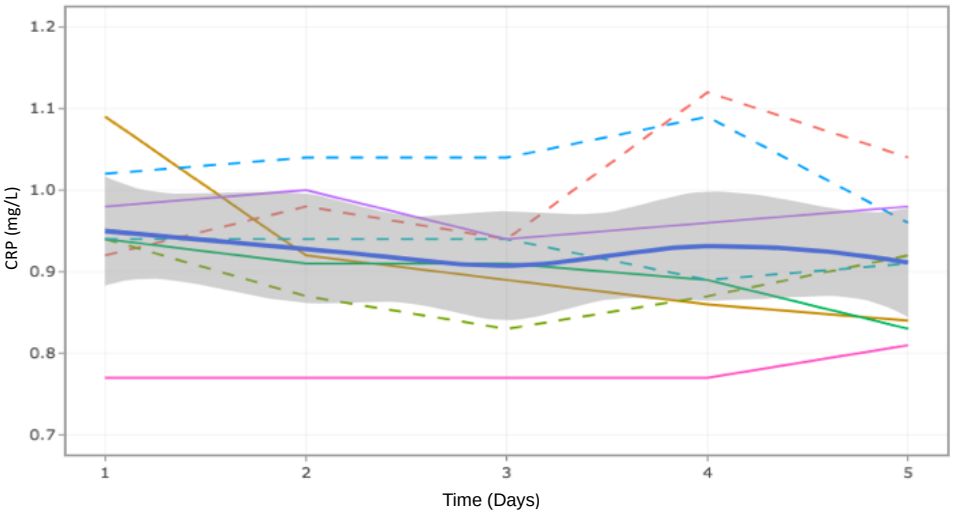

Figure 4.5 Case profile plot of Eurolyser C-reactive protein (CRP) (mg/L) over time with smoothed trajectory (solid blue line) and 95% confidence interval displayed (dark shaded area). *Solid lines* represent females and *dashed lines* represent males.

4.5. Discussion

The primary aims of this study were threefold: (1) to investigate the level of agreement between the standard laboratory method and a POC test for CRP, (2) to calculate the AV for the POC measurement of CRP in well trained participants, and (3) to calculate the BV and CDV for the POC measurement of CRP in well trained participants. The Bland-Altman analysis indicated that the mean difference between methods was 0.27 mg/L, with the limits of agreement of -1.7 mg/L to 2.24 mg/L. However, our analysis shows that the level of agreement is different at low and high values for CRP concentrations <5.1 mg/L (**Figure 4.3**). The within subject variation (CVw) presented in **Table 4.1** for this assay suggests that repeated CRP measurements in the same individual and in the same conditions will lead to comparable results. The repeatability (CVA) of the POC test for the Eurolyser CRP (5.2%) is greater than that reported by the manufacturer (2.83%) for whole blood. Finally, the CDV value reported suggests that relative changes exceeding 20.1% are required for Eurolyser CRP before physiological significance can be confidently stated.

Some lack of agreement between different methods of measurement is inevitable (Bland and Altman, 1983). What is important is the amount by which methods disagree, in order to avoid problems in interpretation and investigate whether the two methods can be used interchangeably (Bland and Altman, 1983). In practice, from an exercise physiologists' perspective the magnitude of the mean bias between methods would appear to be acceptable. However, the wide limits of agreement reported suggest that an athlete's Eurolyser CRP could increase by 2.24 mg/L or decrease by -1.7 mg/L despite there being no change in the athletes laboratory CRP level. It could be argued that this is not clinically important, and athlete management is not likely to change based on this difference (from a clinical perspective). However, when we consider the context in which these biomarkers are deployed in an applied field setting (i.e. to monitor physiological stress/homeostatic perturbations and assist in determining the appropriate hormetic "stimulus" for recovery/adaptation in the context of the athletes own historic data) (Lewis et al., 2020a); the magnitude of the difference detectable is quite large in this cohort. For example, a laboratory CRP level of 3mg/L could result in Eurolyser CRP values of 1.3-5.24 mg/L. If we consider the CDV value reported here (>20.1%), then any change exceeding 0.6 mg/L for the athlete presenting with a CRP of 3 mg/L would be deemed physiologically significant, and therefore the difference between the methods appears to be physiologically relevant. Therefore, although the difference may be acceptable from a clinical perspective for assessing an individual's physiological state when there is only one observation recorded, biomarkers are typically collected longitudinally for athletes, thus we suggest that athletes should always be measured with the same method when collecting serial measurements on the individual's biomarker.

The determination of the CDV for CRP in well trained individuals may be a useful tool to monitor the adaptive effects of exercise and individualize recovery interventions when necessary (Nunes et al., 2010). In the only other study reporting BV and CDV data for CRP in well trained individuals, Nunes et al. (2010) collected blood samples monthly from male subjects undergoing 4 months of strong physical training preparation for a career in the army. The authors reported a CDV of 206% for CRP, notwithstanding the stark differences in methodology; contrasts markedly with the 20.1% CDV reported

in the current study. The CVw values of CRP obtained in the majority of published studies are quite high (Braga et al., 2012), inevitably causing very high CDV. Braga et al. (2012) suggest that the CVw values exceeding 33.3% for CRP represent incorrect estimates which may be due to acute inflammatory episodes in the evaluated sample group. Indeed, the high CVw (74.4%) reported by Nunes et al. (2010) suggests evidence of impaired inflammatory regulation in the study group, and it is plausible that the individuals in question were not adapted to the training volume undertaken during the 4 month observational period. This may lead to disruptions in the balance between pro- and anti-inflammatory cytokines (Capo et al., 2016), and increase the hepatic production of CRP. Furthermore, Braga et al. (2012) suggest that extending the duration of BV studies beyond 3 months is questionable, and may increase the risk of introducing additional sources of variability beside the biologic one, such as seasonal variation, which in turn may amplify the CVw. Therefore, the low CVw (**Table 4.1**) observed in the current study (5.03%) may be due to a multitude of factors including the short study duration (Braga et al., 2012), the level of pre-analytical control (e.g. abstaining from vigorous exercise, controlling for posture) and the training status of the participants. It can be expected that well trained individuals, who are adapted to training, will display greater "inflammatory control" when compared to non-trained individuals, likely due, among other factors, to the protective effect of regular exercise training on the pro/anti-inflammatory cytokine balance (Capo et al., 2016). Moreover, analytical precision is important in order to ensure that analytical "noise" does not confound the biological "signal" (Fraser, 2004). Desirable imprecision is suggested to be equal to or less than half of the CVw. This was not the case in the current study. The imprecision observed is likely the result of combined variables, including antibody affinity, specimen dilution, instrument performance and operator technique (Ledue et al., 2003). However, it has been suggested that for CRP, the within-laboratory total imprecision should be <10% (Fraser, 2004), and therefore, the analytical imprecision observed in the current study meets this requirement.

There are several limitations to the current study. Subject participation within professional soccer research is a challenge due to numerous external influences (Owen

et al., 2019). The level of methodological pre-analytical control applied to minimize sources of variability in the BV study is certainly a strength, however, the intensive analytical effort required to adopt this protocol does limit the number of specimens and subjects which can be studied (Fraser and Harris, 1989). A larger cohort would have strengthened the current study. Furthermore, the method agreement analysis has not addressed the level of agreement at higher CRP concentrations that may occur as a result of acute infection for example (Sproston et al., 2018). Whilst this is certainly a limitation, the purpose of our investigation was to assess the utility of the POC test as a monitoring tool in healthy athletes as opposed to a diagnostic tool. Moreover, anecdotally, at CRP concentrations of >5 mg/L in this cohort, athlete management is likely to be led by the team physician. Nonetheless, future studies should investigate agreement beyond 5 mg/L and assess the diagnostic accuracy of the POC test in capturing sport specific medical problems. Finally, the inclusion of female athletes (Part 2) extends the utility of our findings beyond male athletes, however, failure to capture menstrual cycle phase and the use of hormonal contraception is a limitation given the link between oral contraceptive pill use, menstrual cycle symptoms and low grade inflammation in female athletes (Puder et al., 2006; Cauci et al., 2017). However, it is important to note that we observed no significant differences in inflammation levels between male and female participants (**Figure 4.5**), a finding which is corroborated by previous research (Rifai et al., 2003; Orri et al., 2010).

4.6. Conclusion

The results of this study show that there is considerable variation in agreement between the CRP POC test in comparison with a laboratory reference standard, and that the level of agreement is different at low and high values for CRP concentrations <5.1 mg/L, suggesting that the methods are not interchangeable in this cohort. However, the POC test did perform adequately, AV was 5.26%, BV was 5.03% and CDV was 20.1%. Furthermore, the CDV reported here may be used to enhance interpretation of meaningful changes in CRP in well-trained individuals.

The results of this study have shown that the application of a POC device for measuring inflammation has potential utility in elite sport worthy of scientific investigation. The next experimental chapter will investigate its practical application for monitoring inflammation in the professional setting of the EPL.

CHAPTER 5: THE LONGITUDINAL ANALYSIS OF CAPILLARY BLOOD BIOMARKERS OF INFLAMMATION AND OXIDATIVE STRESS IN CONJUNCTION WITH WORKLOAD AND WELLNESS

Chapter 5

5.1. Abstract

Longitudinal studies examining biomarkers of inflammation in the EPL are absent from the literature. PURPOSE: To examine blood biomarkers of inflammation and oxidative stress in conjunction with workload and wellness data in response to weekly match play across the first half of an EPL season. METHODS: We conducted a longitudinal analysis of 18 EPL games during the 2019-2020 season (n=22). CRP, a marker of systemic inflammation, and plasma HPX, an indirect measure of reactive intermediary by products of in vivo lipid, protein, and nucleic acid oxidation, were collected twice weekly (pre-game and post-game) via point-of-care blood tests. Relationships between biomarkers and co-variates were examined using linear mixed effects models. RESULTS: Post-game CRP responses were significantly associated with CRP measured at GD-1 (0.4434 mg/L; $p<0.001$) and testing day, with CRP measured at GD+2 (-1.4520 mg/L; $p=0.041$), significantly lower in comparison with measures taken at GD+1. Post-game HPX responses were significantly associated with HPX values at GD-1 (0.1977 mmol/L H_2O_2; $p=0.002$). CRP levels were significantly higher at GD+1 (0.7481 mg/L; $p= 0.0447$) and significantly lower at GD+4 (-1.0769 mg/L; $p= 0.048$) in comparison with CRP measured at GD-1. HPX levels (2.0166 mg/L; $p<0.001$), presence of illness symptoms (2.5989 mg/L; $p<0.001$), “low” ratings of energy levels (1.7791 mg/L; $p=0.001$), and “Not Sore” ratings of muscle soreness (-0.5911mg/L; $p=0.041$), were significantly associated with overall variability in CRP levels. HPX levels were significantly higher at GD+4 (0.2918 mmol/L H_2O_2 ; $p<0.001$) in comparison with HPX measured at GD-1. Time (0.0018 mmol/L H_2O_2 ; $p<0.001$), and CRP (0.0337 mmol/L H_2O_2 ; $p<0.001$), were significantly associated with overall variability in HPX levels. CONCLUSION: An EPL game induces time-dependent changes in circulating markers of inflammation and oxidative stress. HPX levels display a significant increase during the first half of the EPL season. POC measurement of CRP is sensitive to the changes in subjective wellness and may assist in protecting player health and availability for team selection.

Chapter 5

5.2. Introduction

Professional soccer players are subjected to considerable muscle damage and inflammation as a result of a match (see section 2.4.2). During the inflammation process, the transcriptional activity of NF-κB leads to the increased production of pro-inflammatory cytokines including IL-6 (Souglis et al., 2015), which act as the major stimuli to the secretion of CRP by the liver (Kasapis et al., 2005). CRP levels have been shown to increase by threefold the next morning after a match (Souglis et al., 2015), peaking approximately 24 hours post, and remaining elevated for approximately 48 hours (Souglis et al., 2015). With increasing fitness and adaptation, the magnitude of this acute CRP response is attenuated (Kasapis et al., 2005), likely due to modification of cytokine production to drive the resting cytokine balance to an anti-inflammatory state (Ostrowski et al., 1999). However, although elite players are well adapted to their sport, the long season with consistent exposure to acute inflammatory episodes, and the limited periods of rest, places the soccer player at risk of under recovery, potentially leading to a loss of inflammatory control.

To the knowledge of the authors, longitudinal data describing inflammation in the EPL are absent from the literature. The POC Free Oxygen Radical Test (FORT) is an indirect measure of reactive intermediary by products of in vivo lipid, protein, and nucleic acid oxidation (hydroperoxides; HPX) (Lewis et al., 2016a). To date, only one case study has explored the application of the FORT assay in elite soccer (Catterson et al., 2014). Similarly, only one study has explored the application of a POC test for CRP in elite soccer (Becker et al., 2020). Moreover, it is critical to contextualize assessment of inflammation (Lee et al., 2017). Pedlar et al. (2019) suggest that modelling biomarkers jointly over time using suitable multivariate statistical techniques in conjunction with workload and wellness data could be of value for the purposes of objectively managing training load and identifying injury and illness risk.

Therefore, this study was designed to examine, for the first time, longitudinal alterations in blood biomarkers of inflammation and oxidative stress simultaneously with workload

and wellness data in response to weekly match play across the first half of an EPL season. We hypothesized that match workload and negative ratings of subjective wellness would be positively associated with these biomarkers.

5.3. Methods

5.3.1. Participants

Twenty two players (age = 25.4 [3] y, height = 181.8 [7.8] cm, weight = 75.1 [6.2] kg, and body mass index (BMI) = 22.7 [1.5]) were recruited to participate. Athletes were also classified as injured and ill by the medical staff, to protect anonymity the specific details of the injuries and illnesses are not disclosed. All athletes were tested in the competition phase of the EPL.

5.3.2. Design overview

We conducted a longitudinal analysis of 18 EPL games during the 2019-2020 season. Over the course of the first half of the competitive season (August to December), repeated measurements of CRP and HPX were taken from these players before and after 18 games. Data were collected twice weekly (pre-game and post-game) at the same time of day and included the following: CRP and plasma HPX via point-of-care blood tests, and subjective assessments of wellness and illness symptoms. Match load (volume, intensity, minutes) were collected and recorded for each game. Testing was carried out between 9 am and 11 am, with pre-game measurements taken on game day -1 (GD-1) and post-game measurements taken on either game day +1 (GD+1), game day +2 (GD+2), game day +3 (GD+3), game day +4 (GD+4) or a combination thereof. Players were asked to report for testing in a fasted and hydrated state, where players reported not being fasted, this was noted and factored into the analysis. Training was prescribed by coaching staff and was deliberately not influenced by the study design or the research personnel. The athletes training load varied in intensity and volume depending on factors including but not limited to the following: player status (starter/bench player), time of season, upcoming games, injuries and illnesses.

5.3.3. Methodology

Wellness and Symptoms:

Perceived ratings of wellness were measured as described in Chapter 3.

Blood Sampling:

Capillary blood samples were obtained as described in Chapter 3. Biomarkers of inflammation and redox homeostasis were measured as described in Chapter 3.

Match Load Assessment:

Match workload were measured as described in Chapter 3.

5.3.4. Data analysis:

A LME was used to model the change in mean post-game CRP responses using testing time, pre-game CRP, pre-game HPX and match workload as covariates. We chose high speed running (km) as our measure of match workload to avoid multicollinearity as game minutes, total distance (km) and high speed running (km) were highly correlated. We performed a separate LME for post-game HPX responses. Furthermore, another LME was used to explain the variability in mean CRP using testing time, date, subjective wellness and presence of symptoms as covariates. We performed a separate LME to explain variability in mean HPX. In this study we include a random intercept for each game and subject. We report effect estimates for the association between each co-variate and biomarker responses, along with standard error and p-values. A number of candidate models were tested in both scenarios where Akaike's Information Criterion (AIC) was used to pick the final model. All statistical analyses were carried out using R (Version 4.0.1). The significance level was set at $p < 0.05$.

5.4. Results

A total of 22 players completed the study. Summary statistics for HPX and CRP responses by testing time are detailed in **Table 5.1**.

Table 5.1: Summary statistics for C-reactive protein (CRP) (mg/L) and hydroperoxides (HPX) (mmol/L H_2O_2) by testing time.

	GD-1 (n=297)	**GD+1 (n=70)**	**GD+2 (n=82)**	**GD+3 (n=14)**	**GD+4 (n=27)**
CRP					
Mean (SD)	1.92 (2.55)	3.42 (3.71)	2.32 (2.72)	1.75 (1.63)	1.53 (1.63)
Median	1.02	2.40	1.44	1.14	0.950
[Min, Max]	[0.740, 25.5]	[0.830, 24.0]	[0.800, 19.1]	[0.810, 6.65]	[0.760, 8.97]
HPX					
Mean (SD)	1.69 (0.391)	1.66 (0.315)	1.77 (0.355)	1.70 (0.381)	2.01 (0.518)
Median	1.64	1.63	1.72	1.60	1.92
[Min, Max]	[1.22, 3.58]	[1.22, 2.44]	[1.22, 2.64]	[1.22, 2.46]	[1.30, 3.41]

CRP *C-Reactive Protein* ***HPX*** *Hydroperoxides* ***SD*** *Standard Deviation* ***Min*** *Minimum* ***Max*** *Maximum* ***GD*** *Game Day*

Relationships between explanatory variables and post-game CRP responses are detailed in **Table 5.2**. Post-game CRP responses were significantly associated with CRP measured at GD-1 (0.4434 mg/L; $p<0.001$) and testing day, with CRP measured at GD+2 (-1.4520 mg/L; $p=0.041$), significantly lower in comparison with measures taken at GD+1. No significant relationships were observed for all other explanatory variables and post-game CRP.

Table 5.2: The estimated effect of explanatory variables on post-game C-reactive protein (CRP) (mg/L) responses adjusted for the effect of game and subject by fitting random intercept mixed effect models. [a]: in comparison to Game Day (GD)+1;

Explanatory Variable	Estimate	Standard Error	P-Value
Pre-CRP (GD-1)	0.4434	0.0082	**<0.001**
Pre-HPX (GD-1)	1.223	0.6572	0.065
THIR (km)	0.0009	0.0013	0.441
GD+2[a]	-1.4520	0.6011	**0.042**
GD+3[a]	-1.5240	1.1250	0.207

CRP *C-reactive protein* ***HPX*** *hydroperoxides* ***GD*** *Game Day* ***THIR*** *Total high intensity running*

Relationships between explanatory variables and post-game HPX responses are detailed in **Table 5.3**. We found a significant association between post-game HPX responses and HPX values at GD-1 (0.1977 mmol/L H_2O_2; p=0.002). No significant relationships were observed for all other explanatory variables and post-game HPX.

Table 5.3: The estimated effect of explanatory variables on post-game hydroperoxides (HPX) (mmol/L H_2O_2) responses adjusted for the effect of game and subject by fitting random intercept mixed effect models. [a]: in comparison to Game Day (GD)+1;

Explanatory Variable	Estimate	Standard Error	P-Value
Pre-HPX (GD-1)	0.1977	0.0634	**0.002**
Pre-CRP (GD-1)	0.0059	0.0084	0.483
THIR (km)	0.0000	0.0001	0.789
GD+2[a]	0.1012	0.0669	0.165
GD+3[a]	-0.0042	0.1250	0.974

CRP *C-reactive protein* ***HPX*** *hydroperoxides* ***GD*** *Game Day* ***THIR*** *Total high intensity running*

Relationships between explanatory variables and total variability in CRP are detailed in **Table 5.4.** CRP levels were significantly higher at GD+1 (0.7481 mg/L; p= 0.045) and significantly lower at GD+4 (-1.0769 mg/L; p= 0.048) in comparison with CRP

measured at GD-1. HPX levels (2.0166 mg/L; p<0.001), presence of illness symptoms (2.5989 mg/L; p<0.001), "low" ratings of energy levels (1.7791 mg/L; p=0.001), and "Not Sore" ratings of muscle soreness (-0.5911 mg/L; p=0.041), were significantly associated with overall variability in CRP levels. No significant relationships were observed for any other explanatory variables and CRP.

Table 5.4: The estimated effect of explanatory variables on total variation in C-reactive protein (CRP) (mg/L) (Pre and Post-game) adjusted for the effect of game and subject by fitting random intercept mixed effect models. [a]: in comparison to Game Day (GD)-1; [b]: in comparison to perceived muscle soreness ('Average'); [c]: in comparison to perceived energy levels ('Good');

Explanatory Variable	Estimate	Standard Error	P-Value
GD+1[a]	0.7481	0.3714	**0.045**
GD+2[a]	0.0276	0.3018	0.927
GD+3[a]	0.2561	0.6645	0.701
GD+4[a]	-1.0769	0.5378	**0.048**
HPX (mmol/L H_2O_2)	2.0166	0.3432	**<0.001**
Time (Days)	0.0037	0.0034	0.296
Illness Symptoms (Yes)	2.5989	0.3138	**<0.001**
Muscle Soreness (Feel Great)[b]	-0.7788	0.4967	0.117
Muscle Soreness (Not Sore)[b]	-0.5911	0.2889	**0.041**
Muscle Soreness (Sore)[b]	-0.4836	0.3950	0.221
Muscle Soreness (Very Sore)[b]	-0.4425	1.0000	0.658
Energy Levels (High)[c]	-0.1128	0.6915	0.870
Energy Levels (Low)[c]	1.7791	0.5453	**0.001**
Energy Levels (OK)[c]	0.3094	0.3069	0.314
Energy Levels (Very Low)[c]	0.4904	1.2486	0.695

***HPX** hydroperoxides **GD** Game Day*

Relationships between explanatory variables and variability in HPX are detailed in **Table 5.5**. HPX levels were significantly higher at GD+4 (0.2918 mmol/L H_2O_2 ; $p<0.001$) in comparison with HPX measured at GD-1. Time (0.0018 mmol/L H_2O_2 ; $p<0.001$), and CRP (0.0337 mmol/L H_2O_2 ; $p<0.001$), were significantly associated with overall variability in HPX levels. No significant relationships were observed for all other explanatory variables and HPX levels.

Table 5.5: The estimated effect of explanatory variables on hydroperoxides (HPX) (mmol/L H_2O_2) (Pre & Post-game) adjusted for the effect of game and subject by fitting random intercept mixed effect models. [a]: in comparison to Game Day (GD) -1;

Explanatory Variable	Estimate	Standard Error	P-Value
GD+1[a]	-0.0265	0.0393	0.499
GD+2[a]	0.0204	0.0362	0.573
GD+3[a]	-0.0303	0.0828	0.714
GD+4[a]	0.2918	0.0679	**<0.001**
CRP (mg/L)	0.0337	0.0051	**<0.001**
Time (Days)	0.0018	0.0004	**<0.001**

CRP *C-reactive protein* ***GD*** *Game Day*

5.5. Discussion

This is the first study, to our knowledge, to investigate the application of a POC test for inflammation, and to report longitudinal blood biomarker data in the EPL. The primary aim of this study were to observe the impact of professional soccer matches on capillary blood biomarkers of inflammation and pro-oxidant status during the in-season competitive phase of the EPL. The principal findings were that (1) a competitive EPL game induces time-dependent changes in circulating markers of inflammation and oxidative stress, a phenomenon likely related to exercise-induced muscle damage, (2) a time specific pattern emerged with significantly higher mean HPX values observed across the first half of the EPL season, (3) the point of care measurement of

inflammation was found to be highly sensitive in detecting subjective feelings of fatigue, muscle soreness and the presence of illness symptoms. The present study findings could have important implications for protecting athlete health and recovery, as blood monitoring, in conjunction with wellness data may offer an objective tool for identifying fatigue and illness risk in professional soccer and can therefore be used to enhance the management of high performance athletes.

We report for the first time that a competitive EPL game induces time-dependent changes in circulating HPX and CRP levels. CRP plays a key role in the regulation of post-exercise inflammatory disturbance (Cox et al., 2009). In agreement with previous research in elite soccer players, significant increases in CRP concentrations were observed at GD+1 (Ispirlidis et al., 2008; Silva et al., 2013; Souglis et al., 2015a; Souglis et al., 2015b; Romagnoli et al., 2016), before declining thereafter at GD+2, GD+3 (**Table 5.2**). Indeed, following eccentric exercise, circulating anti-inflammatory cytokines respond to local production of pro-inflammatory cytokines and restrict systemic inflammation (Peake et al., 2005). CRP may contribute to this increased expression of anti-inflammatory cytokines during late recovery from exercise by enhancing the release of interleukin-1 receptor antagonist (IL-1ra) from monocytes (Fischer, 2006). The delayed increase in HPX levels detected in the blood at GD+4 (significantly higher than HPX levels at GD+1) may be due to RONS production by invading phagocytes, shown to occur 2–4 days after eccentric actions in humans (Margaritelis et al., 2014). Nikolaidis et al. (2012) showed that muscle-damaging exercise including a high aerobic component resulted in a biphasic response, inducing both early (immediately after to 4h post exercise) and delayed (96 h post exercise) perturbations in redox homeostasis. Furthermore, we report a significant relationship between HPX levels and CRP (**Table 5.4)**. RONS regulate the translocation of NF-κB to the nuclei in a redox-sensitive manner (Mohr et al., 2016; Ji, 2015), which modulates key players involved in the inflammatory process such as pro-inflammatory cytokines. Therefore, it is plausible that the delayed perturbations in redox homeostasis evident in the current study, may have led to higher circulating levels of CRP and HPX in some athletes at GD-1. Our results indicate that the magnitude of the post-game biomarker

responses were more pronounced when circulating levels were higher at GD-1 in apparently healthy players (i.e. no illness symptoms) (**Figure 5.1**), a finding which is corroborated by research in runners (Cox et al., 2009).

