THE EFFECTS OF A SINGLE DOSE OF ARONIA MELANOCARPA EXTRACT ON BIOMARKERS ASSOCIATED WITH DELAYED ONSET MUSCLE SORENESS (DOMS).

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Copyright statement

Abstract

Background. Delayed-onset muscle soreness (DOMS) is the pain felt in skeletal muscle in the days following a bout of exercise. Associated with eccentrically biased resistance exercise, DOMS pain can restrict activities of daily living, is a barrier to engagement in further exercise and can hinder sports performance. From a public health point of view exercise is an important mediator of disease. To effectively facilitate exercise engagement, it is important to understand the mechanisms that underpin DOMS and identify interventions that alleviate the symptoms. Current theories of DOMS are based around the inflammatory response caused by exercise induced muscle damage (EIMD) leading to the sensitisation of pain receptors. It has been shown that DOMS and other indices of EIMD can be blunted by ingestion of polyphenol compounds. *Aronia Melanocarpa* is an antioxidant rich berry, the concentrated dried powder of this berry has been shown to have powerful antioxidant properties. Establishing an evidence based exercise protocol that consistently elevates biomarkers is required for DOMS intervention testing, this research was created to establish such a protocol. Ingesting polyphenols may attenuate indices of exercise-induced muscle damage. Thus, this research investigated whether consuming a single dose of polyphenol rich *Aronia Melanocarpa* would attenuate biomarkers of DOMS. The endocannabinoid Anandamide (AEA) has been shown to have pain modulating capability and is of interest in this research.

Purpose. The purpose of this study was to design a protocol to determine the optimum level of exercise to induce measurable changes in the biomarkers associated with DOMS. Once the protocol was established, it was then used to investigate the effect of a single dose of polyphenols rich *Aronia melanocarpa* on those biomarkers.

Design. Phase one utilised a randomised independent group design. Twenty-five healthy men (31.6 ± 13.2 years; 83.2 ± 13.0 kg; 179.0 ±6.9 cm) were randomly assigned to perform either 48, 60 or 72 repetitions of maximal eccentric elbow flexion exercises. Phase two was conducted using a randomised, single blind, placebo controlled independent group design. Seventeen healthy male participants (27.1 ± 10.2 years; 77.8 ±10.3 kg; 177.7 ± 7.0 cm) were randomly assigned to ingest either a placebo drink or polyphenol rich *Aronia melanocarpa* drink after a bout of elbow flexion exercises. In both phase one and phase two, levels of the following biomarkers were assessed before, immediately after and 48 hours after exercise; DOMS, CK, IL-6, AUC, ROM and Anandamide (AEA).

Results. Phase one: After 48 hours, the highest mean DOMS scores (VAS) were recorded in the 60 repetition group; 5.1cm (± 2.2) compared to 48 repetitions 4.3cm (± 2.1) and 72 repetitions 4.6cm (± 2.6). However, the group differences did not reach statistical significance ($p=0.766$). The UAC was significantly increased over the 48-hour period for 48 and 60 repetitions only ($p=0.005$ and $p=0.008$ respectively). A significant 48-hour time effect on the ROM was apparent in the 60 repetition group ($p=0.013$) and 72 repetition group ($p=0.018$), but not 48 repetition group ($p=0.262$). There was no statistical difference between AEA concentrations at any time-point. Phase two: After 48 hours, participants in the *Aronia melanocarpa* group reported 14% less pain than in the control group with mean pain scores of 4.4cm (±2.2) and 5.1cm (±2.2) respectively. However, the difference in pain scores did not reach statistical significance ($p=0.556$). After 48 hours, plasma CK had significantly increased by 462% ($p=0.036$) in the placebo group. There was no significant increase in CK activity in the *Aronia melanocarpa* group ($p=0.173$).

Despite this, no significant difference between the two groups was found (p=0.248). There was a significant reduction in the ROM of the elbow joint over 48 hours of 16.5° (p=0.012) in the control group, but no change in ROM in the *Aronia melanocarpa* group over the same time period (p= 0.122). Despite this, no significant group x time interaction (p=0.111) was found for ROM.

Conclusions. The developed exercise protocol consistently elevated biomarkers of DOMS and was therefore used to test the polyphenol intervention. The findings indicate that a single dose of polyphenol rich *Aronia melancarpa* does not attenuate exercise-induced DOMS in healthy men. However, *Aronia melanocarpa* may confer a small blunting effect on other indices of EIMD, specifically ROM and CK.

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Dedications and Acknowledgements

I would like to dedicate this thesis to my children William and Sophie who are my world.

Secondly, I would like to say a special thank you to Pauline Balac for giving me the courage to attempt this research in the first place. Were it not for her belief in me, this research would never have been written. A thankyou also to my supervisors Michael Fish and Farideh Javid, for being helpful and patient.

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List of abbreviations

DOMS – Delayed-Onset Muscle Soreness

EIMD – Exercise-induced Muscle Damage

VAS – Visual Analogue Scale

CK – Creatine Kinase

IL-6 – Interleukin 6

ROM – Range of Motion

UAC – Upper Arm Circumference

RPE – Rate of Perceived Exertion

MPS – Muscle Protein Synthesis

RBE – Repeated Bout Effect

ADP – Adenosine Di-Phosphate

ATP – Adenosine Tri-Phosphate

NGF – Nerve Growth Factor

ECS – Endocannabinoid System

AEA – Anandamide

2-AG – 2-Arachidonylglycerol

CB1 – Cannabinoid receptor 1

CB2 – Cannabinoid receptor 2

ELISA – Enzyme-Linked Immunosorbent Assay

EDTA - Ethylenediaminetetraacetic acid

ROS – Reactive Oxygen Species

RNS – Reactive Nitrogen Species

RNOS – Reactive Nitrogen & Oxygen Species

IMP – Intramuscular Pressure

CRP – C Reactive Protein

TAC – Total Antioxidant Content

FRAP – Ferric Ion Reducing Antioxidant Power

DPPH - 2,2-diphenyl-2-picrylhydrazyl

ORAC – Oxygen Radical Absorbance Capacity

TNFα – Tumor Necrosis Factor Alpha

SC – Satellite Cell

NSAID's – Nonsteroidal Anti-Inflammatory Drugs

ANOVA – Analysis of Variance

IQR – Interquartile Range

SD – Standard Deviation

HPLC – High Performance Liquid Chromatography

UPLC – Ultra High Performance Liquid Chromatography

Thesis Introduction

Evidence that exercise modulates disease states is well established and still accumulating (Cordero, Masiá, & Galve, 2014; Floegel & Perez, 2016; Pesta, Goncalves, Madiraju, Strasser, & Sparks, 2017; Swift, Johannsen, Lavie, Earnest, & Church, 2014), despite this only 26% of adults in the UK meet the Department of Health exercise guidelines (NHS, 2017). The negative physiological effects of exercise can be demotivating and there is a clear need to evaluate strategies that moderate those effects, one such negative effect being exercise-induced pain. Unaccustomed, strenuous exercise often results in the sensation of pain that appears within a couple of days (Hough, 1900). Given that the pain is delayed, this phenomenon has been termed 'delayed-onset muscle soreness' or DOMS. This pain causes mild discomfort to debilitation depending on the intensity of the exercise bout, and can lessen a person's motivation to perform further exercise (Grant, Zondi, Janse van Rensburg, & Jansen van Rensburg, 2015). The underlying biochemical mechanisms underpinning the pain of DOMS have yet to be fully elucidated (Grant et al., 2015). Despite uncertainty of the underlying cause of DOMS, it is clear that the research community have invested considerable time investigating a whole variety of interventions to alleviate the symptoms. There is a suggestion that exercise-induced increases in reactive oxygen species (ROS) sensitise pain receptors. ROS are a natural by-product of energy metabolism and are important in cell signalling. However, ROS are chemically reactive, and become detrimental as high levels cause cell damage. As ROS may sensitise pain receptors, is follows that nutritional interventions targeting ROS have been investigated as an alleviator of DOMS, antioxidant vitamins and polyphenols in particular have become a focus. Evidence suggests there is some merit in the efficacy of polyphenol interventions in the reduction of DOMS pain, however the evidence is equivocal (J.

Kim & Lee, 2014). This may in some measure be because each polyphenol intervention has a unique profile of polyphenolic compounds and therefore confer differing magnitudes of physiological effects. To the authors knowledge the effect of polyphenol rich *Aronia melanocarpa* on the biomarkers associated with DOMS has yet to be examined. From an experimental standpoint however, it is difficult to compare DOMS intervention studies because of the variety of methods employed within the research community, in particular, disparity in the number of exercise repetitions. A standardised exercise protocol with a set number of repetitions that can be used to test several different interventions would make comparison of intervention studies possible. The present study sets out to examine the physiological effects of three levels of exercise (48, 60 and 72 repetitions of elbow flexion), in order to set a level of exercise to test a polyphenol intervention on biomarkers associated with DOMS.

Main Aims and objectives

The overall aim of this thesis is to investigate biomarkers associated with exercise-induced DOMS in humans. The research intends to address the inconsistencies in DOMS inducing experimental protocols. And further, investigate the effects of a single antioxidant dose on the biomarkers associated with DOMS. These aims will be achieved by the following objectives:

1. To design an evidence based DOMS induction exercise protocol.
2. To investigate biomarkers of DOMS following three levels of exercise.
3. To investigate the effects of a dietary polyphenol antioxidant intervention on biomarkers associated with DOMS.

This thesis first addresses in chapter two the relevant background literature on the aetiology of DOMS and modalities to alleviate it. A two phase experimental

approach is used to look at the effects of the three levels of exercise and then the effects of a polyphenol intervention. Chapter three outlines phase one where biomarkers were measured to assess three levels of exercise. Chapter four then outlines the second phase, which assesses the effects of a polyphenol intervention. Lastly, a short conclusion is outlined in chapter five.

Chapter 1 Background

The phenomenon of delayed onset muscle soreness (DOMS), first described by Hough (1900), is characterised by pain and soreness in exercised skeletal muscle, and develops in the days following exercise. Hough described muscle soreness, with a gradual onset, which began eight to ten hours after a bout of unaccustomed strenuous exercise. After an initial delay, a gradual increase in pain occurs which peaks between 24 and 48 hours after cessation of an exercise bout (Plattner, Lambert, Tam, & Baumeister, 2014). Though there is evidence that DOMS occurs with concentric and isometric contractions, the association between DOMS and unaccustomed strenuous exercise containing a significant number of eccentric contractions has been well documented (R. Armstrong, 1984).

DOMS pain rated as 5, 6 or 7 out of 10 (a score of zero represented no pain and ten represented the worst pain imaginable) are commonly recorded following a number of eccentric contractions (Lau, Blazevich, Newton, Wu, & Nosaka, 2015a; Levers et al., 2016; Matsuda, Kan, Uematsu, Shibata, & Fujino, 2015; Pumpa, Fallon, Bensoussan, & Papalia, 2014; Ra et al., 2013). This level of pain is unpleasant and results in functional limitations and restricts exercise performance (Grant et al., 2015). Indeed, simply the fear of exercise-induced pain may reduce subsequent participation in physical activity (Wingo et al., 2011).

The link between exercise-induced muscle damage (EIMD) and DOMS has for many years received focused attention. It is apparent that the eccentric contractions associated with DOMS also cause a significantly greater amount of EIMD compared to isometric or concentric contractions (Fridén, Sjöström, & Ekblom, 1983; Gibala, MacDougall, Tarnopolsky, Stauber, & Elorriaga, 1995; Newham, McPhail, Mills, & Edwards, 1983). It would seem to follow that EIMD and DOMS pain would be

related as they are both associated with heavily eccentric exercise. However, direct association between indicators of EIMD and DOMS pain is often weak indicating that there may be an interplay with other biological processes, exercise-induced reactive oxygen species (ROS) for example. Looking at the evidence comparing EIMD and DOMS suggests that an association is present but not fully understood. Four decades ago, a weak correlation between exercise-induced ultrastructural injury and DOMS pain was demonstrated using muscle biopsies and electron microscopy (Nurenberg et al., 1992). Later however, using joint angles, upper arm circumference and Creatine Kinase (CK) activity as indicators of EIMD, DOMS did not correlate well (Nosaka, Newton, & Sacco, 2002). In contrast, more recent research reported a correlation between DOMS and CK, though the correlation was a weak one (Damas, Nosaka, Libardi, Chen, & Ugrinowitsch, 2016). Overall, the evidence suggests the presence of DOMS can be considered an indicator that muscle damage has occurred (Schoenfeld & Contreras, 2013). And further, that DOMS is likely to be a secondary consequence of EIMD following microtrauma to the muscle tissue (Lewis, Ruby, & Bush-Joseph, 2012).

The negative consequences of DOMS include pain, reduced motivation to train and reduced performance (Grant et al., 2015). It is therefore important to further understand the underlying biological mechanisms and discover strategies to reduce the soreness, particularly for athletes. A diverse multitude of treatment modalities to alleviate the severity of DOMS pain have been explored (Cheung, Hume, & Maxwell, 2003). However, the complexity and individual variation of the human body's response to a bout of exercise makes research in this area a challenge and a clear DOMS attenuation strategy evasive. Though evidence of attenuation

strategies have often been equivocal, they have provided insight for more focussed research directions into the aetiology and management of DOMS.

1.1 *DOMS and exercise engagement*

DOMS as a negative consequence of exercise can reduce engagement in further exercise. DOMS is an unfortunate consequence of unaccustomed strenuous exercise and has been experienced by most people at some point in their lives. Exercise though is an important part of a healthy lifestyle. There is a large body of evidence documenting the health benefits of being physically active and the role exercise plays in many chronic conditions (Bouchard, Blair, & Haskell, 2007; Cordero et al., 2014; Floegel & Perez, 2016). For example, the UK Department of Health emphasised the clear inverse relationship between exercise and cardiorespiratory, metabolic and musculoskeletal health (DoH, 2011). Regular exercise also reduces the risk of certain cancers, osteoporosis and the symptoms of anxiety and depression (Bouchard et al., 2007). Regular exercise has a positive effect on metabolism and modifiable cardiovascular risk factors, specifically; dyslipidaemia, hypertension and type 2 diabetes (Cordero et al., 2014). Though the Department of Health actively encourage exercise behaviours and disseminate information of the positive health effects of exercise, adherence to exercise programmes is still lacking, particularly in low socioeconomic status groups (NHS, 2018). Self-motivation has been shown to be inversely associated with adherence to exercise programmes (Dishman, Ickes, & Morgan, 1980). Unfortunately, some individuals that are motivated to adhere to the exercise programme reduce physical activity in other areas. The phenomenon of increasing physical activity in one area whilst decreasing physical activity in another area is known as compensation. The principal causes of these compensation behaviours in middle-aged adults has been

reported as fatigue and delayed-onset muscle soreness (DOMS) (Gray, Murphy, Gallagher, & Simpson, 2018).

1.2 *DOMS and obesity*

Exercise-induced DOMS pain can be a barrier to exercise. Overweight and obese individuals report an 'exaggerated' physiological response to exercise, including a reduced tolerance to exercise-induced pain, such as DOMS, compared to normal weight individuals (Wingo et al., 2011). It is important therefore to further understand DOMS pain and research ways to alleviate the exaggerated pain in this group. With pain reduction strategies, this group would be more able to manage weight and disease risk. The prevalence of obesity in the UK is at a record high and is currently increasing, in 2016, 57% of women, 66% of men and 28% of children (2-15yrs) were overweight or obese (NHS, 2017). The metabolic consequences of being obese increase the risk of cardiovascular disease, type 2 diabetes and some cancers. Weight loss *per se* has been shown to reverse these cardiometabolic health risks (Patkar et al., 2017). In addition, for every kilogram of weight loss there was found to be a 16% reduction in type 2 diabetes risk (Hamman et al., 2006). Even a relatively modest 5% reduction in body weight reduces co-morbidities by 8% in the severely obese (Agborsangaya, Majumdar, Sharma, Gregg, & Padwal, 2015). Weight loss protocols usually contain a caloric restriction element along with an exercise element. In 2016, 47% of adults in the UK were trying to lose weight, of those, nearly 30% were using exercise as a weight loss aid (NHS, 2017). Resistance training, during and following weight loss, mitigates the reduction in muscle mass occurring during the weight loss period (B. Kim, Tsujimoto, So, & Tanaka, 2015). The exercise element usually includes an exercise protocol with eccentrically biased, and therefore associated with DOMS, exercises to protect muscle mass.

Though physical activity after weight loss is a determinant of subsequent weight maintenance (Beavers et al., 2014). Following initial weight loss, a reduction in physical activity level is associated with weight regain, and is therefore considered an essential element of weight maintenance (Catenacci et al., 2014). Exercise is an important element of a healthy lifestyle and in the management of disease risk, DOMS can be a barrier to that exercise. For obese individuals the need for exercise to reduce disease risk is even more important as they are often at increased risk compared to normal weight individuals (Jago, Mendoza, Chen, & Baranowski, 2013). Given also that obese individuals experience an exaggerated pain response, reducing DOMS pain in this group is pertinent. In addition, the DOMS pain reduction strategies should not be detrimental to health, repeated use of pain relieving drugs could be considered such. Pain reduction strategies with positive health effects should be considered, such as nutritional interventions containing antioxidant substances.

1.3 *DOMS, physical activity and resistance exercise.*

People may simply be finding exercise physically and mentally challenging and consequently are less motivated to engage. Understanding the processes behind the degenerative/regenerative process of muscle repair after exercise has important implications in exercise engagement. The 2018 physical activity statistics reveal a relatively positive picture for the UK with the majority of adults classified as active (NHS, 2017). The Department of Health developed evidence based physical activity recommendations of 150 minutes aerobic exercise and two bouts of resistance training per week (Bull, 2010). In 2016, 66% of men and 58% of women met the aerobic exercise recommendation. Even though the importance of resistance training is widely known, only 26% of adults meet the Department of

health aerobic and resistance training recommendations combined (NHS, 2017). Pain is an important factor in exercise engagement, and can reduce the likelihood of an individual performing exercise on a regular basis (Vincent, Raiser, & Vincent, 2012). And further, DOMS reduces willingness to perform normal activities of daily living (Francis & Hoobler, 1987). The link between DOMS and increases in the rate of perceived exertion may indicate part of the reason behind reduced exercise engagement (Marcora & Bosio, 2007).

1.4 *DOMS, injury risk and immunosuppression*

In the days following exercise there are a number of acute negative physiological effects including increased injury risk and immunosuppression. The phenomenon of DOMS in itself, has been known to disrupt training schedules for athletes, and reduce their ability to compete (Cheung et al., 2003). Athletes are subject to intensive training programmes, exposure to high frequency and high intensity training increases the risk of injury (Stojmenovic, Malic, Vukasinovic-Vesic, Andjelkovic, & Dikic, 2017). Though athletes become accustomed to their exercise, DOMS is still present albeit at a lower intensity (Mero et al., 2010). Alterations in muscle recruitment patterns as a consequence of DOMS can cause stress to ligaments and tendons (Cheung et al., 2003). This compensatory behaviour is likely to be the cause of the increased risk of injury associated with the DOMS phenomenon (Cheung et al., 2003). Injury risk can be reduced by including recovery time for exercised muscles before the next bout of exercise, which also allows for recovery from DOMS pain. During the recovery period important adaptations occur within the exercised muscles. However, individuals react differently to exercise and recovery. Some individuals require longer to recover and are consequently at greater risk of injury. Understanding the underlying biological processes, including those of DOMS, allow for tailored exercise and

nutrition programmes which accelerate recovery while maximising adaptations to exercise.

While an increase in training load leads to increases in DOMS and increased injury risk, longer training duration leads to increased rates of illness (Gabbett, Whyte, Hartwig, Wescombe, & Naughton, 2014). Immunosuppression is not limited to long training schedules, depression of the immune system also occurs with acute increases in training load (Jones, Griffiths, & Mellalieu, 2016). Adequate recovery time is required to maximise adaptations, e.g. hypertrophy, and minimise the negative effects of DOMS, increased injury risk and immunosuppression. In addition, given that the immune system is sensitive to oxidative damage (Hughes, 2000), for example exercise-induced oxidative stress, antioxidant interventions during the exercise recovery period could be seen as advantageous. The last decade has seen a growing body of research into recovery modalities of training, especially in the athletic community. The recent interest in recovery strategies is based on evidence that inadequate recovery can lead to accumulated fatigue and underperformance in a chosen discipline (Tavares, Smith, & Driller, 2017).

1.5 *DOMS and the repeated bout effect*

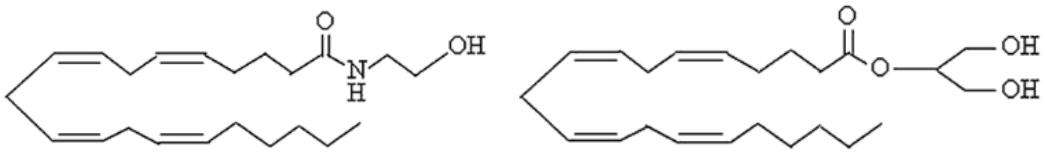
There is a blunting of the EIMD response after the initial bout of resistance training, this is known as the 'repeated bout effect'. The initial bout of resistance training infers a protective effect against EIMD on subsequent exercise bouts (McHugh, 2003). The protective effect is not limited to EIMD, the initial bout also reduces symptoms of DOMS from subsequent bouts of resistance training. Protection against DOMS has been shown to last a few days following two maximal contractions and more than a week after 30 low intensity contractions (H.-L. Chen, Nosaka, & Chen, 2012; T. C. Chen et al., 2013). And further, the protection

against DOMS can last several months if the same muscles are regularly exercised (T. C. Chen, Chen, Lin, Wu, & Nosaka, 2009). DOMS is most pronounced after eccentric unaccustomed maximal contractions, but is often apparent to a lesser extent within a demanding training schedule common among athletes (Mero et al., 2010). The action of lengthening while contracting in eccentric contractions causes significant EIMD due to physical damage to the muscle fibres (Fridén & Lieber, 2001; Newham et al., 1983; Yu et al., 2004). This type of contraction initiates a physiological response which manifest as inflammation, loss of muscle function, and is significantly associated with DOMS (Malm et al., 2004; Molina & Denadai, 2012).

1.6 *The Endocannabinoid System (ECS)*

Endocannabinoids are implicated in the control of several systems including mood, appetite and pain (Watkins, 2018). The pain relieving activity of endocannabinoids stems from work in animal models (Piomelli, Giuffrida, Calignano, & Rana, 1998). This pain relieving activity is present because endocannabinoids reduce nociception, which is the nervous system's response to pain (Marzo, Bifulco, & Petrocellis, 2004). Exercise is known to create an anti-nociception (blocking the detection of pain) response and the underlying mechanism has been suggested to be partly due to endocannabinoids (Galdino et al., 2014). Galdino et al. (2014) demonstrated in a rat model that anti-nociception following exercise was mediated by endocannabinoids. The endocannabinoid system (ECS) in humans consists of cannabinoid receptors and endogenous endocannabinoids. Of the known endocannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG) have been the most extensively studied (figure 1). At present two main receptors have been studied, CB1 and CB2, but there are known to be more. These receptors are expressed in the human uterus (Iuvone et al., 2008), gut (Di Sabatino et al., 2011), central nervous system (Gerard, Mollereau, Vassart, & Parmentier, 1991;

Liu et al., 2009), immune cells (Klein et al., 2003) and muscle (Cavuoto, McAinch, Hatzinikolas, Cameron-Smith, & Wittert, 2007) to name but a few. These receptors are present in some cancer cells and cannabinoids have been demonstrated to exhibit anti-cancer activity which gives this system relevance in cancer research. Interestingly, CB2 receptors have been found to be upregulated in disease states such as inflammation and cancer (Iuvone et al., 2008; Lakiotaki et al., 2015). Serum endocannabinoid levels have been found to be associated with disease progression. For example, AEA was found to be significantly lower in cancer patients compared to controls (Sailler et al., 2014). It is therefore not surprising that cannabinoid receptors have become a focus for pharmacological treatment research for inflammatory disease states and cancers (Pacher, BÃitkai, & Kunos, 2006).