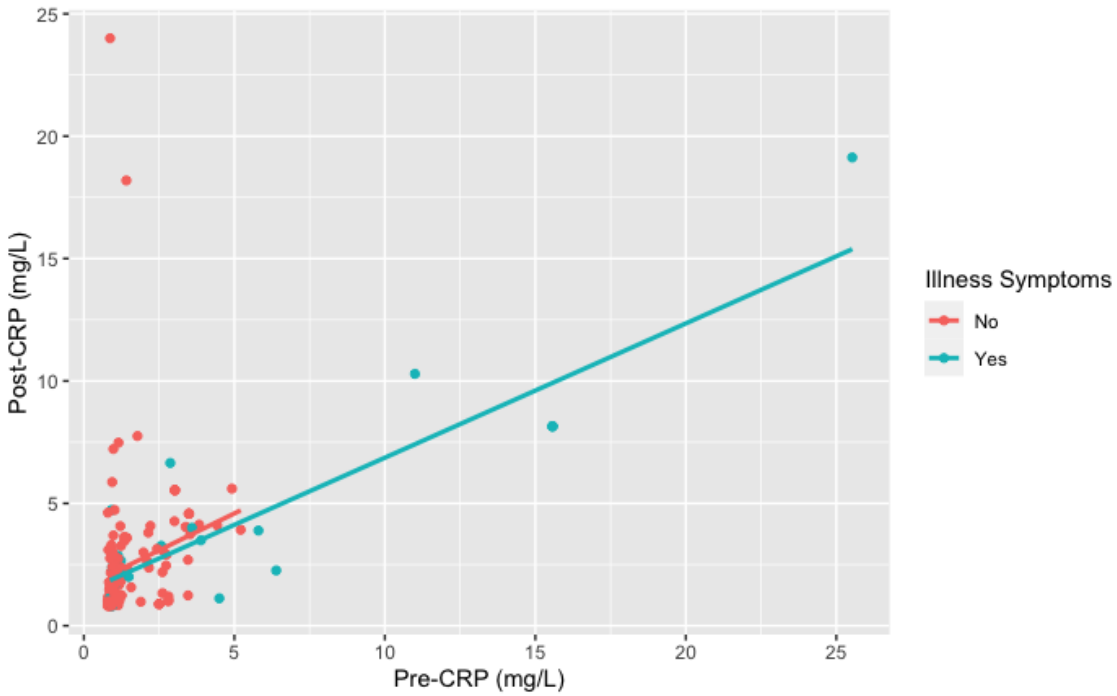

Figure 5.1: Scatter plots of pre-game C-reactive protein (CRP) (mg/L) and post-game CRP (mg/L). *Coloured by presence of illness symptoms at Game Day (GD)-1 (Yes/No).*

These data suggest that recovery time in-season may provoke continual OS during a competition season. Although NF-κB activation is a necessary event in response to oxidative stress in order to induce antioxidant adaptation, chronic activation could lead to systemic inflammation and overwhelm endogenous defence mechanisms of the body (Ji, 2015). The cumulative effect of muscle damaging episodes likely contributed to the sustained HPX concentrations observed across time (**Figure 5.2**). Increased in-season levels of RONS production have been observed during intensive periods in team sports (Bresciani et al., 2010; Silva et al., 2014; LeMoal et al., 2015; Becatti et al., 2017), however, in contrast to our findings, Becatti et al. (2017) observed a decrease in RONS production in the middle of the competitive season in Serie A, likely due to the preceding winter break in Italy. Indeed, the absence of a scheduled winter break has

been found to be associated with both a higher injury burden and higher incidence of severe injuries and non-contact injuries in professional soccer (Ekstrand et al., 2019). Moreover, high levels of oxidants have been described to play a role in the pathophysiology of overuse injuries (Lewis et al., 2020a), illness risk (Lewis et al., 2020a), and muscle fatigue (Powers et al., 2011). Although speculative, the accumulated levels of HPX observed might have predisposed athletes with an inability to cope with the competitive demands of the subsequent winter period in the EPL, which is characterized by an intensive fixture schedule with very limited recovery time (often only 48 hours) (Morgans et al., 2014), and may have led to a greater susceptibility to illness.

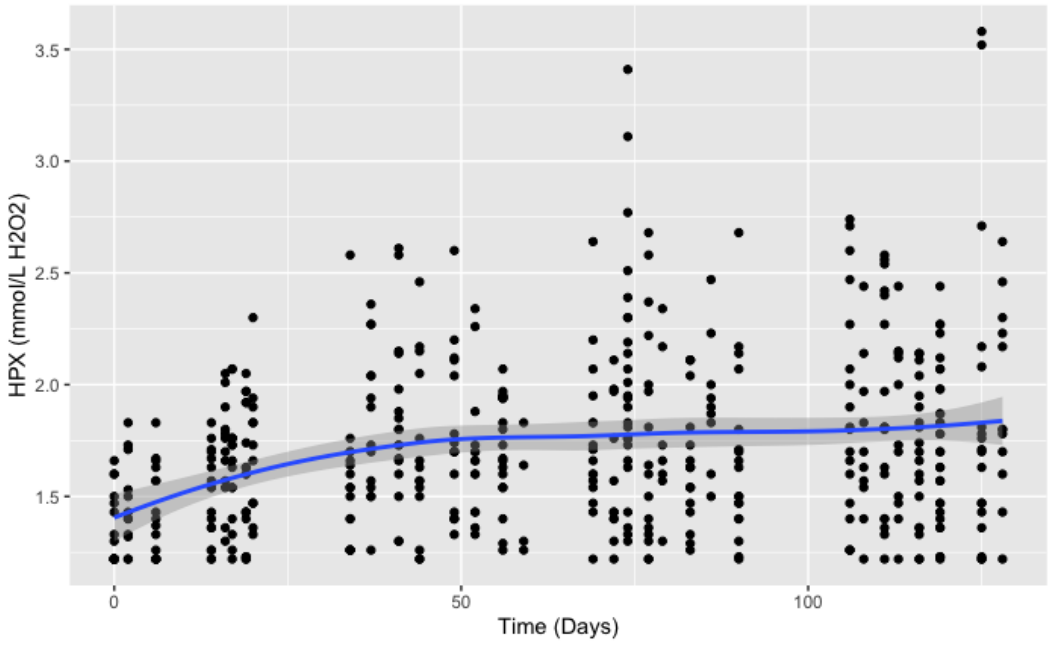

Figure 5.2: Scatter plot of hydroperoxides (HPX) (mmol/L H_2O_2) levels across time. *Blue line denotes group mean, grey shaded area denotes standard error.*

The highly significant association between CRP values and subjective wellness evident in the current study suggests that the POC monitoring of CRP may help detect the presence of infection (**Figure 5.3**), fatigue and muscle soreness in the elite soccer player. The presence of illness symptoms in athletes most likely involves both infectious and non-infectious causes (Cox et al., 2008). Indeed, data suggest that some episodes of

URS in elite athletes appear to have an inflammatory origin. High ventilatory loads experienced by athletes during intense exercise induce trauma to the upper airways, resulting in the local production of inflammatory mediators, and triggering the appearance of URS, confused by the athlete as a URTI (Cox et al., 2008). Nevertheless, it is noteworthy, that a raised CRP and the presence of fever, cough, and fatigue are among the dominant clinical features of COVID-19 (Fu et al., 2019). These findings suggest that alongside collection of body temperature (Sproston et al., 2018), and illness symptoms, CRP could be used as an indicator of infection and may be a high priority for tracking athlete health in the post-COVID-19 era.

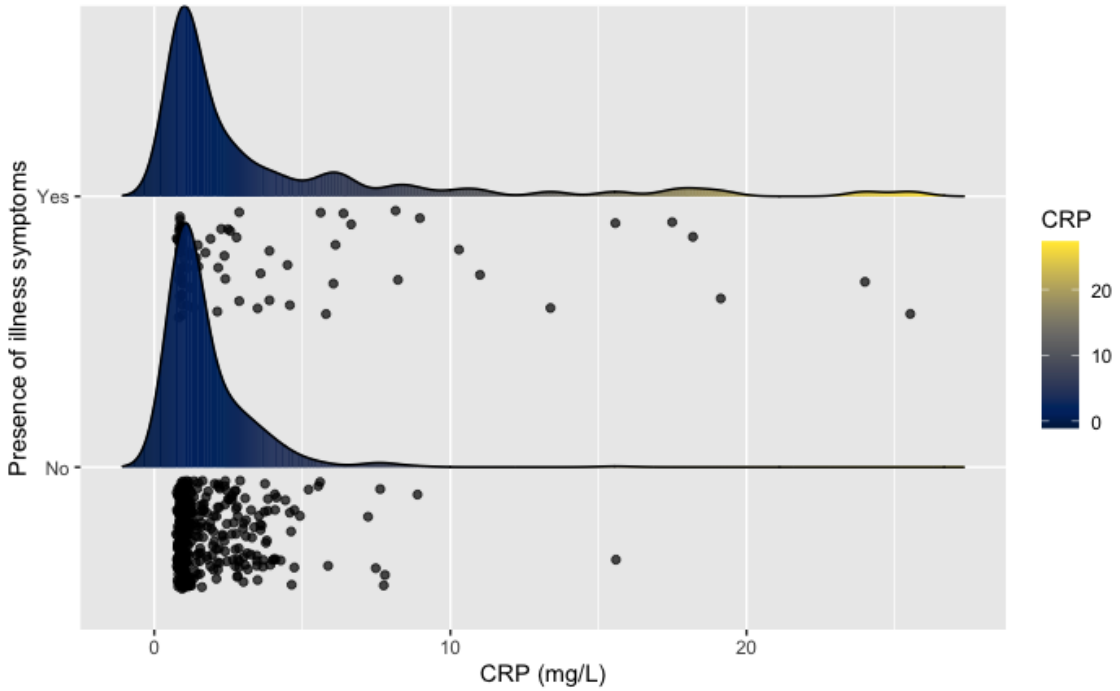

Figure 5.3: Density plot of pre and post-game C-reactive protein (CRP) (mg/L) by the presence of illness symptoms (Yes/No) (assessed via subjective wellness questionnaire).

Accumulating fatigue may persist post-infective episode (Lewis et al., 2015b), and excessive cytokine release is implicated in the pathophysiology of fatigue (Robson et al., 2003). Indeed, the slope of the LME (**Table 5.4**) indicated that players reporting low energy levels were associated with a 1.779 mg/L higher CRP (in comparison to those reporting good energy levels) adjusted for covariates. The significant association between CRP and muscle soreness is an important one given its potential to impair

performance (Owens et al., 2019). The synthesis and release of inflammatory agents by inflammatory cell infiltrates post-exercise has been described to trigger sensitization of nocioceptors and produce soreness (Fatouros et al., 2016). Furthermore, CRP has been found to be sensitive in assessing muscle damage in impact activities (Singh et al., 2011), and the significant relationship observed may also have occurred as a result of muscle contusions resulting from direct impact activities like tackling. Anecdotally, elite players are often hesitant to declare poor wellness prior to a game for fear it may effect team selection, which may explain why we only found a significant negative relationship between CRP concentrations and positive ratings of muscle soreness (**Table 5.4**).

Muscle fatigue and soreness may adversely affect sleep (Doherty et al., 2019), particularly after late night kick off times (Fullagar et al., 2016), and sleep disturbances are associated with elevated inflammation markers (Irwin et al., 2016). Moreover, elite athletes are susceptible to depression, particularly in relation to failed performance and the progression of depressive symptoms is linked to elevated pro-inflammatory cytokines (Ostapiuk-Karolczuk et al., 2015) and CRP levels (Osimo et al., 2019). Failure to capture sleep and psychological stress information is therefore a limitation of the current study. Additionally, capturing dietary data and training load data prior to GD-1 would have strengthened our analysis. Nevertheless, CRP and HPX data were repeatedly measured in the same athletes over time, which allows for stronger inferences to be made compared with a cross-sectional study (Lewis et al., 2020a), and the nature of this sample, i.e. highly-trained EPL players in a ‘real-world’ setting strengthens the ecological validity and novelty of our data.

5.6. Conclusion

We provide novel data demonstrating that pre-match inflammation status predicts post-match inflammatory response, which suggests that the pre-game may be an important window for practitioners to reduce inflammation, since it is unlikely they can influence player match selection. Our data are practically relevant as they demonstrate POC

measurement of CRP provides a non-invasive assessment that is sensitive to the changes in subjective feelings of wellness and illness and may assist in protecting player health and availability for team selection. Additionally, we demonstrate that HPX levels significantly increase within-season in the EPL. It is currently unknown whether the biomarker changes observed across time represent major deviations from optimal ranges, impact upon adaptation, and if the repeated episodes of muscle damage and inflammation in-season will ultimately be beneficial or detrimental across the career of the elite soccer player. High intra- and inter-individual variability in data mean that individualised approaches to biomarker monitoring may be of higher potential value in protecting player health and adaptation. Future studies should explore the generation of athlete specific ranges to assess individual tolerance of training and competition.

The observation that the pre-game may be an important window for practitioners to reduce inflammation provides further rationale for investigating the effect of anti-inflammatory strategies during periods of frequent match-play. The next experimental chapter will assess the efficacy of an acute protective protocol (curcumin supplementation) on reducing capillary blood biomarkers of inflammation and pro-oxidant status during the in-season competitive phase of the EPL.

CHAPTER 6: THE EFFECT OF CURCUMIN SUPPLEMENTATION ON CAPILLARY BLOOD BIOMARKERS OF INFLAMMATION AND OXIDATIVE STRESS

Chapter 6

6.1. Abstract

Reducing inflammation during periods of frequent match play may be an important objective to protect player health. PURPOSE: To investigate the effect of curcumin supplementation on biomarkers of inflammation and oxidative stress in response to weekly match play during an EPL season. METHODS: We conducted a longitudinal analysis of 18 EPL games during the 2019-2020 season (n=22), and used an interrupted time-series design to analyse the effect of curcumin supplementation on biomarkers of oxidative stress and inflammation. Relationships between biomarkers and co-variates were examined using linear mixed effects models. RESULTS: No significant effect was found for the intervention on CRP responses. No significant relationships were observed for all other explanatory variables on CRP responses. We found a significant association between HPX responses and time (0.005 mmol/L H_2O_2; p=0.020). HPX responses were lower compared with measures taken at GD-1 during Case 1 (-0.1811mMol/L H_2O_2; p=0.057), and higher at Control 2 (0.2550 mmol/L H_2O_2; p=0.055), albeit of borderline significance. No significant relationships were observed for all other explanatory variables and HPX. CONCLUSION: HPX levels display a significant increase during the first half of the EPL season in apparently healthy players. Curcumin ingestion at the dose prescribed is not effective, at least at a group level, for attenuating these responses within the applied setting of professional soccer.

6.2. Introduction

During periods of frequent match play, the recovery between successive games may not be adequate for the restoration of normal homeostasis and the resolution of the inflammatory response (Mohr et al., 2016), potentially leading to the development of low grade inflammation. Long-term supplementation with anti-oxidant or anti-inflammatory compounds is not recommended (Owens et al., 2019). However when adaptation is inconsequential, for instance, during periods of heavy training load whilst 'in-season', or during periods of fixture congestion when the ability to recover in sufficient time is compromised, nutritional supplementation may be beneficial in aiding the timely recovery of the player. Furthermore, if we know that an inflammatory episode/insult will occur by virtue of competition, pre-competition acceleration of inflammation resolution via targeted nutritional supplementation may represent a practical approach for preventing the development of low-grade inflammation and promoting the timely recovery of the elite soccer player in-season.

Curcumin is a polyphenol found in the spice turmeric and is often used to reduce exercise induced inflammation (Suhett et al., 2020; Fernández-Lázaro et al. 2020). Moreover, a recent review concluded that curcumin has good potential as a nutritional intervention to accelerate recovery in athletes by attenuating muscle damage and its associated signs and symptoms (Bongiovanni et al., 2020). Specifically, it appears that curcumin supplementation taken prior to exercise may be beneficial for athletes participating in high-intensity exercise with a significant eccentric load (McFarlin et al., 2016; Heaton et al., 2017; Tanabe et al., 2018). However, data in elite athletes are generally absent from the literature, with only one study conducted to date (Delecroix et al., 2017). To the knowledge of the authors, research investigating the efficacy of an anti-oxidant/anti-inflammatory intervention in the EPL are absent from the literature. Therefore, this study was designed to investigate the efficacy of curcumin supplementation on biomarkers of inflammation and oxidative stress during an EPL season. We hypothesized that biomarker responses could be reduced by accelerating the resolution of inflammation using curcumin supplementation prior to competition.

6.3. Methods

6.3.1. Participants

Twenty two players (age = 25.4 [3] y, height = 181.8 [7.8] cm, weight = 75.1 [6.2] kg, and body mass index (BMI) = 22.7 [1.5]) were recruited to participate from the first team of an EPL club.

6.3.2. Design overview

We used an interrupted time-series design to analyse the effect of curcumin supplementation on biomarkers of oxidative stress and inflammation.
Following a seven-game control period, participants completed a four-game supplementation block, followed by a second control period of two games and another supplementation block of five games. Only fit, healthy and training players were included in the experimental trial, with athletes reporting illness symptoms or injury to the medical staff and research personnel excluded from the experimental trial.

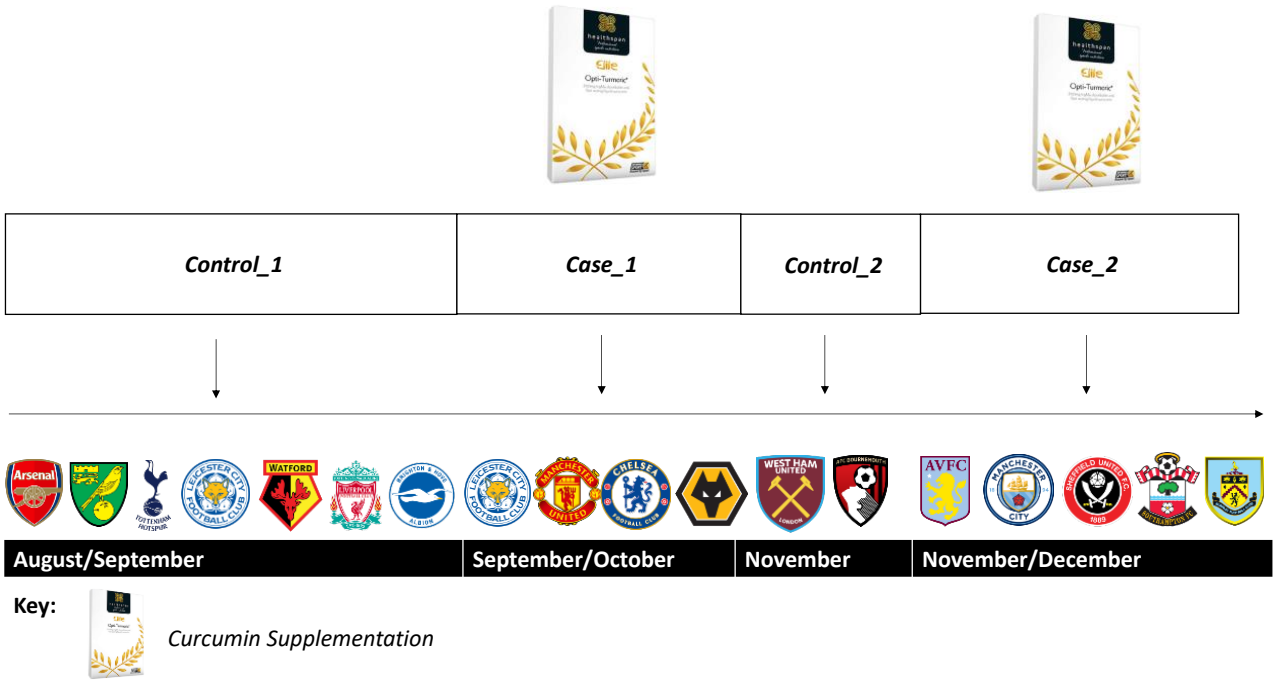

Figure 6.1: Schematic showing interrupted time series design across first half of English Premier League (EPL) season. Fixtures represented by respective club crests. Control (No curcumin), Case (Curcumin). *Stages: Control_1 (August/September), Case_1 (September/October), Control_2 (November), Case_2 (November/December).*

6.3.3. Methodology

Capillary blood samples were obtained as described in Chapter 3. Biomarkers of inflammation and redox homeostasis were measured as described in Chapter 3.

Match workload was measured using a multi-camera computerised tracking system as described in Chapter 3.

Supplementation was initiated two days prior to each game. Participants consumed 2 × 500 mg capsules (Elite Opti-Turmeric, Healthspan, Ltd) for a total dose of 1000 mg of curcumin (as recommended by the manufacturer), the morning of game day -2 (GD-2), game day -1 (GD-1) and immediately post-game during each supplementation period. Each capsule contained: NovaSOL® curcumin 500 mg), vitamin C (20 mg). Participants

maintained their habitual diet routine throughout each supplementation period. Supplement adherence was monitored via verbal confirmation by the same research personnel. To ensure compliance, the supplement was given only during the supplementation block, and on the days specified (GD-2, GD-1, Game Day). Where players reported non-compliance, this was noted and factored into the analysis. Subject non-compliance was broadly defined as subjects that did not adhere to the study protocol (i.e. missed supplement doses, missed testing appointments).

6.3.4. Data analysis

Summary statistics were calculated for each response variable and for the high speed running covariate by testing time (Pre/Post) and stage (Control or Case). The stage variable was coded as a factor (with 4 levels) to account for the interrupted time series study design used (i.e. control / case / control / case). LME were used to estimate the effect of the intervention on each response variable while controlling for stage, testing time, the number of days post game where the post measurement was taken (i.e. GD+1, GD+2, GD+3, GD+4) and the amount of high speed running recorded in the game in question. In order to account for the hierarchical structure in the design random effects were included for players, games and stage in order to account for the correlation within players over time, and for players within games and stages. The analysis excluded goalkeepers. Candidate models that were considered *a priori* included models with an interaction term to adjust for potential differences in the mean response at the pre-testing time in each stage, models that included distance as an additional covariate and models using the gamma distribution to account for the strictly positive responses. All analyses were performed using R (version 4.0.1). A P value of less than 0.05 was considered to indicate statistical significance.

6.4. Results

A total of 22 athletes completed the study (characteristics detailed in section 5.3). Summary statistics for HPX and CRP responses by stage are detailed in **Table 6.1**. The effect of intervention on CRP responses are detailed in **Table 6.2**. No significant effect was found for the intervention on CRP responses. No significant relationships were observed for all other explanatory variables on CRP responses. The effect of intervention on HPX responses are detailed in **Table 6.3**. We found a significant association between HPX responses and time (0.005 mmol/L H_2O_2; p=0.020). HPX responses were lower compared with measures taken at GD-1 during Case 1 (-0.1811 mmol/L H_2O_2; p=0.057), and higher at Control 2 (0.2550 mmol/L H_2O_2; p=0.055), albeit of borderline significance. No significant relationships were observed for all other explanatory variables and HPX.

Table 6.1: Summary Statistics for C-reactive protein (CRP) (mg/L), hydroperoxides (HPX) (mmol/L H_2O_2) and total high intensity running (THIR) (km) by Study Stage and Testing Time.

Stage	Control_1 (August/September)		Case_1 (September/October)		Control_2 (November)		Case_2 (November/December)	
	Pre (n=87)	Post (n=59)	Pre (n=55)	Post (n=41)	Pre (n=33)	Post (n=7)	Pre (n=72)	Post (n=45)
CRP								
Mean (SD)	1.62 (1.52)	2.15 (1.60)	1.48 (0.92)	1.91 (1.30)	1.49 (0.98)	2.72 (1.40)	1.50 (0.92)	2.21 (1.42)
Median [Min, Max]	0.98 [0.76, 8.89]	1.25 [0.80, 7.75]	1.04 [0.81, 5.21]	1.44 [0.77, 7.22]	1.07 [0.74, 4.64]	3.10 [0.94, 4.10]	1.06 [0.79, 4.73]	1.65 [0.91, 7.48]
HPX								
Mean (SD)	1.55 (0.31)	1.64 (0.29)	1.75 (0.37)	1.74 (0.38)	1.67 (0.34)	1.95 (0.34)	1.79 (0.46)	1.78 (0.37)
Median [Min, Max]	1.54 [1.22, 2.61]	1.60 [1.22, 2.46]	1.70 [1.22, 2.68]	1.70 [1.26, 3.11]	1.63 [1.22, 2.68]	1.94 [1.50, 2.47]	1.71 [1.22, 3.52]	1.73 [1.22, 2.46]
THIR								
Mean (SD)	446 (207)	454 (217)	391 (233)	473 (194)	446 (331)	468 (369)	388 (199)	415 (200)
Median [Min, Max]	477 [49.0, 920]	450 [66.3, 920]	479 [14.6, 713]	525 [38.1, 713]	473 [7.83, 1100]	456 [7.83, 970]	420 [10.2, 822]	409 [17.2, 822]

SD *Standard Deviation* ***Min*** *Minimum* ***Max*** *Maximum* ***CRP*** *C-reactive protein* ***HPX*** *hydroperoxides* ***THIR*** *total high intensity running*

Table 6.2: The estimated effect of intervention on C-reactive protein (CRP) (mg/L) responses adjusted for the effect of game and subject by fitting linear mixed effects models. [a]: in comparison to Control 1; [b]: in comparison to Game Day (GD)+4;

Explanatory Variable	Estimate	Standard Error	P-Value
Pre (vs Post) game	-0.8603	1.1320	0.453
Stage (Case 1) [a]	-0.3802	0.5641	0.512
Stage (Control 2) [a]	-0.2992	0.7345	0.691
Stage (Case 2) [a]	-0.6712	0.9408	0.491
GD +1days [b]	2.0210	1.1350	0.084
GD +2days [b]	1.1470	1.1320	0.319
GD +3days [b]	1.2290	1.1970	0.311
Time (Days)	0.0100	0.0094	0.311
THIR (km)	0.0004	0.0003	0.276

THIR *Total high intensity running* ***GD*** *Game Day*

Table 6.3: The estimated effect of intervention on hydroperoxides (HPX) (mmol/L H_2O_2) responses adjusted for the effect of game and subject by fitting linear mixed effects models. [a]: in comparison to Control 1; [b]: in comparison to Game Day (GD)+1;

Explanatory Variable	Estimate	Standard Error	P-Value
Pre (vs Post) game	0.0954	0.1196	0.426
Stage (Case 1) [a]	0.0001	0.1194	0.999
Stage (Control 2) [a]	-0.2774	0.1525	0.089
Stage (Case 2) [a]	-0.2715	0.1957	0.187
Time (Days)	0.0050	0.0019	**0.020**
THIR (km)	-0.0000	0.0000	0.613
Post Test Day [b]	-0.0159	0.0044	0.721
Post (vs Pre): Stage (Case 1) [a]	-0.1811	0.0949	0.057
Post (vs Pre): Stage (Control 2) [a]	0.2550	0.1323	0.055
Post (vs Pre): Stage (Case 2) [a]	-0.0775	0.0073	0.288

THIR *Total high intensity running*

6.5. Discussion

The primary aim of this study was to assess the efficacy of an acute protective protocol (curcumin supplementation) on capillary blood biomarkers of inflammation and pro-oxidant status during the in-season competitive phase of the EPL. The principal findings were that curcumin ingestion had no significant effect on CRP, and had borderline significant effects on HPX responses. However, the magnitude of this change does not appear to be physiologically relevant, suggesting that at a group level curcumin ingestion using the dose and protocol prescribed may not be effective for attenuating inflammation and pro-oxidant status within the real world, applied, field setting of elite soccer. To our knowledge, this is the first study to investigate the efficacy of a nutritional intervention aimed at reducing blood biomarkers of inflammation and oxidative stress in professional soccer players competing in the EPL.

We report that HPX levels significantly increase within-season in apparently healthy players and that curcumin ingestion at the dose prescribed is not effective for attenuating biomarker responses in elite soccer. Curcumin has been reported to downregulate the transcriptional activity of NF-κB, a key regulator of the inflammatory response, and upregulate Nrf2, the 'master regulator' of antioxidant enzymes (Sahin et al., 2016). Therefore, by simultaneously blocking the pro-inflammatory response and activating endogenous anti-oxidants, curcumin ingestion may act to control the regulation of inflammation during periods of high demand in the elite soccer player. However, in the current study, we report no significant differences between conditions for mean CRP values (**Figure 6.2**). Indeed, reports are inconsistent with regards to exercise induced inflammation in the literature (Suhett et al., 2020). In agreement with our findings, some exercise trials have shown that whilst post-exercise increases in markers of inflammation have tended to be lower in curcumin treatment groups, significant reductions in inflammation with curcumin supplementation are lacking (Drobnic et al., 2014; Sciberras et al., 2015). In contrast, we observed reductions in mean HPX levels during the Case 1 phase (**Figure 6.3**), albeit of borderline significance, suggesting that curcumin supplementation can attenuate exercise induced oxidative stress, a finding

which is corroborated by previous research (Chilelli et al. 2016; Takahashi et al. 2013). However, when we consider the magnitude of this change (**Table 6.3**), physiological relevance appears questionable based on the previously published CDV for this assay (Lewis et al., 2016a). The discrepancy between our findings and previous literature demonstrating statistically significant changes may be due to a combination of factors including the training and dietary status of the population investigated, the biomarker assays measured, the timing of the draw and several limitations associated with the current study.

To the authors knowledge, this is the first study investigating the utility of curcumin supplementation in elite soccer players. Indeed, given the recognised differences in inflammatory responses between athletes and non-athletes (Gokhale et al., 2007; Handzlik et al., 2013), caution is necessary when extrapolating findings from previous research investigating the utility of curcumin in non-athletes to the professional soccer player. Evidence suggests that professional soccer players upregulate anti-oxidant enzymes during a season (Silva et al., 2014), thus potentially protecting well recovered players with more training/match exposure from excessive oxidative damage during subsequent competitions and training sessions. It is plausible that this upregulation may be sufficient to limit excessive inflammatory mediator production and therefore elite soccer players may have less need to use anti-oxidant supplements than non-athletes, especially when athletes may be consuming high polyphenolic diets. Indeed, focusing on a well-balanced diet including fruits and vegetables to counteract inflammation and oxidative stress may be more appropriate for the elite soccer player than supplementing with individual antioxidants/anti-inflammatory compounds, as there seems to be no evidence at this time to suggest that consumption of fruits and vegetables blunts exercise-induced adaptations (Heaton et al., 2017). Furthermore, human studies have shown that the intake of fruits and vegetables is associated with a decrease in the levels of systemic inflammatory markers, such as IL-4, IL-6, IL-8, IL-13, TNF-α and CRP (Sureda et al., 2014) and increases in anti-inflammatory cytokines in well trained athletes (McAnulty et al., 2011).

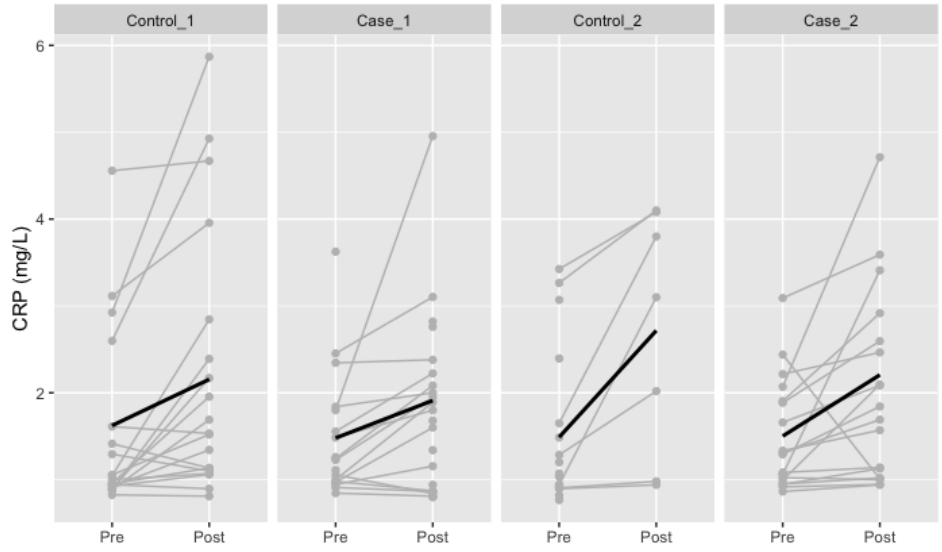

Figure 6.2: Plots of players average C-reactive protein (CRP; mg/L) trajectory over stage. *Black lines represent group mean, grey lines represent player averages. Stages: Control_1 (August/September), Case_1 (September/October), Control_2 (November), Case_2 (November/December).*

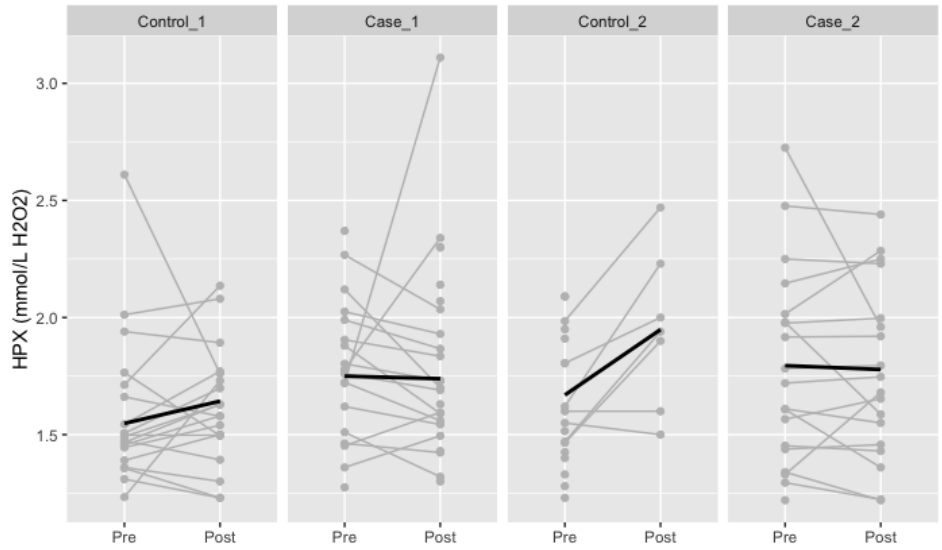

Figure 6.3: Plots of player's average hydroperoxides (HPX; mmol/L H_2O_2) trajectory over stage. *Black lines represent group mean, grey lines represent player averages. Stages: Control_1 (August/September), Case_1 (September/October), Control_2 (November), Case_2 (November/December).*

The wide heterogeneity in CRP (**Figure 6.2**) and HPX responses (**Figure 6.3**) may also provide important clues about why the study results are inconsistent. Indeed, studies have shown that the efficacy of antioxidants is affected by inter-individual variability in redox state, which is dependent, among other factors, on the training and nutritional status of the athlete (Kawamura et al., 2018). Recent reports have recognised the importance of identifying and correcting redox deficiencies in athletes which may occur as a result of low fruit and vegetable intake (Plunkett et al., 2010; Watson et al., 2005; Pedlar et al., 2019). Plunkett et al. (2010) observed increased resting and exercise induced inflammation alongside increased RPE in endurance trained males in response to a 2 week dietary anti-oxidant restriction. Moreover, emerging evidence suggests that antioxidant supplements improve performance and oxidative stress only when administered to deficient individuals (Margaritelis et al., 2020). Therefore, the optimal intervention approach should involve the individualized examination of redox and inflammation status in athletes. This would allow for the identification of responsive phenotypes and the detection of meaningful physiological changes in individual data, by constructing personalised adaptive reference ranges, resulting in the administration of the appropriate anti-oxidant supplementation at the appropriate time. Future studies should look to implement this targeted approach in elite sport. An example of this approach is presented in Chapter 8. Based on previously reported CDVs for HPX (Lewis et al., 2016a) and CRP (Chapter 4), physiologically relevant changes in CRP levels were observed for only 3 athletes in the study group in response to the intervention, with no athletes displaying substantial decreases in HPX levels.

The lack of significant changes in biomarker responses may also be explained by the assays measured in the current study. Higher concentrations of individual antioxidant enzymes as opposed to total anti-oxidant capacity have been found in rodents following curcumin supplementation (Avci et al. 2012), suggesting that curcumin's anti-inflammatory effects may potentially be tissue and/or enzyme specific (Basham et al., 2020). Furthermore, despite observing no significant reduction in serum levels of IL-6, McFarlin et al. (2016) reported a 25% decrease in circulating levels of the inflammatory cytokines TNF-α and IL-8 following muscle damaging exercise in a group of healthy

participants. Thus, the lack of measurements of a wider range of inflammatory biomarkers and anti-oxidant enzymes is a limitation in the current study.

For practical reasons (i.e. financial and time constraints), we did not measure anti-oxidant status, thus, we potentially missed capturing the impact of curcumin on the players' plasma anti-oxidant capacity. Similarly, we did not assess whether curcumin's serum concentration had sufficiently increased after the supplementation regimen so as to produce biological effects (Suhett et al., 2020). Considering the field-based nature of the present study, obvious limitations include the inability to blind the players and staff to the supplementation regimen and control the players' dietary intake. However, the use of control versus intervention groups is generally un-feasible at the elite level as only a single population benefits from the intervention (Carling et al., 2018; Bongiovanni et al., 2020), and restricting an athlete of important dietary nutrients during a competitive season is counter-intuitive in a high performance environment if the goal is to conduct applied research. Indeed, Bongiovanni et al. (2020) suggest that applied research should replicate how athletes actually utilize supplements in a real-world scenario in order to inform practice, and thus, the nature of the study, i.e. highly-trained EPL players in a 'real-world' setting strengthens the ecological validity and novelty of our data. However, capturing dietary data would have strengthened our analysis and allowed us to highlight individuals with dietary deficiencies. Athlete belief is another important consideration in the application of an intervention (Halson et al., 2013; Howatson et al., 2016). Failure to capture the athletes perception of the supplementation regimen and whether or not they felt it was effective in reducing the symptoms of EIMD and inflammation (e.g. muscle soreness) is therefore another limitation in the current study. Future studies with larger samples, and multiple curcumin dosages are warranted to investigate if different curcumin regimens can lead to meaningful changes in CRP and HPX levels in the elite soccer player.

6.6. Conclusion

To our knowledge, this is the first study to investigate the efficacy of a nutritional intervention aimed at reducing blood biomarkers of inflammation and oxidative stress in professional soccer players competing in the EPL. Our data demonstrate that curcumin ingestion at the dose and protocol prescribed is not effective for attenuating biomarker responses in elite soccer players competing in the EPL. Future studies should look to implement a more targeted approach to anti-oxidant supplementation in elite sport, involving the individualized examination of redox and inflammation status in athletes to allow for the identification of responsive phenotypes and the detection of meaningful physiological changes in individual data.

Our data demonstrate that curcumin ingestion, at a group level, was not effective for attenuating biomarker responses in elite soccer players competing in the EPL. Emerging evidence suggest that interventions which are “pro-resolving” in nature (i.e. facilitate the natural resolution of inflammation), such as increased consumption of omega-3 fatty acids, may more effectively enhance exercise recovery than anti-inflammatory interventions. Therefore, the next experimental chapter in the thesis examined concurrent alterations in EMFAs and CRP, and investigated the strength of their association, during the season in a cohort of professional soccer players competing in the EPL.

CHAPTER 7: EVIDENCE OF A RELATIONSHIP BETWEEN ERYTHROCYTE MEMBRANE FATTY ACID COMPOSITION AND INFLAMMATION AMONG HEALTHY PROFESSIONAL SOCCER PLAYERS

Chapter 7

7.1. Abstract

Data describing inflammation, an important component of the stress-adaptation cycle, in athletes competing in the EPL are scarce. PURPOSE: To investigate the seasonal variation in blood biomarkers of inflammation and EMFAs and to examine the strength of their association. METHODS: We conducted a longitudinal observational study in EPL players (n=35) over the course of three seasons. Data collection was conducted on eight occasions; 2017-18 season (September), 2018-19 season (July, November, January, March), 2019-20 season (July, November, January). Seasonal time points were defined as: July (T1), September (T2), November (T3), January (T4), and March (T5), with multiple measures taken at some time points (i.e. July, November, January). Relationships between biomarker variables and differences between seasonal time points were examined using linear mixed effects models. RESULTS: We report a mean Omega-3 Index of 6.8 (1.9)% for the squad. Compared to baseline (T1); C-reactive protein (CRP) levels were significantly higher at T4 (0.312 mg/L; p=0.052). Adjusted for the effect of time, we found a significant negative association between CRP and the omega-3 index (OM3I) (-0.126 mg/L; p=0.001), and a significant positive association between CRP and the omega-6:omega-3 ratio (0.163 mg/L; p=0.009). CONCLUSION: The competitive phase of the EPL induces time dependent changes in markers of inflammation and EMFA status. There was convincing evidence that the EMFA composition influences this change in CRP observed across time in the elite soccer player.

Chapter 7

7.2. Introduction

Within a season, professional teams often compete up to three times a week, particularly during the winter period in the EPL, and the recovery time between successive games may not be adequate for the restoration of normal homeostasis and the resolution of the inflammatory response (Owen et al., 2019; Mohr et al., 2016). Although elite players are well adapted to their sport, the long season with consistent exposure to acute inflammation and limited periods of rest, places the soccer player at high risk of under recovery, potentially leading to the development of low grade inflammation. Furthermore, the kinetics and resolution of inflammation may be influenced by the nutritional status of the athlete. A recent UEFA consensus statement emphasised the importance of nutrition and its role in optimising performance, facilitating post-exercise recovery and maintaining general health of the elite soccer player throughout the season (Collins et al., 2020). However, there is very little data in elite players (Collins et al., 2020). The production of pro-inflammatory eicosanoid end products from omega-6 polyunsaturated fatty acids, namely AA (Calder, 2006), and the actions of pro-resolving lipid mediators produced from EPA and DHA (Calder, 2012), suggest that measuring the composition of the EMFA and examining its association with inflammation could clarify the role of fatty acids in development of low-grade inflammation in soccer players. This in turn may provide rationale for augmenting the EMFA as a potential "pro-resolving" strategy during periods of limited recovery. It is noteworthy, that a recent systematic review reported positive effects for fish oil supplementation (FS) on skeletal muscle recovery in team sport athletes (Lewis et al., 2020).

Currently, there are limited longitudinal data examining inflammation levels of professional soccer players during a season in the EPL. This type of analysis might help us understand the physiologic demands and biochemical impact of competing in the EPL, inform practitioners regarding the time-course of inflammatory responses during key stages of the in-season period and in turn assist in adopting a periodized approach to the implementation of anti-inflammatory strategies to protect player health and recovery. This study was designed to examine, for the first time, concurrent alterations

in EMFAs and inflammatory markers during the season in a cohort of professional soccer players competing in the EPL. We also wanted to determine whether inflammatory status is associated with certain fatty acid variables in professional soccer players. We hypothesized that omega-6 fatty acid variables would be positively associated with CRP and that the omega-3 fatty acid variables would be negatively associated with CRP.

7.3. Methods

6.3.1. Participants

Participant characteristics detailed in section 4.3.1.

7.3.1. Design overview

We conducted a longitudinal, observational study to examine the relationship between inflammation status and red blood cell fatty acids in male professional soccer players who participated in biochemical monitoring through the measurement of many relevant biomarker variables over the course of 3 EPL seasons. Data collection was conducted on eight occasions; 2017-18 season (September), 2018-19 season (July, November, January, March), 2019-20 season (July, November, January) (**Figure 7.1**). In order to assess variation in biomarkers across stages of a season, seasonal time points were defined as: July (T1), September (T2), November (T3), January (T4), and March (T5), with multiple measures taken at some time points (i.e. July, November, January). Players were tested at the same time of day, in a fasted and rested state, with no game or heavy training undertaken the day prior to testing. To be included in the study, players had to be healthy, with no known infection or injury during the week preceding blood sampling. These criteria were assessed through the administration of a brief questionnaire and confirmed with the medical staff. Athletes presenting with illness/injuries were removed from the analysis in order to minimise the effect of acute inflammation.

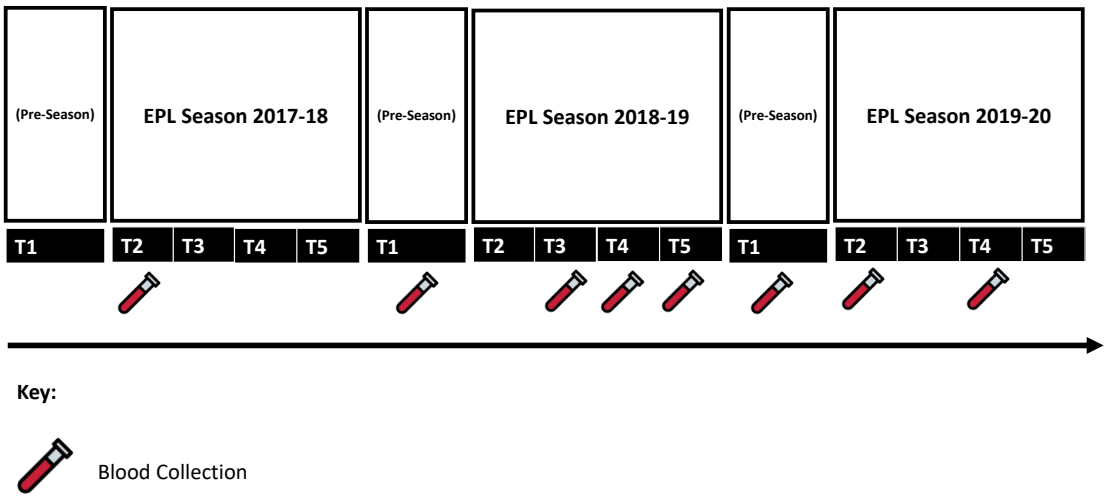

Figure 7.1: Schematic showing testing time points across the 3 English Premier League (EPL) seasons. T1 (July), T2 (September), T3 (November), T4 (January), T5 (March).

7.3.2. Methodology

Capillary blood samples were obtained as described in Chapter 3. Biomarkers of inflammation and EMFAs were measured as described in Chapter 3.

7.3.3. Data analysis

Since data were collected repeatedly on the same group of players, biomarker data are correlated over time. To account for this dependency, we use linear mixed effect models (LME). In this study we include a random intercept to allow for different mean CRP values between players. LME were used to describe biomarker differences across time where the estimated effect for the association between each biomarker (Inflammatory markers: CRP; EMFA variables: AA:EPA ratio, AIFAI, OM3I, omega6:omega3 Ratio, DHA, EPA) and time was reported, along with standard error and p-values. LME were also used to investigate how sets of biomarkers representing similar functions (i.e EMFA variables) relate to the change in CRP over time. A number of candidate models

were tested (with and without an interaction term) using a likelihood ratio test. We report effect estimates for the association between each biomarker and CRP, along with standard error and p-values. Model assumptions were tested using suitable residual plots. All statistical analyses were carried out using R (Version 3.4.6) and the significance level was set at $p < 0.05$.

7.4. Results

A total of 35 athletes completed the study. Mean (standard deviation) for biomarker values across each time point are detailed in **Table 7.1**. Longitudinal data for CRP are presented in **Figure 7.2**. EMFA and CRP biomarker relationships presented in **Figure 7.3**. CRP levels were significantly higher at T4 compared to T1 (0.312 mg/L; p=0.052). The OM3I was significantly higher at T2 (0.831 %; p=0.001), T3 (0.485 %; p=0.015) and T4 (0.565 %; p=0.010), compared to T1. The AA:EPA Ratio was significantly lower at T2 (-0.576; p<0.001), T3 (-5.62; p<0.001), T4 (-5.19%; p<0.001), and T5 (-4.85; p<0.001), compared to T1. The AIFAI was significantly higher at T2 (5.09; p=0.047), T3 (6.08; p=0.002), T4 (8.59; p<0.001) and T5 (7.42; p=0.004) compared to T1. The omega-6:omega-3 ratio was significantly lower at T2 (-0.931; p<0.001), T3 (-0.513; p<0.001), T4 (-0.605; p<0.001), and T5 (-0.674; p<0.001), compared to T1. DHA was significantly higher at T2 (0.502 %; p=0.011) compared to T1, and EPA was significantly higher at T2 (0.295 %; p=0.008), T3 (0.211 %; p=0.014) and T4 (0.276 %; p=0.004) compared to T1.

We found a significant negative association between the OM3I and CRP, with a one unit increase in the OM3I associated with a -0.126 mg/L (p=0.001) decrease in average CRP, a significant negative association between the AIFAI and CRP, with a one unit increase in the AIFAI associated with a -0.009 mg/L (p=0.020) decrease in average CRP, a significant negative association between EPA and CRP, with a one unit increase in EPA associated with a -0.253 mg/L (p=0.014) decrease in average CRP, and a significant negative association between DHA and CRP, with a one unit increase in DHA associated with a -0.233 mg/L (p<0.001) decrease in average CRP. We found a

significant positive association between the omega-6:omega-3 ratio and CRP, with a one unit increase in the omega-6:omega-3 ratio associated with a 0.163 mg/L($p<0.009$) increase in mean CRP. We found a positive association between the AA:EPA Ratio and CRP (albeit not significant), with a one unit increase in the AA:EPA Ratio associated with a 0.018 mg/L ($p<0.094$) increase in mean CRP.

Table 7.1: Mean (Standard Deviation) for biomarker values across time (T) in professional soccer players

Biomarker	**July (T1) (n=42)**	**September (T2) (n=17)**	**November (T3) (n=46)**	**January (T4) (n=31)**	**March (T5) (n=17)**
Arachidonic acid: Eicosapentaenoic acid **(**AA:EPA) Ratio	17.39 (8.44)	11.98 (4.38)	10.95 (4.73)	11.65 (5.68)	12.36 (5.33)
Anti-Inflammatory Fatty Acid Index (AIFAI)	70.19 (17.77)	75.25 (16.73)	80.12 (17.42)	82.58 (20.54)	80.58 (16.60)
C-reactive protein (mg/L)	0.83 (0.86)	0.91 (0.83)	0.80 (0.63)	1.10 (0.84)	1.10 (0.63)
Docosahexaenoic acid (DHA) (%)	4.28 (1.28)	4.81 (1.15)	4.68 (1.30)	4.66 (1.28)	4.72 (1.10)
Eicosapentaenoic acid (EPA) (%)	0.96 (0.78)	1.21 (0.60)	1.31 (0.71)	1.30 (0.85)	1.12 (0.45)
Omega6:Omega3 Ratio	5.28 (1.38)	4.32 (0.84)	4.62 (1.17)	4.54 (1.10)	4.58 (1.10)
Omega-3 Index (%)	6.22 (2.04)	7.05 (1.72)	7.06 (1.94)	7.04 (2.01)	6.80 (1.51)

Table 7.2: The estimated effect of Omega-3 Index on C-reactive protein (CRP) adjusted for the effect of time (T) by fitting random intercept mixed effect models.

Time Point	Effect	Standard Error	P-Value
T2	0.173	0.190	0.362
T3	0.083	0.142	0.560
T4	0.395	0.159	**0.014**
T5	0.304	0.190	0.112
Omega-3 Index (%)	-0.126	0.037	**0.001**

7.5. Discussion

The primary aims of this study were to determine the strength of the association between a host of fatty acid variables and a marker of systemic inflammation measured concurrently in professional soccer players and to evaluate the seasonal variation in these biomarkers. A time specific pattern emerged with significantly higher mean CRP values, at T4 (mid-season) compared with T1 (pre-season). Moreover, time-dependent changes were also observed for Omega-6 and Omega-3 fatty acid variables, with the former decreasing and the latter increasing from baseline (T1). Although we cannot demonstrate causality, we report significant relationships between inflammation and fatty acid variables suggesting that EMFA composition is a significant driver of low-grade inflammation in professional soccer players. As a whole, the squad presented with an adequate mean OM3I, however, our findings suggest an increased systemic inflammatory environment in the presence of Omega-3 ‘insufficiency’. Together these data provide an insight into the biochemical impact of professional soccer across different stages of a season, and suggest that measuring and manipulating the EMFA composition of professional soccer players may be a useful endeavour in order to decrease inflammation and protect player health and recovery.