Anandamide (AEA)

2-arachidonoylglycerol (2-AG)

Figure 1. Endogenous endocannabinoids

Physical activity *per se* has been shown to modulate the endocannabinoid system. More specifically, circulating levels of AEA have been shown to significantly increase to nearly double their baseline values in response to a bout of exercise and nearly triple following a bout of high intensity exercise (Raichlen, Foster, Gerdeman, Seillier, & Giuffrida, 2012; Sparling, Giuffrida, Piomelli, Rosskopf, & Dietrich, 2003). Interesting results using an *in vitro* animal model showed that 2-AG increased

excitation in the spinal cord leading to increased locomotive activity, suggesting that increases in 2-AG lead to increases in speed and force of contraction. Song, Kyriakatos, and El Manira (2012) also demonstrated that excitation was further increased by the presence of the Reactive Nitrogen Species (RNS) Nitric Oxide. Taken together, the importance of endocannabinoids in exercise biochemistry would seem to be increasingly relevant.

1.7 *The Aetiology of DOMS*

Based solely on observations following eccentric exercises of the finger without the use of blood or biopsy biomarkers, Hough (1900) believed the aetiology of delayed onset muscle soreness may be "due to lesions in the organ, either ruptures of the muscle fibres or of the connective tissue or of the nerves. It may even involve inflammation of the interstitial connective tissue." He based these conclusions on the muscles' reduced capacity for work and inability to reach a full voluntary contraction and summated that this phenomenon was distinctly different from fatigue. Since then the animal and human response to exercise has been extensively investigated and knowledge surrounding the physiological and biochemical aspects has expanded. More recently, competing theories of the underlying cellular mechanisms of DOMS have been broadly categorised into six groups: lactic acid formation, muscle spasm, electrolyte/enzyme efflux, microtrauma, connective tissue damage and inflammation (Lewis et al., 2012). However, there is substantial biochemical overlap between these theories and the aetiology of the DOMS response to exercise is complex.

1.7.1 Lactic Acid formation

There is a persistent misconception that lactic acid formation causes DOMS. First postulated by Asmussen (1956), the theory of muscular acidosis as the cause of DOMS pain has long been discounted (Hill, 1951). Though lactate is formed as a

by-product of energy metabolism, the timecourse and magnitude of this response significantly differs from that of DOMS. More specifically, blood lactate is cleared within an hour of exercise and therefore does not correlate with DOMS development over the next 48 hours. The energy cost of concentric contractions is considerably higher than the energy cost of the eccentric contractions that are associated with DOMS. Consequently, by-products of energy metabolism such as lactate are lower for eccentric contractions. In the light of energy cost and lactate clearance, lactate is highly unlikely to be a factor in the causation of DOMS. To fully disprove the hypothesis that lactic acid was implicated in the development of DOMS, Schwane, Watrous, Johnson, and Armstrong (1983) demonstrated that DOMS can develop without an increase in blood lactate.

1.7.2 Muscle Spasm

Decades after Hough's observations, De Vries (1966) hypothesised that 'The delayed, localised soreness which occurs following unaccustomed exercise is caused by tonic localised spasm of motor units, whose number varies with the severity of the pain'. Described as a vicious cycle of pain caused by a tonic contraction brought about by continuous spasms of muscle fibres. The rationale for this theory was based on the presence of exercise-induced ischemia and the knowledge that ischemia causes pain. De Vries also suggested that pain in the muscle is caused by substances passing across cell membranes and gaining access to nerve endings. Interestingly, this part of his theory ties in with more recent research into DOMS and nociceptor sensitisation (Ota et al., 2018). De Vries further reported that increased electrical activity represented increased contraction, and that periodic static stretching resulted in a reduction in electrical activity and a corresponding reduction in the symptoms of DOMS. These results led to the conclusion that spasm of muscle fibres had been reduced (measured as electrical activity) which led to the

alleviation of pain. However, there are other possible reasons that stretching could alleviate pain, for example the dispersion of oedema (Bobbert, Hollander, & Huijing, 1986). De Vries suggested that the positive correlation between the level of pain and level of electrical activity was evidence for a low level tonic contraction. However, pain was rated on a three-point scale only which is unlikely to be a very sensitive instrument of pain severity: 1 slight soreness only on palpation, 2 moderate soreness on movement or 3 limited the range of motion and interfered significantly with normal use. A number of studies have subsequently shown no decrease in DOMS with stretching before (Johansson, Lindström, Sundelin, & Lindström, 1999), after (Wessel & Wan, 1994) or before and after exercise (Lund, Vestergaard-Poulsen, Kanstrup, & Sejrsen, 1998) exercise. The theory of muscle spasm as a cause of DOMS and evidence that stretching eases the spasm does not seem to hold up to scrutiny in the light of more recent evidence.

1.7.3 Creatine Kinase efflux and Calcium influx

There is a large variability in the human CK response to exercise, including sex differences as a result of differing hormones (Oosthuyse & Bosch, 2017). The CK response also differs with intensity and type of exercise (Tiidus, Tiidus, Ianuzzo, & Ianuzzo, 1983), therefore this enzyme is problematic as a marker for muscle damage and as a measure of DOMS. The efflux of cytosolic CK as a consequence of increased membrane permeability is progressive and occurs in the days following eccentric exercise usually peaking in the blood on day four (a couple of days after DOMS). This delay indicates that the efflux of CK is not a response to the mechanical muscle damage, but a response to a secondary process following that damage (Tidball, 2011). In general though, eccentric exercise produces higher CK activity and more DOMS than other exercise types, which is most likely the reason CK has been linked with soreness intensity. When pre-trained versus naïve

participants were taken through an eight-week protocol, five-fold higher levels of CK were seen only in the group that presented with DOMS (Flann, Lastayo, McClain, Hazel, & Lindstedt, 2011). It is also worth noting that CK and DOMS respond similarly to repeated bouts of exercise, namely the repeated bout effect (RBE). In the RBE, attenuation of increases in both CK and DOMS are seen on subsequent bouts of the same exercise (T. C. Chen, Lin, Chen, Yu, & Nosaka, 2017). Though these two markers of EIMD share similarities in magnitude and timecourse, the consensus is that DOMS peaks a day or so before CK peaks are seen in the blood (Cleak & Eston, 1992). And further, exercise-induced elevations in CK have been reported with and without DOMS pain (Hagberg et al., 1982; Maxwell & Bloor, 1981; Shumate et al., 1979). One proposed explanation of the expulsion of CK from exercised muscle is that this represents a protective mechanism which limits the depletion of adenosine tri-phosphate (ATP) (Baird, Graham, Baker, & Bickerstaff, 2012). Behringer et al. (2014) suggest that the expulsion of CK allows for relaxation and regeneration within exercised muscle. They proposed that the CK efflux is a survival mechanism for muscle fibres when subjected to very high energy demands. And further, that this process prevents cell death during exhaustive exercise. This theory therefore proposes that if CK has a relationship with DOMS, the relationship is an indirect one.

Though CK efflux is unlikely to be directly implicated in the aetiology of DOMS (Schwane, Johnson, Vandenakker, & Armstrong, 1981), the underlying mechanism by which CK is expelled from cells is of interest. Exercise-induced muscle damage (EIMD) causes disruption of muscle cell membranes thus increasing cell membrane permeability. This increase in permeability allows for the abnormal transit of large proteins and ions through the cell wall. Enzymes, such as CK, are proteins that

usually have a limited capacity to cross the cell membrane. However, as cell permeability increases due to EIMD, CK crosses the cell membrane in a seemingly unregulated efflux (Tidball, 2011). During this period of membrane disruption, some ions also cross the cell wall as they follow their concentration gradient. For example, as a consequence of reduced membrane integrity there is a calcium ion influx into muscle cells (Tidball, 2011). Interestingly, calcium concentrations have been directly implicated in the expulsion of CK from exercised muscle. Duncan and Jackson (1987) showed *in vitro* that the efflux of CK is *dependent* on the exercise-induced rise in intracellular calcium. And further, that the CK efflux was almost completely eliminated by the removal of extracellular calcium i.e. influx of calcium into the cell could no longer occur (Duncan & Jackson, 1987). Though intramuscular calcium concentration are similar in response to both eccentric and concentric contraction, downstream calcium dependent pathway initiation differs between these types of contractions even when intramuscular calcium levels are the same (Vissing, Overgaard, Nedergaard, Fredsted, & Schjerling, 2008). It therefore follows that the initiation of DOMS induced by eccentric contractions is not due to calcium influx *per se*. This however, does not preclude the possibility that DOMS is initiated by a calcium induced downstream pathway.

Type II muscle fibres are more susceptible to membrane damage (t-tubules) when subjected to eccentric contractions, which causes the subsequent effect on calcium homeostasis (Takekura, Fujinami, Nishizawa, Ogasawara, & Kasuga, 2001). Vissing et al. (2008) showed that the hypertrophic effect of exercise-induced calcium homeostasis disruption on downstream proteolysis was absent in concentric contractions and only occurs with the eccentric contractions which produced DOMS. However, though muscle soreness was measured at 48 hours, calcium and

proteolytic calpain were not, missing an opportunity to further understand these relationships. Previously though, it had been shown that simply reducing calcium with a calcium antagonist does not reduce muscle pain (Lane et al., 1986). Taken together the evidence suggests calcium efflux is unlikely to be the cause of DOMS.

1.7.4 Inflammatory response

Exercise-induced muscle damage presents as microtrauma to muscle fibres, specifically myofibrils show z-band streaming, broadening and sometimes total disruption (Fridén et al., 1983). The damage is followed by the inflammatory response and a subsequent shift in fluids and electrolytes. The extra fluid volume manifests as oedema and the exercised limb becomes swollen (Felipe Damas et al., 2016). Earlier research showed that DOMS and swelling shared the same timecourse but no causal link was found(Howell, Chleboun, & Conatser, 1993). The conclusion was that other chemical mediators were present following exercise which altered the threshold for pain, together with the additional pressure was then interpreted as pain.

To investigate increased intramuscular fluid pressure and pain perception, Wolff, Potter, Vermeer, and McEwen (1961) injected saline into the human gastrocnemius. Injecting at higher volumes and at faster speeds caused the most pain leading to the conclusion that volume and pressure were both important in the perception of pain. They further concluded that fluid pressure altered the pain threshold and that the pain threshold differed by age and gender (Wolff & Jarvik, 1965). Given the link between increased fluid pressure and increased pain sensitivity it seemed plausible that inflammatory pressure and DOMS were related. However, DOMS pain is persistent and in the above gastrocnemius injections, the pain was transient, i.e. the pain occurring from the saline injections was relieved after a few

minutes as the fluid dispersed. It seems possible though, that in the case of exercise-induced oedema, the fluid is kept in place by concentration gradients and therefore does not disperse in the same way. And further, that the reduction in DOMS pain on exercising (R. Armstrong, 1984) or stretching (De Vries, 1966) the affected muscle is a temporary dispersal of fluids as they are forced out of the muscle. More recent investigations into fluid pressure and DOMS injected saline directly into DOMS affected muscles (Gibson, Arendt-Nielsen, Taguchi, Mizumura, & Graven-Nielsen, 2009). Pain increased only when saline was injected just inside the muscle fascia versus deep intramuscular injection. This may indicate that pressure increases just beneath the fascia of the muscle is implicated in the aetiology of DOMS, not simply intramuscular pressure (Gibson et al., 2009).

Intramuscular pressure (IMP) during exercise has been shown to be significantly higher for eccentric contractions as opposed to concentric contractions (Friden, Sfakianos, & Hargens, 1986). Furthermore, when the resting IMP was measured two days after the contractions that was still the case. As DOMS was only experienced in the eccentrically exercised limb, it was concluded that IMP was a factor in the aetiology of DOMS (Friden et al., 1986). In contrast to Friden's findings, more recent research has shown that intramuscular pressure (IMP) is not an indicator of DOMS (Crenshaw et al., 1995). Crenshaw *et al* demonstrated the same peak IMP for concentric and eccentric leg exercises while DOMS was only reported for the eccentric group. This led to the assumption that IMP was not an indicator of DOMS. However, IMP was only measured *during* the exercise protocol and not while the participants were experiencing DOMS two days later, which created ambiguous findings. Friden's findings that IMP and DOMS occurred together on day two only for eccentric contractions does not show a causal link.

However, these findings may indicate that the underlying processes associated with oedemic pressure are relevant in the aetiology of DOMS.

Limb volume has been used as an indicator or inflammatory pressure and compared with the timecourse of DOMS. Both DOMS and persistent oedema are associated with eccentric muscular actions. Though limb volume increases are seen for both eccentric and concentric actions immediately after exercise, the volume remains elevated only following eccentric contractions (Talag, 1973). This may explain Crenshaw's IMP findings above. Talag demonstrated that limb volume did not correlate with DOMS pain, in that DOMS pain peaked at 48 hours and limb volume was still increasing at 72 hours (Talag, 1973). Later research confirmed the disassociation of these events (Bobbert et al., 1986).

It has been proposed that this increase in limb volume is two-fold in that there is an increase in fluid volume followed later by an increase in structural components e.g. connective tissue during the regeneration process. The initial increase in fluid volume not being associated with DOMS (Smith, 1991). This seems plausible in the light of Talag's findings that initial oedema present immediately after exercise is present in limbs that do not go on to develop DOMS. There are inherent issues with limb volume as a measure of intramuscular pressure in that there is no differentiation between intra- and extra-cellular volumes. In addition, extra-cellular volume increases may be intravascular due to increased blood flow.

Increased intramuscular pressure present in oedema is a likely contributor to the reduced pressure pain threshold present in DOMS. Palpation and active movement of muscles with DOMS is painful, this may be in part due to pressure increases

activating mechanoreceptor nerve endings (Lewis et al., 2012). The aetiology of DOMS is complex and is not due to one single factor such as intramuscular pressure. It is likely that DOMS is the result of oedemic pressure increases in conjunction with pain sensitisation brought on by another element of the inflammatory response to EIMD (Behringer et al., 2014; Smith, 1991).

1.7.5 Microtrauma and Muscle Regeneration

The link between muscle damage and the pain associated with DOMS is complex. Extensive intramuscular microtrauma caused by eccentric exercise was demonstrated in humans by Fridén et al. (1983). The height of damage to myofibrils on day three corresponded with the development of DOMS. Though the underlying biological mechanism was not yet apparent, their work gave credence to the theory that intramuscular microtrauma is important in the aetiology of DOMS. Since then, individual aspects of this relationship have been investigated using blood biomarkers and muscle biopsy results in both human and animal models. In an animal model Takekura et al. (2001) demonstrated microtrauma to type two fibre membranes caused by eccentric exercise, more specifically, the physical and chemical disruption of membrane systems. In humans, muscle damage following a bout of eccentric exercise caused increases in satellite cell (SC) proliferation as part of the regeneration of myofibres (Chargé & Rudnicki, 2004). Interestingly, whey protein supplementation resulted in significant increases in SC proliferation in exercised muscles and also resulted in significantly higher DOMS scores compared to placebo. These results may indicate that SC proliferation in the muscle regeneration process is involved in the pain associated with DOMS. The aforementioned SC proliferation increases were recorded in Type II muscle fibres, where the number of SC's doubled. SC proliferation peaked at 48 hours post exercise which has been shown to follow the same timecourse as DOMS pain (Farup

et al., 2014). It was later shown that part of the regeneration process, namely, increases in 'glial cell line derived neurotrophic factor' and 'nerve growth factor' produced by muscle fibres/SC's were crucial in the development of DOMS (Mizumura & Taguchi, 2016).

1.7.6 The Immune Response and Pain Sensitisation

The inflammatory response to exercise begins in earnest within minutes of exercise-induced muscle damage with a cascade of molecules released in the acute phase response. Elements of this response last for several days while the repair and regeneration process of the exercised muscles is underway. It is known that the immune response to exercise results in significant increases in circulating leukocytes, lymphocytes and monocytes in the blood (Malm et al., 2000). The release of pro-inflammatory agents by immune cells has been linked to increased pain sensitivity (Fatouros & Jamurtas, 2016). Attempts to elucidate the immune response within the exercised muscle has become a focus in recent years. Although individual immune system biomarkers following a bout of exercise have been extensively investigated, several aspects of this response and the relationship to DOMS remain unclear.

DOMS pain involves activation of pain afferents within skeletal muscle, that is sensations of pain sent via nociceptor sensory neurons. The pain sensations are initiated when potentially harmful stimuli, either mechanical, thermal or chemical act on nociceptors sending pain signals to the brain. There is evidence that elements of the immune response are responsible for a change in the nociception threshold, causing a state of hyperalgesia (Ota et al., 2018). It follows therefore that elements of the immune response are implicated in the aetiology of DOMS. A prevailing theory of DOMS is that damage to sarcomeres leads to cell death and

elements of the subsequent immune response increase nociceptor pain sensitivity due to the presence of chemical mediators (Clarkson & Hubal, 2002; Hyldahl & Hubal, 2014). Specific noxious substances have been implicated, for example, E series prostaglandins and Nerve Growth Factor (NGF) (Murase et al., 2010; Ota et al., 2018).

NGF is upregulated in the muscle following eccentric contractions and has a comparable time course to DOMS, this indicates a possible involvement in DOMS aetiology (Hyldahl & Hubal, 2014). In an animal model upregulation of NGF was completely suppressed by blocking the receptor before exercise. An intramuscular injection of B2 Bradykinin receptor antagonist led to the absence of NGF from this pathway which in turn resulted in the absence of DOMS following eccentric contractions. And further, antibodies to NGF injected intramuscularly two days after the exercise at the height of DOMS pain, completely attenuated DOMS. These results suggest that NGF upregulation through the B2 Bradykinin pathway is essential in the aetiology of DOMS. Interestingly, intramuscular injections of bradykinin alone, i.e. without eccentric contractions, did not produce DOMS, indicating that this biochemical pathway does not work alone to induce pain (Murase et al., 2010). A recent review of neuropathic factors in the aetiology of DOMS concluded that NGF plays a crucial role in DOMS pain (Mizumura & Taguchi, 2016).

Another suggestion is the increases in macrophage activity is related to DOMS as it shares the same 24/48 hour timecourse. Given that macrophages produce prostaglandins, which are implicated in nociceptor sensitisation, there may be credence in this theory (Smith, 1991). A significant correlation has been shown

between prostaglandin E2 and DOMS pain, adding further weight to the theory that the immune response and DOMS are tightly linked (Uchida et al., 2009).

An investigation into leukocyte infiltration into exercised muscle and the symptoms of DOMS showed no significant relationship (Malm et al., 2000). However, Malm et al. (2004) in later research concluded that there was a significant difference in the immune response of participants that had a DOMS score above 3 versus those categorised without DOMS (scores below 3). His later results indicated that one element of the immune response was an essential factor in DOMS pain following a bout of exercise. Specifically, a significant correlation between granulocytes in the epimysium and DOMS pain. However, this was the only immune measure that correlated with DOMS leading to the final conclusion that there was no difference in the immune response between uphill (not eccentric) and downhill (eccentric) exercise, despite only the downhill group experienced DOMS. This may indicate that DOMS is related to the immune response of the individual person and not the type of exercise performed or resultant EIMD. It also has to be said that the inflammatory response to exercise is not the same across the board. In some individuals this response occurs with concentric exercise and does not always occur in response to eccentric exercise.

Much evidence exists both for and against the involvement of the inflammatory response in the aetiology of DOMS. One possible reason for lack of evidence in the causation of DOMS is the difficulty in evaluating the inflammatory response in live human muscle tissue. There are inherent issues in identifying immune cells from a small muscle biopsy sample. Armstrong suggests that inflammation may be localised in muscle tissue and 'missed' when muscle biopsies are taken for analysis

(R. B. Armstrong, 1986). On this point, participants report DOMS pain being more pronounced in different areas of the muscle, distally, proximally or in the middle, and that this changes over time (Bobbert et al., 1986).

In conclusion, DOMS is likely the result of the immune response causing intracellular and extracellular matrix disruptions, however, the exact mechanisms of this reaction remains unclear (Stauber, Clarkson, Fritz, & Evans, 1990).

1.7.7 Reactive Oxygen & Nitrogen Species

Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) are naturally produced in muscle and increases on exercise (figure 2). Collectively known as Reactive Oxygen & Nitrogen Species (RONS), these free radicals are known to be both metabolically essential and physically damaging molecules. Increased oxygen metabolism as a result of exercise increases free radicals and associated damage (Davies, Quintanilha, Brooks, & Packer, 1982). For example, during exercise, leukocyte production increases to release proteolytic enzymes and synthesising cytokines such as interleukin-6 (IL-6). During this process, reactive oxygen species are created (Fatouros & Jamurtas, 2016). ROS reducing enzymes may be unable to keep pace with free radical formation as oxygen usage increases, this leaves the cells vulnerable to oxidative damage (Higuchi, Cartier, Chen, & Holloszy, 1985). Antioxidant nutrient deficiency exacerbates exercise-induced muscle damage because of the body's inability to effectively scavenge the increased ROS (Ji, 1995). Interestingly, this 'under capacity' to scavenge ROS and avoid ROS induced damage may be a contributory factor in individual variation in muscle damage biomarkers.

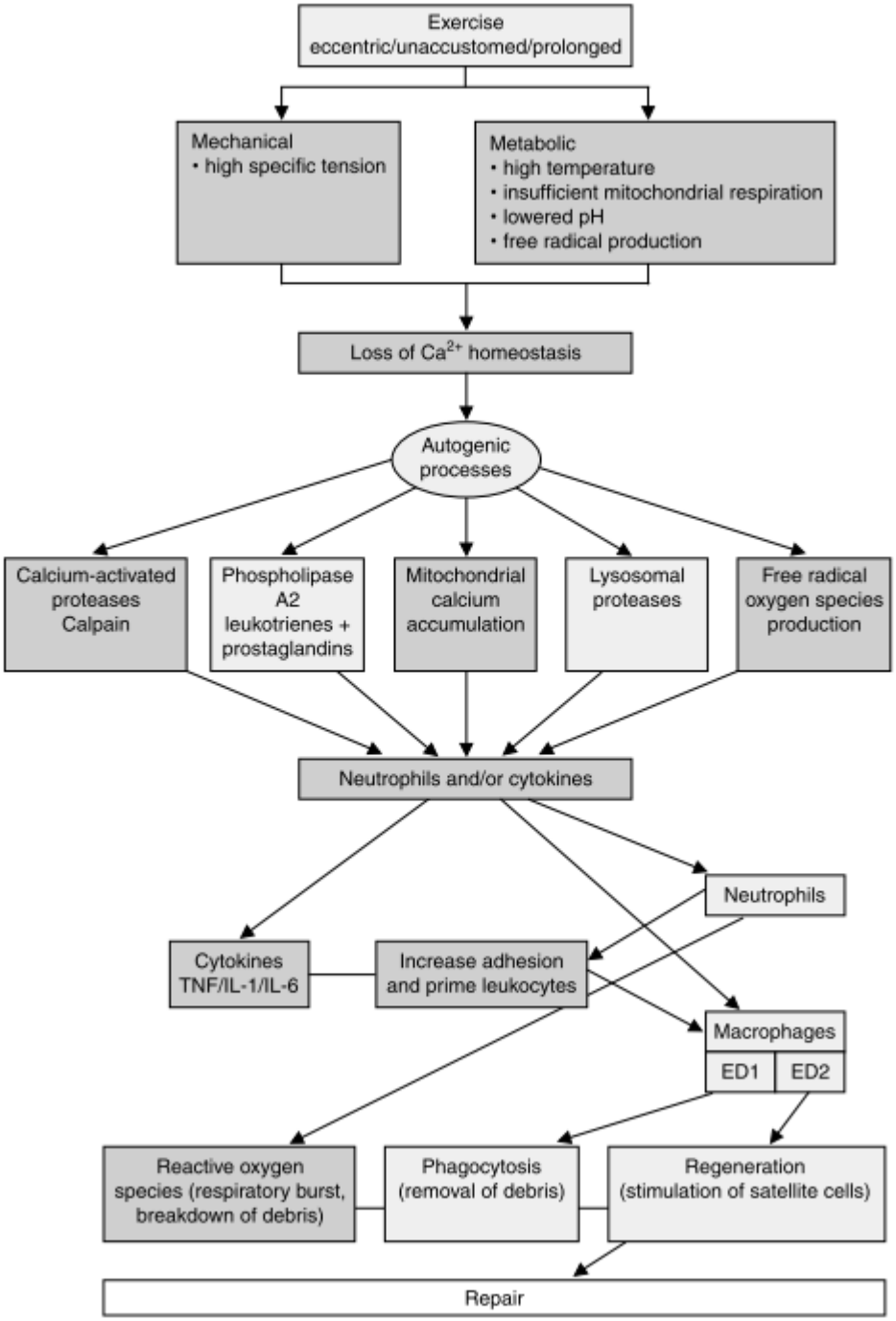


Figure 2. Overview of the regeneration and repair process in exercised muscles (Kendall & Eston, 2002)

While low levels of ROS are essential for normal cell signalling, high levels of ROS promote contractile dysfunction, more specifically, fatigue and force loss (Powers & Jackson, 2008). Increased ROS can also lead to pain, e.g. in an animal model, intramuscular injection of the reactive oxygen species (H2O2) increased nociceptor

activity (p=0.0004) and consequently pain behaviours in rats (Sugiyama, Kang, & Brennan, 2017). The RNS nitric oxide is a signalling molecule that increases in the muscle during exercise. In a review of human studies, it has been proposed that nitric oxide plays a role in the pain associated with DOMS (Radak, Naito, Taylor, & Goto, 2012). Eccentric exercise that produces soreness ratings of 7 out of 10 is accompanied by a threefold increase in Nitric Oxide and a significant increase in DNA oxidation in skeletal muscle (Radák, Pucsok, Mecseki, Csont, & Ferdinandy, 1999).