The present investigation suggests that a competitive soccer season induces time-dependent changes in systemic inflammation (**Figure 7.2**). Significant rises in CRP were evident in the present cohort after the winter period in the EPL (T4), which is characterized by an intensive fixture schedule with very limited recovery time. We observed a 32.5% increase in mean CRP between T1 (July) and T4 (January). It is likely that a combination of repetitive loading and limited recovery due to increased competitive demands led to this rise. Similarly, Silva et al. (2013) investigated seasonal variation in biomarkers of physiological strain in a group of professional soccer players competing in the Portuguese professional soccer league and found CRP to be significantly higher (130% increase) at the middle of the season when compared to measurements taken at the beginning of the pre-season period. The clinical relevance of the changes in CRP are debatable given the mean values are within the reference ranges previously defined for elite soccer players (Meyer et al., 2011). However, the clear intra- and inter-individual differences evident in **Figure 7.2**, highlight the need for an individualised approach to longitudinal biomarker tracking to assist in the interpretation of physiologically meaningful changes in the inflammatory status of the elite soccer player.

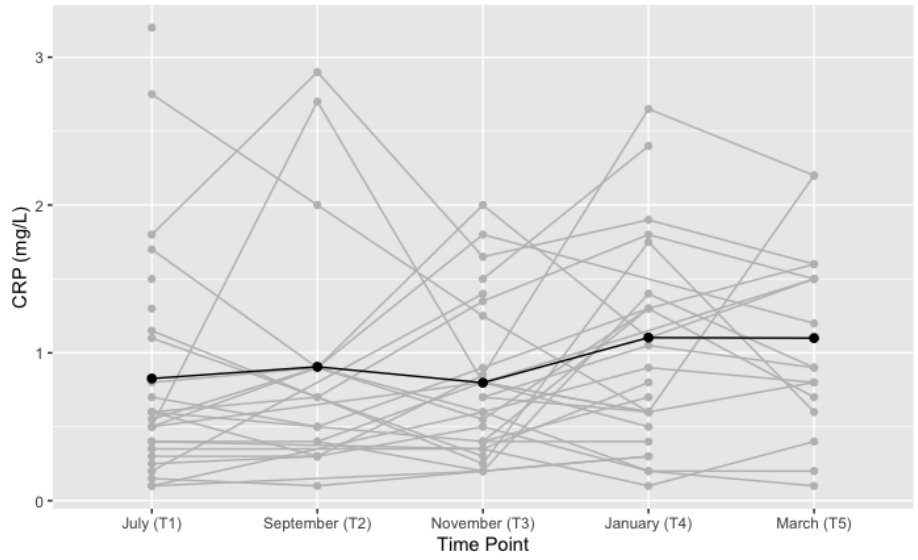

Figure 7.2: Case profile plot of C-reactive protein (CRP) (mg/L) for all players across time. *Black lines* represent group mean at each time point, and *grey lines* represent individual data.

The mean OM3I observed in the current study was 6.8 (1.9)%, and is higher than previously observed in elite winter endurance athletes 4.9 (1.2)% (von Schacky., 2014), collegiate American footballers 4.4 (0.8)% (Anzalone et al., 2019) and Summer Olympians 5.1 (1.0)% (Drobnic et al., 2017), but below the target range of 8 to 11% suggested for the OM3I (Drobnic et al., 2017). However, further research is required to substantiate this precise range in the context of the health and performance of the elite athlete (Pedlar et al., 2019). Mean values for the Omega-6:Omega-3 Ratio, 4.75 (1.2), were lower in this cohort than previously reported in a group of professional soccer players (6.79) (Tepsic et al., 2009). These findings may be related to a number of factors, including the dietary practices (Takkunen et al., 2014), genetics (Malerba et al., 2008), compliance with FS (Bloomer et al., 2009), and training history of the athletes, as previous research in footballers suggest that long-term intensive exercise has significant influence on phospholipid fatty acid profile in erythrocytes (Tepsic et al., 2009). Therefore, it is likely that a combination of alterations in exogenous and endogenous fatty acid supply will have contributed to the EMFA composition observed at each time point.

We can speculate that significant changes in the EMFA composition are more likely to be observed after an off-season period (where athletes may be detrained, consuming a diet less rich in Omega-3 fatty acids and not supplementing with fish oils), as evidenced by the higher Omega-6:Omega-3 Ratio at T1 (**Table 7.1**). Indeed, athletes presenting with a lower baseline OM3I have been found to be more successful at incrementing their OM3I levels in response to FS (Drobnic et al., 2017). It is important to note that FS was administered by the high performance staff from the start of the pre-season across all squad players. The increased bioavailability of Omega-3 from FS will have influenced the extent of incorporation of EPA and DHA into erythrocytes (Buckley et al., 2009), and as such, displaced AA, reflected in the significant decreases in the Omega-6:Omega-3 observed across time. Moreover, the OM3I concentrations displayed significant increases across time points, before plateauing at T5. Indeed, it has been reported that it takes approximately five months for the EMFA to reach a new steady state (Flock et al., 2013). It is plausible that the Omega-3 concentrations in the

erythrocyte membrane reached a saturation point by T4 (Flock et al., 2013; Drobnic et al., 2017), resulting in a slower incorporation rate across the rest of the season. Moreover, endurance training has been shown to increase DHA content, and decrease the Omega6:Omega3 ratio (Helge et al., 2001; Andersson et al., 2002). Therefore, in addition to the influence of exogenous fatty acid supply, the consistent training and frequent competition over the winter period may have induced an adaptive response in muscle membrane phospholipid fatty acid composition and contributed to the changes observed across time.

There was convincing evidence that the EMFA variables influenced the change in CRP observed across time. Some omega-6 fatty acids may aggravate low-grade inflammation because of their pro-inflammatory eicosanoid end-products (Calder, 2006). Consistent with our hypothesis, we found a significant relationship between the omega-6:omega-3 ratio and CRP (**Figure 7.3A**). We also found a significant relationship between the proportion of omega-3 fatty acids in EMFA and inflammation, with the OM3I shown to be inversely associated with circulating concentrations of CRP (**Figure 7.3B**). Omega-3 fatty acids can directly decrease production of inflammatory mediators by inhibiting production of AA-derived eicosanoids, replacing AA in the membrane phospholipids (Calder, 2006), which may explain many of their anti-inflammatory actions (Calder, 2009; Kohli et al., 2009; Fritsche, 2015). Additionally, the discovery of resolvins and protectins produced from DHA and the potency of those mediators in resolving inflammation (Calder, 2009) may also explain the significant relationship observed between OM3I and CRP. Nonetheless, it has been suggested that eicosanoid-independent actions are the most important mechanism underlying this relationship (Calder, 2009). The effects of fatty acids on inflammation appear to be related to their effects on transcription factors, namely NF-κB and peroxisome proliferator activated receptor (PPAR)-g, which regulate inflammatory cytokine gene expression (Calder, 2009). Calder (2009) suggests omega-3 fatty acids act in a way that modifies inflammatory gene expression by inhibiting activation of NF-κB, as well as enhancing PPAR-g activity, which is believed to act in an anti-inflammatory manner (Kliewer et al., 1997).

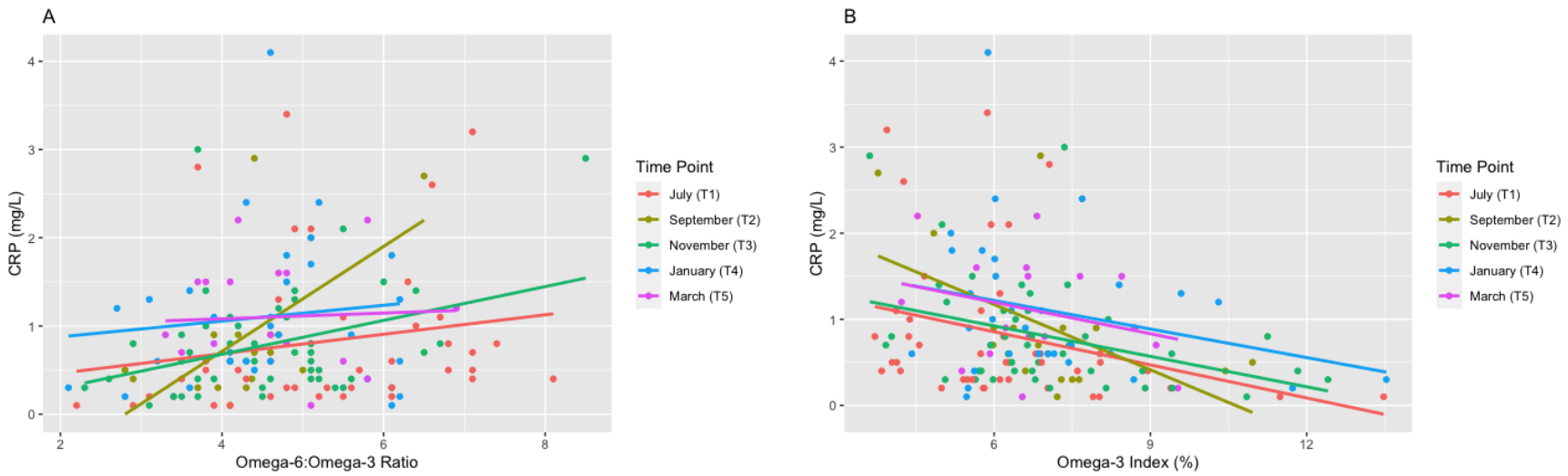

Figure 7.3: Scatter Plots of (A) Omega-6:Omega-3 Ratio (B) Omega-3 Index (%) versus C-reactive protein (CRP) (mg/L) for Time Point (T)1, T2, T3, T4 and T5 (with line of best fit).

The use of anti-inflammatory drugs that strive to control inflammation via antagonism of pro-inflammatory pathways, whilst currently commonplace in elite sport (Lippi et al., 2006), may impair the timely resolution of inflammation (Markworth et al., 2016). EPA and DHA, the long-chain fatty acids present in FS, are precursors for pro-resolving lipid mediators which collectively function as endogenous 'stop signals' to control and resolve inflammation (Markworth et al., 2016). Our findings provide rationale that augmenting the EMFA composition of professional soccer players may facilitate the natural resolution of inflammation during periods of frequent match play. Increased consumption of oily fish has been shown to increase biomarkers of omega-3 status (Grimstad et al., 2011), and epidemiological studies suggest an inverse relationship between CRP and a diet rich in marine products (Niu et al., 2006). However, it has been suggested that in adult humans, an EPA plus DHA intake >2 g/d may be required to elicit anti-inflammatory actions (Fritsche, 2015), and in the case whereby this is not obtained through dietary means, supplementation may be necessary to support the elite athlete.

It is beyond the scope of this paper to provide detailed discussion on FS and inflammation in athletes, and readers are directed towards a recent systematic review by Lewis et al. (2020). Briefly, however, Flock et al. (2013) showed that healthy individuals with a low average OM3I of 4.3% increased their average OM3I to 9.5% in response to 1.8g/d of EPA + DHA for five months. In the current study, adjusted for the effect of time, the slope of the linear mixed effects model indicated that for every 1% increase in the OM3I, CRP decreased on average by -0.126 mg/L, meaning that a 5.2% increase in the OM3I, as observed above, could equate to a 0.65mg/L decrease in CRP in individuals presenting with a low OM3I. However, it is important to note, supplementation would need to commence several months prior to the congested winter period in order to maximize incorporation into the muscle membrane.

The present observational study has many limitations: The absence of dietary data did not allow for detailed dietary analysis, and therefore, we cannot determine whether fish intake, supplementation compliance or workload induced adaptive responses were

responsible for the alterations in the EMFA composition found. The sporadic nature of our observational periods is certainly a limitation. Increasing the number of observational periods across each season would be beneficial in order to add greater strength to the association demonstrated between EMFA status and inflammation across time. However, this may not always be feasible when conducting scientific studies at the elite level during a competitive season. Numerous external influences such as suspensions, team selections, illness and injury (Owen et al., 2019), and the logistical challenge of gathering data in the interval between successive matches, realistically reduce opportunities for monitoring during a competitive season (Carling et al., 2018). We only used one biomarker to assess inflammation status, and a wider range of inflammatory biomarkers would have strengthened our analysis. Future work may address these limitations.

This study has several strengths. The use of validated analytical methods to determine biomarker levels of Omega-3 fatty acids incorporated into the red cell membrane is considered superior to serum measures which are more dependent on recent dietary intake (Harris, 2013). This study uniquely tracks both inflammation and EMFA status together in professional soccer players competing in the EPL, and therefore, the research can be considered to have high ecological validity.

7.6 Conclusion

In line with our hypothesis, we report a significant a relationship between inflammation and EMFA variables in healthy professional soccer players. Future research should involve the individualized examination of inflammation status in athletes, should measure biomarkers of Omega-3 fatty acid status, and investigate the impact of FS and the dose–response on inflammation during periods when the recovery time between successive games may be insufficient to resolve inflammation. This may have important implications for player health given the link between inflammation and a host of clinical issues.

Chapter 7

We present collective data for biomarker responses in Chapter 5, Chapter 6 and Chapter 7. The clear intra- and inter-individual differences evident in the data highlight the need for an individualised approach to longitudinal biomarker monitoring. The last experimental chapter of the thesis explored the generation of athlete specific ranges and a more targeted approach to anti-oxidant supplementation as discussed in Chapter 6.

CHAPTER 8: THE APPLICATION OF INDIVIDUALIZED ADAPTIVE REFERENCE RANGES IN THE LONGITUDINAL BLOOD MONITORING OF THE ELITE SOCCER PLAYER

Chapter 8

8.1. Abstract

Blood biomarkers play an important role in understanding the physiological state of an individual. In the context of elite soccer, the utilisation of POC tests for biomarkers of inflammation and oxidative stress via capillary sampling, facilitates more frequent testing in order to assess adaptation/recovery from disturbances in homeostasis. Large between-subject variability impedes the practical value of population-based reference ranges. Individualised approaches to biomarker monitoring may therefore be of higher potential value in protecting player health. In this study, we describe the use of dynamic individualised reference ranges to evaluate biomarker data collected longitudinally from elite soccer players competing in the EPL. Secondly, we aimed to investigate whether the observed changes in blood as a result of curcumin supplementation are greater than the previously reported CDV for the aforementioned biomarkers and therefore of physiological significance. Our study confirms that athletes deemed "healthy" or "non-healthy" present markedly different within- and between-subject variations in biomarkers of CRP and HPX values, with healthy athletes displaying negligible heterogeneity and non-healthy athletes displaying wide heterogeneity in biomarker responses, which has a significant impact on their respective individualized ranges. Physiologically relevant changes in CRP levels were observed for only 3 athletes in the study group in response to the intervention, with no athletes displaying substantial decreases in HPX levels. The application of individualized reference ranges for blood biomarker monitoring data may assist the practitioner in identifying periods where an individual may require a nutritional, performance or clinical review. However, further research is warranted in order to transform this promising strategy into an efficient monitoring tool in a real world applied setting.

8.2. Introduction

Blood biomarkers play an important role in understanding the physiological state of an individual. Indeed, the discovery of novel biomarkers is an active area of interest in the field of "personalized medicine" (Sottas et al., 2011). In the context of elite soccer, the utilisation of POC tests for biomarkers of inflammation and oxidative stress via capillary sampling, would allow for more routine testing in order to assess ongoing adaptation/recovery from disturbed homeostasis, and may be used to enhance the load management of the high performance athlete (Pedlar et al., 2019). However, Lewis et al. (2016a) outline that the successful application of biomarker monitoring programs in elite sport is not only hindered by the need for the generation of "real time" results but also an understanding of what is 'normal' and what constitutes a meaningful deviation from normal for each individual athlete.

Population based reference ranges, known as 'static' or 'normative' reference ranges are often used when interpreting a set of biomarker test results. Although these ranges may be valuable in assessing an individual's physiological state when there is only one observation recorded, biomarkers are typically collected longitudinally for athletes, and they have limited applicability when there are serial measurements on the individual's biomarker, as consideration of changes in the biomarker over time are ignored (Roshan, 2019). This is particularly true when biomarkers present higher between-subject biologic variations than within-subject biologic variations (Roshan, 2019). Interestingly, Heikura et al. (2018) recently reported that bone injuries were 4.5 fold more prevalent in male athletes with testosterone values in the lower quartile of the sample, but notably all values were within the clinical range. Anecdotally, this is common with repeated blood samples in athletes, but examples documenting this in the research literature are rare. This large between subject variability impedes the practical value of population based reference ranges. Individualised approaches to biomarker monitoring may therefore be of higher potential value in protecting player health or detecting an under-recovered state. Indeed, the elimination of the inter-individual component by deriving reference ranges from repeated measurements in a given athlete may lead to a significant

improvement of the biomarker signal, and thus the diagnostic accuracy of blood monitoring tools (Hecksteden et a., 2017). This individualisation is used in the Athlete Biological Passport, as implemented by WADA (Sottas et al., 2011). Moreover, although blood samples are routinely drawn with the athlete in a rested state, testing administered after competition could be valuable when resting values of biomarkers might not reveal any concerns, but biomarker responses to an acute stress would more sensitively detect potential health/recovery issues (Lee et al., 2017), and may allow for the identification of heavily fatigued states (Lewis et al., 2015b).

During periods of regular competition when recovery is the priority, as opposed to adaptation, nutritional strategies may be appropriate to alleviate muscle soreness and restrict secondary tissue injury associated with these inflammatory episodes (Peake et al., 2015). Curcumin has been described to moderate the inflammatory response and essentially act as a booster of the body's endogenous antioxidant response (Drobnic et al., 2014), therefore, supplementation in-season could be beneficial in modulating any further exacerbation of damage. Recent studies have shown that the efficacy of antioxidant supplementation is affected by inter-individual variability in redox state (Kawamura et al., 2018), meaning that athletes may be either low-, moderate-, or high-responders to a given supplementation regimen. The individualized examination of redox and inflammation status in response to curcumin supplementation using previously reported CDV, for biomarkers of inflammation (see Chapter 4) and RONS (Lewis et al., 2016a) in athletes, may be useful in understanding whether a 'true' physiological change has occurred as a result of the supplementation regimen for the individual athlete in question.

The current study aimed to explore the application of individualized adaptive reference ranges in professional soccer players competing in the EPL using two POC biomarkers: CRP as a marker of inflammation, and HPX a marker of pro-oxidant activity. Secondly, we aimed to investigate whether the observed changes in blood as a result of curcumin supplementation are greater than the previously reported CDV for the aforementioned biomarkers and therefore of physiological significance.

8.3. Methods

8.3.1. Participants

Data from six players (age = 22.9 [2.3] y, height = 178.8 [7.1] cm, weight = 73.9 [6] kg, and BMI = 23.1 [0.75]) from the first team of an EPL club are discussed.

8.3.2. Design overview

Data derived from Chapter 5 and Chapter 6 was subsequently used for the purpose of this chapter. A series of individual cases of longitudinal point of care biomarker data are described.

8.3.3. Methodology

Wellness and Symptoms:

Perceived ratings of wellness were measured as described in Chapter 3.

Blood Sampling:

Capillary blood samples were obtained as described in Chapter 3. Biomarkers of inflammation and redox homeostasis were measured as described in Chapter 3.

Match Load Assessment:

Match workload were measured as described in Chapter 3.

Curcumin Supplementation:

Supplementation protocol was as described in Chapter 6.

8.3.4. Data analysis: Generation of Individualized Adaptive Reference Ranges

In this study, we focus on discussing the benefits of using dynamic approaches to create individualised reference ranges by implementing the methods on biomarker data collected longitudinally from elite soccer players competing in the EPL. The aim is to combine prior information (i.e. knowledge about the biomarker distribution) with individual measurements in order to construct reference ranges where they can be

adapted every time a new measurement is recorded for the individual (Roshan, 2019). Roshan et al. (2019) proposed generating such adaptive reference ranges using both the Bayesian approach and an approximate Expectation-Maximisation (EM) algorithm where they concluded despite the negligible difference between the two methods in terms of performance, the latter outperforms the former in terms of the computation time. Thus, the approximate EM algorithm was employed in the current study for rapid construction of individualised adaptive reference ranges for CRP and HPX. When athletes reported the presence of any illness/injury symptoms, to the research personnel or medical team at the club, these data were excluded from the generation of the adaptive reference ranges.

8.4. Case Studies

To our knowledge, this is the first set of case studies to investigate the application of individualized adaptive reference ranges in elite soccer. The purpose of our investigation was to evaluate the utility of the adaptive ranges in detecting health/recovery concerns in the elite soccer player and differentiating between normal and abnormal profiles. The approach taken was to construct static and dynamic reference ranges for each athlete to not only compare their test results with the rest of the athletes but also to see if there were any meaningful changes in their CRP and HPX levels. Whereby abnormal data were flagged via the application of a calculated dynamic reference range, the focus of the case discussion will be on evaluating its abnormality and the potential causes. Our study confirms that athletes categorized as “healthy” or “non-healthy” present markedly different within- and between-subject variations in biomarkers of CRP and HPX, which will be presented below. Moreover, based on previously reported CDVs for CRP (Chapter 4) and HPX (Lewis et al., 2016a), physiologically relevant changes in CRP levels were observed for only 3 athletes in the study group in response to the intervention, with no athletes displaying substantial decreases in HPX levels. The following set of case studies describe the application of individualized adaptive reference ranges, together with the visualization of post-game biomarker responses, and

discuss how they may be used to optimize the practical application of blood monitoring and allow for the identification of health/recovery concerns in the elite soccer player.

Table 8.1: Clinical Laboratory Results (October, 2019):

Biomarker	**Range (Unit)**	**Player 1**	**Player 2**	**Player 3**	**Player 4**	**Player 5**	**Player 6**
White Blood Cells	3.5-10 (x10^9/L)	3.5	6.3	4.6	5.8	4.3	4.2
Neutrophils	1.7-7.5 (x10^9/L)	1.9	2.7	1.9	2.7	1.5	1.9
Monocytes	0.3-1 (x10^9/L)	0.3	0.6	0.4	0.4	0.4	0.4
Lymphocytes	1-3.5 (x10^9/L)	1.3	2.7	2	2.5	2.3	1.3
Eosinophils	<0.4 (x10^9/L)	0.1	0.2	0.2	0.1	0.1	0.5
Basophils	<0.1 (x10^9/L)	0.0	0.0	0.0	0.0	0.0	0.0
Red Blood Cells	4.25 - 5.75 (x10^12/L)	5	5.08	5.05	5.64	5.6	5.35
RDW	<14.5 (%)	13.3	12.3	14.4	12.1	12.3	12.3
MCV	84-98 (fL)	88	89	89	89	81	86
Haematocrit	0.4-0.5 (L/L)	0.44	0.45	0.45	0.50	0.45	0.46
Haemoglobin	130-170 (g/L)	146	158	150	174	153	158
MCH	27.5-32 (pg)	29.2	31.1	29.7	30.9	27.3	29.5
MCHC	300-360 (g/L)	333	349	336	347	337	345
Ferritin	25-200 (μg/L)	46	148	54	56	46	91
Serum Iron	10-30 (μmol/L)	14.9	16.9	19.2	12.6	19.2	20.4
Transferrin Saturation	23-57 (%)	23	25	27	16	26	31

***MCV**, mean cell volume; **MCH**, mean corpuscular haemoglobin; **MCHC**, mean corpuscular haemoglobin concentration; **RDW**, red cell distribution width*

Table 8.2: Dietary Analysis: Biomarkers of fruit and vegetable intake, vitamins and RBC fatty acids (October, 2019).

Biomarker	Range (Units)	Player 1	Player 2	Player 3	Player 4	Player 5	Player 6
Hydroperoxides (FORT)	1.22–2.5 (mmol/L H_2O_2)	1.94	2.19	2.3	1.76	2.01	1.66
Total Anti-oxidants (FORD)	1.1–2.0 (mmol/L Trolox)	1.05	0.97	0.8	1.12	1.08	0.94
Oxidative Stress Index (FORT/FORD)	< 2	1.85	2.26	2.88	1.57	1.86	1.77
RBC GSH	1.6–2.8 (mmol/L)	2.15	1.87	2.15	1.85	2.15	1.89
RBC SOD	1102–1601 (U/gHb)	2650	2487	2015	2220	2756	2,322
Serum Folate	3-20 (µg/L)	13.1	6.5	6.4	5.1	6.1	15
Vitamin B12	200-910 (ng/L)	772	526	313	373	293	791
25-Hydroxy Vitamin D	50-200 (nmol/L)	93	107	59	113	67	127
Omega-3 Index	4-13 (%)	8.85	6.73	7.35	5	6.69	7.44
Anti-Inflammatory Fatty Acid Index	No range	82.32	73.34	92.25	74.41	81.79	94.95
Omega-6:Omega-3 Ratio	2.3-8.1	3.7	4.6	3.7	5.5	4.9	3.6
AA:EPA Ratio	1.4-52.6	6.1	10.3	9.3	13.5	11.8	8.4

***RBC GSH**, red blood cell glutathione; **RBC SOD**, red blood cell superoxide dismutase. **AA:EPA**, Arachidonic acid: Eicosapentaenoic acid*

The use of post-exercise measures may improve the identification of heavily fatigued states in athletes (Lewis et al., 2015b). In accordance, significant disturbances in the athletes' inflammatory response are observed post-game, but not at rest, from the period between the 28th of October and November 30th (**Figure 8.1A**). Excessive cytokine release is implicated in the pathophysiology of fatigue, a response which may be amplified upon repeated exposure to subsequent bouts of exercise (Robson, 2003). Player 1 experienced an increase in workload during this period, with THIR ranging from 0.7-0.97 km across the 4 games measured, the highest experienced by the player up to that point (player average THIR = 0.68km). Increases in THIR have been shown to be associated with augmented sensations of fatigue in elite soccer players (Thorpe et al., 2015), and the athlete persistently reported low energy levels during this period. Susceptibility to illness and infection is exacerbated during periods of heavy competition (Gunzer et al., 2012), and this increase in workload may have contributed to the URTI observed in the upper right quadrant of **Figure 8.1A**. The GD-1 measure of CRP proved valuable in this case, with the athlete considerably outside his individualized range of 0.76-1.08mg/L, but notably within the clinical range of <5.1 mg/L for soccer players previously defined by Meyer et al. (2011). Of note, the athletes 19th and 23rd HPX test results were found atypical for the athlete while the static reference range treated that as a 'typical' observation (**Figure 8.1B**).