Downhill running is an exercise that increases ROS production (G. L. Close et al., 2005). To evaluate the relationship between ROS and DOMS, the response between downhill running versus flat running were compared, i.e. eccentrically biased downhill running is known to produce soreness. As expected, significant increases in both ROS and DOMS occurred following the eccentric exercise protocol and a temporal relationship between ROS production and DOMS was demonstrated. However, there was a timecourse disassociation; ROS was still increasing at 72hrs and DOMS peaked at 48hrs. These results lead to the conclusion that; though increases in soreness and ROS resulted only from eccentric exercise, there was a disassociation in the temporal relationship between these two responses (Close, Ashton, Cable, Doran, & MacLaren, 2004). Despite the dissociation, it is possible to reduce DOMS with substances that target ROS (Udani, Singh, Singh, & Sandoval, 2009).

1.8 *Attenuation of DOMS*

Proposed strategies to reduce the symptoms of DOMS have met with limited success. Interventions that did not reduce the symptoms of DOMS include: cold water immersion (Glasgow, Ferris, & Bleakley, 2014), massage before exercise

(Garrido, Oliveira, Mendes, Sousa, & Sousa, 2013), topical arnica (Pumpa et al., 2014), ginger supplementation (Matsumura, Zavorsky, & Smoliga, 2015; Tanabe et al., 2015), curcumin supplementation (Tanabe et al., 2015), and Omega 3 supplementation (Jakeman, Lambrick, Wooley, Babraj, & Faulkner, 2017). Interventions where reductions in pain scores reached significance include; contrast water therapy (Bieuzen, Bleakley, & Costello, 2013), neuromuscular electrical stimulation (Taylor et al., 2015), exercised limb occlusion (Page, Swan, & Patterson, 2016), NSAIDs (Morelli, Brown, & Warren, 2018) and Montmorency cherry supplementation (Bell, Stevenson, Davison, & Howatson, 2016; Connolly, McHugh, & Padilla-Zakour, 2006). Post-exercise massage reduces the symptoms of DOMS but the effect is minimal, and further, under which conditions the massage is effective is still unclear (Poppendieck et al., 2016).

1.8.1 Supplementation strategies

Interventions using fruit extracts such as Montmorency cherry supplementation have been showing promising results. However, the effect of antioxidant interventions may be limb specific, e.g. chronic supplementation of pomegranate juice has been shown to significantly reduce DOMS for knee extensors but not elbow flexors in the same participants (Ammar et al., 2016). Interestingly, DOMS scores were more than double for knee extensors compared to elbow flexors, which may have contributed to the non-significant result (Ammar et al., 2016). It is clear that there is merit in the theory that antioxidant interventions reduce oxidative stress and have a reducing effect on DOMS soreness. The exact mode of action, limb specificity and which particular antioxidant interventions are most likely to have an effect require further investigation.

Oxidative stress, from increased reactive oxygen species (ROS), following a bout of intense exercise can be moderated by dietary phytochemical supplementation (McLeay et al., 2012). In addition, DOMS has been reduced by targeting ROS with polyphenols (Ammar et al., 2016). The reduction of exercise-induced oxidative stress with polyphenols has received focussed attention in recent years. Polyphenols, an important group of phytochemicals, are found naturally in plants and known for their antioxidant capacity. Berry fruits contain anthocyanins and proanthocyanidins, two important classes of polyphenols which confer their reputed antioxidant capacity. Antioxidant rich food and drink may moderate the depression of the immune system common during a demanding training schedule without interfering with training adaptations (Nieman & Mitmesser, 2017). Known for their antioxidant qualities, *Aronia melanocarpa* berries have high concentration of anthocyanins which reduce oxidative stress. For example, Aronia in juice form has been shown to reduce oxidative stress in triathletes (García-Flores, Medina, Cejuela-Anta, et al., 2016; García-Flores, Medina, Oger, et al., 2016), and positively modulate the immune system (in vitro) (Ho et al., 2014). And further, there is a synergistic effect of combining exercise with the ingestion of Aronia juice on the improvement of oxidative stress (Medina et al., 2012). The anthocyanins reduce the exercise-induced oxidative stress while the exercise increases bioavailability of the anthocyanins in the Aronia juice (García-Flores, Medina, Oger, et al., 2016; Villaño et al., 2016).

Though anthocyanins mop up RNOS *per se*, this has a further effect on inflammatory markers such as interleukin-6 (IL-6). The immune system is complex and responds to a bout of exercise with an acute phase response characterised by a cascade of biological molecules to modulate the repair and regeneration process.

During the recovery period elevated levels of the inflammatory cytokine interleukin-6 (IL-6) is apparent. This marker usually returns to baseline within a few days (Van De Vyver & Myburgh, 2012). Following a single bout of resistance training the IL-6 cytokine peaked between 4 and 8 hours (Louis, Raue, Yang, Jemiolo, & Trappe, 2007). Dietary interventions can attenuate the IL-6 inflammatory response, for example, anthocyanin rich cherry juice has been shown to reduce the IL-6 response compared to a placebo. Interestingly, the cherry juice intervention also attenuated DOMS, pain scores returned to baseline levels by 72hrs for the cherry juice group whilst pain scores for the placebo group did not (Bell et al., 2016; Clifford, Bell, West, Howatson, & Stevenson, 2016). The reduction of pain and attenuation of elements of the immune response are both considerations for exercise recovery.

1.9 *Conclusions*

It is important to rest and recover following a bout of exercise and get to the next bout as quickly and optimally as possible, including recovery from the pain of DOMS. The pain of DOMS interferes with activities of daily living and a person's willingness to participate in exercise and rehabilitation. It is important to discover approaches that alleviate the soreness and restore a person's willingness to perform exercise and activities of daily living without compromising the intensity of these activities (Gulick & Kimura, 1996) . However, techniques designed to speed up recovery time do not always produce the desired long term effects and are sometimes poorly understood. Specifically, techniques that minimise recovery time by moderating the underlying biological processes involved in EIMD and DOMS may be detrimental to the positive recovery process, the hypertrophic effects of exercise for example may be interfered with (Schoenfeld, 2012). It is vital to discover approaches that reduce DOMS without losing the beneficial adaptive

responses of the preceding exercise session. An increased understanding of the complex relationship between the biochemical processes involved in EIMD, inflammation, oxidative stress and DOMS, will assist athletes and recreational gym users to generate positive physiological adaptations whilst alleviating the negative effects of exercise.

Interventions to reduce this uncomfortable physiological response to exercise whilst maintaining the body's ability to maintain/increase muscle mass are receiving much attention. However, underlying mechanisms and attenuation strategies for DOMS pain remains unclear which impacts on the efficacy of these treatment modalities. Understanding the degeneration/regeneration cycles are crucial to elucidate of the aetiology of the DOMS phenomenon (Hyldahl & Hubal, 2014). As knowledge in this area develops, approaches to facilitate healthy muscle repair while alleviating the symptoms of DOMS will become clearer. It will then be possible to personalise exercise and rehabilitation programmes to suit the individual with consideration to the increases in injury risk and metabolic load. Discovering how a person will physiologically respond to an exercise regime can be used to tailor programmes that maximise recovery, maximise positive adaptations and minimise injury risk.

The application of DOMS biochemistry research to the 'real world' is a challenge as the human physiological response to exercise is complex. In addition, evaluating exercise biochemistry research is complicated by varying methodologies, making inferences and generalisations problematic. There is a wealth of research using these markers following muscle damaging exercise, however, differences in methodology make direct comparison a challenge (Kerksick, Willoughby, Kouretas, & Tsatsakis, 2013). There is also a large variability of response to muscle damaging exercise. On an individual level, muscle damaging exercise causes

varying degrees of change in each biological element, DOMS, CK and IL-6 for example. Furthermore, the response varies depending on a number of factors including: age, sex, training status, body composition etc. There is now a growing body of evidence suggesting that genetics also play a role in the human response to exercise (Baumert, Lake, Stewart, Drust, & Erskine, 2016). On a cellular level, the biochemistry involves an interplay between several systems with a vast network of signalling activity. The 'crosstalk' between these systems is complicated which makes intramuscular changes a challenge to accurately measure in vivo, particularly in humans. Secondary biomarkers such as Creatine Kinase and Interleukin-6 have been used as proxy markers for muscle damage and the associated biochemistry.

From a research perspective, it would be useful to establish an evidence based testing protocol which can be used to test new DOMS relieving approaches. There have been many antioxidant interventions tested, including polyphenol interventions. However, the effects of polyphenol rich *Aronia melanocarpa* on the symptoms of DOMS have yet to be examined.

Chapter 2 Phase one: DOMS Induction Protocol Development

2.1 *Introduction*

A valid protocol to consistently induce muscle damage and initiate measurable changes in associated biomarkers is required to elucidate the physiological and biochemical aspects of delayed-onset muscle soreness (DOMS). For example, changes in soreness following exercise-induced muscle damage (EIMD) can be compared to clarify the effects of nutritional and physical interventions. EIMD is associated with changes in limb circumference, DOMS, range of motion (ROM), creatine kinase (CK) and interleukin-6 (IL-6) concentrations to name but a few.

2.1.1 DOMS induction

There is a significant time effect on EIMD biomarkers following a bout of exercise. In response to muscle damaging exercise, the pain associated with DOMS, CK concentrations and limb circumference increase as the ROM decreases. The magnitude and timecourse of these changes however are not uniform and subject to variability in the human response and differences in experimental protocols. Measurable changes in these biomarkers, large enough to record significant differences is required to test nutritional and exercise interventions. Isokinetic dynamometry is one of the safest and most effective ways to perform exercise testing. Strength testing results using the Cybex Norm isokinetic dynamometer have been shown to be highly reliable (Impellizzeri, Bizzini, Rampinini, Cereda, & Maffiuletti, 2008). Once the angles and velocity of the dynamometer are correctly pre-set to isolate a single muscle group, joint overload, muscle overload, and risk of injury are reduced. A targeted amount of exercise-induced muscle damage (EIMD) can be produced by performing eccentric contractions within an isolated muscle group. Maximally resisting the rotating action of the dynamometer loads

the isolated muscle group to maximum capacity over the full range of motion (Payton, Burden, British Association of, & Exercise, 2018). The mean range of motion (ROM) of the elbow joint in a large sample of adult men 20-44 years was reported as 144.6º (Soucie et al., 2011). This 'normative value' provides a guide for dynamometry settings which are then set at a slightly reduced ROM to ensure safety, usually 120º. For research purposes, elbow flexion exercises begin at 10º from full extension through to 130º (towards full flexion but not fully flexed).

There appears to be a difference in magnitude of exercise-induced muscle damage (EIMD) between the upper and lower extremities. A comparison between the response to eccentric exercise performed by legs versus arms shows the percentage reduction in strength and the CK increase was significantly greater for arms. This response was most notably 72 and 96 hours post exercise. These results suggest that muscle damage is greater and recovery slower in arms versus legs, this may in part be due to normal use of the legs in daily living (Jamurtas et al., 2005). The magnitude of the increased muscle damage in the arms compared to the legs has been suggested to be 5-fold, based on the differences in plasma CK levels (Morelli et al., 2018).

To evaluate differences in response to eccentric exercise by arm dominance, eighteen men performed 60 maximal eccentric contractions of elbow flexors four weeks apart using the contralateral arm. There was found to be no significant differences between the dominant and non-dominant arm for CK, upper arm circumference and muscle soreness. That being said, a weak correlation was recorded for the magnitude of these changes between collateral arms (Newton, Sacco, Chapman, & Nosaka, 2013). However, even though dominant/non

dominant arm bout order was randomly assigned and the bouts were separated by four weeks, a repeated bout effect was also reported which could have confounded the results. Despite the unlikeliness of a different response between the dominant and non-dominant arm, it seems prudent to randomise or stratify by arm dominance. And further, when EIMD and DOMS is induced in only one arm the non-dominant arm is used as the soreness interferes less with daily tasks.

Quantification of pain is difficult as pain perception is subjective and individual (Ohnhaus & Adler, 1975). The pressure pain threshold or a visual analogue scale (VAS) are in common use for the evaluation of pain in DOMS research. Of the two, the 10cm VAS is less invasive and easier to compare across studies. There are however limitations of using a closed-ended VAS scale, in that a person may mark 10 for severe pain on day one and have nowhere to mark the scale if the pain is worse the following day. And further, one person may mark a 5 when their pain is severe and another may mark a 5 when their pain is moderate. To alleviate that issue VAS pain scales have descriptive terms for pain alongside the numbered scale; 0 - no pain, 6 – severe pain and 10 – worst pain imaginable. Notwithstanding some limitations, the 10cm VAS has been successfully and consistently used to quantify pain in DOMS research (Ammar et al., 2016; Matsuda et al., 2015; Pumpa et al., 2014).

2.1.1.1 Eccentric contractions

Since Hough (1900) identified the distinct phenomenon of DOMS which occurs following voluntary eccentric contractions of untrained muscles, DOMS association with exercise bouts containing a high element of eccentric contractions has been well documented (Asmussen, 1956; Molina & Denadai, 2012). The use of dynamometry in exercise research ensures that when investigating DOMS,

contractions are eccentric in nature and the concentric action is eliminated with a passive reset of the dynamometer arm. Combined with instructions to maximally resist the dynamometer arm during eccentric action, muscle damage is initiated. Contractions that are maximal in nature produce the most marked physiological and biochemical response, i.e. the process of eccentrically lengthening while maximally contracting, and the high tensile forces produced, result in a greater level of damage to the muscle fibres on a microscopic level (Yu et al., 2004). The ensuing exercise-induced muscle damage (EIMD) is responsible for a cascade of biochemical responses resulting in measurable increases in DOMS, enzyme activity and inflammation markers, for example, increased concentrations of CK (Tiidus et al., 1983) and IL-6 (Arakawa et al., 2016). The most pronounced pain from eccentric contractions is when those contractions are performed after an 'exercise free' period and are not influenced by the repeated bout effect. In the RBE, exercise-induced changes in biomarkers, such as CK and IL-6, are less pronounced. Therefore, it is normal procedure in exercise research to restrict exercise for a few days before testing takes place in an attempt to partially eliminate the RBE.

2.1.1.2 Speed, set, repetition and rest configuration.

Exercise to induce the greatest changes in biomarkers usually includes a number of maximal eccentric contraction for the reasons outlined above. It is also the case that short inter-set rest intervals of 60s or less may be useful to increase measureable metabolic stress (Grgic, Lazinica, Mikulic, Krieger, & Schoenfeld, 2017). Probably the most important consideration after the type of contraction, is the number of contractions, as the number of contractions heavily influences the physiological and biochemical response. On this point, Nosaka et al. (2002) recorded peak pain scores 48 hours after exercise, and found a significantly less pain after 12 repetitions (2.5cm) compared to 60 (3.7cm) repetitions of elbow

flexion. In DOMS research, several sets of maximal eccentric elbow flexion exercises produce a variety of pain scores, usually ranging between 3cm and 6cm on a 10cm VAS. These scores are likely to be more influenced by the number of repetitions and less influenced by the set, repetition configuration. For example, muscle soreness following 30 repetitions of maximal eccentric elbow flexion exercises did not differ whether the configuration was 10 x 3 repetitions or 3 x 10 repetitions. There were no significant differences in strength, cross sectional area (biceps brachii) or DOMS (Chan, Newton, & Nosaka, 2012). On performing 60 maximal eccentric contractions of the elbow flexors significant changes in biomarkers have been recorded as follows; reduced muscle strength (Panza et al., 2016); and; reduced ROM and increased DOMS UAC and CK (Newton et al., 2013; Nosaka et al., 2002).

In exercise research, the exercise protocol is complex and dependent on factors that may not be the same from one research setting to another. For example, how well the researcher motivates the participant to maximally resist the dynamometer arm and the length of time the contraction is held, both of which are determinants of the level of muscle damage produced. The increase in delayed soreness associated with slower, longer held contractions was first noted by Hough (1900). With the use of modern technology in the form of isometric dynamometry the speed and length of contractions can be quantified and biochemical responses compared. When 36 eccentric contractions were performed at an angular velocity of either 30 degrees/second or 180 degrees per second the physiological response was greater at the slower speed(Paddon-Jones, Keech, Lonergan, & Abernethy, 2005). The inflammatory response (measured as upper arm circumference) for example, was significantly greater at 24 hours in the slower 30 degrees/second

group. And further, measurements of muscle soreness peaked faster (24hrs) and returned to baseline quicker with slow contractions at 30 degrees/second (Paddon-Jones et al., 2005). More recent research reported that dynamometer speeds of 180 degrees/second induces less electrical activity in the muscle compared to 60 degrees/second (Greig & Marchant, 2014). The lower electrical activity was evident in the resulting peak torque measurements which was significantly higher for 60 degrees/second compared to 180 degrees per second (Greig & Marchant, 2014). Greater torque may begin to explain the more marked increases in biomarkers at slower speeds. Maximal eccentric elbow flexion exercises at an angular velocity of 60°/sec provides a slow and more damaging contraction of the biceps brachii (Greig & Marchant, 2014; Hody, Rogister, Leprince, Wang, & Croisier, 2013).

2.1.2 Indirect muscle damage markers

Following a bout of exercise two types of pain may develop, pain immediately after the exercise and the delayed pain of DOMS. The delayed pain of DOMS is generally only present in resting muscles as a dull ache but is intensified by stretching or palpating the muscle. A common method to assess the level of pain is pain on movement of the exercised muscles recorded on a visual analogue scale (VAS), a 10cm (100mm) scale from 'no pain' at one end to 'worst possible pain' at the opposing end. Although pain is subjective, asking participants to indicate their level of pain by marking on a 10cm line in this way has been shown to be a reliable measure (Bahreini, Jalili, & Moradi-Lakeh, 2015; Mattacola, Perrin, Gansneder, Allen, & Mickey, 1997). In DOMS research, VAS scores are used to compare pain between experimental groups to assess the response to an intervention (Glasgow et al., 2014; Matsumura et al., 2015; Romain et al., 2017). Using the VAS method of assessing pain in the biceps brachii, Tanabe et al. (2015) recorded a peak in DOMS pain at 48 hours post exercise. Jubeau, Muthalib, Millet, Maffiuletti, and Nosaka

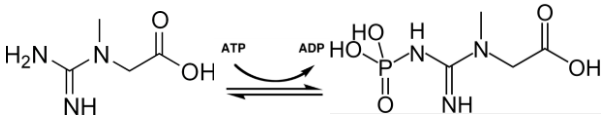
(2012) and Matsuda et al. (2015) concur, recording pain scores that peaked 48 hours after the exercise protocol.

2.1.2.1 Creatine Kinase

Following a bout of strenuous prolonged exercise, the rise in CK is likely the result of muscle damage causing a loss of membrane integrity in the exercised muscles. However, there is controversy concerning how accurately the rise in CK reflects the type and intensity of the exercise performed. It has also been suggested that the release of CK into the blood is an indication of disturbances in energy production processes rather than muscle damage *per se* (Baird et al., 2012). This may explain the some of the disparity between other proxy measures of muscle damage and the CK response. Though the human CK response to exercise has not been fully elucidated, it is recognised as a good sign that EIMD has occurred. CK in itself may not be the best measure of the magnitude of EIMD, and despite human variability, it can be a useful indicator of significant differences following manipulation of other variables, nutritional interventions for example.

Creatine Kinase (CK) is involved in energy production, this enzyme catalyses adenosine di-phosphate (ADP) to adenosine tri-phosphate (ATP) in a reversible reaction (figure 3). This reaction works alongside other energy systems to buffer energy production and thus meet increased energy requirements (Sherwood, 2016). In exercising muscles when energy demands are high, levels of CK in the blood rise exponentially (Kenney et al., 2012). There is a general consensus that increases in CK after strenuous exercise peak within a week and then begin to return to baseline. The magnitude and timing of the CK response however is dependent on the exercise modality and duration. For example, the levels of CK for men subjected to exhaustive running peaked at 24 hours (170 IU/L), was falling at

48 hours and retuned to baseline by 72 hours (Oosthuyse & Bosch, 2017). A slower and much more exaggerated response is apparent for resistance compared to endurance exercise. When EIMD was induced in the biceps brachii of male participants by performing 50 maximal eccentric elbow flexion exercises, CK levels (7,684 IU/L) were still rising 96 hours after cessation of the exercise protocol (Tanabe et al., 2015). The CK response also differs by limb. There is a greater CK response to eccentric arm exercises compared to eccentric leg exercises, with CK increases peaking on day three over 2000 IU/L for elbow flexion and below 500 IU/L for knee extension (Saka et al., 2009). Similar results have been reported for leg exercises more recently with CK peaks at 636IU/L after 100 maximal intensity drop jumps and 270IU/L for 64 barbell squats (Page, Swan, & Patterson, 2017; Romain et al., 2017). There is clearly a more inflated CK response following exercise to upper limbs compared to lower limbs. Interestingly, this more exaggerated response extends to other markers, specifically, DOMS and ROM (Saka et al., 2009).



Creatine Creatine Kinase Creatine phosphate

Figure 3. The Creatine Kinase reaction

The CK response also differs by gender and is congruous in men but not in women, which may be due to menstrual phase (Oosthuyse & Bosch, 2017). A correlation between pre exercise oestrogen and exercise-induced increases in CK levels has been previously reported (Carter, Dobridge, & Hackney, 2001). It has been postulated that this is a result of the protective effect of oestrogen on exercise-

induced muscle damage (Kendall & Eston, 2002). Given gender differences in the physiological response to eccentric exercise and the role that oestrogen may play in CK activity, it is common to exclude female participants to limit inter-individual variations in response. Therefore, DOMS research is often gender specific and restricted to male participants (Pumpa, Fallon, Bensoussan, & Papalia, 2013; Ra et al., 2013; Vaile, Halson, Gill, & Dawson, 2008).

Increases in CK activity can be a useful measure in exercise research when it correlates with other measures. For example, a significant correlation has been demonstrated between CK and the reduction in muscle function, more specifically; torque (Hicks, Onambélé, Winwood, & Morse, 2016). And further, the rise in blood CK has been shown to significantly correlate with DOMS pain following a bout of strenuous exercise (Ammar et al., 2016; J. Kim & Lee, 2015).

It is clear that the CK timecourse variability is due to differences in the type, duration and intensity of each exercise protocol. It has also been shown that inter-individual variability is common and the CK response to exercise is biphasic and exaggerated in some individuals (Vyver & Myburgh, 2014). That being said, the most consistently exaggerated response would seem to be in men with a protocol using eccentric exercises of the upper limbs. It seems prudent therefore to use eccentric elbow flexion exercises with male participants in exercise biochemistry research.

2.1.2.2 Interleukin 6

Interleukin 6 (IL-6) is a cytokine released by t-cells and macrophages in the immune response. This cell signalling protein can be synthesised within skeletal muscle and is therefore also known as a myokine. High levels of circulating IL-6

are generated during exercise predominantly in fast twitch muscle fibres (Hiscock, Chan, Bisucci, Darby, & Febbraio, 2004). The increases in muscle generated IL-6 following a bout of exercise are dependent on the type, duration and intensity of that exercise (Morettini, Palumbo, Sacchetti, Castiglione, & Mazzà, 2017). And further, exercise-induced increases in IL-6 are associated with the symptoms of DOMS (MacIntyre, Sorichter, Mair, Berg, & McKenzie, 2001).