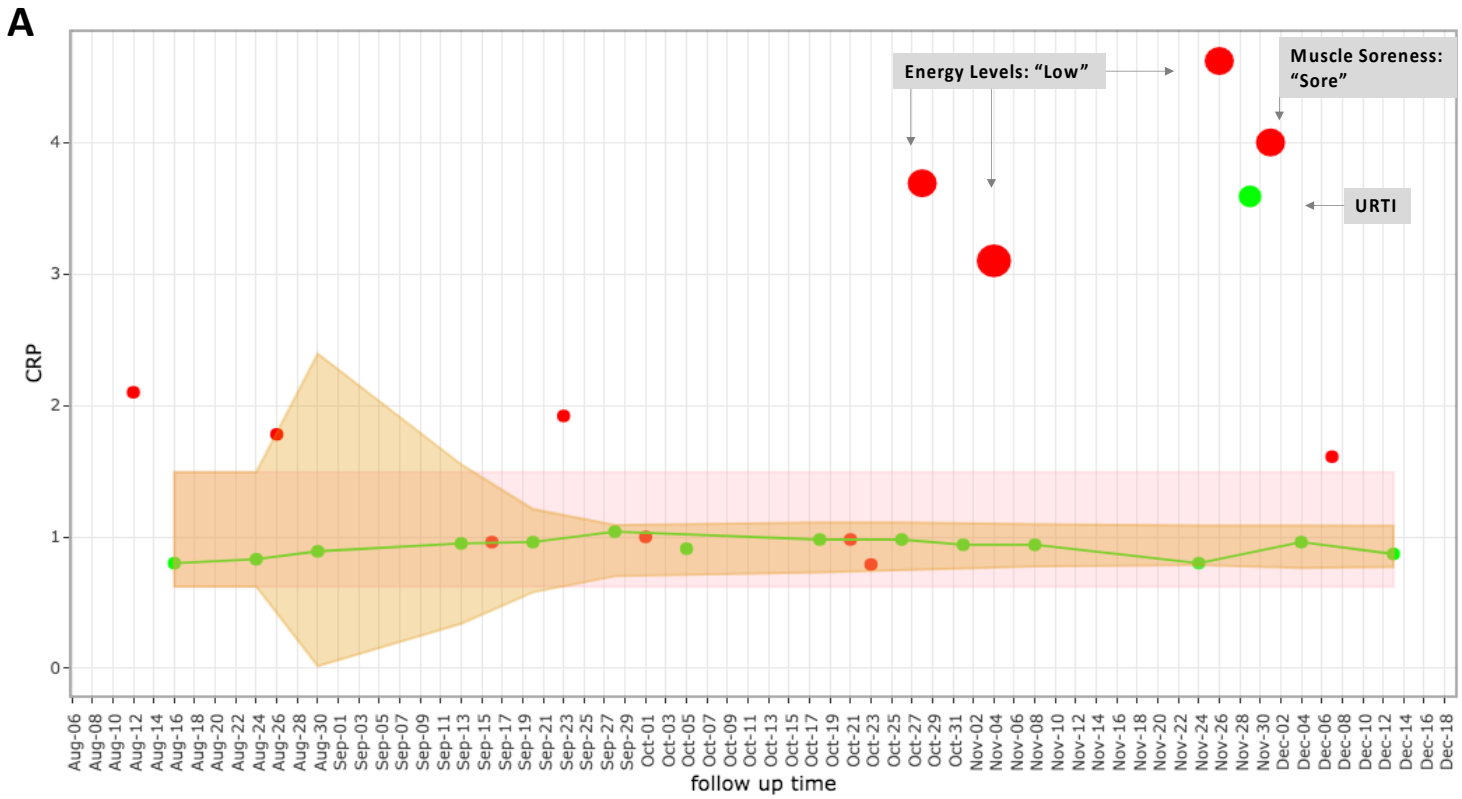

Figure 8.1A: Player 1: A series of longitudinal biomarker values (applied to pre-game measures) with static reference ranges (pink) and dynamic reference ranges using Approximate Expectation-Maximisation (brown) (applied to pre-game measures). *Green represent pre-game measures. Red represent post game-responses*. C-reactive protein (CRP) (mg/L) CRP levels. Sized by CRP.

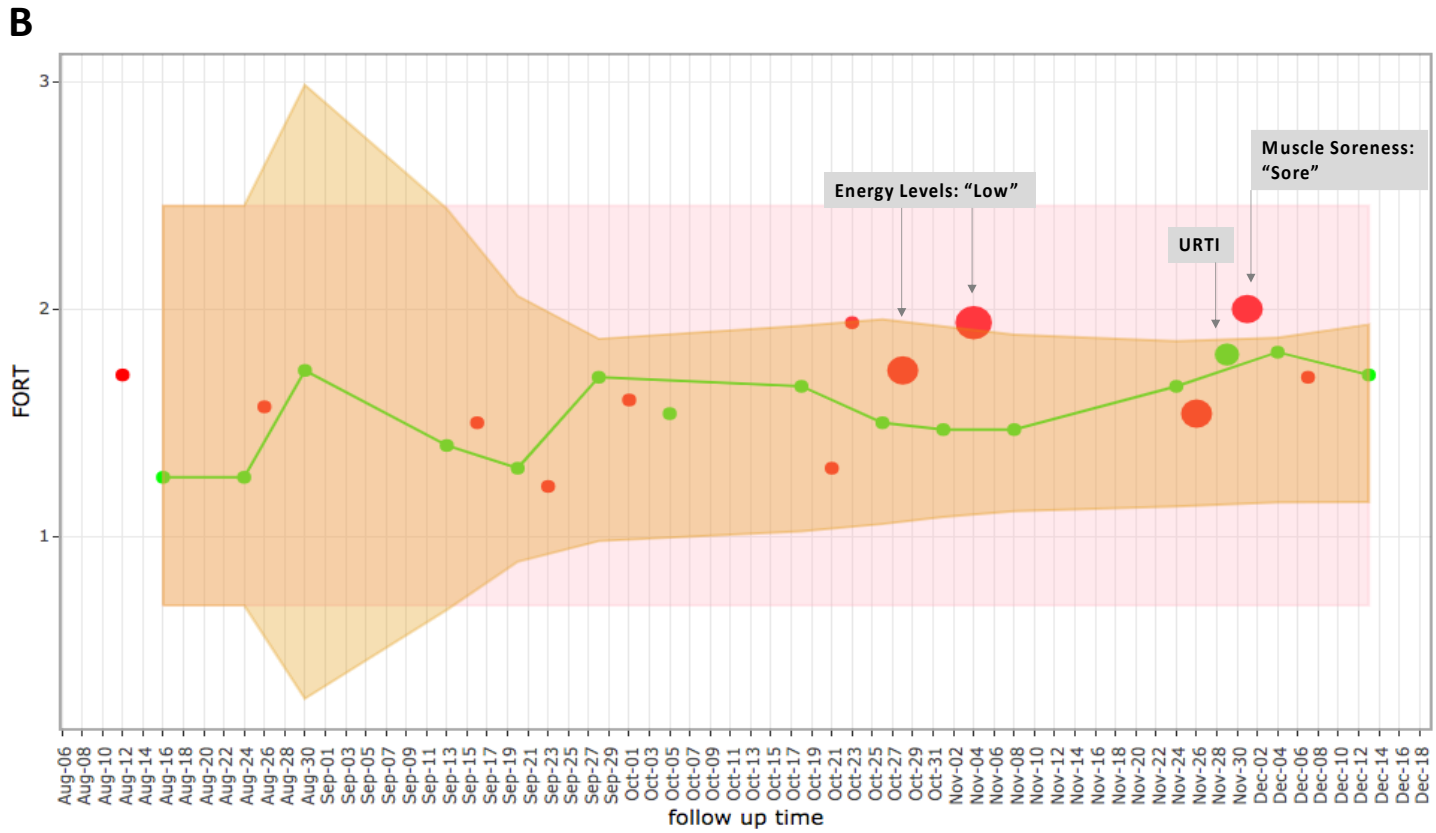

Figure 8.1B: Player 1: A series of longitudinal biomarker values (applied to pre-game measures) with static reference ranges (pink) and dynamic reference ranges using Approximate Expectation-Maximisation (brown) (applied to pre-game measures). *Green represent pre-game measures. Red represent post game-responses*. FORT (hydroperoxides; HPX) (mmol/L H_2O_2) levels. Sized by CRP.

Player 2 generally presented with low within subject variation for CRP (**Figure 8.2A**). However, of note, abnormal values were detected post-game on October 28th, with the athlete presenting with CRP levels of 24 mg/L, due to an URTI, and reported low energy levels and increased levels of muscle soreness. It is plausible that the recovery period post-game led to a short term suppression of the immune system, giving pathogens a chance to gain a foothold, and leading to a greater susceptibility to infection, as described in the "open window theory"(Kasanis et al., 2010). The athlete reported URS a week prior to the infective episode that were outside his adaptive range but notably within the static reference range. Moreover, accumulating fatigue may persist post-infective episode, particularly in the athlete who continues to compete (Lewis et al., 2015b), as was the case here, evidenced by the augmented levels of CRP in the week that followed. Systemic inflammation was subsequently resolved after an enforced 2 week recovery period. Furthermore, we observed a trend for cumulative HPX levels prior to the infective episode (**Figure 8.2B**), suggesting that HPX concentration may be an early indicator of illness susceptibility. Indeed, previous research has shown that athletes with elevated pro-oxidant levels were more prone to illness (Schippinger et al., 2009). However, it is important to note, that this rise in HPX levels prior to infection was within the athletes adaptive reference range, which highlights a limitation with the individualized reference range in not detecting a gradual change of concern and emphasises the importance of human interpretation of trends in the data.

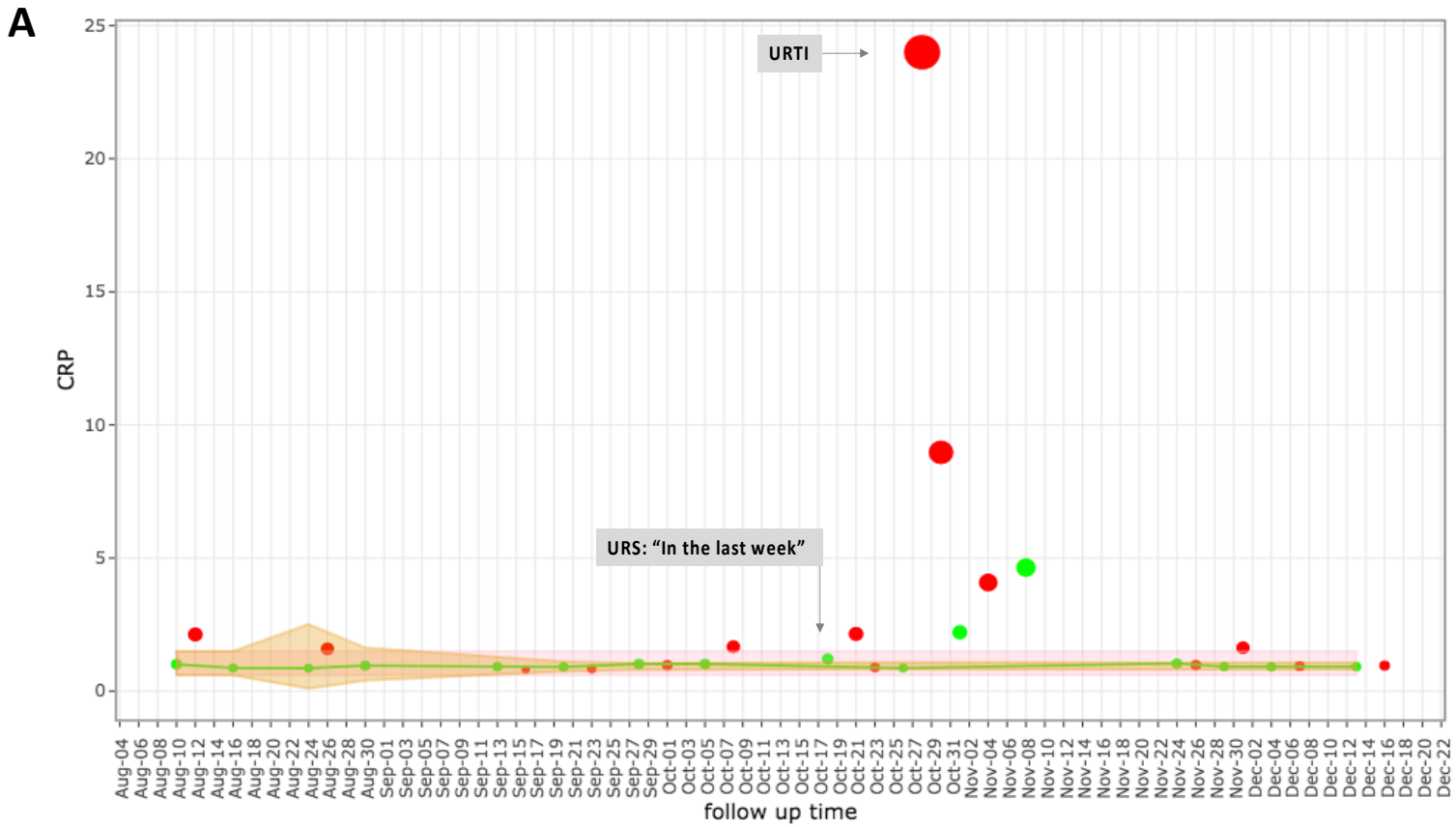

Figure 8.2A: Player 2: A series of longitudinal biomarker values (applied to pre-game measures) with static reference ranges (pink) and dynamic reference ranges using Approximate Expectation-Maximisation (brown) (applied to pre-game measures). *Green represent pre-game measures. Red represent post game-responses.* C-reactive protein (CRP) (mg/L) levels. Sized by CRP.

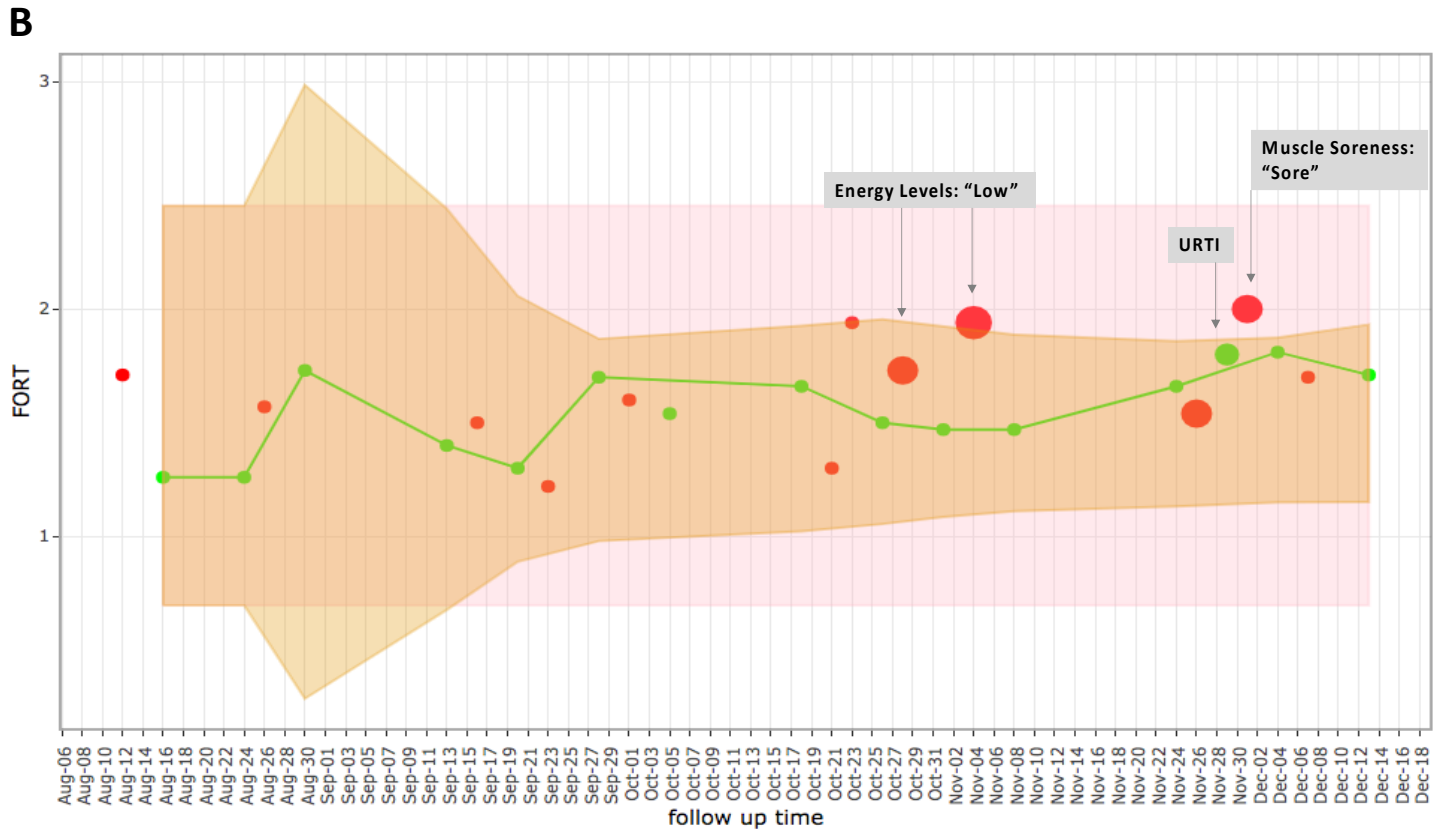

Figure 8.2B: Player 2: A series of longitudinal biomarker values (applied to pre-game measures) with static reference ranges (pink) and dynamic reference ranges using Approximate Expectation-Maximisation (brown) (applied to pre-game measures). *Green represent pre-game measures. Red represent post game-responses.* FORT (hydroperoxides; HPX) (mmol/L H_2O_2) levels. Sized by HPX.

We present evidence suggestive of impaired inflammatory regulation in an illness-prone athlete in **Figure 8.3A** and **Figure 8.3B**. The athlete reported feeling healthy (i.e. no illness symptoms) only 5 times out of the 15 measures taken at GD-1. Nevertheless, the dynamic reference ranges were still trying to adapt whenever a new observation was collected for the individual. This is an example where dynamic methods identified most of the test results as typical for that individual resulting in a wide individualized range for both CRP (**Figure 8.3A**) and HPX (**Figure 8.3B**). Disruptions in the balance between pro- and anti-inflammatory cytokines may lead to a loss of inflammatory control in illness prone athletes (Cox et al., 2007). Indeed, Cox et al. (2007) presented evidence of lower resting IL-8, IL-1ra, and IL-10 concentrations and a more potent IL-6 response to treadmill running in illness-prone compared with healthy athletes. Of note, the lower IL-8, known as neutrophil chemotactic factor, would suggest a lesser propensity for neutrophil recruitment in the illness prone athlete, which may be dis-advantageous in instances of infection, and explain why resistance to infection was reduced. The athlete participated in biomarker screening in October, and presented with evidence of borderline neutropenia (**Table 8.1**) (Parisotto et al., 2003), which corroborates the observation of regular infections.

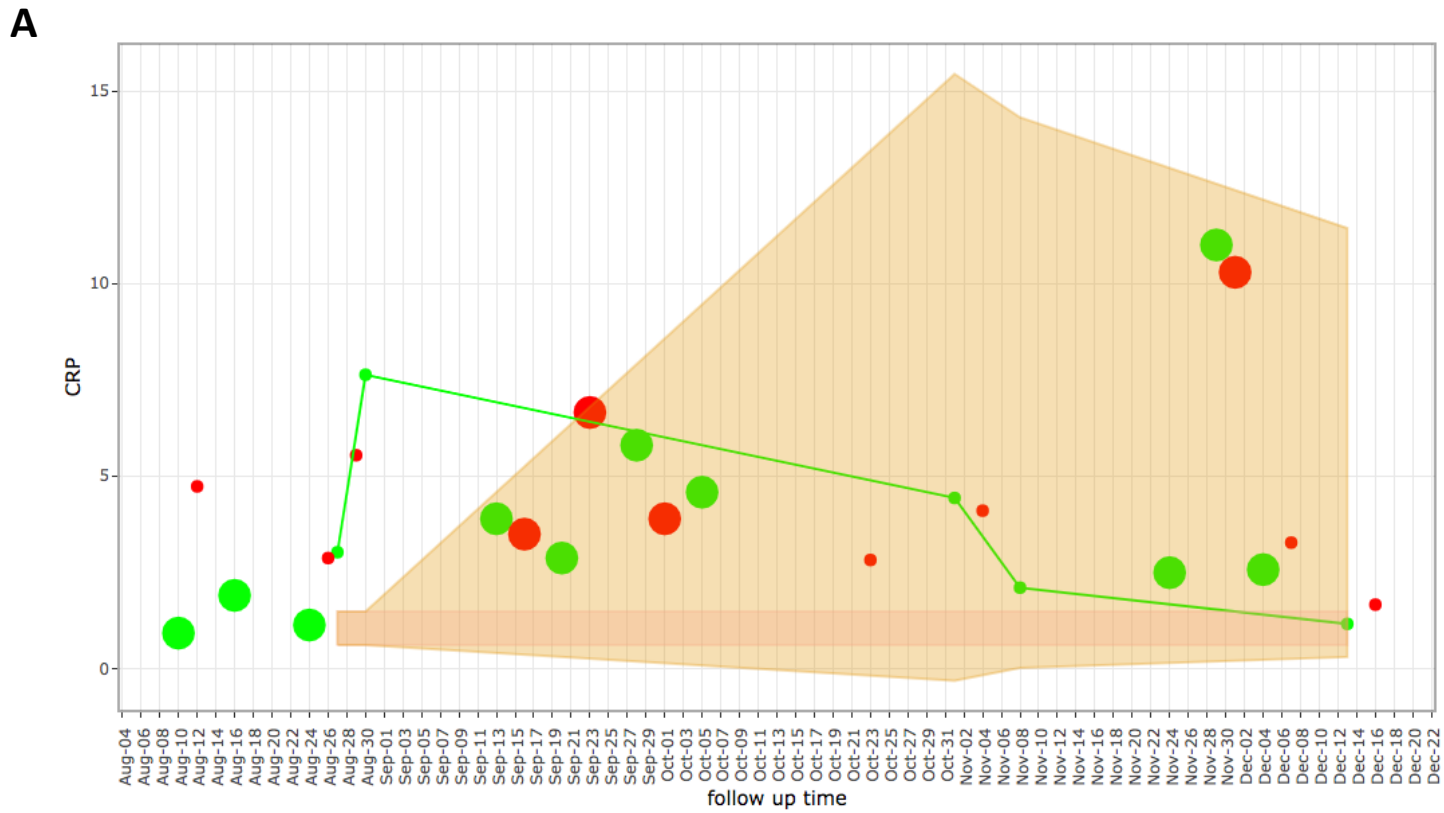

Figure 8.3A: Player 3: A series of longitudinal biomarker values (applied to pre-game measures) with static reference ranges (pink) and dynamic reference ranges using Approximate Expectation-Maximisation (brown) (applied to pre-game measures). *Green represent pre-game measures. Red represent post game-responses.* C-reactive protein (CRP) (mg/L) levels. Sized by Illness Symptoms (Yes).

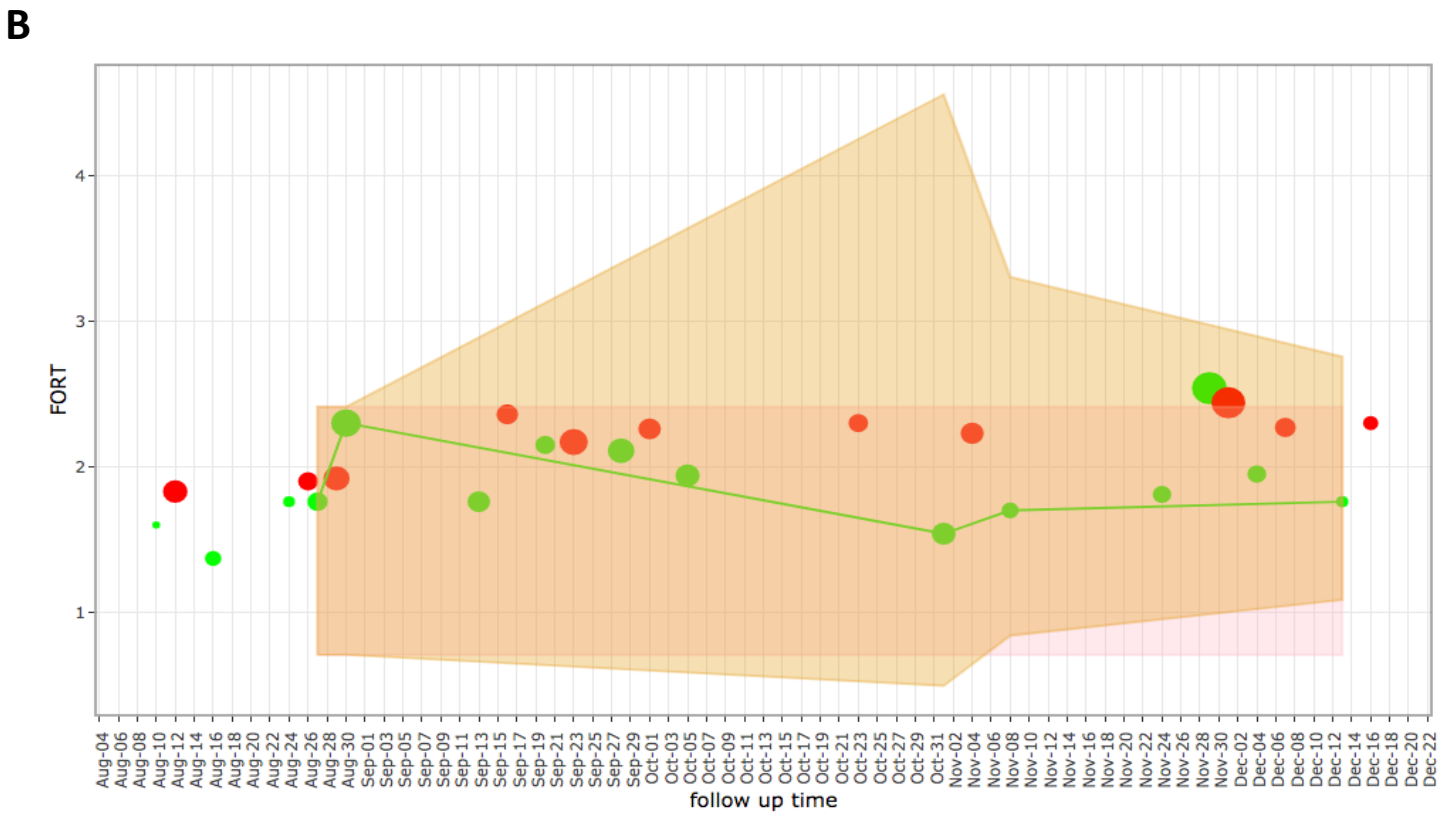

Figure 8.3B: Player 3: A series of longitudinal biomarker values (applied to pre-game measures) with static reference ranges (pink) and dynamic reference ranges using Approximate Expectation-Maximisation (brown) (applied to pre-game measures). *Green represent pre-game measures. Red represent post game-responses.* FORT (hydroperoxides; HPX) (mmol/L H_2O_2) levels. Sized by Illness Symptoms (Yes).

Figure 8.4A reveals that, the athlete in question had relatively higher CRP results compared to the rest of individuals in the sample and as a result the majority of his test results were outside the defined static reference ranges. On the other hand, as can be seen from **Figure 8.4B**, the athlete's HPX results have relatively smaller variability compared to the squad athlete which results in a more narrow individualized reference range. Baseline levels were already outside the static range, and the consistent exposure to competition led to further development of a chronic state of low grade inflammation. Exercise duration is an important factor determining the magnitude of the post-exercise inflammatory response (Fischer, 2006). Indeed, CRP levels declined when the athletes' game minutes were reduced (**Figure 8.4A**), before exhibiting a similar upward trend upon return to competition. It is noteworthy, that the athlete presented with an OM3I of 5% during the October biomarker screening (**Table 8.2**), as a result, the increased proportion of Omega-6 fatty acids in the athletes' membrane phospholipids may have aggravated low-grade inflammation (Calder, 2009), thereby further inhibiting the resolution of inflammation. A prolonged state of low grade inflammation has been described to predispose susceptible individuals to the development of chronic overuse tendinopathies (Battery et al., 2011). Although speculative, it is plausible that the demands of the winter period may have further exacerbated the athletes' chronic state of inflammation leading to a greater susceptibility to injury, and Player 4 suffered a tendon rupture in January. It is noteworthy that the athlete consistently reported the same positive ratings of subjective wellness throughout this period, which highlights an important limitation in our study that will be discussed below.

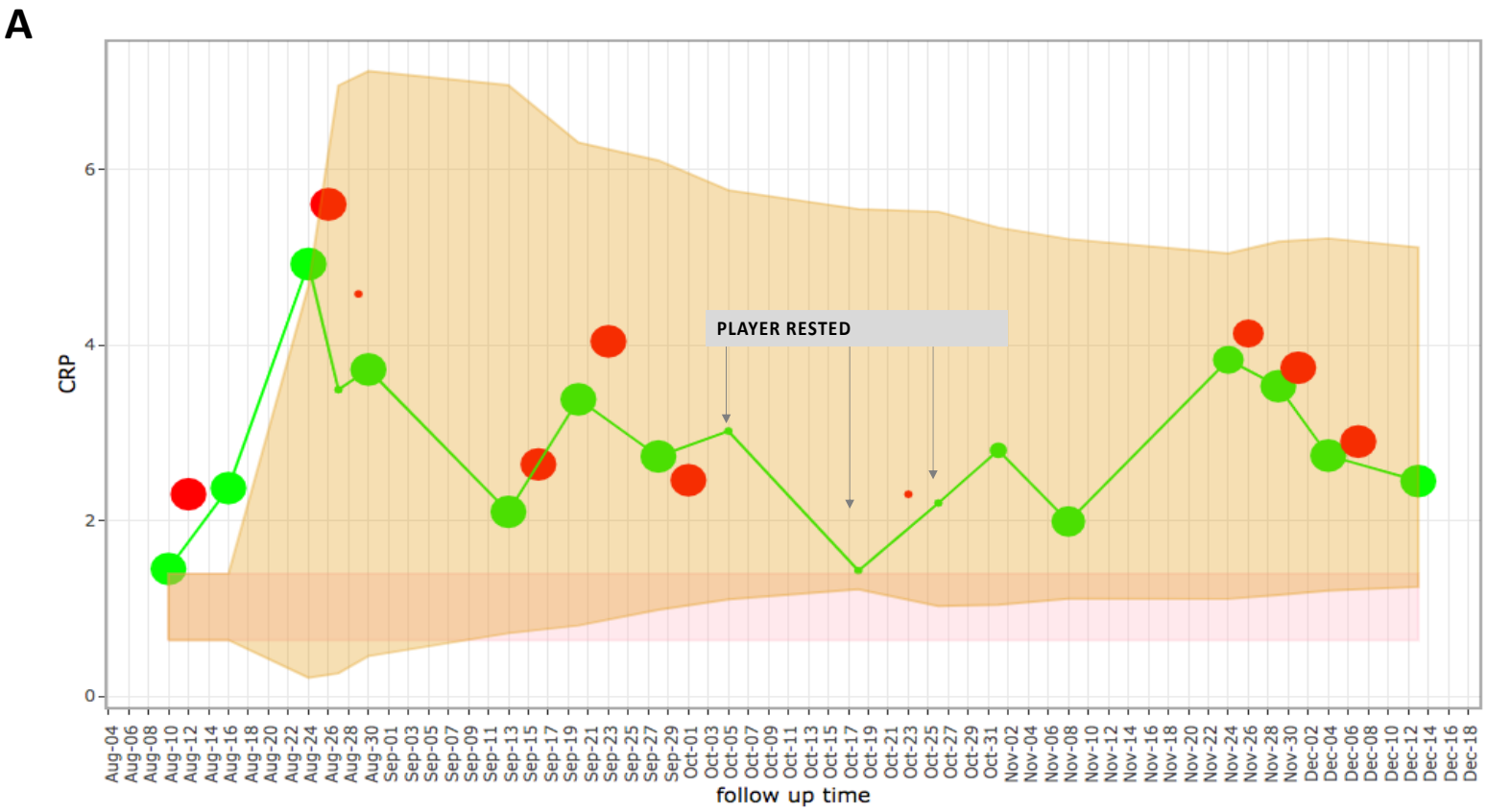

Figure 8.4A: Player 4: A series of longitudinal biomarker values (applied to pre-game measures) with static reference ranges (pink) and dynamic reference ranges using Approximate Expectation-Maximisation (brown) (applied to pre-game measures). *Green represent pre-game measures. Red represent post game-responses.* C-reactive protein (CRP) (mg/L) levels. Sized by Game Minutes.

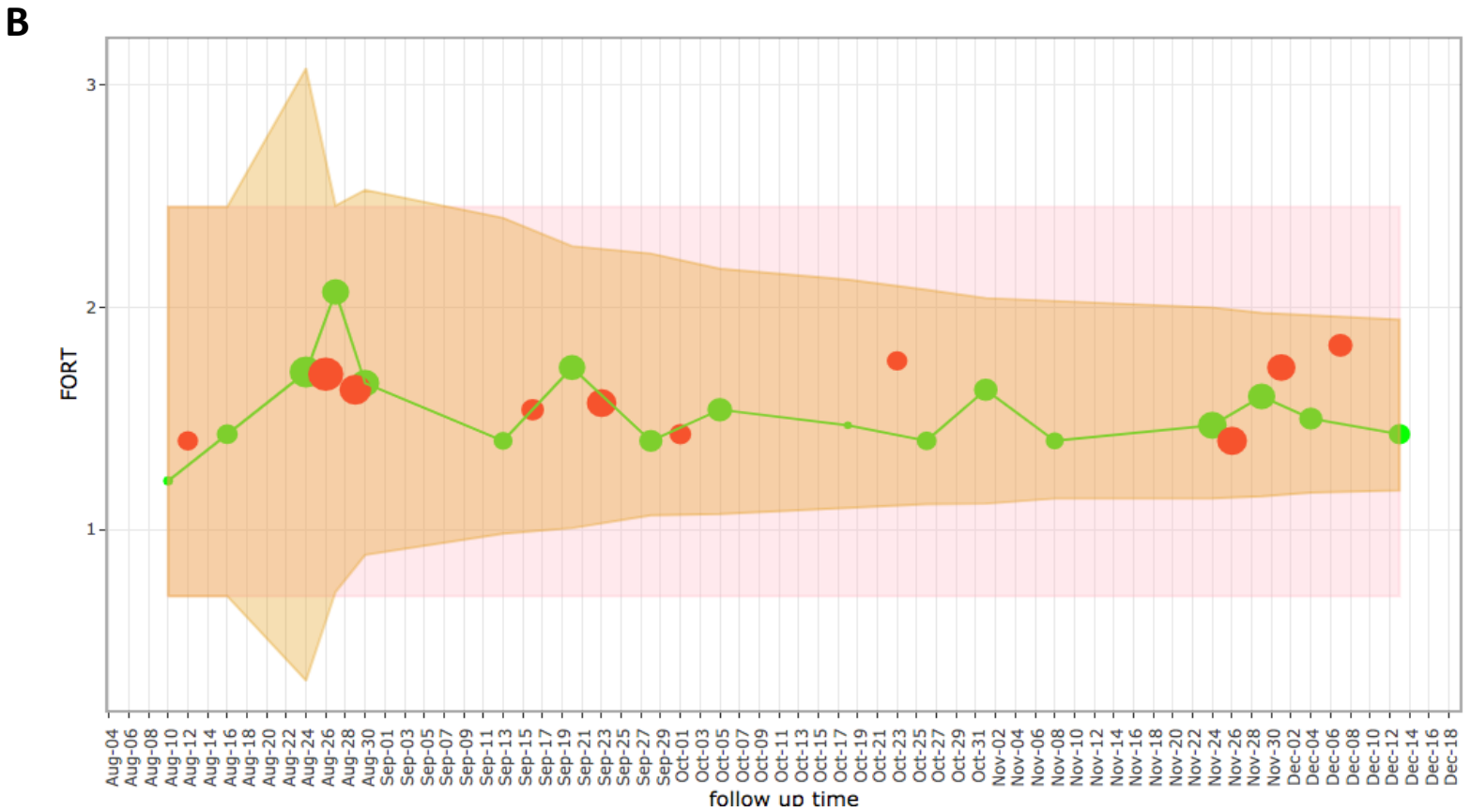

Figure 8.4B: Player 4: A series of longitudinal biomarker values (applied to pre-game measures) with static reference ranges (pink) and dynamic reference ranges using Approximate Expectation-Maximisation (brown) (applied to pre-game measures). *Green represent pre-game measures. Red represent post game-responses.* FORT (hydroperoxides; HPX) (mmol/L H_2O_2) levels. Sized by HPX.

The observed decreases in CRP for Player 2 (32.7%) (**Figure 8.5A**), Player 5 (60.5%) (**Figure 8.5B**), and Player 6 (51.9%) (**Figure 8.5C**), in response to the supplementation protocol are greater than the previously reported CDV (Chapter 4) and therefore of physiological significance. The individuals displaying significant reductions in inflammation represents a deviation from the squad trend. The following factors may have collectively contributed to the meaningful reductions observed in the athletes inflammation levels: Basal CRP levels prior to supplementation, player fitness and workload at different time points (i.e. the timing of the control/case blocks), training history, dietary deficiencies, and genetic factors (see **Table 8.3**).

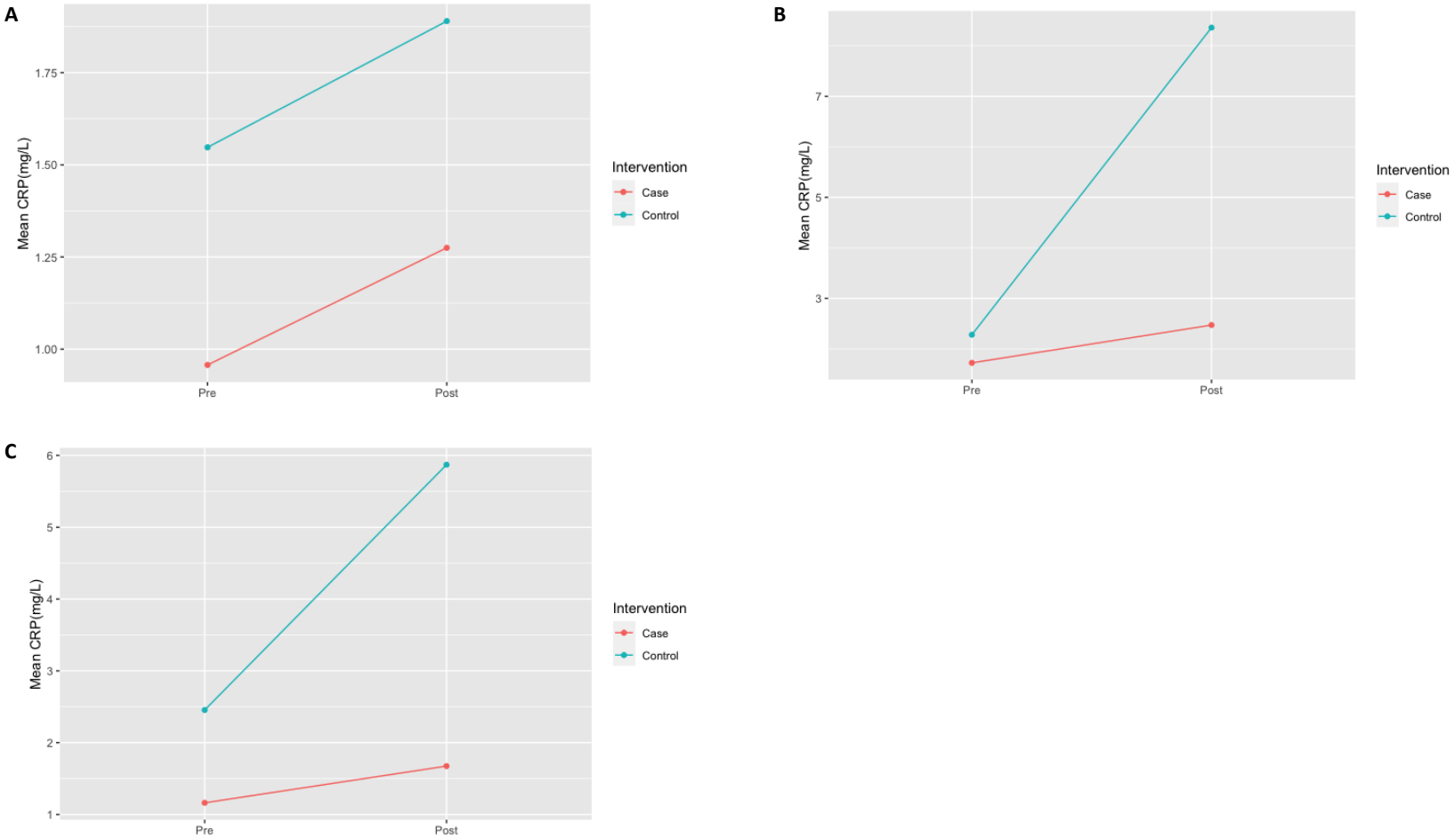

Figure 8.5: Plots of mean biomarker responses for C-reactive protein (CRP) (mg/L). *Coloured by Intervention (Case/Control).* (A) Player 2, (B) Player 5, (C) Player 6.

Table 8.3: Potential factors explaining meaningful reductions observed in players inflammation levels.

Potential factors explaining meaningful reductions observed in players C-Reactive Protein (CRP) levels
Athletes with higher basal CRP may be more prone to decrease their levels. Block et al. (2009) found that anti-oxidant supplementation significantly reduced CRP in healthy individuals only when levels were elevated at baseline. Player 5 and Player 6 had average CRP levels above the squad average (1.85 mg/L) during the control period.
Although, the acute phase response to competition does not appear to be related to the amount of high intensity activity performed in elite soccer players at a group level (see Chapter 5). Player 2, 5 and 6 displayed higher average THIR during the control condition and more variability in inflammation is to be expected when an athlete may be unaccustomed to the workload (i.e. during the initial control period at the start of the season), which may have contributed to the differences observed between conditions.
Athletes with a better training background and history show better oxidative stress-associated adaptations, manifested by a higher concentration of endogenous anti-oxidant enzymes (Martinovic et al., 2009). Player 6 displayed poor inflammatory regulation during the initial control period after being introduced to the first team for the first time. Curcumin has been described to downregulate inflammation and upregulate the Nrf2, the master regulator of antioxidant enzymes (Sahin et al., 2016). Therefore, by simultaneously blocking the pro-inflammatory response and activating endogenous anti-oxidants, curcumin ingestion may have acted to enhance the athletes' regulation of inflammation during the subsequent case condition.
Previous research has reported that a diet chronically deficient in antioxidants aggravates exercise-induced OS, which is involved in the mediation of the inflammatory process. Furthermore, evidence suggests that antioxidant supplements improve OS only when administered to deficient individuals (Margaritelis et al., 2020). The 3 athletes in question all presented with blood antioxidant capacity <1.08 mmol·L−1 (**Table 8.2**), and deemed insufficient when tested in October. However, we did not measure anti-oxidant status longitudinally in response to match play, and thus, are unable to ascertain the impact of curcumin on the correction of this deficiency.
The likelihood that biological variability due to hereditary differences may also determine those who are responders and those who do not respond to nutritional intervention (Syrotuik et al., 2004) is quite possible and may have contributed to the observed results.

8.5. Reflections & Future Directions

Although CRP displayed negligible heterogeneity of within-subject variation in healthy subjects, these case studies demonstrate that athletes with chronic low grade inflammation present markedly different within-subject variations, which has a significant impact on their respective individualized ranges. Nonetheless, the individualized approach presented here may help guide the practitioner's interpretation of the data, for example, identifying athletes who could benefit from a nutritional, performance (i.e. review of workload) or clinical review. Furthermore, the URTI observed for Player 1 in **Figure 8.1B**, and the rise in HPX levels prior to the URTI observed for Player 2 in **Figure 8.2B**, were within the athletes respective adaptive reference ranges. This highlights an important point. While such statistical methods are potentially useful for the individualization procedure, it is important to keep in mind that human interpretation is required in order to intuitively observe trends in the data and make informed decisions regarding athlete management. Moreover, the individualized examination of redox and inflammation status in response to curcumin supplementation revealed no substantial decreases in HPX levels. HPX levels were only slightly increased after exercise during both conditions, which does not allow a comparison of the effects of case (curcumin) versus control, and therefore the timing of the HPX sample may be a limitation in our study design. A prerequisite for the effective application of adaptive reference ranges is the inclusion of "healthy" athlete data. Accordingly, we set the exclusion criteria as athletes' self-reporting the presence of any illness/injury symptoms, to the research personnel or medical team at the club. The success of this approach is, however, underpinned by the willingness of the athlete to detail such information, and anecdotally this will largely be dependent on the individual in question, with some athletes consistently reporting the same subjective wellness throughout the study period regardless of time of measurement. Furthermore, ideally, frequent POC testing should be performed when the athlete returns from the off-season period in order to observe normal changes in biomarkers during healthy states. This may assist the practitioner in identifying meaningful deviations from normal ranges once the season commences. The absence of baseline data collected prior to the season can

therefore be considered a limitation in the current study. Future studies are clearly warranted in order to transform this promising strategy into an efficient monitoring tool in a real world setting.

CHAPTER 9: THESIS SYNTHESIS

Chapter 9

9.1. Realisation of Aims

9.1.1. Aim 1: To assess the precision of a POC test for CRP against the gold standard method and quantify the BV and AV in order to create an individual CDV for CRP.

This aim was addressed in Chapter 4. Values for CRP were obtained from 35 professional soccer players over the course of 3 EPL seasons in order to evaluate the precision of a POC test for CRP against the gold standard method. The study focused on healthy, well trained individuals and professional soccer players with physiological CRP concentrations below 5mg/L, in line with the target of monitoring athletes during normal training micro- and macro-cycles. The results of this study show that there is considerable variation in agreement between the CRP POC test in comparison with a laboratory reference standard, and that the level of agreement is different at low and high CRP concentrations <5mg/L. Therefore, the results indicate that the level of agreement between methods is concentration dependent, and the magnitude of the difference suggests that the methods are not interchangeable in this cohort. Additionally, the AV (via duplicate capillary sampling), BV (via capillary sampling on 5 consecutive days) and CDV were calculated for the POC measurement of CRP in 8 well trained participants. These data suggest that repeated Eurolyser CRP measurements in the same individual and in the same conditions will lead to comparable results and the CDV reported here (>20.1%) may be used to enhance interpretation of meaningful changes in CRP in well-trained individuals.

9.1.2. Aim 2: To examine longitudinal alterations in blood biomarkers of inflammation and oxidative stress in conjunction with workload and wellness data in response to weekly match play during an EPL season.

This aim was addressed in Chapter 5 by assessing the impact of professional soccer matches on capillary blood biomarkers of inflammation and pro-oxidant status in 22 players across 18 EPL games during the first half of the 2019-2020 season. The principal findings of the study were (1) a competitive EPL game induces time-dependent changes in circulating markers of inflammation and oxidative stress, (2) the magnitude of the post-game biomarker responses were more pronounced when circulating levels

were higher at GD-1, (3) significantly higher mean HPX values were observed across the first half of the EPL season, (4) that the point of care measurement of inflammation was found to be highly sensitive in detecting subjective feelings of fatigue, muscle soreness and the presence of illness symptoms. These data suggest that blood monitoring, in conjunction with wellness data may offer an objective tool for identifying fatigue and illness risk in professional soccer and highlight that the pre-game (i.e. GD-1) may be an important window for practitioners to reduce inflammation during a competitive season.

9.1.3. Aim 3: To investigate the efficacy of curcumin supplementation on biomarkers of inflammation and oxidative stress during an EPL season.

This aim was addressed in Chapter 6. The study used an interrupted time-series design to analyse the effect of curcumin supplementation on reducing biomarkers of oxidative stress and inflammation during an EPL season in 22 players consuming their habitual diets. To the authors knowledge, this is the first study to investigate the efficacy of a nutritional intervention aimed at reducing blood biomarkers of inflammation and oxidative stress in professional soccer players competing in the EPL. The main findings were that curcumin ingestion had no significant effect on CRP, and borderline significant effects on HPX responses, although in the case of the latter finding, the magnitude of this change does not appear to be physiologically relevant. Therefore, these data question the assumption that curcumin ingestion is effective for attenuating inflammation and pro-oxidant status within the applied, field setting of elite soccer, at least at a group level.

9.1.4 Aim 4: To investigate the seasonal variation in blood biomarkers of inflammation and EMFAs and to examine the strength of their association.

This aim was addressed in Chapter 7. The relationship between inflammation status and red blood cell fatty acids were examined longitudinally in male professional soccer players who participated in biochemical monitoring through the measurement of many relevant biomarker variables over the course of 3 EPL seasons. The study hypothesis was that Omega-6 fatty acid variables would be positively associated with CRP and that

the Omega-3 fatty acid variables would be negatively associated with CRP. Significant rises in CRP were observed after the winter period in the EPL, and the findings present convincing evidence that the EMFA variables influenced the change in CRP observed across time. Together, the findings suggest that measuring and manipulating the EMFA composition may be a potential "pro-resolving" strategy during periods of frequent match play in professional soccer players.

9.1.5. Aim 5: To explore the application of individualized adaptive reference ranges and the individualized examination of redox and inflammation status in response to curcumin supplementation in professional soccer players competing in the EPL.
This aim was addressed in Chapter 8. A series of individual cases describing the use of dynamic individualised reference ranges on longitudinal point of care biomarker data were presented. The study also assessed whether the observed changes in blood as a result of curcumin supplementation for each player were greater than the previously reported CDVs. The study confirms that athletes categorized as "healthy" or "non-healthy" present markedly different within- and between-subject variations in biomarkers of inflammation and pro-oxidant status, with healthy athletes displaying negligible heterogeneity and non-healthy athletes displaying wide heterogeneity in biomarker responses, which has a significant impact on their respective individualized ranges. Physiologically relevant changes in CRP levels were observed for only 3 athletes in the study group in response to the intervention, with no athletes displaying substantial decreases in HPX levels. As such, these data contradict the assumption that meaningful reductions in markers of inflammation and pro-oxidant status are possible in a large number of elite soccer players following the ingestion of curcumin and the novel approach to biomarker monitoring presented may have translational potential to the individualisation of player recovery.