2.1.2.3 Swelling

EIMD causes inflammation which usually manifests in a measurable swelling of the muscles (Felipe Damas et al., 2016). The swelling can be quantified using a constant-tension measuring tape to determine the limb circumference, at baseline and at other timepoints after exercise when the inflammation is at or near its peak. In this way, the change in limb girth can then be used as an indicator of increased inflammation (Plattner et al., 2014; Ra et al., 2013). Following an eccentric elbow flexion protocol, significant increases of upper arm circumference in the magnitude of 9% have been recorded (Howell et al., 1993). More recently a cluster analysis looking at high, moderate and low responders to EIMD after elbow flexion recorded increases of 3%, 6% and 7% respectively (F. Damas et al., 2016).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are a class of pain relieving drugs that reduce inflammation. The efficacy of NSAIDs in reducing the pain of DOMS is equivocal and where evidence exists, the effect size is small to medium (Morelli et al., 2018). While NSAIDs do very little to reduce DOMS, they do interfere with the biochemical response to exercise (Urso, 2013). Most notably, NSAIDs inhibit swelling caused by exercise-induced inflammation and have a negative impact on the repair and adaptation process (Ziltener, Leal, & Fournier, 2010). Interestingly, inhibiting the inflammatory response to EIMD with NSAID's in turn reduces other

biomarkers, CK for example (Morelli et al., 2018). The magnitude of the response to these drugs in skeletal muscle is also dependent on the individual drug administered, timing and dose (Connolly, Sayers, & McHugh, 2003). Without clarity on the effect of NSAIDs on DOMS pain, swelling and CK responses, for the purposes of the present study, NSAID use was restricted.

2.1.2.4 Range of Motion

The movement potential of the elbow joint can be determined using a goniometer to measure the number of degrees between full flexion and full extension. The normal range of motion (ROM) for the elbow joint in men has been reported as 144.6º (Soucie et al., 2011). Following prolonged unaccustomed eccentric exercise of the biceps brachii, the ROM of the elbow joint decreases due to swelling and DOMS pain. Reductions in ROM have been recorded in the magnitude of -7º and -20º following 24 and 50 maximal eccentric contractions respectively (O'Fallon et al., 2012; Tanabe et al., 2015).

2.1.3 Endocannabinoids

Exercise in itself has been shown to have analgesic affects, reducing pain sensitivity. Recently, endocannabinoids have been shown in animal models to have exercise-induced nociception capabilities (Galdino et al., 2014). Suggesting that endocannabinoids have a role to play in exercise-induced analgesia in humans. Certainly, there appears to be some 'cross-talk' between exercise and the endocannabinoid system (ECS). It is likely that the ECS mediates some of the effects of exercise and that exercise has a modulatory effect on the ECS (Tantimonaco et al., 2014). For example, increased concentrations of AEA in the blood were reported after jogging or running on a treadmill compared to walking. And further, significantly greater increases in AEA were recorded at higher exercise intensities (Raichlen, Foster, Seillier, Giuffrida, & Gerdeman, 2013). The pain

relieving, antinociceptive effects of the endocannabinoid AEA was documented two decades ago (Piomelli et al., 1998). Taken together, it seems reasonable to assume that circulating levels of endocannabinoids have a bearing on pain perception after a bout of exercise.

2.1.4 Summary

Experimentally, physiological responses to exercise vary and do not consistently correlate with the level of exercise performed. This may in part be due to the nature of varying exercise protocols and differing equipment use in randomised controlled trials. Minor disparity in study methods and high variability in the human response mean that it is a challenge to compare intervention studies.

There is a need for a standardised exercise protocol where there are expected outcomes for EIMD biomarkers and other variables such as nutritional interventions can be tested. An evidence based exercise protocol that consistently induces measurable changes in the biomarkers associated with DOMS can be used to test DOMS intervention strategies.

2.1.5 Aims

To determine the optimum level of exercise to produce a physiological response that generates the greatest measurable changes in biomarkers of DOMS. To design an evidence based protocol that can consistently induce DOMS for experimentally testing nutritional interventions.

2.1.6 Hypothesis

H_0: There is no significant difference in biomarkers of DOMS in the biceps brachii induced by 48, 60 and 72 maximal eccentric repetitions of elbow flexion in men.

2.2 *Materials and Methods*

2.2.1 Participant Volunteers

A convenience sample of volunteers were recruited though presentations, emails, flyers and word of mouth. Following an expression of interest, volunteers were screened for eligibility. Participants were ineligible to take part if they: were taking anticoagulant or anti-inflammatory medications; had type 2 diabetes; or had muscle/joint disorders of the upper body. These exclusion criteria and details of the study were provided by verbal briefings and participant information sheets (Appendix 1). Participants were fully informed of procedures and risks before providing written informed consent (Appendix 2). Ethical approval was granted by the University of Huddersfield, Human and Health Sciences, School Research Ethics Panel (SREP). This study was carried out in accordance with the declaration of Helsinki (WMA, 2013).

A total of 28 male university staff and students volunteered to participate in the DOMS protocol development. Previous studies have reported differences in baseline measures and the magnitude of the biological response to exercise between genders (2 x citations). To minimise inter-individual variability, the present study used only male volunteers. Volunteers were randomly assigned into one of three groups using a random number generator (www.graphpad.com). Height and weight measurements were self-reported or measured using a Seca 213 portable stadiometer (Seca GMBH) (Figure 4) and the Salter compact analyser scale (Salter Housewares Ltd).

Figure 4. Seca Stadiometer.

2.2.2 Experimental Design

This study utilised a randomised independent group design. Participants attended the Exercise Physiology Laboratory at the University of Huddersfield to perform an eccentric exercise intervention on an isokinetic dynamometer (Cybex, USA) (figure 5). Biomarkers (including DOMS scores) were taken at three time points: before the exercise intervention (time point A), after exercise intervention (time point B) and at follow up 48 hours later (time point C) (figure 1).

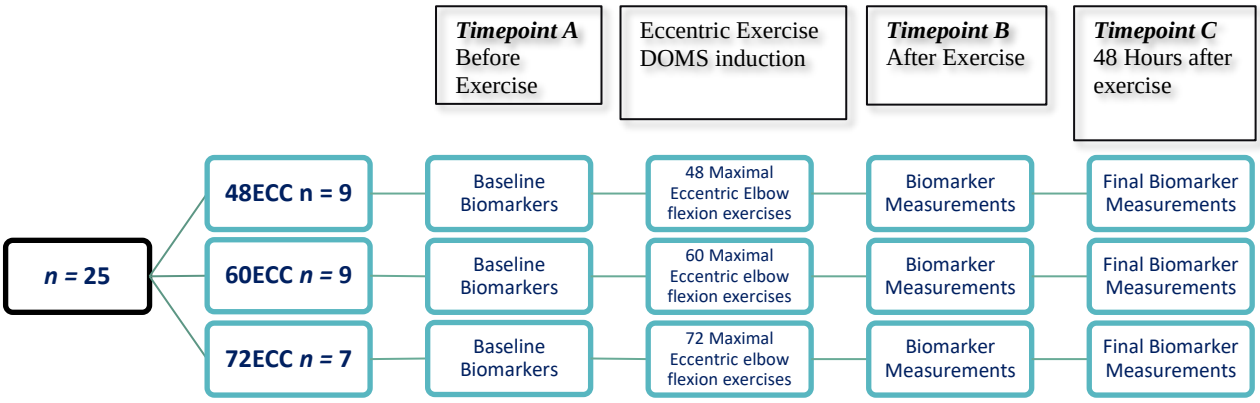


Figure 5 Overview of experimental design. The exercise intervention to induce DOMS was performed immediately following baseline biomarker measurements taken at time point A. Biomarker measurements were then repeated at time points B and C. Measurements recorded at each timepoint: upper arm circumference (UAC), range of motion (ROM) of the exercised arm, a recording of pain (VAS) in the biceps brachii and blood sampling took place.

2.2.3 Pilot

Three participants were invited to take part in the DOMS protocol pilot to assess feasibility and timing requirements for blood sampling, analysis and the exercise intervention.

2.2.4 Health Screening

Participants were asked to complete a Health Questionnaire (Appendix 3), to provide background information and assess each participant for inclusion. The Health Questionnaire included questions about dietary and lifestyle habits that may interfere with biomarker results.

2.2.5 Dietary, exercise and drug Restrictions

Participants restricted exercise, the use of over-the-counter pain relievers, and aspects of diet during the experimental phase. The experimental phase began two days prior to the exercise intervention and finished after the follow up (48 hour) blood samples had been taken. Participants desisted from exercise throughout the four-day experimental period, and did not eat or drink anything except water for

the two hours prior to each blood sampling appointment. Participant were requested to do without over-the-counter pain relievers during the experimental phase where possible, and report use.

2.2.6 Eccentric Exercise Intervention

The exercise intervention was performed on a Cybex NORM isokinetic dynamometer (Cybex, USA) in an Exercise Physiology Laboratory at the University of Huddersfield (figure 6). All tests were carried out on the non-dominant arm, the dominant arm was defined as the writing arm. The exercise intervention required participants to adopt the supine position and the elbow joint was aligned with the axis of rotation of the dynamometer. The length of lever arm was adjusted to provide a comfortable grip. Anatomical zero was set, and a range of motion restricted to 10° from anatomical zero through to 130° of elbow extension (120° ROM). Familiarisation with the dynamometer equipment consisted of submaximal elbow flexion exercises, and an explanation of the Humac Norm on screen graph. Participants then performed sets of 12 maximal voluntary eccentric contractions at 60^0/s with 1-min rest periods between sets. Following each contraction, the dynamometer arm passively reset allowing a 2s rest between contractions. To ensure maximal contractions, participants were verbally encouraged to maximally resit the downward action of the dynamometer arm throughout the entire 120^0 range of motion (ROM). Participants were given visual feedback via the dynamometer screen graph, which has been shown to have a positive effect on effort (Baltzopoulos, Williams, & Brodie, 1991).

Figure 6. Cybex NORM dynomometer

2.2.7 Biomarkers

Biomarkers were recorded on a participant testing record (Appendix 4)

2.2.7.1 Perceived Muscle Soreness

A Likert-like visual analogue scale (VAS) was used to evaluate perceived muscle soreness. A 10cm sliding scale ruler with 0 at one end and 10 at the other, 0 representing 'no pain' and 10 representing 'extremely painful'. Participants were asked to move the slide on the ruler to the corresponding level of pain in the bicep brachii when the elbow joint was passively extended and flexed. Scoring pain on a visual analogue scale is widely used method to determine pain intensity (Lau et al., 2015; Matsumura et al., 2015).

2.2.7.2 Upper Arm Circumference

The upper arm circumference (UAC) provided a measure of swelling within the biceps brachii, changes in the UAC was used as a marker of inflammation. The participant stood with a relaxed arm while the mid-belly of the biceps brachii was measured in millimetres (mm) using a constant-tension measuring tape (brand).

2.2.7.3 Range of Motion

The range of motion (ROM) of the elbow joint was quantified by taking two joint angle measurements using a standard goniometer. The flexed joint angle was

taken when each participant attempted to fully flex the elbow joint by touching the shoulder on the same side. The extended joint angle taken with the participant's arm proximal to the body and maximally extended. The ROM was calculated as the extended joint angle minus the flexed joint angle as previously described (H.-L. Chen et al., 2012).

2.2.8 Blood Biomarkers; Sampling & Analysis

Blood biomarkers were measured before the exercise intervention (at baseline); immediately after the exercise intervention; and finally 48 hours later. Standard venepuncture techniques and fingerprick techniques were used to collect blood samples at the three time points: timepoint A (immediately before exercise), timepoint B (immediately after exercise) and timepoint C (48 hours after exercise). Finger-prick capillary blood samples for the assessment of CK were obtained using ACCU-CHEK Plus (Roche Diagnostics USA) disposable lancets. Capillary blood was then collected into 30µl capillary action micropipettes for analysis. Venous blood samples were obtained from an antecubital vein using the vacutainer system (BD Vacutainer). Each 6ml vacutainer tube was inverted 8 times to mix the anticoagulant ethylenediaminetetraacetic acid (EDTA) with the sample. Within one hour of collection, the sample was centrifuged (1,000 x g for 10 mins) to separate cells from plasma. The supernatant plasma was pipetted into 1.5ml microcentrifuge tubes for storage until analysis for IL-6, AEA, and 2-AG. Participants were given an aftercare sheet (Appendix 5)

2.2.8.1 Equipment list for blood sampling (figures 7 & 8)

Finger prick sampling:

Paper towels

Alcohol hand gel

70% Alcohol Pre-injection swabs (Medisave)

Safe-T-Pro plus lancets (Accu-Check)

Sharps Bin

30µl capillary action pipettes

Cotton Wool

Plasters

Venepuncture:

Paper Towels

Alcohol hand gel

70% Alcohol Pre-injection swabs (Medisave)

Tourniquet

Medical Gloves (find Name)

6ml EDTA venepuncture tubes

Vacutainer single use holders

21g Vacutainer Eclipse needles

Cotton Wool

Sharps Bin

Cotton Gauze

Non-allergy Medical Tape

Eppendorf MiniSpin Microcentrifuge (figure 7)

Figure 7. Eppendorf Mini Spin Microcentrifuge

Figure 8. Blood sampling table layout and equipment.

2.2.8.2 Creatine Kinase

The 30µl sample of capillary blood was applied to the Roche Creatine Kinase (CK) test strip and inserted into the clinical analyser immediately after collection. The Reflotron clinical analyser (Roche Diagnostics USA) (figure 9) then measured CK

within detection limits of 24-1400 International Units (U/L).

Figure 9. Reflotron Plus (Roche Diagnostics USA)

2.2.8.3 Interleukin-6

The analysis of plasma levels of interleukin 6 (IL-6) was then determined using a commercially available enzyme linked immunosorbent assay (ELISA) kit (Biolegend Inc, USA). All ELISA analyses were performed according to the manufacturers recommended instructions and in duplicate. Sample concentrations were determined relative to the standard solutions provided in the ELISA kit.

Procedure for Human IL-6 Enzyme Linked Immunosorbent Assay (ELISA)

Equipment and solutions for Biolegend Human IL-6 ELISA (figure 10)

Microplate Reader

Microcentrifuge (Eppendorf UK)

Adjustable pipettes

Test tubes

2ml Microcentrifuge Tubes (Eppendorf UK)

Wash Bottle

Plate sealers

Paper towels

Human IL-6 ELISA Capture Antibody (200X)

Human IL-6 ELISA Detection Antibody (200X)

Human IL-6 Standard

Avedin-HRP (1000X)

Substrate solution A

Substrate solution B

Coating Buffer A (5X)

Coating Buffer B (5X)

96 Microwell Plates (Nunc™)

Phosphate Buffered Saline (PBS)

Wash Buffer

Stop Solution

Deionised water

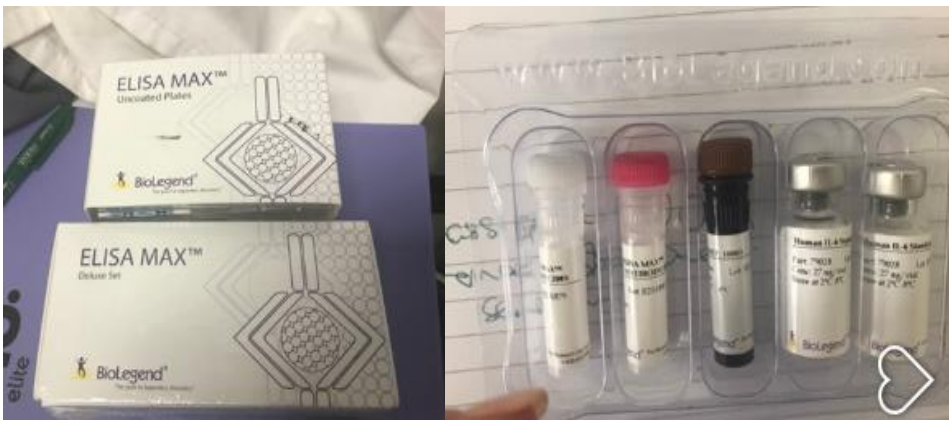


Figure 10. ELISA kit and solutions

Laboratory procedure for Human IL-6 Enzyme Linked Immunosorbent Assay (ELISA)

1. Coating buffer A (5X) was diluted to 1X with deionised water.
2. Capture antibody was diluted to 1:200 with 1X coating buffer A.

3. The PBS solution was made up by adding 1 tablet per 200ml of deionised water and stirred using a magnetic stir plate and magnetic stirrer until clear with no particles.
4. Assay diluent A (5X) was diluted to 1X with PBS.
5. The IL-6 standard solution was reconstituted with 1X assay diluent A, then allowed to stand for 15 minutes and gently agitated.
6. Standard solutions were prepared as follows: A six two-fold serial dilution of 500pg/ml top standard with 1X assay diluent A gave concentrations of – 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.3pg/ml, 15.6pg/ml and 7.8pg/ml. 1X assay diluent A served as a 'blank well' zero concentration standard (Figure 11).

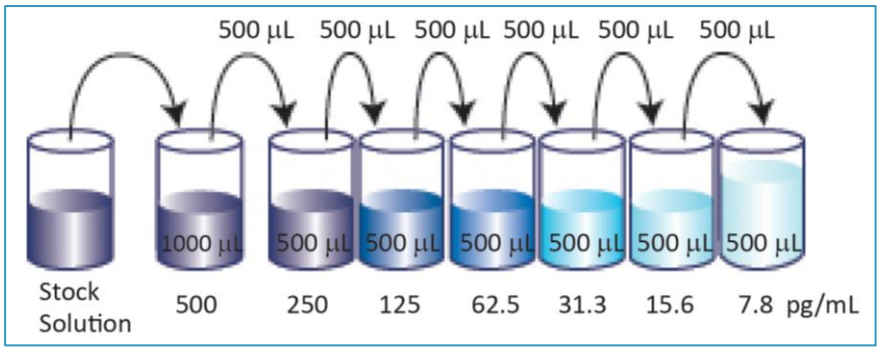


Figure 11. Serial Dilution of Human IL-6 standard solutions.

7. IL-6 detection antibody was diluted 1:200 with 1X assay diluent A.
8. Avidin HRP was diluted 1:1,000 in 1X assay diluent A.
9. One day prior to running the ELISA the plates were pre-coat with the capture antibody solution (see 2. above). Each well of the 96-well plate was inoculated with 100µl of capture antibody solution. The plate was then sealed and incubated overnight (16-18hours) between 2ºC and 8ºC.

10. Samples were removed from the freezer (-18ºC) and refrigerated overnight with the plate.
11. All reagents were brought to room temperature before being used.
12. The plate was washed 4 times by pipetting 300µl of wash buffer per well, emptied, then blotted by firmly tapping the plate upside down on an absorbent paper towel (subsequent washes were performed in the same way).
13. Background and non-specific binding was reduced by 'blocking' the plate with 200µl of assay diluent A per well and shaken on a shaker plate for 1hour at room temperature (all subsequent incubation with shaking was performed in this way)
14. The plate was washed 4 times with wash buffer.
15. 100µl of each standard solution (7 + blank) or plasma samples was pipetted into the wells in duplicate (figure 12). The plate was then sealed and incubated with shaking for two hours.

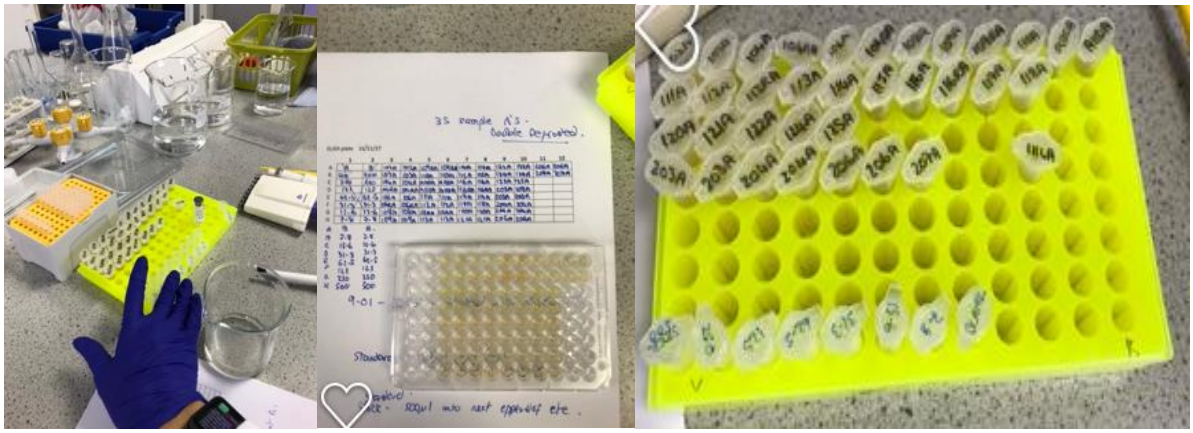


Figure 12. Samples and standards preparation

16. The plate was washed 4 times with wash buffer.

17. 100µl of diluted detection antibody solution (see 7. above) was pipetted into each well and then the plate was sealed and incubated with shaking for 1 hour.
18. The plate was washed 4 times with wash buffer.
19. 100µl of Avidin HRP solution (see 8. above) was pipetted into each well and then the plate was sealed and incubated with shaking for 30 minutes.
20. The plate was washed 5 times with wash buffer. For this wash cycle the wash buffer was allowed to sit in the wells for 1 minute before blotting each wash to help minimise background.
21. TMB substrate solution was made up by mixing equal volumes of Substrate solution A & B and used immediately. 100µl TMB substrate solution was pipetted into each well then the plate was incubated in the dark for 15 minutes until cells began to turn blue.
22. The reaction was stopped by adding 100µl of stop solution to each well, positive cells now turned from blue to yellow.
23. Immediately following the addition of the stop solution, the absorbance was read at 450mn using a microplate reader (figure 13).

Figure 13. Microplate reader

2.2.8.4 Anandamide

Circulating levels of Anandamide (AEA) was determined using HPLC triple quadrupole mass spectrometry (HPLC-QQQ-MS). Following venepuncture, blood samples were centrifuged and the supernatant plasma was removed, pipetted into 1.5ml aliquots and stored at -80ºC until analysis. Levels of Anandamide was established using a validated method for human plasma outlined by Zoerner et al. (2012).

Laboratory procedure for HPLC-QQQ mass spectrometry

Equipment and solutions for HPLC-QQQ-MS

Agilent 1290 Infinity II UPLC machine

Agilent 6470 Triple Quadrupole LC/MS system (figure 14)

Zorbax Eclipse Plus C18 HPLC column (Agilent Technologies)

TurboVap Evaporator (Biotage EU)

Refrigerated Centrifuge

Auto pipettes

10ml volumetric flask

100ml volumetric flask

Test tubes

HPLC vials

HPLC inserts

2ml Microcentrifuge Tubes (Eppendorf UK)

Ice bowl

Water bath Sonicator

LCMS grade Acetonitrile (Fisher Scientific)

HPLC grade Toluene (Fisher Scientific)

HPLC grade Formic Acid

LCMS grade 3:1 Ethenol:water (Fisher Scientific)

A 50,000ppm solution containing 10 mg of AEA in 200µl using ethanol as a solvent (Caymen Chemicals).

A 1,000ppm solution of 500µg of D4-AEA in 50 µl using ethanol as a solvent (Caymen Chemicals).

Figure 14. Agilent 1290 Infinity II UPLC and Agilent 6470 Triple Quadrupole LC/MS system.

Sample and standards preparation

1. A 1ppm internal standard solution of D4-AEA was prepared by adding 10µl of the D4-AEA standard to a 10ml volumetric flask and making to volume with acetonitrile.
2. A 32 ppb internal standard solution of D4-AEA was prepared by adding 320µl of the 1ppm standard to a 10 ml volumetric flask and making to volume with acetonitrile.
3. A 100ppm internal standard solution of AEA solution was prepared by adding 20µl of the 50,000ppm standard to a 100 ml volumetric flask and making to volume with acetonitrile.

4. Samples were removed from the freezer and allowed to thaw at room temperature before 1ml of plasma was pipetted into 2ml microcentrifuge tubes (Eppendorf).
5. 10µl of 32ppp standard D4-AEA solution was added to each sample with 1ml of toluene (Fisher Scientific).
6. The Sample solutions were sonicated for 30 minutes in a water bath then centrifuged for 5 minutes at 12,000rpm.
7. The organic phase was pipetted out and transferred to a test tube. The samples were then evaporated (TurboVap) under Nitrogen.
8. 100µl of 3:1 menthanol:water was added to each sample and sonicated for a further 5 minutes in a water bath.
9. Final sample solutions were transferred to 200µl HPLC vials and analysed by HPLC triple quadrupole mass spectrometry (figure 14). 3:1 Methonol:water served as a blank.