9.2. General Discussion

The timely and rapid POC measurement of a capillary blood biomarker of inflammation would allow for the collection of longitudinal data and importantly may inform athlete management decisions at the time of sampling. However, considerations must be made regarding the validity of the POC device, in order to avoid problems in interpretation and investigate whether the POC test can be used interchangeably with the gold standard method (Bland and Altman, 1983). Furthermore, accounting for the analytical and biological variation of the biomarker so that meaningful changes in biomarker responses can be distinguished from natural variations in the biomarker is a key issue. Ultimately, the POC measurement of CRP coupled with knowledge of the BV may help sport scientists support the adaptation and recovery needs of elite athletes (Lewis et al., 2016a). Studies investigating the AV, BV and CDV for the POC measurement of CRP in well trained individuals were absent from the literature. To this end, the first aim of the thesis was to evaluate a point of care test for assessing inflammation in healthy athletes (Aim 1). The Bland-Altman analysis indicated that the mean difference between methods was 0.27 mg/L, with the limits of agreement of -1.7 mg/L to 2.24 mg/L, which is quite large when we consider the context in which these biomarkers are deployed in an applied field setting (i.e. to monitor physiological stress; Lewis et al., 2020a), suggesting that the methods are not interchangeable in this cohort. The results also show that the level of agreement between methods is different at low and high CRP concentrations <5.1 mg/L, where the bias is positive for low values (which may be explained by the smaller limits of detection for laboratory based CRP vs Eurolyser CRP) and negative by a consistently small amount for values larger than 2 mg/L, suggesting that a different calibration is needed at low values compared to high values. The repeatability of the POC test for the Eurolyser CRP (5.2%) is greater than that reported by the manufacturer (2.83%) for whole blood, but within the 10% previously suggested for CRP (Fraser, 2004), and therefore of sufficient analytical precision to be used in the field. The low CVw observed in Chapter 4 contrasts markedly with previous research in well trained individuals (Nunes et al., 2010), and may be due to a multitude of factors including the short study duration (Braga et al., 2012), the level of pre-analytical control

(e.g. abstaining from vigorous exercise, controlling for posture) and the training status of the participants. These data suggest that repeated Eurolyser CRP measurements in the same individual and in the same conditions will lead to comparable results, therefore applicable for future experimental work, and the CDV reported here (>20.1%) may be used to enhance interpretation of meaningful changes in CRP in well-trained individuals.

In addition to considerations regarding the validity and test-retest reliability of the POC device, investigations into its practical application in the professional setting of the EPL were warranted in order to understand the real-world meaningfulness of the biomarker data, and its sensitivity/utility as positive or negative outcome indicator. Most studies investigating inflammatory disturbances induced by a competitive game have done so in response to a single match and contextual information (i.e. workload and wellness data) surrounding these biomarker responses were absent from the literature. To this end, Chapter 5 aimed to examine longitudinal alterations in blood biomarkers of inflammation and oxidative stress simultaneously with workload and wellness data in response to weekly match play during an EPL season (Aim 2). The findings showed that a competitive EPL game induces time-dependent changes in circulating markers of inflammation and oxidative stress. In agreement with previous research in elite soccer players (Ispirlidis et al., 2008; Silva et al., 2013; Souglis et al., 2015a; Souglis et al., 2015b; Romagnoli et al., 2016), CRP concentrations at GD+1 were significantly higher than GD-1, and at a group level were not significantly higher at GD+2 compared to GD-1, returning near pre-match levels at GD+3. This may have implications for the prescription of training volume and intensity after a soccer match, as practitioners may seek to adjust training load in individuals with pronounced responses beyond GD+1. In contrast to previous research in elite soccer players which has shown redox markers generally return to near baseline levels at GD+3 (Fatouros et al., 2010; Ispirlidis et al., 2008; Ascensao et al., 2008), a delayed increase in HPX levels was detected in the blood at GD+4 (significantly higher than HPX levels at GD-1). The discrepancy between our findings and previous literature may be due to a combination of factors including the biomarker assays measured, the timing of the post-game sample and differences in study

design (i.e. the observational periods in the studies presented above did not extend beyond GD+3). One could speculate that the delay in the appearance of pro-oxidants in the blood and the significant relationship observed with CRP indicate that the redox perturbations in the current study are largely attributable to muscle damage and inflammation (RONS production by invading phagocytes). Importantly, the results of the study showed that the magnitude of the post-game biomarker responses were more pronounced when circulating levels of CRP and HPX were higher at GD-1, which suggests that the pre-game may be an important window for practitioners to monitor biomarkers of inflammation during the competitive season. Moreover, significantly higher mean HPX values were observed across the first half of the EPL season, a finding which is corroborated by research in the French League (Le Moal et al., 2015) and Portuguese League (Silva et al., 2014). Whether the accumulation in HPX levels observed across time were damaging or beneficial to the athlete likely depends on the balance between the players' levels of RONS production and the competency of their cellular antioxidant systems to protect cells against this pro-oxidant challenge.

Although post-exercise CRP and HPX levels showed an upward trend with higher THIR, a significant relationship was not detected. It has been suggested that THIR will underestimate the true load incurred by the athlete since it does not account for the stress and mechanical load associated with the frequent explosive acceleration and deceleration type movements inherent in match play (Thorpe et al., 2017; Gaudino et al., 2013). Moreover, inflammatory disturbances in response to a game may be highly individual, and be more player- than load-dependent. Indeed, the external and internal load experienced by each player is likely influenced by a number of intrinsic (e.g. age, training history) and extrinsic (e.g. opposition standard, tactics and the 'recovery window' i.e. number of recovery days from previous match) factors (Silva et al., 2018). Additionally, the professional soccer player is subject to various forms of stress (physical, psychological, lifestyle) over the course of a season and the results of this study showed that the POC measurement of inflammation was highly sensitive in detecting subjective feelings of fatigue, muscle soreness and the presence of illness symptoms. To the authors knowledge, this is the first study to investigate the strength of

the relationship between blood biomarker and wellness data in elite soccer, and these findings could have important implications for protecting athlete health and recovery, as blood monitoring, in conjunction with wellness data may be used to identify fatigue/illness risk and enhance the management of high performance athletes.

The observation that pre-match inflammation status predicts post-match inflammation response (Chapter 5) suggests that the pre-game may be an important window for practitioners to reduce inflammation, since it is unlikely they can influence player match selection. Curcumin is a component of the spice Turmeric, and its anti-oxidant and anti-inflammatory properties suggest that it could modulate the negative effects of the secondary damage cascade during periods of high demand in the EPL. Moreover, anecdotally speaking, at the time of the intervention, curcumin supplements were garnering attention from players in the EPL, and thus scientific investigation into its efficacy in a cohort of EPL players were warranted. To this end, Chapter 6 aimed to investigate the efficacy of curcumin supplementation on reducing biomarkers of inflammation and oxidative stress during an EPL season (Aim 3). The study findings suggest that curcumin ingestion had no significant effect on CRP. In agreement, previous research has shown that whilst post-exercise increases in markers of inflammation have tended to be lower in curcumin treatment groups, significant reductions in inflammation with curcumin supplementation are lacking (Nicol et al., 2015; Drobnic et al., 2014; Sciberras et al., 2015). Indeed, reports are generally inconsistent with regards to exercise induced inflammation in the literature (Suhett et al., 2020). Borderline significant effects on HPX responses were observed, a finding which is corroborated by previous research (Chilelli et al. 2016; Takahashi et al. 2013). However, in the case of the latter finding, the magnitude of this change does not appear to be physiologically relevant, based on previously published CDV for this assay (Lewis et al., 2016a).

To the authors knowledge, this was the first study to investigate the efficacy of a nutritional intervention aimed at reducing blood biomarkers of inflammation and oxidative stress in professional soccer players competing in the EPL. Indeed, elite soccer

players with a better training background display enhanced oxidative stress-associated adaptations, manifested by a higher concentration of endogenous anti-oxidant enzymes (Silva et al., 2014), and therefore the requirement for anti-oxidant supplementation may be less compared to non-athletes. The wide heterogeneity in CRP and HPX responses may also provide important clues about why the study results are inconsistent. It has been shown that the efficacy of antioxidant supplementation is affected by inter-individual variability in redox state (Kawamura et al., 2018), meaning that athletes may be either low-, moderate-, or high-responders to a given supplementation regimen. As such, the effect of curcumin supplementation on redox and inflammation status using previously reported CDVs, for CRP (see Chapter 4) and HPX (Lewis et al., 2016a) in players were presented in Chapter 8, in order to understand whether a 'true' physiological change had occurred as a result of the supplementation regimen for each individual athlete (Chapter 8). Physiologically relevant changes in CRP levels were observed for only 3 athletes in the study group in response to the intervention, with no athletes displaying substantial decreases in HPX levels. The individuals displaying significant reductions in inflammation represents a deviation from the squad trend, and may be due to a number of factors including basal CRP levels prior to supplementation (Block et al., 2009), training history (Martinovic et al., 2009) and dietary deficiencies (Margaritelis et al., 2020). These data therefore questions the efficacy of curcumin ingestion for the purpose of reducing inflammation and pro-oxidant status in elite soccer players when following their habitual diets, which may be rich in anti-oxidants from dietary sources. Indeed, in athletes consuming a well-balanced diet with adequate fruit and vegetable intake, supplementation may only be beneficial in instances when the exercise stressors might outweigh the benefits provided by whole foods (e.g. periods of intense fixture congestion) (Bongiovanni et al., 2020).

Chapter 4 presented novel data examining biomarker response to match play across the first half of the EPL season. However, data describing inflammation across the latter stages of the season were absent from the literature, and recovery time during periods of frequent match play may be insufficient for the resolution of inflammation (Mohr et al., 2016). Furthermore, the kinetics and resolution of inflammation may be influenced by

the nutritional status of the athlete and emerging evidence suggest that interventions which are “pro-resolving” in nature (i.e. facilitate the natural resolution of inflammation), such as increased consumption of omega-3 fatty acids, may more effectively enhance exercise recovery than anti-inflammatory interventions which target reductions in the expression of COX enzymes (Markworth et al., 2016). To this end, Chapter 7 aimed to examine, for the first time, concurrent alterations in EMFAs and inflammatory markers and the strength of their association during the season in a cohort of professional soccer players competing in the EPL (Aim 4). Significant rises in CRP were observed after the winter period in the EPL (T4), a finding which is corroborated by research in the Portuguese professional league (Silva et al., 2014). The study findings present convincing evidence that the EMFA variables influenced the change in CRP observed across time, suggesting that EMFA composition is a significant driver of low-grade inflammation in professional soccer players and provide rationale for augmenting the EMFA as a potential “pro-resolving” strategy during periods of limited recovery. Practitioners may therefore seek to prescribe FS during a season, however, there are a number of factors which must be considered including: The underlying fatty acid status of the athlete in question, as athletes presenting with a lower baseline level have been found to be more successful at incrementing their levels (Drobnic et al., 2017); The timing/periodization of the supplementation regimen, as Flock et al. (2013) showed that healthy individuals with a low average OM3I of 4.3% increased their average OM3I to 9.5% in response to 1.8g/d of EPA + DHA for five months, meaning the supplementation would need to commence several months prior to the congested winter period in order to maximize incorporation into the RBC membrane.

Collective data for elite soccer players were presented in Chapter 5. However, large inter- and intra-individual variability in biomarker responses exist. The clear intra- and inter-individual differences evident in Chapter 5, Chapter 6 and Chapter 7 highlight the need for an individualised approach to longitudinal biomarker tracking to assist in the interpretation of meaningful changes in the inflammatory status of the elite soccer player. Large between subject variability impedes the practical value of population based reference ranges (Pedlar et al., 2019) and individualised approaches to biomarker

monitoring may therefore be of higher potential value in protecting player health or detecting an under-recovered state. This individualisation approach is used in the Athlete Biological Passport, as implemented by WADA (Sottas et al., 2011). To this end, Chapter 8 aimed to explore the application of individualized adaptive reference ranges in professional soccer players competing in the EPL (Aim 5). CRP displayed negligible heterogeneity of within-subject variation in healthy subjects, however, these case studies confirm that athletes with chronic low grade inflammation present markedly different within-subject variations, which has a significant impact on their respective individualized ranges. Furthermore, whilst such statistical methods may assist in differentiating between normal and abnormal profiles for a given athlete (Schumacher et al., 2012), the results of this study highlight that human interpretation is still required in order to intuitively observe trends and emphasise the importance of subjective evaluation of the results in view of possible causes by the sports science and medical staff.

In summary, the present thesis has evaluated the validity of a POC device for assessing inflammation, has reported the analytical and biological variability for the POC biomarker CRP and calculated the CDV to enhance interpretation of meaningful changes. We longitudinally analysed POC biomarkers for inflammation and oxidative stress and assessed their utility and sensitivity as positive or negative outcome indicators during an EPL season. We evaluated the effectiveness of a nutritional intervention on attenuating biomarker responses in-season at a group and individual level and identified the potential for augmenting the EMFA composition as a potential “pro-resolving” strategy. Finally, we have explored the intra- and inter-individual variability between players by applying individualised adaptive reference ranges to longitudinal point of care biomarker data. As such, the data has made meaningful contributions to the existing soccer literature and the information presented here may help sport scientists in supporting the health and recovery needs of the professional soccer player.

9.3. Limitations and Future Directions

9.3.1. Chapter 4

Subject participation within professional soccer research is a challenge due to numerous external influences (Owen et al., 2019), and a larger sample size would have strengthened the method agreement analysis. We aimed to focus our study on healthy, well trained individuals and professional soccer players with physiological CRP concentrations below 5 mg/L, in line with the target of monitoring athletes during normal training micro- and macro-cycles. Therefore, the statistical analysis of the data is only applicable to that limit and our study has not addressed the level of agreement at higher CRP concentrations that may be important from a clinical perspective. Future studies should be performed on a larger cohort, investigate agreement beyond 5 mg/L and assess the diagnostic accuracy of the POC test in capturing sport specific medical problems. Subject participation in the BV study was also a challenge due to the intensive analytical effort required (Fraser and Harris, 1989), and the small sample size in Chapter 4 is certainly a limitation. A larger sample size with a greater number of individuals presenting with a broader range of CRP concentrations, would provide valuable information on the assay's working range and allow for the construction of an imprecision profile across specimens of various concentrations (LeDue et al., 2003). Future research should address this limitation.

Previous research has demonstrated similar distribution of CRP concentrations among apparently healthy men and women (Rifai et al. 2003; Orri et al. 2010), however, failure to capture menstrual cycle phase and the use of hormonal contraception is a limitation in our study given the link between OCP use, menstrual cycle symptoms and low grade inflammation in female athletes (Puder et al., 2006; Cauci et al., 2017). Additionally, extrapolating findings from well-trained individuals to the professional soccer player may be considered a limitation given that training status is a factor dictating the magnitude of the inflammatory response (Fischer, 2006). Indeed, in order to minimise physiological differences as a source of error the CDV should ideally be calculated in the athletic group of interest (Pedlar et al., 2019). Conducting such a study in a group of

professional soccer players may not be feasible given the intensive schedule of elite soccer and the level of pre-analytical control required in BV studies, however future studies should be conducted in soccer players where feasible and account for additional sources of variability in the female athlete. The CDV we report may be used to enhance interpretation of meaningful changes in CRP in well trained individuals, however, future work is required to quantify the real world meaningfulness of changes exceeding the CDV in longitudinal biomarker tracking (i.e. the robustness of the relationship between changes exceeding the CDV and relevant negative outcome indicators). For example, future studies should examine the degree to which a 20.1% change in an athletes CRP promotes subsequent changes in negative outcome indicators such as performance, fatigue, illness, and injury. Indeed, a greater understanding of the functional relevance of the CDV in the real world applied environment may assist the practitioner in validating the decision to reduce or restrict an athlete's training in order to, for example, resolve a >20.1% increase in CRP in the interval between successive matches.

9.3.2. Chapter 5

Chapter 5 provides novel data for the literature, however, inherent limitations exist as a consequence of collecting data on professional soccer players. We evaluated inflammation through a single biomarker and not via a battery of biomarkers such as inflammatory cytokines commonly assessed in the literature, which would have provided further context into the inflammatory response to competition. Measures of inflammation and oxidative stress were taken from circulatory blood markers. OS biomarkers have been criticized in the literature (Sies, 2007; Cobley et al., 2017), as they provide little mechanistic insight into the complexity of cellular redox pathways. However, Margaritelis et al. (2014) outline that whilst blood has largely been considered as a "sink" that passively accepts reactive species produced from tissues, some of the effects of exercise on muscle redox homeostasis are partially due to changes in the redox composition of blood, and therefore, investigating the effects of exercise on blood redox homeostasis has scientific value in itself. Nonetheless, the absence of muscle biopsy data mean that it is difficult to ascertain the magnitude of oxidative stress and inflammation that was localized to the skeletal muscle from the information provided in

the current study. These laboratory assessments clearly cannot be employed in the field, however, future controlled laboratory studies in a cohort of soccer players would provide a greater understanding of the mechanisms underpinning how inflammatory mediators exert their effects in response to soccer specific activity. Furthermore, when examining redox responses to exercise, consideration must be given to the timing of the post-exercise sample. Using the same POC redox test, Lewis et al. (2016b) found increases in HPX occurred immediately and 20 minutes after maximal exercise. Selecting sampling points closer to exercise cessation may prove more effective in detecting early perturbations in redox homeostasis and establishing the association, if any, between HPX levels, workload, and perhaps even adaptation in the elite soccer player, given the recent data demonstrating that the magnitude of the post-exercise response in redox biomarkers was predictive of adaptation (Margaritelis et al., 2018).

Capturing training load data would have provided greater context into the potential causes of higher levels of CRP and HPX reported for some in athletes at GD-1. Methodological limitations exist for optical based tracking systems as utilised in the current study, as they do not provide information on force load and stride characteristics which may better assess the mechanical demands of match play (Carling et al., 2018; Bucheit et al., 2017). THIR will underestimate the true load incurred by the athlete since it does not account for the frequent explosive acceleration and deceleration type movements (Gaudino et al., 2013; Thorpe et al., 2017). Furthermore, the present study uses an absolute speed threshold (> 21km/h) to identify THIR, and previous research in professional soccer players has highlighted the need for individualized high-intensity speed thresholds (based on the performance metrics; Apt et al., 2009). Finally, RPE has previously been demonstrated to be correlated to changes in CRP across a pre-season (Coppalle et al., 2019) and OS biomarker changes across various time points of a season in a group of elite soccer players competing in the French league (Le Moal et al., 2015). Therefore, future work should investigate the relationship between post-game biomarker response and a variety of workload measures, both objective and subjective, and apply individual thresholds where feasible.

Given the association between inflammation and disturbed sleep (Irwin et al., 2016) and psychological stress (Ostapiuk-Karolczuk et al. 2015), failure to capture this information in Chapter 5 is a limitation. Indeed, investigations are warranted in order to determine the extent to which sleep disturbances and psychological stress occurs in the elite soccer player and the influence of these environmental stressors on the time course of biomarker responses post-match. Whilst we demonstrate a relationship between subjective ratings of muscle soreness and fatigue on CRP levels, we did not perform any field based physical tests (such as maximal jump testing), and therefore cannot demonstrate a link between inflammation, oxidative stress and more objective measures of muscle function, as has been previously observed (Ispirlidis et al. 2008; Silva et al., 2013; Mohr et al., 2016). Performing such tests on already 'fatigued' players during the post-game recovery period, as well as the logistical challenges associated with player availability and willingness to participate mean that capturing this information during a competitive season may prove difficult. However, future research may address these limitations.

Whilst we provide novel data into the longitudinal changes in biomarkers across the first half of the EPL season, future studies should attempt to capture the effect of the congested winter fixture schedule on POC biomarker responses, as it has been shown to be associated with both a higher injury burden and higher incidence of severe injuries in professional soccer (Ekstrand et al., 2019). Additionally, although the study demonstrates a cumulative effect for HPX levels across time, the absence of a measure of anti-oxidant status means we cannot ascertain whether changes in OS (i.e. an imbalance between pro-oxidant and anti-oxidant alterations) occurred across time. Furthermore, it is currently unknown whether the biomarker changes observed across time represent major deviations from optimal ranges, and if the repeated episodes of inflammation and oxidative stress in-season will ultimately be beneficial or detrimental across the career of the elite soccer player. Given the association reported between some sports and disease continuums post career (Lincoln et al., 2018; Pedlar et al., 2019), future work is required to demonstrate the effects of biomarker changes on career longevity and post-career health.

9.3.3. Chapter 6

As with chapter 5, limitations with the timing of the HPX sample is also a limitation in Chapter 6. HPX levels were only slightly increased after match play during both conditions, which does not allow a comparison of the effects of case (curcumin) versus control, and therefore a limitation in our study design. For practical reasons (i.e. financial and time constraints), we did not measure anti-oxidant status, thus, we potentially missed capturing the impact of curcumin on the players' plasma anti-oxidant capacity. The lack of measurements of a wider range of inflammatory biomarkers and antioxidant enzymes could be a limiting factor in the current study as curcumin's anti-inflammatory effects may potentially be tissue and/or enzyme specific (Basham et al., 2020). Considering the field-based nature of the present study, an obvious limitation is the inability to blind the players and staff to the supplementation regimen. However, the use of control versus intervention groups is generally un-feasible at the elite level as only a single population benefits from the intervention (Carling et al., 2018). The use of an interrupted time series design may therefore be considered novel, however, it is also possible that the training status of the athlete during the different stages of the intervention may have influenced the regulation of inflammation to a greater extent than the supplementation regimen. Whilst supplement adherence was monitored via verbal confirmation, visual supervision of the athlete consuming the supplement did not occur, which may also be considered a limitation. Furthermore, athlete belief is another important consideration in the application of an intervention strategy (Halson et al., 2013; Howatson et al., 2016). Failure to capture data on the players perception of the intervention is therefore a limitation in the current study. Lastly, athletes consumed their habitual diets during the study period, which adds to the ecological validity of the study, however, it is possible some players were consuming higher polyphenolic diets than others. Dietary data would have allowed us to highlight individuals with dietary deficiencies, and given the evidence suggesting that antioxidant supplements improve performance and oxidative stress only when administered to deficient individuals (Margaritelis et al., 2020), this information would have strengthened our analysis. Future work may address these limitations.

9.3.4. Chapter 7

As with Chapter 6, the absence of dietary data did not allow for detailed dietary analysis, and therefore, we cannot determine whether fish intake, supplementation compliance or workload induced adaptive responses were responsible for the alterations in the EMFA composition found. Similarly, we did not investigate the impact of FS on inflammation and therefore cannot demonstrate causality between inflammation and the EMFA composition. The sporadic nature of our observational periods is certainly a limitation. More frequent measures of inflammation alongside EMFA composition would be beneficial in order to add greater strength to the association demonstrated between EMFA status and inflammation across time. Future research should measure the EMFA composition, and investigate the impact of FS and the dose–response on inflammation during periods when the recovery time between successive games may be insufficient to resolve inflammation and restore normal homeostasis. Moreover, future research investigating the effect of FS on inflammation in elite soccer players should also consider:

1. The ratio of EPA to DHA present within the fish oil supplement, as it has been argued that the velocity of incorporation may differ depending on that percentage (Drobnic et al., 2017).
2. The composition of the supplement itself, as Lewis et al. (2020b) showed that the strongest effects for FS on accelerating muscle recovery were most evident when administered alongside other nutrients known to impact on skeletal muscle remodelling (i.e., vitamin D, whey protein) as part of a recovery drink.
3. The timing of the supplementation regimen to determine if FS should be periodized in order to maximize EPA and DHA incorporation into the RBC membrane, and in turn significantly impact inflammatory responses at targeted periods of a season.

9.3.5. Chapter 8

The set of case studies presented in Chapter 8 illustrate the application of individualized adaptive ranges, together with the visualization of post-game biomarker responses. In the case of the latter, testing administered after competition may be valuable when

resting values of biomarkers might not reveal any concerns, but biomarker *responses* to an acute stress would more sensitively detect potential recovery issues (Lee et al., 2017), as was the case for Player 1 (**Figure 8.1A**). However, whilst visualizations of biomarker responses may aid the high performance practitioner in subjectively evaluating the athletes profile, we did not apply an adaptive range to the post-game biomarker measure, as previously conducted by Hecksteden et al. (2017). Future research should look to apply two individual reference ranges for each player (fatigued and recovered) instead of only detecting deviations from the recovered state (i.e. GD-1).

A prerequisite for the effective application of adaptive reference ranges is the inclusion of "healthy" athlete data. As such, we set the exclusion criteria as athletes' self-reporting the presence of any illness/injury symptoms, to the research personnel or medical team at the club. However, self-reporting is influenced by a number of factors such as match outcome (Fessi et al., 2018) and social influences (Carling et al., 2018), which may result in under-reporting of unfavourable responses to the sports scientist, although, anecdotally speaking this is largely dependent on the individual in question. Therefore, the success of our approach was limited by the willingness of the athlete to detail such information. Another limitation associated with Chapter 8 was the absence of baseline data collected prior to the commencement of the season. Ideally, baseline data should be collected when the athlete returns from the off-season period and is in a rested and healthy physiological state. This approach would allow the practitioner to gather baseline values, observe normal changes in biomarkers during healthy states, and may help the practitioner identify meaningful deviations in data during a season. Future studies investigating this individualised approach to longitudinal biomarker monitoring are warranted in elite soccer.

9.4. Practical Applications

The results of this thesis have demonstrated the utility of a POC device for the measurement of inflammation in elite soccer players. The POC measurement of CRP was found to be reliable (Chapter 4) and sensitive to player wellness and the recovery phase from competition (Chapter 5) and thus may be a suitable strategy to help practitioners monitor recovery status in elite soccer players. Importantly, the timely and rapid POC measurement of a capillary blood biomarker of inflammation informs athlete management decisions at the time of sampling. Furthermore, it allows for the collection of frequent longitudinal inflammation data in professional sport thus providing an opportunity for future research to greater understand healthy vs damaging levels of inflammation and the effects of repeated inflammatory episodes on player health.