2.2.9 Variables

The independent variable (IV) consisted of three levels of exercise represented as different numbers of repetitions of eccentric elbow flexion exercises: 48 repetitions (48ECC), 60 repetitions (60ECC) and 72 repetitions (72ECC). Dependent variables comprised of the subsequently recorded biomarkers at time point A (before exercise), time point B (after exercise), and time point C (48 hours later). Dependant variables (DV's) were as follows: DOMS, UAC, ROM, CK, IL-6, and AEA.

2.2.10 Statistical methods

Statistical analysis was performed using SPSS version 24 (IBM Corp., USA) with the significance level set at $p>0.05$ for all analyses. To determine normality, these data were assessed both visually (histograms) and statistically using the Shapiro-Wilk test for normality. Assumptions of homogeneity of variance were tested using

the Levene's test for equality of variances. Central tendencies and measures of spread were derived for the DV's and expressed as mean ± standard deviation (SD) or median with interquartile range (IQR).

Parametric tests were used on DOMS and UAC data. Significant main time effects for DOMS, and UAC was assessed using paired sample t tests. To detect significant differences between conditions (48, 60, 72 repetitions) at baseline and at 48 hours, data was compared using a one-way between groups analysis of variance (ANOVA). Where a significant experimental effect existed, Bonferroni's post hoc test was used to establish specific group differences. The effect of time was assessed within each condition using paired sample t tests, a within group comparison between baseline and 48 hours was performed for each variable. A one-way between groups ANOVA was then used to assess the time x condition effect of the level of exercise (48, 60 or 72 repetitions) on the change in DOMS and UAC over the 48 hour period.

For non-parametric CK, IL-6, ROM and AEA data the following tests were used to assess; baseline differences (Kruskal-Wallis test), main time effect (wilcoxen Signed Rank test) and group x time effect (Kruskal-Wallis test). Where significant differences were identified, 95% confidence intervals were used to present the estimation of mean differences.

A Pearson product moment correlation was used to assess the relationship between the 48 hour changes in DOMS and UAC scores. Finally, a Spearmans Rho correlation was used to assess the relationships between 48 hour changes in DOMS, IL-6, ROM and AEA.

2.3 *Results*

2.3.1 Study population

Twenty-eight volunteers were assessed for eligibility before taking part in the study. Of these volunteers, one reported to the physiology laboratory after inadvertently taking part in an additional bout of exercise and was excluded from further participation in the study. Two participants had implausibly high CK at baseline and were excluded from further analysis. Also, full blood samples were not possible for two participants and were therefore excluded from blood biomarker analysis, but included in all other measures. A total of 25 volunteers (31.6 ± 13.2 years; 83.2 ± 13.0 kg; 179.0 ±6.9 cm) completed the experimental protocol and were included for statistical analysis.

2.3.2 Indicators of Muscle Damage

There were no significant differences between groups for any baseline measures. All three exercise protocols (4, 5 and 6 sets) were sufficient to induce significant changes in muscle damage biomarkers. The data for DOMS and UAC followed a normal distribution. The data for ROM, CK, IL-6 and AEA did not follow a normal distribution both visually (histogram) and statistically (Shapiro-Wilk), non-parametric tests were therefore used for these data. The data for DOMS and ROM returned a non-significant result for the Shapiro Wilk test, indicating a normal distribution of these data. A significant time effect between baseline measures and 48 hours were found for DOMS ($p=0.000$), upper arm circumference ($p=0.001$), ROM ($p=0.000$), CK ($p=0.004$), IL-6 ($p=0.007$). Table 1 shows mean and median values for biomarkers for muscle damage; DOMS, UAC, ROM, CK activity and circulating IL-6.

Table 1. Biomarkers of Exercise-induced Muscle Damage

Biomarker	Time point A Baseline	Time point B After exercise	Time point C 48 hours
Soreness (VAS 0-10)	0.8 ± 0.3	2.6 ± 1.7*	4.7 ± 2.2***
Creatine Kinase (IU/L)	124.0 (206.0)	141.5 (184.5)	254.0 (503.0)**
Interleukin 6 (pg/ml)	8.2 (4.4)	-	10.86 (7.8)**
Upper Arm Circ (cm)	32.7 ± 3.0	33.3 ± 3.0*	33.2 ± 3.2**
Range of motion (Degrees)	145.0 (17.0)	132.0 (15.0)*	133.0 (11.0)***

Mean (SD)/median (IQR) values at baseline, immediately after and 48 hours after exercise for all participants (*n*=25). Asterisk superscript ***, **** and ******* denotes a significant difference relative to baseline $p<0.05$, $p<0.01$and $p<0.001$ respectively.

2.3.2.1 Perceived Muscle Soreness

There was a significant main time effect on DOMS ratings following the exercise protocol, all participants reported an increase in soreness at 48 hours (p=0.000) (table 1). Ratings of perceived muscle soreness did not differ between the three groups at baseline (p=0.685), immediately following the exercise protocol (p=0.195) or 48 hours after exercise (p=0.766). At 48 hours, the greatest changes in DOMS scores were recorded for the 60 repetition group (5.0 ± 2.1) compared to 48 repetitions (4.2 ± 2.1) and 72 repetitions (4.6 ± 2.6) (Figure 15). However, no significant differences in soreness ratings were found between groups after 48 hours (p=0.766) (Table 2).

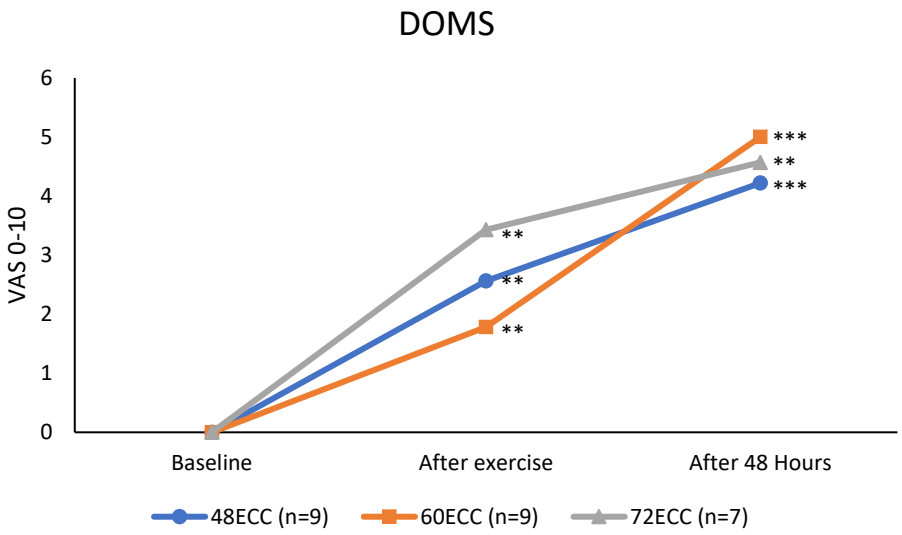


Figure 15. Increases in DOMS pain scores relative to baseline by exercise volume (displayed as a mean); 48 eccentric elbow contraction, 60 eccentric elbow contractions and 72 eccentric elbow contractions. No significant differences between groups were found (p=0.766). Asterisk superscript ** and *** denotes a significant difference relative to baseline within a group p<0.01 and p<0.001 respectively.

2.3.2.2 Creatine Kinase

There was a significant main time effect at 48 hours (p=0.004) compared to baseline (table 1). CK activity did not differ between the three groups at baseline (p=0.494), immediately following the exercise protocol (p=0.405), or at 48 hours (p=0.632). There was no group x time interaction (p=0.829) (table 2). Significant increases in CK concentrations were recorded in the 48 (p=0.028) repetition group only. However, increases in CK in the 60 repetition group neared significance (p=0.069). There was no significant increase in CK for the 72 repetition group (p=0.310). Median (IQR) increases in CK at 48 hours were highest for the 48 repetition group of 61.5 (651.4) compared to the 60 repetition group of 55.5 (476.7) and 72 repetitions 34.0 (218.0) (figure 16).

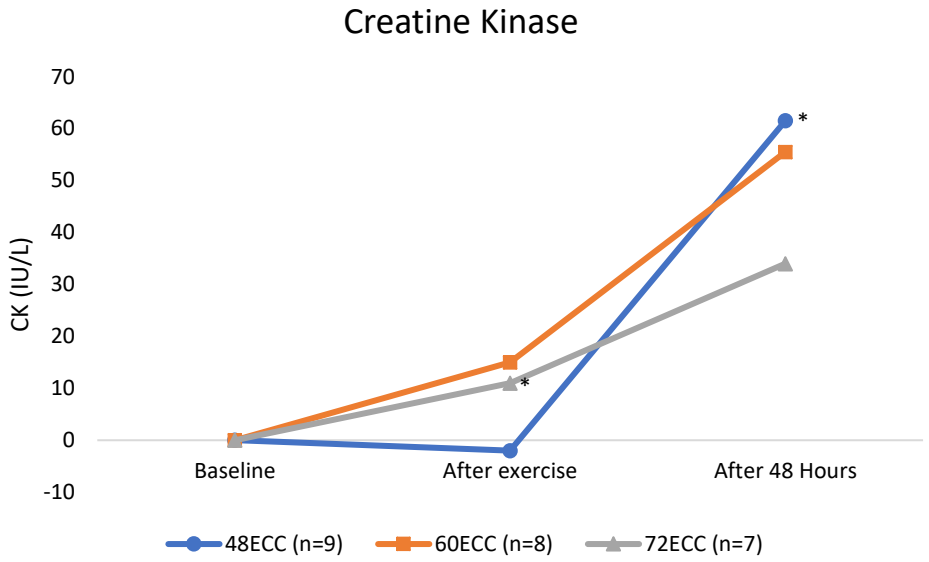


Figure 16. Change in Creatine Kinase activity relative to baseline by exercise volume; 48 eccentric elbow contraction, 60 eccentric elbow contractions and 72 eccentric elbow contractions (displayed as a median). No significant differences between groups were found (p=0.829). *Significantly different to baseline p<0.05.

Table 2. Biomarker outcomes according to number of repetitions of elbow flexion. Displayed as mean (SD) or median (IQR).

	48 Reps *(n=9)*		60 Reps *(n=9)*		72 Reps *(n=7)*		between groups; *p*
	Baseline	48 hours	Baseline	48 hours	Baseline	48 hours	
DOMS (VAS 0-10)	0.1 ± 0.3	4.3 ± 2.2***	0.1 ± 0.3	5.0 ± 2.1***	0.00 ± 0.00	4.6 ± 2.6**	*p*=0.766
UAC (cm)	33.4 ± 2.0	34.0 ± 2.4**	33.6 ± 3.9	34.2 ± 3.7**	30.7 ± 2.2	30.7 ± 2.1	*p*=0.069
ROM (Degrees)	145.0 (22.0)	138.0 (21.0)	137.0 (19.0)	129.0 (29.0)**	146.0 (18.0)	136.0 (11.0)**	*p*=0.111
CK (IU/L)	124.0 (61.0)	189.0 (737.0)**	158.5 (444.5)	447.0 (867.3)	121.0 ± 421.0	246.0 (393.0)	*p*=0.829
IL-6 (IU/L)	9.5 (7.5)	15.1 (21.7)*	9.6 (6.7)	10.9 (5.4)	5.4 (2.5)	9.0 (7.4)	*p*=0.247

Asterisk superscript ***, **** and ******* denotes a significant difference relative to baseline within a group $p<0.05$, $p<0.01$ and $p<0.001$ respectively. Significance assessed by ANOVA for Soreness, and UAC. Significance assessed for CK, IL-6 and ROM with the Kruskal-Wallis Test.

2.3.2.3 Interleukin-6

There was a significant main time effect at 48 hours (P=0.006) compared to baseline (table 1). There was no significant group x time interaction for the change in circulating IL-6 (p=0.247) (table 2). After 48 hours, a significant time effect was evident for 4 sets only (p=0.021). After 48 hours, there was no significant time effect for 5 sets (p=0.484) or 6 sets (p=0.080) (figure 17).

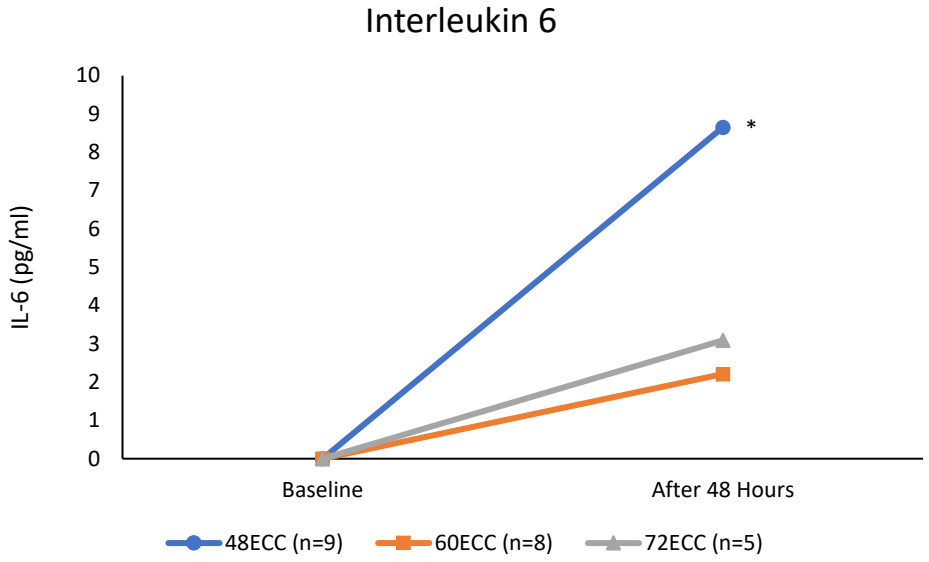


Figure 17. Change in Interleukin-6 levels relative to baseline by exercise (displayed as a median); 48 eccentric elbow contraction, 60 eccentric elbow contractions and 72 eccentric elbow contractions. No significant differences between groups were found (p=0.247). *Significantly different to baseline p<0.05.

2.3.2.4 Upper Arm Circumference

Baseline data did not differ between groups. Homogeneity of variance was violated at baseline according to Levene's test (p=0.028). However, using more robust tests of equality of means, Welch (p=0.066) and Brown-Forsythe (p=0.111), homogeneity of variance can be assumed. There was a significant main time effect immediately after the exercise protocol (p=0.000) and at 48 hours (p=0.001) compared to baseline (table 1). More specifically within each group, significant

time effect existed from baseline to 48 hours for 48 and 60 repetitions only (p=0.005 and p=0.008 respectively). There was no significant increase in upper arm circumference 48 hours after the exercise protocol where 72 repetitions (p=0.914) were performed (Table 2). There was no significant group x time interaction, however, this did approach significance (p=0.069). Mean changes in UAC were higher for 48 repetitions (0.69 ± 0.53) and 60 repetitions (0.61 ± 0.53) compared to 72 repetitions (0.03 ± 0.67) (figure 18).

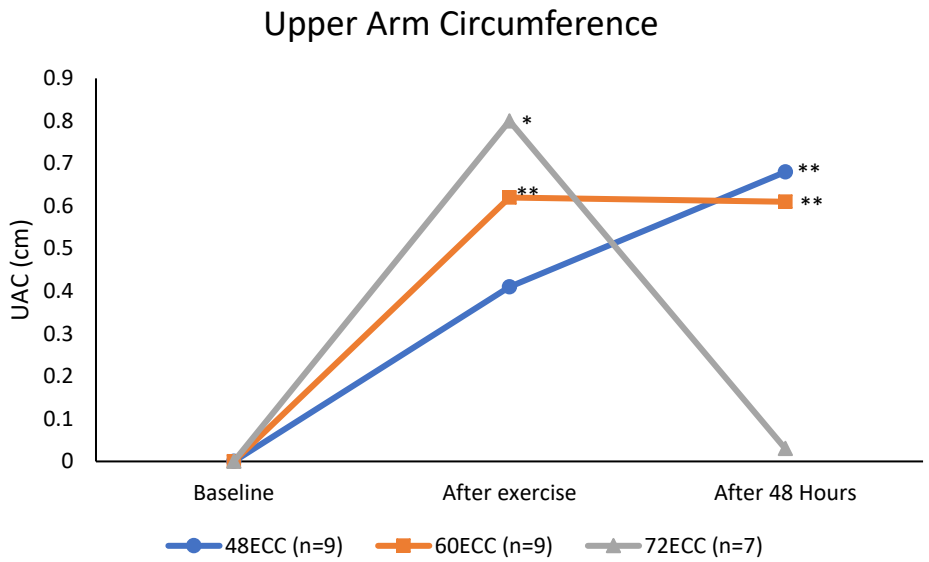


Figure 18. Changes in upper arm circumference (UAC) relative to baseline by exercise volume (displayed as a mean); 48 eccentric elbow contraction, 60 eccentric elbow contractions and 72 eccentric elbow contractions. No significant differences between groups were found (p=0.069). Asterisk superscript * and ** denotes a significant difference relative to baseline within a group p<0.05 and p<0.01 respectively.

2.3.2.5 Range of Motion

There was a significant main time effect immediately after the exercise protocol (p=0.000) and after 48 hours (p=0.000) compared to baseline (table 1). This was represented by a significant reduction of 8° in the range of motion of the elbow joint over 48 hours. There existed a significant time effect from baseline to 48

hours for the 60 repetition group (p=0.013) and 72 repetition group (p=0.018), but not 48 repetition group (p=0.262). However, there was no significant group x time interaction (p=0.111) found. Within the individual groups, there was a 48 hour ROM reduction of 3° in the 48 repetition group, 12° in the 60 repetition group and 8° in the 72 repetition group (figure 19).

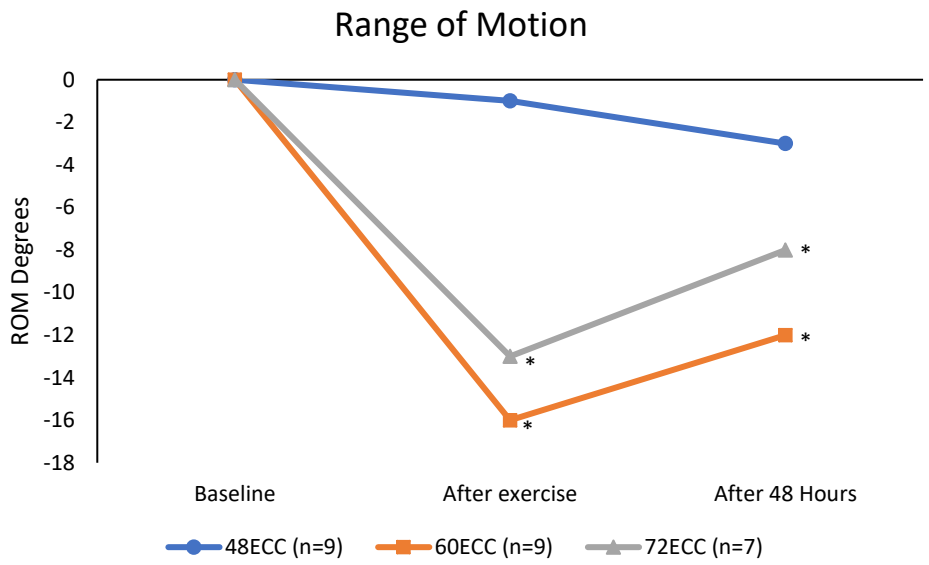


Figure 19. Decreases in range of motion (ROM) relative to baseline by exercise volume (displayed as a mean); 48 eccentric elbow flexion contraction, 60 eccentric elbow flexion contractions and 72 eccentric elbow flexion contractions. No significant differences between groups were found (p=0.111). Significantly different to baseline p<0.05*.

2.3.3 Anandamide

Anandamide (AEA) data did not follow a normal distribution, therefore non-parametric tests were used to evaluate differences of this variable. There was no statistical difference between the groups at baseline (p=0.319), or at 48 hours (p=0.435). A reduction in circulating AEA was evident with a significant main time effect over 48 hours (p=0.033). Median 48 hours decreases in AEA were as follows: 48 repetition group -0.54 (n=6), 60 repetition group -1.67 (n=3), 72 repetition group -1.32 (n=2) (figure 20). However, these reductions did not reach

statistical significance for any of the three groups. There was no significant group x time interaction (p=0.149). Due to QQQ mass spectrometry issues, the AEA data results were reduced to between 2 and 6 data points for each analysis. Therefore, the endocannabinoid results should be interpreted with caution.

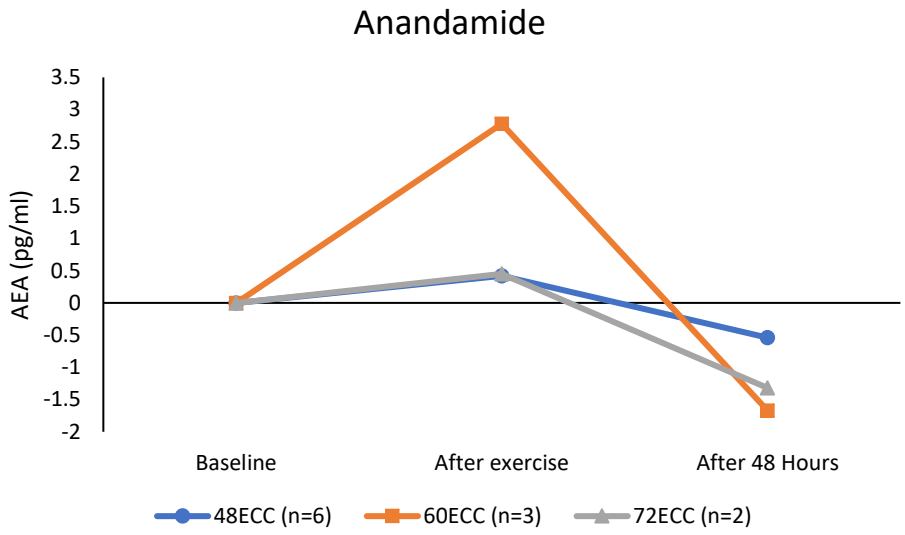


Figure 20. Changes in Anandamide (AEA) concentration relative to baseline by exercise volume (displayed as a median); 48 eccentric elbow contraction, 60 eccentric elbow contractions and 72 eccentric elbow contractions. No significant differences between groups were found (p=0.149). Anandamide concentrations did not significantly change from baseline for any level of exercise volume.

2.3.4 Correlations between variables

Significant negative Spearman's Rho correlations were observed between; the 48-hour decrease in increase in DOMS and the decrease in ROM (r=-0.425; p=0.034); and the increase in circulating CK levels and decrease in ROM (r=-0.427; p=0.037). A significant positive Spearman's Rho correlation between the increase in DOMS and the increase in CK (r=0.436; p=0.033).

2.4 *Discussion*

2.4.1 DOMS induction

The present study evaluated the effects of three levels of exercise on DOMS related indices of muscle damage 48 hours after an acute bout of exercise. The exercise protocol was sufficient to induce DOMS pain in the biceps brachii, and increases in the blood markers CK and IL-6 confirmed EIMD. The magnitude of post exercise alterations in the indices analysed were similar to those reported in other studies (Guilhem et al., 2013; Lau, Blazevich, Newton, Wu, & Nosaka, 2015b; Nosaka et al., 2002; Tanabe et al., 2015).

2.4.2 Muscle soreness.

Maximal voluntary contractions are unlikely to be maximal throughout a high number of repetitions. From previous dynamometry research using a 10cm VAS to measure DOMS in the biceps brachii, it is apparent that a lower number of eccentric contractions usually, but not always, results in lower mean pain scores 48 hours later. For example, 20 voluntary maximal eccentric contractions resulted in a mean soreness score of less than 3cm (Lima et al., 2017), and 40 repetitions resulted in 4.5cm (Connolly et al., 2006). This is broadly in line with 4.3cm (48 contractions) and 5.1cm (60 contractions) in the present study. Conversely, a high DOMS score of 8.5cm have been recorded following a protocol involving a large number of maximal eccentric contractions of the biceps brachii (Jeon, Kang, Park, & Lee, 2015). However, in that case the exercises were performed to exhaustion, which would undoubtedly have increased the DOMS scores. When a large number of maximal eccentric contractions are performed *without* reaching exhaustion, DOMS pain is less intense. For example, performing 125 maximal eccentric elbow flexor exercises resulted in peak pain scores of less than 4cm (Plattner et al., 2014).

Given the large number of contractions, motivation to make each contraction maximal may have been compromised. It has been shown that the perception of effort correlates with motor command during a contraction (de Morree, Klein, & Marcora, 2012). The contractions in the present study were voluntary and therefore the strength of each contraction was dependent on the motivation and effort of each participant. It is therefore possible that participants reduce effort to a manageable level knowing they were expected to repeat each maximal contraction many times. Thus resulting in lower DOMS scores in the present study for the 72 contractions group (4.6cm) compared to the 60 contraction group (5.1cm). It is likely that motivation to contract every repetition maximally was sustained at 60 repetitions but not 72 repetitions, leading to moderation of the increases in muscle damage indices including DOMS pain. Based on the pain scores alone, the optimum number of repetitions to test an antioxidant intervention using the same protocol is 60.

The length of time each contraction is sustained has a bearing on resulting soreness. After 30 maximal eccentric contractions, a relatively high peak soreness on day two was recorded as 4cm (Ochi, Tsuchiya, & Nosaka, 2016). Though only 30 contractions were performed, each contraction lasting 3 seconds which likely increased the pain score for such a low number of contractions. This level of pain is broadly in line with that produced in the 48 contractions group in the present study where each contraction lasted 2 seconds. When 30 contractions were performed with each contraction lasting 5 seconds the resulting mean DOMS score has been reported as almost 6cm. Study participants were allowed a 3s rest between contractions which seems to have a bearing on DOMS intensity (Ra et al., 2013).

For example, the same 6cm DOMS score was reported after 50 contractions lasting only 1 second each, crucially, participants were allowed a 12s rest and reset between contractions compared to 3s above (Tanabe et al., 2015). The relatively long rest period of 12s allowed for a 'biochemical reset', e.g. enabling the replenishment of ATP and phosphocreatine and removal of lactate. Without this 'metabolic reset' the ability to sustain high force muscular contractions is compromised (Grgic, Schoenfeld, Skrepnik, Davies, & Mikulic, 2017). As expected in the present study, the relatively short 2 second rest period and 2 second contraction time resulted in slightly lower DOMS scores than those above.

While significant statistical differences between groups is important, in DOMS research where small sample sizes are common, considering the magnitude of the effect can be more useful in evaluating the data. In the present study there was no statistical difference between the DOMS scores for the three groups. However, the highest mean DOMS score of 5.1cm was recorded in the 60 repetition group. This DOMS score is in agreement with previous work for 60 repetitions of maximal eccentric elbow flexion exercises, 5.2cm (Guilhem et al., 2013) and 4.8cm (Lau et al., 2015b). Given that the 72 repetition group had a lower DOMS score than the 60 repetition group, 60 repetitions is an adequate number to test an antioxidant intervention.

2.4.3 Creatine Kinase

In the present study, the greatest increases in CK enzyme activity were seen following 48 repetitions (306.3 IU/L), with a lower response after 60 repetitions (447 IU/L) and the lowest response after 72 repetitions (246 IU/L). Similar 48hr responses have been recorded following maximal eccentric elbow flexion exercises

with CK increases in the magnitude of the present study (Howatson & van Someren, 2007; Matsumura et al., 2015; Newton et al., 2013). In some cases a seemingly similar protocol brings about a more exaggerated CK response (Coratella & Bertinato, 2015; Ra et al., 2013). The CK results in the present study were a little lower than expected for 72 repetitions. However, it is likely that CK was still rising at 48 hours, for example, CK was recorded as still increasing on day 5 after 42 eccentric contractions of the elbow flexor (Shirato et al., 2016). In the present study, CK was likely to still be rising at 48 hours. It is possible that the response was slower after 72 repetitions compared to less repetitions. In a less strenuous protocol using the same biceps brachii muscles, a less exaggerated and more delayed response occurs. Eccentric contractions performed at 80% of the maximum isometric contraction torque, resulted in a peak in CK that did not occur until day five, 108 hours after cessation of exercise (Plattner et al., 2014). For other types of contraction, isometric for example, the CK response can be absent altogether. Following 50 isometric elbow flexion exercises, no significant time effect for CK was recorded. Interestingly, in the same participants, when the biceps brachii muscles were electrically stimulated in the contralateral arm, CK levels were still rising at 96 hours in a similar response to Tanabe above (Jubeau et al., 2012). The timecourse has been demonstrated using a cluster analysis (n=286) which showed that CK gradually rises in the days following maximal eccentric elbow flexion exercises and generally peaks on day 4. Interestingly the cluster analysis also demonstrated clear evidence for high and low responders for increases in CK enzyme activity (F. Damas et al., 2016). It is likely that there were high and low responders within the 25 participants in the present study. It should also be noted that a relatively small group of participants performed 72 repetitions

(n=7). And further, given that the CK response can be biphasic (Vyver & Myburgh, 2014), and does not necessarily correlate well with exercise intensity (Malm et al., 2000) recording CK at more than one timepoint would be advantageous.

2.4.4 Interleukin 6

A bout of maximal eccentric elbow flexion exercises does not usually significantly increase IL-6 concentrations (Guilhem et al., 2013; Tanabe et al., 2015). However, in contrast to previous work, the present study reported significant increases in IL-6 in the 48 repetition group (Guilhem et al., 2013; Trombold, Barnes, Critchley, & Coyle, 2010). This is unusual for a bout of exercise with a relatively low metabolic cost compared to high metabolic cost activities such as running e.g. downhill running, half marathon or an ultramarathon, activities which significantly increase IL-6 concentrations (Arakawa et al., 2016; Vyver, Engelbrecht, Smith, & Myburgh, 2016). Interestingly there were two peaks following the ultramarathon, one on day one and a second on day five (Arakawa et al., 2016). Following a half marathon IL-6 peaked 60 minutes after exercise and had returned to baseline by 24hours and was still around baseline at 48 hours (Levers et al., 2016). Compared to the lower metabolically costly maximal eccentric elbow flexion exercises, where there are no significant increases in IL-6 over 48 hours; 30 contractions (Conceição et al., 2012), 40 contractions (Trombold et al., 2010) or 60 contraction (Guilhem et al., 2013). Though not significant, levels of IL6 after 50 maximal eccentric contractions of the elbow flexor shows a trend upwards over a four day period, and was still rising on day four (Tanabe et al., 2015). It is possible therefore, that participants in the 60 and 72 repetition groups had increases in IL-6 that occurred after 48 hours which were not captured. However, the follow up to 60 eccentric elbow flexion contractions in Guilhem et al.

(2013) was 14 days with no significant increases in IL-6 at any time point. This suggests that the non-significant increases in IL-6 in the 60 and 72 repetition group was to be expected.

2.4.5 Swelling

It is likely that one or more elements of inflammation, with the corresponding muscle swelling, is responsible for the pain of DOMS. In a DOMS affected muscle, pain sensitivity changes, measured by the electrical pain threshold (EPT), do not occur uniformly across the whole muscle structure. When comparing the electrical pain threshold (EPT) of the skin, fascia and muscle tissue after eccentric contractions of the finger, the EPT was only significantly reduced in the fascia. And further, that the pressure pain threshold (PPT) was reduced in fascia in a mirrored fashion with the increase in DOMS (Itoh, Okada, Kawakita, White, & Cummings, 2004). More recent evidence indicates that the same is true for the biceps brachii. Decreases in EPT have been shown to be significantly greater in fascia compared to muscle tissue in a DOMS affected biceps brachii. It was concluded that the increased pain sensitivity in DOMS is associated with inflammation in the fascia rather than muscle fibres (Lau et al., 2015a). This seems plausible given that nociceptors are sensitised by the inflammation caused by muscle damaging exercise (Ota et al., 2018) and the density of nociceptors in the fascia is higher than in muscle tissue (Andres, von Düring, Von During, & Schmidt, 1985). In the present study there was a relatively small but significant increase in UAC, indicating that the eccentric contractions had elicited an inflammatory response in the biceps brachii, albeit a minor one. It is likely that the swelling would have continued to increase over the subsequent 5-day period (Tanabe et al., 2015). In the present study the inflammatory response and corresponding increases in UAC only occurred

in the 48 (0.69mm) and 60 (0.61mm) repetition groups and was not present after 72 (0.03mm) repetitions. These were unexpectedly low 48 hour increases in UAC compared to studies using similar protocols: 5mm after 50 repetitions (Tanabe et al., 2015), 8ml after 60 repetitions (Newton et al., 2013).

2.4.6 Range of Motion

As expected, the eccentric exercise protocol resulted significant reductions in the ROM of the exercised arm. The decreases in ROM were significant after 60 repetitions (8°) and 72 repetitions (10°), but not after 48 repetitions (7°). These were unexpectedly moderate decreases in ROM compared to other work. For example a reduction in ROM of the elbow flexors of nearly 20° has been recorded after 24 maximal eccentric contractions (Nosaka et al., 2002) and 50 maximal eccentric contractions (Tanabe et al., 2015). After 60 a more marked reduction of 25° has been recorded (Lau et al., 2015b).

2.4.7 Anandamide

In humans, AEA has been shown to at least double in response to endurance exercise, running for example (Raichlen et al., 2013; Sparling et al., 2003). However, in the present study there was no significant increase in AEA following a resistance exercise protocol. Perhaps in a similar way to IL-6 the response differs between activities depending on metabolic cost.

2.4.8 Methodological considerations

Significant group differences may not have been detected because of the small sample size with 9 participants in the 48 and 60 rep groups but only 7 in the 72 rep group. Group differences may have been underpowered, i.e. there may not be enough statistical power in a small sample size to detect significant effects (Cohen & Cohen, 1988). In particular, the AEA results were based on 6, 3 & 2 participants

in the 48, 60 and 72 repetition groups respectively. It should also be noted that disparity in trained versus untrained participants may have interfered with the results for some biomarkers, controlling for exercise in the 48 prior to the biceps curls may not have been enough.

2.5 ***Conclusions***

A large number of maximal eccentric elbow flexion exercises produces measurable changes in biomarkers associated with DOMS, whether that be 48, 60 or 72 contractions. The null hypothesis can be accepted as no significant differences were found between the three groups for any biomarker. However, the within group response differed depending on the number of contractions and the biomarker involved showing more subtle effects. If we were to consider the pain of DOMS in itself to be the most relevant biomarker then the highest mean score for the 60 repetition group would seem important. And further, the increase in DOMS score for the 60 repetition group was highly significant ($p=0.000$). In addition, DOMS significantly correlated with ROM, which significantly decreased for the 60 and 72 repetition groups only. Significant increases in UAC were only recorded for the 48 and 60 repetition groups, with no significant increase for the 72 repetition group. Taken together the most appropriate number of repetitions for further testing would be the minimum number with the most response. Therefore 60 repetitions of maximal eccentric elbow flexion exercises were chosen for a nutritional intervention study.

Chapter 3 Phase two: Polyphenol Intervention.

3.1 *Introduction*

Antioxidants may be useful to reduce the oxidative stress response brought about by strenuous exercise. *Aronia melanocarpa* plants are widely found in Europe and the berries are considered to be one of the richest natural sources of antioxidants. The widely known antioxidant capacity of these berries is due to the concentrated levels of a group of bioactive compounds known as polyphenols. Exercise-induced muscle damage as a result of contractional stresses give rise to tissue damage and a stress response. This stress response results in oxidative stress and the production of reactive oxygen species (ROS). Antioxidants scavenge ROS and may attenuate some biomarkers of exercise-induced muscle damage (EIMD), for example, it has been shown to attenuate DOMS (Udani et al., 2009).

3.1.1 Polyphenolic compounds and health

Supplementation with berry extracts has been extensively studied of late for positive health effects, among them, *Aronia melanocarpa* for being one of the most active and richest sources of bioactive compounds (Lou-Bonafonte, Gabás-Rivera, Navarro, & Osada, 2017). Polyphenols, from *Aronia melanocarpa* have been shown *in vitro* to have a powerful antioxidant and anti-inflammatory effect (Gao et al., 2018). Particularly high levels of three classes of polyphenols: anthocyanins, proanthocyanidins and flavonols establish *Aronia melanocarpa* berries as a food supplement of interest to the research community. Consequently, research to further strengthen the association between these berries and anti-cancer, antibacterial and anti-inflammatory effects among others, is ongoing (D'Archivio et al., 2008; Dayem et al., 2016; Shukla & Mehta, 2015; Strugała, Gładkowski, Kucharska, Sokół-Łętowska, & Gabrielska, 2016). In an animal model,

supplementation of *Aronia melanocarpa* juice reduced chemically induced oxidative stress by 92% (Kujawska, Ignatowicz, Ewertowska, Oszmiański, & Jodynis-Liebert, 2011). On a cellular level polyphenols reduce oxidative stress in skeletal muscle by scavenging reactive oxygen species (ROS) which are known to cause cell damage (Goutzourelas et al., 2014).

The damaging effects of ROS have been well researched since being discovered in the 1950's (Gerschman, Gilbert, Nye, Dwyer, & Fenn, 1954). However, the role of ROS is more complex than originally understood, alongside the seemingly detrimental effects of ROS in skeletal muscle, these reactive molecules also have a positive effect on force production and regulate cell signalling pathways (McClung, Judge, Powers, & Yan, 2010; Powers & Jackson, 2008). In the exercise arena a few recent studies of athletes ingesting an Aronia-citrus drink reported an improvement in oxidative stress and inflammation. Furthermore, a positive effects on DNA repair, the central nervous system and the cardiovascular system were noted (García-Flores, Medina, Cejuela-Anta, et al., 2016; García-Flores et al., 2018; García-Flores, Medina, Oger, et al., 2016).

3.1.2 Antioxidants, exercise and DOMS

The limited understanding of the relationship between exercise, DOMS and ROS is partly due to the complex nature of ROS, the many species involved and the difficulty in accurately assessing those species (Jackson, 2016). Increased oxidative stress typically occurs in response to exercise, particularly prolonged endurance exercise or heavy bouts of resistance training (Howatson et al., 2010; Tomeleri et al., 2018). The oxidation of fatty acids and carbohydrates to meet the energy demands of exercise increase ROS in muscle tissue (Bailey et al., 2007;

Meisenberg & Simmons, 2012). The increase in ROS is generated by many biochemical processes, increases in the xanthane oxidase enzyme for example. In an animal model, an eccentric exercise protocol producing significant increases in xanthane oxidase also produced a concomitant increase in DOMS pain behaviours (Retamoso et al., 2016). Oxidative stress research *in vivo* in animals can be challenging, in humans more so (Jackson, 2016). Despite these challenges, understanding of exercise-induced oxidative stress has expanded over the last two decades (Powers, Radak, & Ji, 2016). Most notably, the discovery that; susceptibility to EIMD was increased when an antioxidant deficiency was present, and that; the endogenous antioxidant system protected against EIMD (Powers et al., 2016).

It has previously been observed that ROS elevations following a bout of exercise leads to an increased demand for antioxidant substances (Ji, 1995), and that antioxidant supplementation increases antioxidant status and mediates exercise-induced oxidative stress (Del Bo, Martini, Porrini, Klimis-Zacas, & Riso, 2015). Therefore, the effects of dietary antioxidant interventions on exercise-induced changes in biomarkers has been extensively studied, however, results can be somewhat conflicting. On a simple level, a reduction in exercise-induced oxidative stress following antioxidant vitamin supplementation has been shown with vitamins C & E (Machefer et al., 2007). Furthermore, in one study, acute vitamin C supplementation resulted in a reduction of DOMS of up to 44% (Kaminski & Boal, 1992). However, conclusions drawn from Kaminski and Boal (1992) are limited by the small sample size and the lack of an untreated control group. More recent research that used vitamin C, has shown a reduction in ROS without a reduction in

DOMS (Close et al., 2006). In a recent review of the effects of vitamin C & E on EIMD, it was concluded that there was no direct evidence that these vitamins reduce exercise-induced ROS. The final conclusion in that review was that it was still worth considering other methods of reducing ROS with alternative antioxidant interventions (Cobley, McHardy, Morton, Nikolaidis, & Close, 2015), polyphenolic compounds for example. Supplementation with polyphenols as opposed to antioxidant vitamins, has also been shown to reduce the oxidative stress brought about by exercise (Levers et al., 2016). In addition, the polyphenolic compound Curcumin has been shown to significantly reduce DOMS, though the mechanism of action remains unclear (Drobnic et al., 2014). Other agents that have a direct effect in exercised muscle have been tested as DOMS reducing agents. Ginseng for example interferes with the calcium efflux from exercising muscles and has been successfully used to reduce DOMS pain (Pumpa et al., 2013). Bromelain in pineapple juice, which inhibits pro-inflammatory agents and stimulates anti-inflammatory agents in muscle has also been shown to be a more successful DOMS reducing agent. Interventions using bromelain have resulted in a significant reduction of DOMS pain (Miller, Bailey, Barnes, Derr, & Hall, 2004; Udani et al., 2009). However, the evidence is equivocal, others have found no significant effect on DOMS pain with Bromelain (Beck et al., 2007; Stone, Merrick, Ingersoll, & Edwards, 2002). It is important to note that each substance tested has its own biochemical properties and specific mode of action on ROS in vivo (Murphy et al., 2011). The biochemical effect of each nutritional intervention will effect indices of EIMD, including the pain of DOMS, in a specific and unique manner depending on the dose.

Chronic supplementation with a polyphenol rich compound can be effective for increasing exercise performance (Deley, Guillemet, Allaert, & Babault, 2017) and reducing unfavourable symptoms of EIMD, e.g. DOMS (Levers et al., 2016). That being said, the effects of polyphenols on individual markers of EIMD, particularly DOMS, is not uniform across exercise research. Research involving polyphenols from Montmorency cherries have produced mixed results, successfully prevented some of the symptoms of EIMD but not others. For example, a polyphenol rich Montmorency cherry juice blend reduced self-reported pain scores for DOMS but had no effect on ROM (Connolly et al., 2006). In other research, cherry juice attenuated the rises in IL-6 and CRP but did not reduce DOMS in marathon runners (Howatson et al., 2010). Bell et al. (2016) later demonstrated the same attenuation of the inflammatory marker IL-6 *with* a corresponding reduction in DOMS following a sprint protocol. Similarly, Levers et al. (2016) reported attenuation of DOMS soreness alongside a 47% reduction of inflammation markers after a half marathon following 10 days of powdered Montmorency cherry supplementation. However, following a plyometric protocol with cherry juice supplementation, while the antioxidant capacity of the blood was increased by 67% there was no effect on CK or DOMS (Hillman, Taylor, & Thompkins, 2017). One explanation of the differences in effect and magnitude of the above interventions on EIMD is the variety of exercise protocols employed. Moreover, individual studies provide a different Montmorency cherry supplement, each with a unique polyphenolic profile.

Cherries are known for their numerous polyphenolic anti-inflammatory and antioxidant agents, and have been shown to significantly reduce biomarkers of

inflammation (Kelley, Rasooly, Jacob, Kader, & Mackey, 2006). The consumption of cherries is therefore receiving attention in the exercise physiology and biochemistry arena. How consumption of these berries specifically affect individual biomarkers of oxidative stress and EIMD has been investigated. For example, haematological results following 28 days of sweet cherries consumption showed a reduction in the inflammatory markers; Nitric oxide (NO), C-reactive protein (CRP), and tumour necrosis factor alpha (TNF-α). The cytokine Interleukin-6 (IL-6) however, was unaffected. Though these results demonstrated the effect of cherries on inflammatory markers, clear individual differences in response to the sweet cherry intervention were noted (Kelley et al., 2006). In contrast, Lynn et al. (2014) reported no effect of Montmorency cherry (*Prunus cerasus*) juice consumption on CRP as a biomarker for inflammation (Lynn et al., 2014). No influence on CRP was also reported in a later study using Montmorency cherry, in this case though, IL-6 and DOMS were both attenuated (Bell et al., 2016). When Elite weightlifters consumed either pomegranate juice containing a total of 20,480mg of polyphenols or a placebo, the pomegranate juice caused a suppression of oxidative stress markers which was not seen with the placebo (Ammar et al., 2016). In this case, the pomegranate juice did not attenuate DOMS, however, the juice significantly reduced CK ($p<0.001$). In addition, there was a highly significant correlation between DOMS and CK ($p<0.001$, $r=0.8$).

Each berry species has a unique polyphenolic profile and subsequent antioxidant activity. Polyphenols, from *Aronia melanocarpa* for example, have been shown to have a powerful antioxidant and anti-inflammatory effect (Gao et al., 2018).

Most antioxidant interventions in exercise research involved chronic or acute supplementation with a polyphenol rich compound, however, few involving *Aronia melanocarpa*. There has been some success with chronic supplementation of *Aronia melanocarpa* juice on markers of exercise-induced oxidative stress. For example, a daily Aronia-citrus juice reduced biomarkers of inflammation and oxidative stress in triathletes during a 21week training programme (García-Flores et al., 2018).

3.1.3 Polyphenols in berry fruits

Powdered berry fruits are more a more concentrated source of polyphenols than berry juice products. In addition, the phenolic content significantly differs between commercially available fresh juice products with up to four times the polyphenol content in some products compared to others (Sosnowska et al., 2016). Juice production from berry fruits creates 70-80% juice and 20-30% by-product known as pomace. The pomace by-product contains high levels of fibre and polyphenolic compounds and is therefore a valuable source of antioxidants. Berry pomace comprises mostly of fruit skin where concentrations of polyphenolic compounds are high, drying the pomace condenses and preserves much of the antioxidant compounds that remain in the skin following juice production (Brazdauskas, Montero, Venskutonis, Ibañez, & Herrero, 2016). The pomace as a by-product also contains especially high levels of the class of polyphenols known as anthocyanins (Oszmiański & Lachowicz, 2016). Powdered berry products are therefore exceptional as a nutritional source of polyphenol compounds

The levels of phenolic compounds found in berry fruits depends on the stage of ripening, e.g. total phenolic compounds decrease as the fruit ripens and total

anthocyanin content increases (Karaaslan, Yılmaz, Karaaslan, & Vardin, 2016). Following harvesting of *Aronia melanocarpa* the drying process can significantly reduce the total phenolic content (TPC), freeze drying is a key method which preserves 90% of the phenolic compounds. This process results in a total polyphenol content (TPC) of 7265mg/100g dry mass and 2227mg/100g dry mass of whole dried berries (Samoticha, Wojdyło, & Lech, 2016). However, the processing method of berry products can actually increase the resulting Polyphenol content. A combination of drying, using pectinolytic enzymes and milling the pomace increases the available polyphenols by more than double. And further, milling the pomace into smaller particles (Micro-milling) increases the TPC by a further 25%. In that study, *Aronia melanocarpa* pomace after course milling contained total polyphenols of 3,100–6,300mg/100g dry mass (dm) and 1190-1950mg/100g dm of anthocyanins. However, with micro milling this increased to 10,800mg/100g dm and 2,280mg/100g dm for TPC and anthocyanins respectively (Mayer-Miebach, Adamiuk, & Behsnilian, 2012). An integrated extraction-absorption processing maximises the extraction rate of almost all of the extractable polyphenols (Vauchel, Galván D'Alessandro, Dhulster, Nikov, & Dimitrov, 2015).

The polyphenol content of cherry fruit depends on the variety, tart cherry (prunus cerasus) tend to have substantially higher levels than sweet cherry (prunus avium) varieties. Compared to the sweet cherry variety Bing (prunus avium), the TPC content of the tart cherry Montmorency (prunus cerasus) was three times higher. That being said, the anthocyanin content was similar in both varieties (Picariello, De Vito, Ferranti, Paolucci, & Volpe, 2016). The highest polyphenol content of eight cherry varieties analysed (as fresh weight) was found to be the white variety of

prunus tomantosa at 8.33mg/g (fw), followed by the popular prunus cerasus with 6.06mg/g (fw) (Cao et al., 2015). Measured on a dry mass basis Wojdyło, Nowicka, Laskowski, and Oszmiański (2014) reported the TPC of prunus cerasus cherries as 29.83mg/g (dm) and total anthocyanin content (TAC) as 9.94mg/g (dm). *Aronia melanocarpa* on the other hand contain more than double the TPC at 72.65mg/g (dm) and TAC at 22.27mg/g (dm) (Samoticha et al., 2016). The most abundant anthocyanin in *Aronia melanocarpa* determined as cyanidin-3-galactoside (Ćujić et al., 2016).