The individualized approach to biomarker monitoring presented in Chapter 8 represents a promising strategy to assess ongoing recovery from disturbed homeostasis in the professional soccer player. However, a challenge for sports scientists is to identify meaningful changes in biomarker data in order to make informed decisions regarding athlete management. Often these decisions need to be made within 1 to 2 hours before training. Indeed, there is a need to establish baseline values whilst the athlete is in a rested and healthy physiological state (e.g. frequent POC testing could be performed when the athlete returns from the off-season period). This approach would allow the practitioner to gather baseline values, observe normal changes in biomarkers during healthy states and help establish individualized reference ranges for each athlete. These ranges can then be adapted every time a new measurement is recorded for the individual, assisting the practitioner in continually building up individual player profiles prior to the commencement of the season. This may assist in differentiating between normal and abnormal profiles for a given athlete during the competitive season, however, evaluation of the results in view of possible causes by the sports science and medical staff is still required. Indeed, the highly contextualized nature of biomarker testing and analysis means that communication amongst key stakeholders including the sports scientist, team doctor and the athlete themselves is vital in understanding the

biomarker results. Anecdotally, initially players may be reluctant to accept blood sampling as it can be considered invasive, therefore, communication as to the why/what is being measured to the athlete is fundamental in order to offset any negative connotations the player may have towards so called “load management” tools and in turn improve player engagement with the monitoring process. Clearly, further work applying individualized adaptive reference ranges is required in a real world setting as highlighted in Chapter 8.

The significant relationships observed between the EMFA composition and CRP in Chapter 7 provides rationale for augmenting the EMFA status of the elite soccer player. Measuring the EMFA status of the athlete during the pre-season may assist the practitioner in establishing a fish oil supplementation regimen for each athlete in order to maximize incorporation into the RBC membrane and reduce inflammation during the congested winter period. Data derived from Chapter 5 revealed that pre-match inflammation status predicts post-match inflammation response, which suggests that the pre-game may be an important window for practitioners to monitor inflammation during the competitive season. This pre-game approach to biomarker monitoring may be advantageous when monitoring during the acute phase is not feasible due to the logistical challenges associated with travel and instances whereby competition is followed by a rest day. Post-game assessments may also be used to assess player recovery rates in the days following competition. Chapter 5 showed significantly elevated CRP concentrations occurred at GD+1, and at a group level were not significantly higher at GD+2, compared to GD-1. Practitioners may therefore seek to review training load in individuals with pronounced responses beyond GD+1. Additionally, during periods of fixture congestion, where realistically opportunities to monitor athlete recovery at a squad level may be reduced, the initial use of subjective wellness questionnaires may create opportunities for further assessment of recovery via capillary blood sampling in individuals reporting negative subjective ratings of wellness.

The results of this thesis showed that the POC measurement of inflammation was highly sensitive in detecting subjective feelings of fatigue, muscle soreness and the presence of

illness symptoms (Chapter 5). It is unlikely that subjective wellness and blood biomarker data will influence player match selection, however, this information may be used to better inform the subsequent course of action (e.g. adjustment of training workload) to protect player health, promote timely recovery and ensure players are not overloaded in the lead up to games. When subjective data is combined with blood biomarker data, the practitioner may be able to gather a more holistic understanding of each individual player and gain context into the cause of disturbance in the athlete's physiological state. For example, from a clinical perspective, the POC measurement of CRP alongside the self-reporting of illness symptoms could be used as an indicator of infection and identify athletes that require clinical review. Further assessment by medical staff may include collection of body temperature to assess if URS may be due to infectious or non-infectious causes. This latter approach was identified through the practical application of blood monitoring during the period after which the data had been collected for this thesis. On the other hand, when biomarker 'red flags' of physiological relevance are detected (e.g. CRP changes greater than CDV of 20.1% established in Chapter 4), and the athlete reports no illness symptoms, recovery strategies may be targeted. For example, if during a period where recovery is the priority (as opposed to adaptation), and an athlete presents with an abnormal profile, pre-competition or post-competition acceleration of inflammation resolution via targeted nutritional supplementation may represent a practical approach for preventing the development of excessive systemic inflammation and promote the timely recovery of the player. This may also help progress away from a 'one-size fits all' approach to the use of antioxidant/anti-inflammatory supplementation by identifying periods where an individual may require (or not require) intervention, resulting in the administration of anti-inflammatory interventions at the appropriate time. However, for the pre-game approach to be successful considerations must also be made regarding data feedback in order to minimise disruptions to the players' pre-game routine and any potential anxiety associated with this. Furthermore, investigations into the optimum timing of the supplementation regimen and whether meaningful reductions in inflammation levels are achievable in this recovery window prior to competition are warranted in order to better optimise this process. **Figure 9.1** illustrates how blood monitoring in conjunction with

subjective wellness data may guide prescription of training load, recovery, or medical intervention.

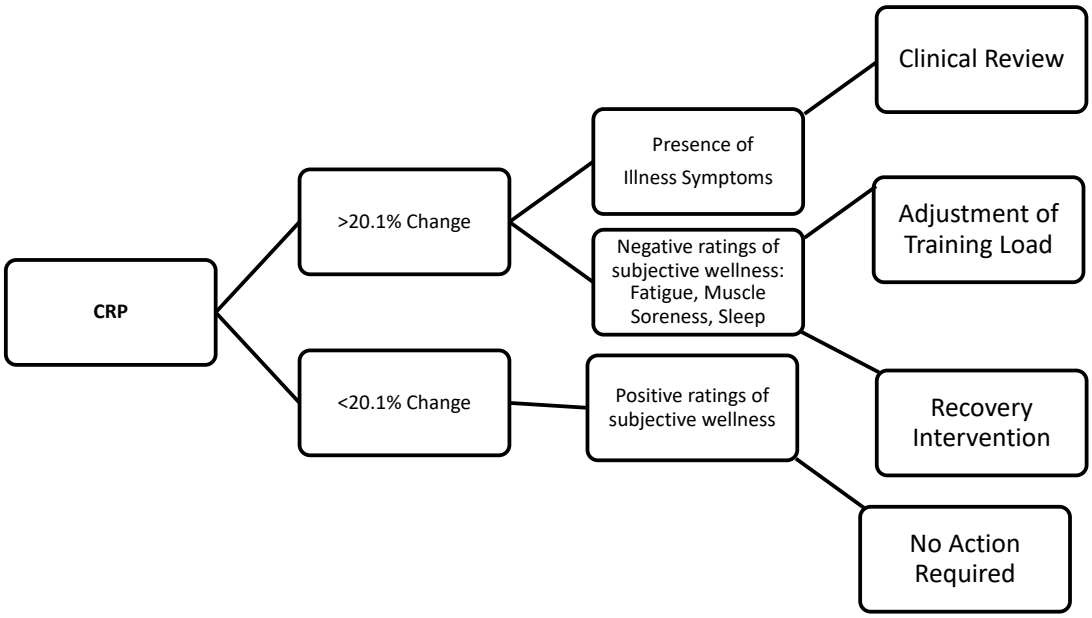

Figure 9.1. Potential framework for blood monitoring illustrating how the point of care measurement of C-reactive protein (CRP) in conjunction with subjective wellness may be used to identify athletes which require clinical, recovery or performance review.

9.5. Conclusion

In conclusion, this thesis is the first investigation to longitudinally examine inflammation in elite soccer players competing in the EPL, and as such has contributed novel data to the soccer literature. We report that that the use of a POC test for CRP in well trained individuals is practical, but not interchangeable with the standard laboratory method, and established a CDV for CRP to enhance interpretation of meaningful changes in well trained individuals (Chapter 4). Data presented in this thesis shows that an EPL game and season induce time-dependent changes in circulating markers of inflammation and oxidative stress, demonstrates that pre-match inflammation status predicts post-match inflammatory response and that the POC measurement of CRP is sensitive to the changes in subjective wellness but not match workload (Chapter 5). Data derived from Chapter 5 are practically relevant as they demonstrate that the pre-game may be an important window for practitioners to reduce inflammation, since it is unlikely they can influence player match selection, and that the POC measurement of CRP in conjunction with wellness data may offer an objective tool for identifying fatigue and illness risk in professional soccer and can therefore be used to enhance the management of the professional soccer player. Chapter 6 documents the first study to investigate the efficacy of a nutritional intervention aimed at reducing blood biomarkers of inflammation and oxidative stress in professional soccer players competing in the EPL. We show that curcumin ingestion is not effective for attenuating biomarker responses at a group level, and based on the CDV established in Chapter 4, report physiologically relevant decreases in CRP levels for only 3 athletes in response to the intervention (Chapter 8). It has been proposed that interventions which facilitate the natural resolution of inflammation may more effectively enhance exercise recovery than anti-inflammatory interventions and we present convincing evidence that the EMFA composition influences seasonal changes in CRP in the elite soccer player (Chapter 7). In light of this finding, this thesis suggests that measuring and manipulating the EMFA composition of professional soccer players may be a useful endeavour in order to decrease inflammation during a competitive season. Finally, we present a set of case studies which explore the application of an individualized approach to biomarker

monitoring, by constructing adaptive reference ranges for each athlete, as implemented by WADA, and confirm that athletes deemed “healthy” or “non-healthy” present markedly different within- and between-subject variations in biomarker responses, which has a significant impact on their respective individualized ranges. This thesis uniquely tracks inflammation in professional soccer players in a ‘real-world’ setting, thereby strengthening the ecological validity and novelty of our data. However, inherent limitations exist as a consequence of collecting data in professional soccer players and the small cohort investigated may limit the application of the thesis findings to the club in question. Future studies should look to implement a more targeted approach to anti-oxidant supplementation in elite soccer and further explore the application of individualized methods for longitudinal blood monitoring in professional soccer.

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Chapter 11

APPENDIX

1. Ethical Approval
2. Informed Consent
3. Contributions to literature

Appendix 1: Ethical Approval

NUI Galway
OÉ Gaillimh

Leas-Uachtarán um Thaighde
Vice President for Research

18 May 2018
Ref: **18-May-01**

Diarmuid Daniels
School of Medicine
NUI Galway

Dear Diarmuid,

Re: 'The Longitudinal Analysis of Redox and Inflammation Biomarkers in Athletes'

I write to you regarding the above proposal which was submitted for ethical review. Having reviewed your response to my letter, I am pleased to inform you that your proposal has been granted **APPROVAL**

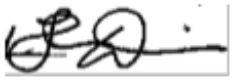
All NUI Galway Research Ethic Committee approval is given subject to the Principal Investigator submitting annual and final statements of compliance. The first statement is due on or before 18 May 2019.

See annual and final statement of compliance forms below. Section 7 of the REC's Standard Operating Procedures gives further details, and also outlines other instances where you are required to report to the REC.

Please note the following:

1. This submission has been reviewed primarily from an ethical perspective. It is the responsibility of the Principal Applicant to ensure and monitor compliance with any relevant legislation/public health guidelines in the country where the study is due to take place or any local policy in the site where the study is due to take place. It is also the researcher's responsibility to undertake this research in accordance with the National and NUI Galway guidelines and protocols regarding Covid-19 which are in effect at the time of data collection.
2. Any significant alterations to an approved proposal must receive prior approval from the REC prior to implementation. Please request an Amendment Form;
3. You are responsible for notifying the REC in the event of serious or unexpected adverse effects, unforeseen circumstances, the termination of the study, and any significant decisions by other Ethics Committees. Section 7 of the REC's Standard Operating Procedures gives further details on instances requiring follow-up reviews, and reporting obligations.
4. Principal Applicants given NUI Galway REC approval must, upon completion of the approved research, submit an End-of Study report. Failure to submit such a report may impact upon future ethics applications.

Yours sincerely



Kevin Davison

Chair, Research Ethics Committee

OÉ Gaillimh,
Bóthar na hOllscoile,
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Appendix 2: Informed Consent

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OÉ Gaillimh
NUI Galway

Participant Information Sheet

Title
Monitoring biomarkers of hormesis during the in-season competitive phase in elite soccer players.

You have been invited to take part in a University study as part of a PhD programme. Please take the time to read the information care fully. This information sheet should help you to decide whether or not you would like to take part in this study. Should you have any questions, please do not hesitate to make contact (details below).

Purpose and value of this study
The purpose of the current study is to individualize the monitoring of athlete recovery using blood biomarkers of Inflammation and Oxidative Stress.

How will the data be used?
The data will be analysed and presented as part of my PhD degree. Data may also be submitted for publication in a peer reviewed academic journal. Individual participants will not be referred to in any publication and every effort will be made to ensure the anonymity of those taking part.

What would be expected of you?
As a participant in this study you will be asked to fill out a consent form to ensure you will be able to take part in the study, this will take approximately 5 minutes. Each participant will be required to attend two testing sessions a week. Testing will be carried out using a capillary blood sample (via pin prick in the ear lobe) and will take approximately 5 minutes each week.

Confidentiality:
General group test data will remain with the investigators following participation, individuals will be shown their own results but all others shall be anonymous. All participant data and results will be anonymous and stored confidentially in accordance with the NUIG Data Protection Policy.

Benefits of participation in this study?
The incentive for the participants would be to provide them with the medical staff with the data collected and an interpretation about their general health.

Possible risks associated with participation?
There are no risks envisioned for the subject in this study. Subjects may feel a slight pricking sensation during capillary blood sample collection (via pin prick in the ear lobe). As participation is purely voluntary, there will be no consequences for not taking part in this study.

Withdrawal from the study?
You are free to withdraw from the study at any time during the process with no adverse effects. This includes withdrawal either before or on the day of testing.

Who is funding the research?
PhD research will provide the funding for this study (co -funded by NUIG, Insight Centre for Data Analytics and Orreco).

Who is carrying out the research?
Diarmuid Daniels (Lead Researcher).
If you have any queries please contact : diarmuid.daniels@orreco.com

Name of Participant: _____

Title of the project: Monitoring biomarkers of hormesis during the in-season competitive phase in elite soccer players.

Main investigator and contact details: Diarmuid Daniels –diarmuid.daniels@orreco.com

Members of the research team:

1. I agree to take part in the above research. I have read the Participant Information Sheet which is attached to this form. I understand what my role will be in this research, and all my questions have been answered to my satisfaction.
2. I understand that I am free to withdraw from the research at any time, for any reason and without prejudice.
3. I have been informed that the confidentiality of the information I provide will be safeguarded.
4. I am free to ask any questions at any time before and during the study.
5. I have been provided with a copy of this form and the Participant Information Sheet.

Data Protection: I agree to the University processing personal data which I have supplied. I agree to the processing of such data for any purposes connected with the Research Project as outlined to me.

Name (Print): _____

Signed: _____ **Date:** _____

4

If you wish to withdraw from the research, please complete the form below and return to the main investigator named above.

Title of Project: Monitoring biomarkers of hormesis during the in-season competitive phase in elite soccer players.

I WISH TO WITHDRAW FROM THIS STUDY

Name:_____

Signed: _____ **Date:** _____

5

Appendix 3: Contributions to Literature

REVIEW

Are There Benefits from the Use of Fish Oil Supplements in Athletes? A Systematic Review

Nathan A Lewis,[1,2,3] Diarmuid Daniels,[2,3,4] Philip C Calder,[5,6] Lindy M Castell,[7] and Charles R Pedlar[2,3,8]

[1] English Institute of Sport, Sports Training Village, University of Bath, United Kingdom; [2] Faculty of Sport, Health and Applied Science, St Mary's University, London, United Kingdom; [3] Orreco, Research & Innovation Centre, National University of Ireland, Galway, Ireland; [4] School of Medicine, National University of Ireland, Galway, Ireland; [5] School of Human Development and Health, Faculty of Medicine, University of Southampton, Southampton, United Kingdom; [6] NIHR Southampton Biomedical Research Centre, University Hospital Southampton NHS Foundation Trust and University of Southampton, Southampton, United Kingdom; [7] Green Templeton College, University of Oxford, Oxford, United Kingdom; and [8] Division of Surgery and Interventional Science, University College London (UCL), London, United Kingdom

ABSTRACT

Despite almost 25 y of fish oil supplementation (FS) research in athletes and widespread use by the athletic community, no systematic reviews of FS in athletes have been conducted. The objectives of this systematic review are to: *1*) provide a summary of the effect of FS on the athlete's physiology, health, and performance; *2*) report on the quality of the evidence; *3*) document any side effects as reported in the athlete research; *4*) discuss any risks associated with FS use; and *5*) provide guidance for FS use and highlight gaps for future research. Electronic databases (PubMed, Embase, Web of Science, Google Scholar) were searched up until April 2019. Only randomized placebo-controlled trials (RCTs) in athletes, assessing the effect of FS on a health, physiological/biochemical, or performance variable were included. Of the 137 papers identified through searches, 32 met inclusion criteria for final analysis. Athletes varied in classification from recreational to elite, and from Olympic to professional sports. Mean age for participants was 24.9 ± 4.5 y, with 70% of RCTs in males. We report consistent effects for FS on reaction time, mood, cardiovascular dynamics in cyclists, skeletal muscle recovery, the proinflammatory cytokine TNF-α, and postexercise NO responses. No clear effects on endurance performance, lung function, muscle force, or training adaptation were evident. Methodological quality, applying the Physiotherapy Evidence Database (PEDro) scale, ranged from 6 to a maximum of 11, with only 4 RCTs reporting effect sizes. Few negative outcomes were reported. We report various effects for FS on the athlete's physiology; the most consistent findings were on the central nervous system, cardiovascular system, proinflammatory cytokines, and skeletal muscle. We provide recommendations for future research and discuss the potential risks with FS use. *Adv Nutr* 2020;00:1–15.

Keywords: performance, injury, inflammation, mood state, recovery, DHA, EPA, exercise

Introduction

The effects of fish oil supplementation (FS) on athlete health and performance have been researched for the past ~25 y. The early research focused on the potential of FS to modify the inflammatory response to exercise. However, subsequent studies have investigated the effects of FS on metabolism, the immune response, and the respiratory and cardiovascular, musculoskeletal, or central and peripheral nervous systems, with most recent research exploring the effect of FS on neuronal injury.

The authors reported no funding received for this study.
Author disclosures: The authors report no conflicts of interest.
Address correspondence to NAL (e-mail: nathan.lewis@eis2win.co.uk).
Abbreviations used: EIB, exercise-induced bronchoconstriction; FS, fish oil supplementation; PBMC, peripheral blood mononuclear cell; PEDro, Physiotherapy Evidence Database; RCT, randomized placebo-controlled trial; SPM, specialized proresolving mediator; URTI, upper respiratory tract illness.

DHA (22:6n–3) and EPA (20:5n–3) are the long-chain polyunsaturated n–3 (ω-3) fatty acids present in FS. They are natural constituents of seafood including algae, crustaceans, and fish, and to a much a lesser extent in dairy and meat (the diet of the animal influencing the n–3 fatty acid content). In addition to both dietary intake (e.g., eating fish) and FS changing n–3 fatty acid status, endurance training is known to alter skeletal muscle membrane composition, leading to changes in the muscle phospholipids including increasing DHA content and decreasing the n–6:n–3 fatty acid ratio (1, 2). By virtue of training, athletes can acquire a "superior" n–3 fatty acid status compared with the nonathlete and therefore have less need to use supplements. For example, endurance training alters muscle DHA content significantly (1). Therefore, dietary requirements for the specific long-chain n–3 fatty acids can differ in athletes, both collectively in terms of recovery and performance effects, or individually

 Adv Nutr 2020;00:1–15; doi: https://doi.org/10.1093/advances/nmaa050.

EVIDENCE OF A RELATIONSHIP BETWEEN DIETARY FAT INTAKE AND INFLAMMATION AMONG PROFESSIONAL SOCCER PLAYERS

Diarmuid Daniels [1,2], Nathan Lewis [2,3], Georgie Bruinvels [2,3], Paul Catterson [4], John Newell [1,2], Micheal Newell [1], Andrew Simpkin [1,2], Andrew Barr [2], Charles R Pedlar [2,3]
[1]National University of Ireland Galway, Ireland, [2]Orreco, Business Innovation Centre, National University of Ireland, Galway, Ireland, [3]St Mary's University, Twickenham, UK, [4]Newcastle United Football Club, UK

ABSTRACT

Reducing background inflammation in athletes may be a medical and performance objective. Data describing the relationship between erythrocyte membrane fatty acids (EMFA) and low grade inflammation in soccer players are absent from the literature. EMFA reflects dietary fat intake in the weeks preceding the blood test.

PURPOSE: To investigate the strength and reproducibility of the relationship between EMFAs and inflammation in a group of professional soccer players.

METHODS: We conducted an observational study, collecting venous blood samples measuring high-sensitivity C-reactive protein (CRP) and EMFA in the early season (T1) and the start of the following pre-season (T2). A total of 47 blood samples were collected from 29 different athletes, with 25 athletes tested at T1, and 22 athletes at T2. A cut off point of >5mg.L-1 was set to minimize the effect of acute inflammation, and these samples were removed from the analysis. Relationships between biomarker variables were examined using Pearson correlations.

RESULTS: At T1, we report significant positive correlations between CRP and the following EMFA variables: Omega6:Omega3 ratio and the Arachidonic Acid: Eicosapentaenoic Acid (AA:EPA) ratio (0.566, p< 0.003, and 0.582, p< 0.002 respectively) and significant negative correlations with the Omega 3 index and the anti-inflammatory fatty acid index (AIFAI; -0.495, p< 0.011, and -0.465, p< 0.018 respectively). However, at T2, there were no significant correlations between EMFA variables and inflammation. The correlation analysis of all the blood samples collected (n=47) showed significant correlations between the Omega-3 Index, the AIFAI and CRP (-0.319, p< 0.028, and -0.299, p< 0.040 respectively).

CONCLUSION: There is a relationship between inflammation and EMFA variables in professional soccer players and the strength of this relationship depends on the sampling occasion. Future research should explore augmenting EMFA as an anti-inflammatory strategy.

INTRODUCTION

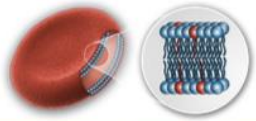
- Soccer is characterized by many high intensity episodes of physical stress each game [1]. As a result, a game produces significant increases in inflammatory response variables, with elevated biomarkers of inflammation shown to persist for 48-72 hours post game [2]. Prolonged increases, however, may indicate the presence of systemic inflammation and imply that the athletes recovery is impaired, and may not be capable of performing.
- Identifying appropriate anti-inflammatory strategies to decrease inflammation may be an important objective of both sports medicine and sports science staff in order to protect player health and training adaptation. Erythrocyte membrane fatty acids (EMFA) are thought to reflect dietary fat intake within the past few months [3].
- However, only few studies have examined their relationship with low-grade inflammation, with data describing this relationship in soccer players absent from the literature.

AIMS & HYPOTHESIS

- We therefore sought to Investigate the strength of the relationship between EMFAs and inflammation, with a particular emphasis on the Omega-3 Index.
- The Omega-3 Index is the red blood cell (RBC) content of the two marine long-chain omega-3 fatty acids, and is expressed as EPA+DHA as a percent of total identified RBC fatty acids.
- We hypothesized that the Omega-3 fatty acid variables would have an inverse relationship with inflammation.

METHODS

- We conducted an observational study to examine the relationship between inflammation status and RBC fatty acid profile in a group of professional soccer players.
- Data collection was conducted at two time points: September 2017 (T1), the start of the 2017-2018 English Premier League season and July 2018 (T2), at the start of pre-season for the 2018-2019 Premier League season.
- Venous blood samples were collected for the analysis of CRP and EMFAs. Blood sampling was conducted at the same time of day (8.30-10.30am). Players were tested in a fasted, rested and hydrated state.
- A cut off point of >5mg.L-1 was set to exclude any athletes with spikes resulting from acute inflammation. Relationships between biomarker variables were examined using Pearson correlations. All statistical analyses were carried out using R (Version 1.0.143). The significance level was set at $p < 0.05$.



RESULTS

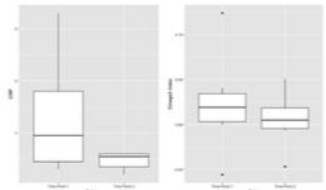


Figure 1: Median (interquartile range) for CRP values in soccer athletes at T1 vs T2 (Figure 1A). Median (interquartile range) for the Omega-3 Index in soccer athletes at T1 vs T2 (Figure 1B).

RESULTS

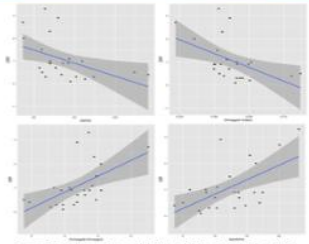


Figure 2: Inflammation vs. EMFA variables at September 2017.

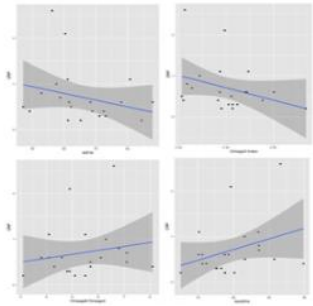


Figure 3: Inflammation vs. EMFA variables at July 2018.

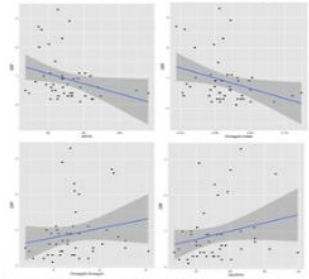


Figure 4: Inflammation vs. EMFA variables for the full dataset (n=47).

SUMMARY

- T1, we report significant correlations between EMFA variables and inflammation suggesting that dietary fat intake may be a significant driver of inflammatory status in soccer players.
- In contrast, however, at T2, we report no significant correlations between EMFA variables and inflammation. This latter finding could be due to the fact that testing time points varied, and both time of season and training status may be important factors to consider when investigating the relationship between inflammation and EMFA biomarkers in soccer players.
- Finally, the analysis of all 47 samples collected showed significant correlations between the AIFAI, Omega-3 Index and inflammation, suggesting that altering dietary fat intake may present an appropriate anti-inflammatory strategy in professional soccer players

CONCLUSION

- Our results suggest there may be a relationship between inflammation and EMFA variables in professional soccer players, but further investigation is warranted.
- Future studies collecting more longitudinal data are needed in order to observe patterns in inflammation and RBC fatty acid profiles and the strength of their relationship across different stages of the season.
- Although players are well adapted to their sport, the long season with consistent exposure to acute inflammation and limited periods of rest, places the soccer player at high risk of under recovery. This is of relevance to this population given that cumulative exposure to inflammation has a number of health implications [4, 5, 6].
- Therefore, establishing the strength of this relationship and investigating appropriate anti-inflammatory strategies may have important implications for player health and adaptation.

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