3.1.4 Antioxidant activity of polyphenols in berry fruits

The antioxidant capacity of berry products as a result of the antioxidant compounds present can be tested *in vitro*. The oxidant scavenging activity of berries is measured in several ways, the most common methods are; oxygen radical absorbance capacity (ORAC), ferric ion reducing antioxidant power (FRAP) and 2,2-diphenyl-2-picrylhydrazyl (DPPH). These methods measure different types of scavenging activity, all three methods are standardised and expressed as Trolox Equivalents (TE).

The ORAC method measures total antioxidant capacity by introducing a substance to free radical generators and recording how much that substances neutralises these radicals. Berry extracts were exposed to peroxyl radicals generated by 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH), and the scavenging activity recorded. Measured on a fresh weight (fw) basis, the ORAC of berry fruits have been reported as follows: gooseberry (*Dovyalis hebecarpa*) 50.1µmol TE/g (Bochi, Barcia, Rodrigues, & Godoy, 2015); Morello cherry (*Prunus cerasus*) 38.9µmol TE/g

(Picariello et al., 2016); chokeberries (*Aronia melanocarpa*) 158.2μmol TE/g (Wu, Gu, Prior, & McKay, 2004).

Results of antioxidant activity measured as FRAP on a dry mass (dm) basis were as follows: tart cherry (*Prunus cerasus*) 12.95mmol TE/100g (Wojdyło et al., 2014); chokeberry pomace (*Aronia melanocarpa*) 47.38mmol TE/100g (Tolić et al., 2015); chokeberry pomace (*Aronia melanocarpa*) 53.78mmol TE/100g (Oszmiański & Lachowicz, 2016). Interestingly (Oszmiański & Lachowicz, 2016) compared *Aronia melanocarpa* juice to pomace on a dry weight basis and recorded DPPH as 127.45μM Trolox/100g and 301.89μM Trolox/100g respectively.

The above results were measured in vitro and as such do not take into account bioavailability and bioactivity within a living organism. *In vivo* studies in humans reveal factors that reduce the scavenging capacity of antioxidant compounds including but not limited to: absorption in the gut, genetics, medication and the food matrix (Holst & Williamson, 2008). Polyphenols after absorption in the gut are extensively modified and as such are chemically, biologically and functionally very different substances by the time the reach target cells and tissues (D'Archivio, Filesi, Varì, Scazzocchio, & Masella, 2010). The metabolites produced *in vitro* can be measured in plasma and urine to indicate antioxidant activity within the body, however, this is again subject to individual variability.

The uptake and excretion of anthocyanin metabolites varies greatly between individuals, excretion varying by a magnitude of ten between only 17 participants (Kalt, McDonald, Vinqvist-Tymchuk, Liu, & Fillmore, 2017). Feliciano, Istas, Heiss,

and Rodriguez-Mateos (2016) illustrated that this variability is due to individual differences in phase II enzymes and gut microbiota involved in the absorption and metabolism of polyphenolic compounds. Add in here polyphenol uptake kinetics section (Myburgh, 2014).

Like antioxidant vitamin C, anthocyanins would seem to be excreted via urine in a matter of hours. (Wiczkowski, Romaszko, & Piskula, 2010) reported that ingesting *Aronia melanocarpa* resulted in the presence of cyanidin metabolites in both blood and urine. Cyanadin-3-galactoside was measure by HPLC and found to make up 66% of the polyphenol profile of the *Aronia melanocarpa* ingested by the participants. The cyanidin metabolites peaked in blood at 1.3h and the urine excretion rate peaked in the first two hours, both measures returned to baseline within 24 hours. Exercise appears to increase bioavailability of polyphenolic compounds. A five-fold increase in urinary anthocyanin metabolites has been shown in athletes who consumed *Aronia melanocarpa* juice 15 minutes after training compared to untrained controls (Medina et al., 2012). Anthocyanin metabolites in urine and plasma roughly correlate (Kalt et al., 2017), and individual anthocyanin metabolites from *Aronia melanocarpa* peak in plasma between 1h and 6h (Xie et al., 2016).

3.1.5 Summary

Generally, antioxidant interventions involving vitamins C and E have little effect on muscle damage or pain (Urso, 2013). However, each oral intervention reduced inflammation in a very specific and complex way, for example; Vitamin C reduces, among other things, superoxide production (Cobley et al., 2015), and NSAIDS reduce prostaglandins by blocking the cyclo-oxygenase (COX) enzyme (Schoenfeld,

2012). There is a great diversity of antioxidant interventions, each with a unique and complex biochemical action. This leaves the possibility that further research has yet to uncover an intervention that reduces pain without detriment to adaptations.

Antioxidant substances such as Aronia melanocarpa may be useful in reducing exercise-induced oxidative stress. Aronia menalocarpa is a potent antioxidant containing high levels of polyphenols. Polyphenols have been shown to affect biomarkers of EIMD including DOMS, however, the evidence is equivocal. A single dose of Aronia melanocarpa has not been tested in an exercise intervention as a DOMS reducing agent.

3.1.6 Aims

To test the effect of a high dose of polyphenol rich *Aronia melanocarpa* on the biomarkers associated with DOMS.

3.1.7 Hypothesis

H_0 A single dose of *Aronia melanocarpa* extract has no effect on the biomarkers of delayed-onset muscle soreness (DOMS).

3.2 *Materials and Methods*

3.2.1 Participant volunteers

See section 2.2.1 above.

A total of 21 male university staff and students volunteered to participate in the DOMS antioxidant intervention study. Details of the second study were provided by verbal briefings and participant information sheets (Appendix 6). Participants were fully informed of procedures and risks before providing written informed consent (Appendix 7).

3.2.2 Experimental Design

This study utilised a randomised, single blind, placebo controlled independent group design. Participants attended the Exercise Physiology Laboratory at the University of Huddersfield to perform an eccentric exercise intervention on an isokinetic dynamometer (Cybex, USA). Biomarkers (including pain scores) were taken at three time points: before the exercise intervention (time point A), after exercise intervention (time point B) and at follow up 48 hours later (time point C) (figure 21). Participants were given an Aronia/placebo beverage after biomarkers were recorded and within half an hour of cessation of the exercise protocol.

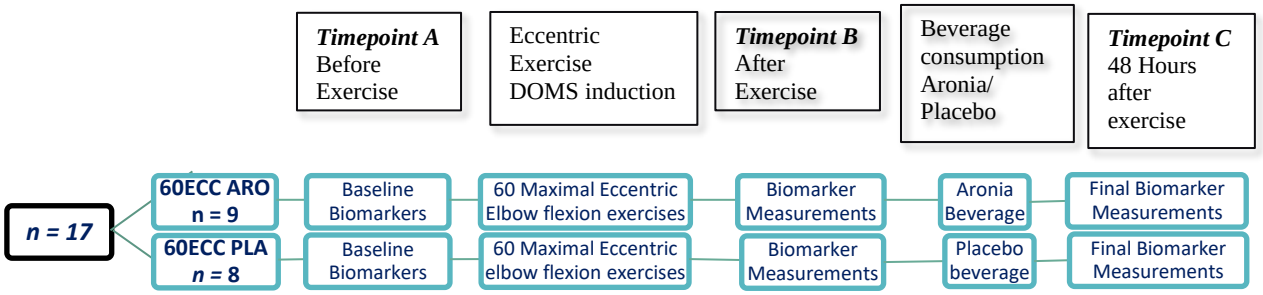


Figure 21. Overview of experimental design. The exercise intervention to induce DOMS was performed immediately following baseline biomarker measurements taken at time point A. Biomarker measurements were then repeated at time points B and C. Measurements recorded at each timepoint: upper arm circumference (UAC), range of motion (ROM) of the exercised arm, a recording of pain (VAS) in the biceps brachii and blood sampling took place.

3.2.3 Beverages

Aronia powder was milled for three minutes in a Nutribullet 600W blender (Nutribullet LLC.) to reduce particle size, then sieved to a particle size of <425µm. The manufactures recommended intake for this product was 1-2 tablespoons per day, the lower end of this recommendation i.e. 1 tablespoon (18.2g) was tolerated well in smoothie taste tests and was subsequently used in the experimental group drink. This quantity of Aronia powder would provide approximately 405.31mg of

polyphenols (Samoticha et al., 2016), and 9.79mmol TE of Ferric Ion Reducing Power (FRAP) (Oszmiański & Lachowicz, 2016). With regard to food allergies, participants were given a list of possible ingredients in their beverage before consumption (Appendix 8). The 'Canderel spoonful' sweetener contained a source of phenylalanine, the label was shown to participants to avoid consumption by participants with phenylketonuria.

Recipes for the Aronia/placebo beverages were as follows (appendix 9)

Experimental Aronia beverage;

60g Fresh banana	49kcal
120ml Sainsbury's Pineapple juice (ambient)	60kcal
18.2g Biojoy Aronia Pomace Powder	51kcal
1g Canderel Spoonful sweetener	4kcal
Total Kilocalories	164kcal

Macronutrient composition;

Energy	164kcal
Carbohydrate	31.66g
of which sugars	25.39g
Fat	0.62g
of which Saturates	0.16g
Protein	2.04g

Isocaloric Placebo beverage;

60g Fresh banana	49kcal
222ml Sainsbury's Pineapple juice (ambient)	111kcal
1g Canderel Spoonful sweetener	4kcal

Total Kilocalories	164kcal

Macronutrient composition;

Energy	164kcal
Carbohydrate	38.43g
of which sugars	34.68g
Fat	0.0g
of which Saturates	0.0g
Protein	0.73g

All beverages were blended in the Nutribullet for 30 seconds and served in a dome topped smoothie cup with three ice cubes and a jumbo straw. The drinks were formulated and made up by a nutritionist. Participants ingested the smoothie drink within 30 minutes of cessation of the exercise protocol, and consumption was confirmed through direct observation by the nutritionist. Calorific values were taken from packaging and recorded in kilocalories (kcal), with the exception of fresh banana. The caloric values for 60g of fresh banana was taken from the McCance and Widdowson nutrient tables.

3.2.4 Perceived Muscle Soreness

See section 2.3.3 above.

3.2.5 Creatine Kinase

See section 2.3.6 above.

3.2.6 Upper Arm Circumference

See section 2.3.4 above.

3.2.7 Range of Motion

See section 2.3.5 above.

3.2.8 Variables

The independent variable (IV) was exposure to the Aronia or placebo beverage. Dependent variables comprised of the subsequently recorded biomarkers at time point A (before exercise), time point B (after exercise), and time point C (48 hours later). Dependant variables (DV's) were as follows: DOMS, UAC, ROM and CK.

3.2.9 Statistical methods

Statistical analysis was performed using SPSS version 24 (IBM Corp. USA) with the significance level set at $p>0.05$ for all analyses. To determine normality, these data were assessed both visually (histograms) and statistically using the Shapiro-Wilk test for normality. Assumptions of homogeneity of variance were tested using the Levene's test for equality of variances. Central tendencies and measures of spread were derived for the DV's and expressed as mean ± standard deviation (SD) or median with interquartile range (IQR).

Significant main time effects were assessed using paired sample t tests for DOMS, and UAC. To detect significant differences at baseline, baseline data for these variables was compared between Aronia and control conditions using independent samples t-tests. Significant time effects within each condition, i.e. Aronia and control, were assessed using paired sample t tests. This entailed performing a within group comparison between baseline and 48 hours for DOMS and UAC. Group x time interactions were assessed using independent samples t test. A paired sample t test was then used to assess the effect of the Aronia beverage on change over 48 hours for DOMS and UAC. Where significant p values were returned for interaction effects (time x condition), Aronia was deemed to have influenced the response. Where significant differences were identified, 95% confidence intervals

were used to present the estimation of mean differences. Finally, Pearson product moment correlations were used to assess the relationship between DOMS scores and other outcomes.

To determine normality, CK data were assessed both visually (histograms) and statistically using the Shapiro-Wilk test for normality. For CK and ROM, non-parametric tests were used to assess baseline data (Wilcoxen Signed Rank Test), main time effects, and main time x condition effects (Mann-Whitney U) of these variables. Correlations with other dependent variables was assessed using a non-parametric Spearman's Rho correlation.

3.3 *Results*

3.3.1 Study population

Twenty-one volunteers were assessed for eligibility before taking part in the study. Of those volunteers, three did not report back to the physiology laboratory for follow up biomarkers. One participant had implausibly high CK at baseline and was excluded from any further analysis. Full blood samples were not possible for two participants and were excluded from blood biomarker analysis only. A total of 17 volunteers (27.1 ± 10.2 years; 77.8 ±10.3 kg; 177.7 ± 7.0 cm) completed the experimental protocol and were included for statistical analysis.

3.3.2 Indicators of Muscle damage

There were no significant differences between the experimental and control groups for any baseline measure. The exercise protocol was sufficient to induce significant changes in muscle damage biomarkers. A significant main time effect between baseline and 48 hours were found for; increases in DOMS (p=0.000) & creatine kinase (p=0.015); and decreases in ROM (p=0.004) & Anandamide (p=0.033).

Main time effect for increases in upper arm circumference approached significance (p=0.061). Table 3 shows mean and median values for soreness, upper arm circumference, range of motion and CK activity. The data for ROM and CK did not follow a normal distribution both visually (histogram) and statistically (Shapiro-Wilk), non-parametric tests were subsequently used for these data. All other variables returned a non-significant result on the Shapiro Wilk test, indicating a normal distribution of these data. Table 4 shows mean biomarker values for all variables by experimental and control group.

Table 3. Biomarkers of Exercise-induced Muscle Damage

Biomarker	Time point A Baseline	Time point B After exercise	Time point C 48 hours
Soreness (VAS 0-10)	0.00 ± 0.0	2.3 ± 1.8***	4.7 ± 2.2***
Creatine Kinase (IU/L)	91.6 (78.6)	89.6 (81.9)	153.0 (1258.6)**
Upper Arm Circ (cm)	31.5 ± 4.0	31.9 ± 3.9**	31.8 ± 3.9
Range of motion (Degrees)	150.0 (13.0)	135.0 (16.0)**	138.0 (13.0)**

Mean (SD)/median (IQR) values at baseline, immediately after and 48 hours after exercise for all participants (*n*=17). Asterisk superscript ** and *** denotes a significant difference relative to baseline within a group p<0.01 and p<0.001 respectively.

3.3.2.1 Perceived Muscle Soreness

There was a significant main time effect on DOMS ratings following the exercise protocol (p=0.000). All participants reported an increase in soreness after 48 hours (figure 22). Ratings of perceived muscle soreness did not differ between the two groups at baseline (p=0.000). After 48 hours, participants in the Aronia group reported 14% less pain than in the control group with mean increases in pain scores of 4.4cm (±2.2) and 5.1cm (±2.2) respectively. However, the difference in pain scores did not reach statistical significance (p=0.556).

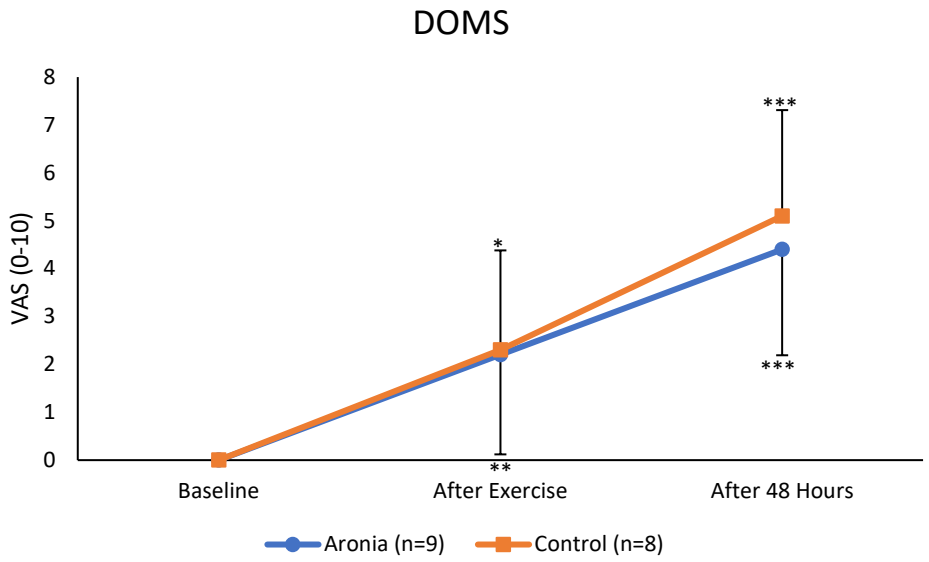


Figure 22. Increases in DOMS pain scores relative to baseline by Aronia melanocarpa (experimental) and control group. Mean ± SD values for each group are shown. No significant differences between groups were found (p=0.556). Asterisk superscript *, ** and *** denotes a significant difference relative to baseline within a group p<0.05, p<0.01 and p<0.001 respectively.

3.3.2.2 Creatine Kinase

There was a significant main time effect for CK activity (p=0.015). Plasma CK had significantly increased by 462% (p=0.036) in the placebo group. Plasma CK increased by 226% in the Aronia group, however this did not reach significance (p=0.173). Though CK activity in the Aronia group showed no significant difference between baseline and 48 hours (compared to a significant time effect in the control group), no significant group x time effect was found (p=0.248). Plasma CK activity was not significantly different between the two groups at any timepoint; baseline (p=0.736), immediately following exercise (p=0.847), and at 48 hours (p=0.191) (figure 23).

Figure 23. Change in Creatine Kinase activity relative to baseline by *Aronia melanocarpa* (experimental) and control group. Median ± IQR values for each group are shown. The Aronia group's CK values remained near baseline for the 48 hour period. No significant differences between groups were found ($p=0.191$). Significantly different to baseline $p<0.05$*.

Table 4. Biomarker outcomes displayed as mean (SD) or median (IQR).

	Aronia *(n=9)*		Control *(n=8)*		between groups; *p*
	Baseline	48 hours	Baseline	48 hours	
DOMS (VAS 0-10)	0.0 ± 0.0	4.4 ± 2.2***	0.0 ± 0.0	5.1 ± 2.2***	*p*=0.556
UAC (cm)	31.0 ± 3.6	31.1 ± 3.5	32.1 ± 4.6	32.5 ± 4.4	*p*=0.348
ROM (Degrees)	148.0 (18.0)	140.0 (6.0)	150.0 (10.0)	133.5 (29.0)*	*p*=0.177
CK (IU/L)	91.6 (51.8)	124.0 (108.6)*	94.6 (286.8)	1305.0 (1276.9)*	*p*=0.248

Significance assessed by t-test for Soreness, and UAC. Significance assessed for CK and ROM with the Mann Whitney U test. Asterisk superscript * and *** denotes a significant difference relative to baseline within a group $p<0.05$ and $p<0.001$ respectively.

3.3.2.3 Upper Arm Circumference

There was no main time effect for UAC, but this did approach significance (p=0.061). The UAC was not significantly different at 48 hours compared to baseline for either the Aronia group (p=0.468), or the control group (p=0.063). Homogeneity of variance was assumed according to Levene's test (p=0.844). There was no significant group x time interaction (p=0.348) (figure 24).

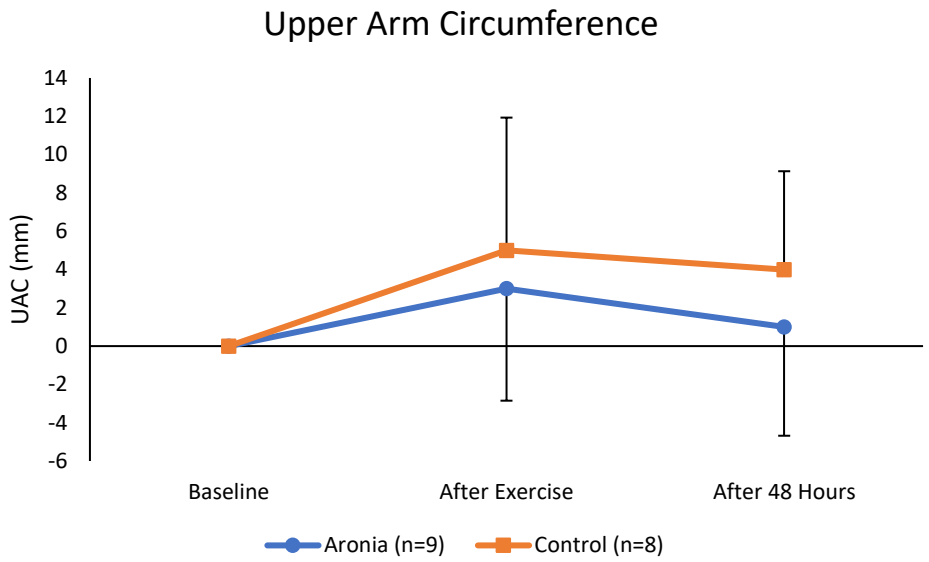


Figure 24. Changes in upper arm circumference (UAC) relative to baseline by *Aronia melanocarpa* (experimental) and control group. Mean ± SD values for each group are shown. No significant differences between groups were found (p=0.348). Upper arm circumferences did not significantly change from baseline for either the *Aronia melanocarpa* (experimental) and control group.

3.3.2.4 Range of Motion

There was a significant main time effect immediately after the exercise protocol (p=0.001) and after 48 hours (p=0.004) compared to baseline (table 3). There was a significant reduction in the range of motion of the elbow joint over 48 hours of 13.5° (p=0.012) in the control group. There was no difference in ROM between baseline and 48 hours in the Aronia group (p= 0.122). No significant group x time interaction (p=0.177) was found (figure 25).

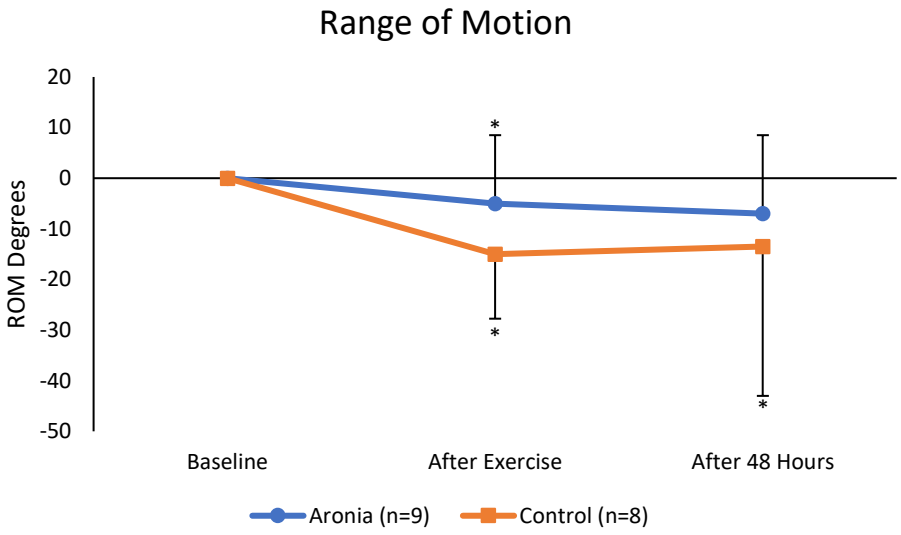


Figure 25. Changes in the range of motion (degrees) of the elbow joint relative to baseline by *Aronia melanocarpa* (experimental) and control group. Median ± IQR values for each group are shown. No significant differences between groups were found (p=0.556). *Significantly different to baseline p<0.05.

3.4 *Discussion*

The present study evaluated the effects of a single dose of polyphenols (*Aronia melanocarpa*) on DOMS related indices of muscle damage 48 hours after an acute bout of exercise. The exercise protocol was sufficient to induce DOMS pain as well as post exercise alterations in the other indices analysed; CK, IL-6, UAC & ROM. Similar changes have been reported in other studies (Guilhem et al., 2013; Lau et al., 2015b; Nosaka et al., 2002; Tanabe et al., 2015). Increases in the skeletal muscle enzyme CK and the cytokine IL-6 confirmed EIMD was sufficient to evaluate

the potential effects of a dietary antioxidant supplement on DOMS. The Aronia group had a lessor response to the bicep curl exercise protocol. The Aronia group reported less pain although this did not reach significance. Increases in CK activity in response to the exercise protocol in the Aronia group were half that of the control group, and a decrease in ROM only occurred in the contro

3.4.1 Polyphenols and DOMS

The effect of polyphenols on DOMS pain is limb specific. Though there is some evidence to suggest that polyphenols have no effect on DOMS pain for either arms or legs (Machin et al., 2014), other research has found that arms and legs respond differently (Ammar et al., 2016). These differences may be explained by the different methodologies. In research conducted by Machin et al. (2014), downhill running versus maximal eccentric elbow flexion was used, which may not have a comparable eccentric element. In research conducted by Ammar et al. (2016), comparison between limbs was possible because the 'clean & jerk with squat' provided resistance exercise that was heavily eccentric on both arms and legs. The polyphenol supplement resulted in a significant improvement in DOMS pain in the legs but this effect was absent in the elbow flexors. This indicates that polyphenols have a lesser effect on DOMS in the elbow flexors, which may be one of the reasons that the effect on DOMS in the present study was minor.

Utilising a similar design to the present study, but using a chronic supplementation strategy, a mixed polyphenol powder containing pomegranate, mangosteen and elderberry was shown to significantly reduce DOMS (Romain et al., 2017). The polyphenol rich powder contained a TPC of 146.1mg/g and anthocyanin content of 3mg/g. This equated to a total dose of 1,095.75mg of polyphenols which is more than twice as much as the 405.31mg single dose given in the present study. With

the pomegranate, mangosteen and elderberry intervention, DOMS pain was significantly lower after 48 hours when DOMS was attenuated by 33% (p=0.008) (Romain et al., 2017). The reduction in pain in the present study with acute supplementation was a much smaller 14% after 48 hours and did not reach significance. The large reductions in pain demonstrated in Romain et al. (2017) is likely due to the chronic versus acute supplementation strategy, in that the 1,095.75mg dose of polyphenols was ingested in 15 equal doses over a five-day period starting on the day of exercise. Similar results were reported following chronic supplementation over an eight-day period starting four days before exercise. This resulted in a 31% reduction in DOMS (p=0.017) 48 hours after cessation of the exercise protocol (Connolly et al., 2006). In Connolly et al. (2006), the total dose of polyphenols was 9,600mg, of which, 640mg was anthocyanins. This again was double the 405.31mg of polyphenols in the present study. It has been shown that anthocyanins reach maximal concentrations between 1 and 6 hours post ingestion after which point, plasma concentration begin to fall (Myburgh, 2014). And further, that the resulting plasma metabolites do not bioaccumulate, and that repeated dosing is required to maintain levels in the blood (Feliciano et al., 2016). It seems likely therefore that chronic supplementation over a period of days using the same quantity of Aronia powder in the present study would significantly reduce DOMS. However, it is also clear that the polyphenolic profiles of each supplement differ which in itself will have an impact on the efficacy of each supplemental regimen. And further, a whole array of metabolites appear in plasma following ingestion of polyphenols and the effects of individual metabolites are not yet clear. What is also interesting about research where DOMS has been significantly reduced with polyphenols is the effect on performance markers, for example; 13% faster marathon (p=0.001) (Levers et al., 2016), strength loss

attenuation of 18% (P<0.0001) (Connolly et al., 2006), and 3% faster agility tests (p=0.017) (Bell et al., 2016).

3.4.2 Polyphenols and Creatine Kinase

The ingestion of polyphenols blunts the CK response to muscle damaging exercise, however, this effect is dose dependent. The present study found no effect on CK activity following the ingestion of a single dose of *Aronia melanocarpa*, this may be because there was not enough bioactives produced in the blood by the dose given. The quantity of polyphenols, the number of doses given and the timing of those doses in relation to the bout of exercise effects the levels of bioactive compounds in the blood. The levels of bioactive compound in the blood must be high enough to elicit an effect, and must coincide with the biochemical response to the exercise bout. The levels of bioactives in the blood also varied depending on bioavailability, absorption kinetics and the polyphenolic profile of the supplement ingested. Maximum concentration in the blood are reached between 1.5hrs and 5.5hrs after ingesting a single dose depending on the polyphenols involved (Manach, Williamson, Morand, Scalbert, & Remesy, 2005). It follows therefore that acute dosing with a single dose is likely to elicit a lesser effect than chronic dosing with several doses. And further, higher doses over a longer period of time are likely to elicit a more powerful effect. For example, chronic supplementation of curcumin resulted in significantly smaller increases in CK activity in the magnitude of -48% (McFarlin et al., 2016). Conversely, Drobnic et al. (2014) found no effect of ingesting curcumin on CK activity. Though these were both chronic doses, the contrast in results is likely to be due to amount and timing of the doses. McFarlin et al. (2016) gave a higher dose over a longer period of time, i.e. 2400mg over 6 days compared to (Drobnic et al., 2014) who gave 1600mg over 4 days. Similar to the present study, the effect on CK can be absent altogether with an acute dose

(Peschek, Pritchett, Bergman, & Pritchett, 2013). The present study found no significant difference between the supplemented and placebo groups for CK activity. It is likely that the polyphenol intervention did not result in sufficient bioactives in the blood to effect CK enough to show a statistically significant difference between the groups.

Though significant differences are not always found, the blunting effect of polyphenols on exercise-induced CK activity may be apparent in subtle differences between experimental and control groups. For example, though no significant difference in CK were found following ingestion of a polyphenol rich extract, the levels of CK returned to baseline quicker in the supplementation group (Romain et al., 2017). Even when significant differences are not detected between groups, CK activity is often lower in the groups that ingest the polyphenols (Clifford et al., 2016; Levers et al., 2016; McLeay et al., 2012). In the current study, though CK activity increased by 226% in the Aronia group, this increase was not statistically significantly different from baseline values. The control group saw an increase in CK activity of 462%, which *was* a statistically significant increase from baseline. Given that the 48-hour increase in CK was not statistically significant in the Aronia group it is likely that the polyphenol intervention produced a dampening effect on CK activity in this group. Is also possible that the effects of the polyphenols would have become apparent after the 48 hour follow up in the present study. The levels of CK are likely to still be rising at 48 hours and the blunting of the CK response may have become apparent *after* that time, i.e. To illustrate this point, in both Tanabe et al. (2015) and McFarlin et al. (2016) where CK was significantly attenuated with curcumin, CK peaks were measured on day four and attenuated by 56% and 69% respectively.

3.4.3 Polyphenols and inflammation

The anti-inflammatory effect of polyphenols has been well documented (Goutzourelas et al., 2014; Howatson et al., 2010; Kujawska et al., 2011). It would therefore seem logical that polyphenols would influence swelling and ROM. However, the evidence is equivocal at best (Urso, 2013). In line with the present study, most evidence shows no effect of polyphenols on swelling of the exercised limb even though inflammation is reduced in the polyphenol groups. Depending on the polyphenols, the suppression of inflammation is at its highest when metabolites peak between 1.5 and 5.5 hours after ingestion. After this time, metabolites are being lost in urine, therefore the effects of a single dose are relatively short lived and repeated doses are required to sustain elevated metabolites capable of reducing inflammation. As chronic supplementation with polyphenols brings inflammatory markers back to baseline quicker than placebo (Ammar et al., 2016), it seems logical that chronic doses would reduce swelling in the same timeframe. However, after chronic supplementation with quercetin, no effect on swelling was found (O'Fallon et al., 2012). This evidence suggests that the anti-inflammatory effects of polyphenols do not extend to the swelling caused by EIMD.

Polyphenols consumption may have a small effect on ROM. ROM in the elbow is reduced as a consequence of an intense bout of elbow flexion exercises, with a loss of around 9°s (T. C. Chen et al., 2009). On the whole, supplementation with polyphenols does not significantly attenuate ROM. It seems evident that even though the differences do not reach significance, the reduction in ROM in the polyphenol groups is lower than the placebo groups (Pereira Panza, Diefenthaeler, & da Silva, 2015). For example, an acute dose of the polyphenol gingerol has been shown to have no effect on ROM. However, the reduction in ROM was lower at all

timepoints in the gingerol group (Tanabe et al., 2015). Even with chronic supplementation with gingerol, no group x time effect on ROM was reported, however, again the supplemented group showed a smaller reduction in ROM (Black, Herring, Hurley, & O'Connor, 2010). The results in the present study were interesting because a small effect on ROM was evident i.e. there was a significant time effect on the range of motion of the elbow joint of -16.5° ($p=0.012$) in the control group but no time effect in the Aronia group ($p= 0.122$). The absence of a time effect in the aronia group could indicate an attenuation of the loss of motion, even when a group effect was not apparent.

3.5 *Conclusions*

The null hypothesis can be accepted as no significant differences were recorded between groups. However, there were subtle within group differences. For the measures of ROM and CK, there was no change in the *Aronia melanocarpa* group. In contrast, there was a significant decrease in ROM and a significant increase in CK in the control group. This suggests that *Aronia melanocarpa* attenuated the response for these two biomarkers, though in such a small sample size group differences were too subtle to detect.

Chapter 4 Thesis conclusion

In this investigation the aim was to design an evidence based DOMS induction protocol that could be used to test an antioxidant intervention using *Aronia melanocarpa* extract. This was successfully achieved in the two phases of this research. To the best of the authors knowledge *Aronia melanocarpa* extract had not been tested as a strategy to reduce the pain of DOMS.

4.1 *Protocol Development*

There is a large variation in exercise protocols used to induce DOMS for experimental testing. For example, the biomarkers vary from one protocol to the

next as does the speed, number and type of contraction. By reviewing the evidence for each aspect of DOMS induction protocols it was possible to construct an evidence based protocol with relevant biomarkers. In phase one, a protocol was successfully developed which produced measurable changes in biomarkers of EIMD. Increases in pain, CK and swelling were recorded alongside a decrease in the elbow's range of motion after 48, 60 and 72 repetitions of maximal eccentric isokinetic elbow flexion exercises. Every participant experienced DOMS following each of the exercise protocols with the highest and most significantly increased ($p<0.001$) DOMS scores being recorded in the 60 repetition group compared to baseline. Given also that no swelling was apparent in the 72 repetition group and no change in ROM in the 48 repetition group, 60 repetitions was the minimum number with the most pronounced response and was therefore deemed appropriate for antioxidant testing in phase two.

4.2 ***Protocol application***

Following the development of a successful DOMS induction protocol in phase one, it was now possible to use the protocol to test the effects of the ingestion of an antioxidant compound in phase two. It was hypothesised that an antioxidant compound would reduce some of the symptoms associated with EIMD, in particular, DOMS. The protocol established in phase one encompassed 60 repetitions of maximal eccentric elbow flexion exercises and a set of biomarkers that indicate the biochemical and physical response. A similar biochemical response was seen in the control group in phase two compared to the 60 repetition group in phase one, e.g. pain 5.1 and 5.0 respectively. The protocol replicated well with the addition of an antioxidant intervention and allowed for the comparison of the biochemical response between the experimental and control (who did not ingest a dose of

antioxidants) groups. This meant that the effects of the antioxidant intervention could be reported.

4.3 *Aronia melanocarpa intervention*

Ingestion of *Aronia melanocarpa* extract successfully altered the biochemical response to exercise. Subtle within group effects suggest a single dose resulted in the blunting of some biomarkers of EIMD, specifically CK and ROM. The normal increases in CK and decreases in ROM in response to a large number of elbow flexion exercises did not occur in the *Aronia melanocarpa* group. This lack of change for ROM and CK in the experimental group suggests that *Aronia melanocarpa* ingestion in some way attenuated these responses. In contrast, significant increases in CK and decreases in ROM were recorded in the control group. And further, higher scores were recorded for swelling and pain in the control group. Based on these results, it can be concluded that a single dose of *Aronia melanocarpa* had a blunting effect on some aspects of the biochemical response to a bout of eccentric exercise. It is possible that several doses of *Aronia melanocarpa* would strengthen these effects and make significant differences between groups more statistically detectable, particularly with respect to DOMS pain.

4.4 *Further Research*

There is still much to be discovered about the pain of DOMS and strategies to reduce that pain. *Aronia melanocarpa* as an antioxidant intervention would seem to hold promise, though chronic rather than acute supplementation should be considered for future research. Furthermore, as the mode of action of this supplement reduces oxidation, a proxy measure of ROS such as Malondialdehyde would be advantageous. The *Aronia melanocarpa* intervention seemed effective on certain biomarkers, however a limited number of participants undoubtedly

underpowered the statistical analysis. Therefore the main limitation of this research was the small sample size, a larger group size in future research would increase the statistical power and potentially be more likely to determine group differences (Cohen & Cohen, 1988).

Appendices

Appendix 1

Participant Information Sheet Phase 1 "DOMS Protocol"

University of HUDDERSFIELD
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Investigation into Delayed Onset Muscle Soreness (DOMS).

INFORMATION SHEET

You are being invited to take part in a study about Delayed Onset Muscle Soreness (DOMS). Before you decide to take part it is important that you understand why the research is being done and what this will involve. Your contribution to this research is entirely voluntary and you are not obliged in any way to participate, you can also withdraw at any time. Please take time to read the following information carefully and ask me if there is anything that is not clear or if you would like more information.

WHAT IS THE RESEARCH ABOUT?

We are investigating Delayed Onset Muscle Soreness (DOMS). The soreness that occurs in the muscle in the hours and days after exercise. We are asking participants to do either 48, 60 or 72 repetitions of bicep curls on an *isokinetic dynamometer* pictured here. We would like to record DOMS signs and symptoms. Symptoms of mild pain, stiffness and inflammation are likely to be felt in your arm between 24 and 72 hours after the exercises. We would like to record the *level of pain* you experience in your arm, measure your *arm circumference* and take *blood samples*. The blood samples will be analysed for biomarkers e.g. Interleukin 6, glucose, endocannabinoids and Creatine Kinase.

Isokinetic dynamometer

EXLUSION CRITERIA. YOU CANNOT TAKE PART IF YOU.....

..... are currently having anticoagulation therapy (blood thinning drugs), e.g. Warfarin, Aspirin.
..... have a bleeding disorder, e.g. haemophilia.
..... have diabetes.
..... are taking anti-inflammatory medicines, e.g. Aspirin, Ibuprofen.
..... have any muscle or joint disorders in your arms, e.g. Arthritis.

WHAT WILL I NEED TO DO?

If you agree to take part you will be asked to attend two sessions, the first taking around half an hour and the second around 15 minutes. In the first session you will be asked to lie down on the isokinetic dynamometer and perform bicep curl exercises in sets of 12 using your non-dominant arm. You will be asked to provide three venous blood samples (18ml in total) and three blood droplet samples from your thumb or finger. The first blood samples *before exercise*, the second *immediately after exercise* and a third *48 hours after exercise*. These samples will be taken from your dominant arm. After a blood test some people experience a little bleeding or bruising. If you experience more serious symptoms contact your GP.
If possible, please do not take pain relief medicines such as Aspirin and Ibuprofen during the research period and let the researcher know if you have. The research period runs from two days before your first visit until you leave after your second visit.

WHAT WILL HAPPEN TO MY INFORMATION?

All information collected during the research will be kept secure. Your name will be removed to ensure you cannot be identified both during the research period and afterwards when the research is published.

WHAT WILL HAPPEN TO MY BLOOD SAMPLES?

Your blood samples *will not* have your name on them. All blood samples will be stored without DNA identifying cells.

WHO CAN I CONTACT FOR FURTHER INFORMATION?

Katie Speirs BSc (Hons). Researcher. katie.speirs@hud.ac.uk	Dr Michael Fish. Lecturer - Sport, Exercise & Nutritional Sciences. m.fish@hud.ac.uk. Office HW3/14

Appendix 2

Informed Consent form 'DOMS protocol'

University of HUDDERSFIELD
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PARTICIPANT ID

Investigation into Delayed Onset Muscle Soreness (DOMS).

CONSENT FORM

It is important that you read, understand and sign this consent form.
If you require any further details please ask or send an email to an address below.

I have been fully informed of the nature and aims of this research as outlined in the Information Sheet version X, dated 00:00:00.	YES ☐	NO ☐
I confirm that I have read and understood the exclusion criteria on the Information Sheet and I am eligible to take part.	YES ☐	NO ☐
I am aged 18 years or over.	YES ☐	NO ☐
I consent to taking part in this research.	YES ☐	NO ☐
I consent to having blood samples taken.	YES ☐	NO ☐
I understand that I have the right to withdraw from the research procedures at any time without giving a reason.	YES ☐	NO ☐
I understand that the information collected will be kept under secure conditions. And that my identity will be protected by the use of a participant identification (ID) number and I will not be identified in any reports or publications.	YES ☐	NO ☐
I understand that no person other than the researchers will have access to the information provided.	YES ☐	NO ☐
I understand that this research will be published.	YES ☐	NO ☐

If you are satisfied that you understand the Information Sheet and are happy to take part in this project please put a tick in the box aligned to each sentence above and print and sign below.

DECLARATION

Name of participant _____ Contact telephone number _____

Signature of participant _____ Contact Email Address _____

Name of Researcher_____ Signature of Researcher_____

BLOOD SAMPLE COLLECTION

Date_____ Time _____

Signed _____ Print name_____

WHO CAN I CONTACT FOR FURTHER INFORMATION?

Katie Speirs BSc (Hons). Researcher.	Dr Michael Fish. Lecturer - Sport, Exercise & Nutritional Sciences.
katie.speirs@hud.ac.uk	m.fish@hud.ac.uk. Office HW3/14

(one copy to be retained by Participant / one copy to be retained by Researcher)

Appendix 3

Health Questionnaire

University of HUDDERSFIELD
Inspiring tomorrow's professionals

PARTICIPANT ID

Investigation into Delayed Onset Muscle Soreness (DOMS).

HEALTH QUESTIONNAIRE

ABOUT YOU……		
Height	Weight	Male/Female
Age	Ethnicity	

ABOUT YOUR HEALTH…..		
Do you smoke? If yes, how many per day?	YES ☐	NO ☐
Do you drink alcohol? If yes, how many units per week?	YES ☐	NO ☐
Do you have any medical conditions? If yes, please list here.	YES ☐	NO ☐
Are you currently taking any medications? If yes, please list here.	YES ☐	NO ☐
Are you taking recreational drugs? If yes, what type?	YES ☐	NO ☐
Do you exercise regularly? If yes, what do you do?	YES ☐	NO ☐
Do you take dietary supplements? e.g. multivitamins, Cod Liver Oil or any other supplements. If yes, which ones?	YES ☐	NO ☐
How many times per week do you eat oily fish? e.g. salmon and mackerel.		

WHO CAN I CONTACT FOR FURTHER INFORMATION?	
Katie Speirs BSc (Hons). Researcher. katie.speirs@hud.ac.uk	Dr Michael Fish. Lecturer - Sport, Exercise & Nutritional Sciences. m.fish@hud.ac.uk. Office HW3/14

Appendix 4

Participant testing record

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PARTICIPANT ID

Investigation into Delayed Onset Muscle Soreness (DOMS).

PARTICIPANT TESTING RECORD

EXPERIMENTAL						
Dominant Arm, throw a ball (CYBEX use non-dominant arm)					**R**	**L**
Elbow flexion exercises (1 min rest between)					**12 REPS X 4/5/6 SETS**	
SETS	**1**	**2**	**3**	**4**	**5**	**6**

UPPER ARM CIRCUMFERENCE (UAC)				
Before exercise	**A**	DATE	TIME	RESULT **cm**
After exercise	**B**	DATE	TIME	RESULT **cm**
48 hours	**C**	DATE	TIME	RESULT **cm**

RANGE OF MOTION (ROM)				
Before exercise	**A**	DATE	TIME	RESULT ^{0}
After exercise	**B**	DATE	TIME	RESULT ^{0}
48 hours	**C**	DATE	TIME	RESULT ^{0}

RATE OF PERCEIVED EXERTION (RPE)				
After exercise	**B**	DATE	TIME	1-10

PAIN SCALE RESULTS (VAS)				
Before exercise	**A**	DATE	TIME	1-10 **cm**
After exercise	**B**	DATE	TIME	1-10 **cm**
48 hours	**C**	DATE	TIME	1-10 **cm**

CREATINE KINASE (CK)				
Before exercise	**A**	DATE	TIME	RESULT **U/L**
After exercise	**B**	DATE	TIME	RESULT **U/L**
48 hours	**C**	DATE	TIME	RESULT **U/L**

CONTACT INFORMATION	
Katie Speirs BSc (Hons). Researcher. katie.speirs@hud.ac.uk	Dr Michael Fish. Lecturer - Sport, Exercise & Nutritional Sciences. m.fish@hud.ac.uk. Office HW3/14

Appendix 5

Participant aftercare sheet

PARTICIPANT ID

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Investigation into Delayed Onset Muscle Soreness (DOMS).

PARTICIPANT AFTERCARE AND TEST RESULTS

AFTERCARE
Please refrain from heavy lifting for a couple of hours after having a blood test. **You are likely to experience mild pain in the muscles of the exercised arm. This mild pain should increase over the next two days.**

YOUR SECOND APPOINTMENT	
Day	Time
Please continue to refrain from exercise until you have been to your second appointment. Do not eat or drink (except water) for two hours before your second appointment.	

UPPER ARM CIRCUMFERENCE	
Before exercise	RESULT **cm**
After exercise	RESULT **cm**
48 hours after exercise	RESULT **cm**

PAIN SCALE RESULTS	
Before testing	1-10 **cm**
After testing	1-10 **cm**
48 hours	1-10 **cm**

CREATINE KINASE NORMAL RESTING AND EXERCISE RANGE AND EXPLANATION???	
Before exercise	RESULT **U/L**
After exercise	RESULT **U/L**
48 hours after exercise	RESULT **U/L**

The accuracy of the above results cannot be guaranteed and are for your interest only.

Appendix 6

Participant Information Sheet Phase 2 "Aronia Intervention"

University of HUDDERSFIELD
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Investigation into Delayed Onset Muscle Soreness (DOMS).

INFORMATION SHEET

You are being invited to take part in a study about Delayed Onset Muscle Soreness (DOMS). Before you decide to take part it is important that you understand why the research is being done and what this will involve. Your contribution to this research is entirely voluntary and you are not obliged in any way to participate, you can also withdraw at any time. Please take time to read the following information carefully and ask me if there is anything that is not clear or if you would like more information.

WHAT IS THE RESEARCH ABOUT?

Delayed Onset Muscle Soreness (DOMS), is the soreness that occurs in the muscle in the hours and days after exercise. Participants will be asked to do 5 sets of bicep curls on an isokinetic dynamometer pictured here. Symptoms of mild pain, stiffness and inflammation are likely to be felt 24 hours after these exercises. Participants will be given a fruit drink to test the effect on the level of mild pain in the exercised arm. We would like to record this level of pain, measure the arm circumference and take blood samples. The blood samples will be analysed for biomarkers e.g. Interleukin 6, endocannabinoids and Creatine Kinase.

Isokinetic dynamometer

EXLUSION CRITERIA. YOU CANNOT TAKE PART IF YOU.....

..... are currently having anticoagulation therapy (blood thinning drugs), e.g. Warfarin, Aspirin.
..... have a bleeding disorder, e.g. haemophilia.
..... have diabetes.
..... are taking anti-inflammatory medicines, e.g. Aspirin, Ibuprofen.
..... have any muscle or joint disorders in your arms, e.g. Arthritis.

WHAT WILL I BE ASKED TO DO?

If you agree to take part you will be asked to attend two sessions, the first taking around half an hour and the second around 15 minutes. During the first visit you will be given a fruit drink made only from natural ingredients. Also during the first visit, you will be asked to lie down on the isokinetic dynamometer and perform 5 sets of bicep curls using your non-dominant arm. You will be asked to provide three venous blood samples (18ml in total) and three blood droplet samples from your thumb or finger. The first blood samples *before exercise*, the second *immediately after exercise* and a third *48 hours after exercise*. After a blood test some people experience a little bleeding or bruising. If you experience more serious symptoms contact your GP.

If possible, please do not take pain relief medicines such as Aspirin and Ibuprofen during the research period and let the researcher know if you have. The research period runs from two days before your first visit until you leave after your second visit.

WHAT WILL HAPPEN TO MY INFORMATION?

All information collected during the research will be kept secure. Your name will be removed to ensure you cannot be identified both during the research period and afterwards when the research is published.

WHAT WILL HAPPEN TO MY BLOOD SAMPLES?

Your blood samples *will not* have your name on them. All blood samples will be stored without DNA identifying cells.

WHO CAN I CONTACT FOR FURTHER INFORMATION?

Katie Speirs BSc (Hons). Researcher. katie.speirs@hud.ac.uk	Dr Michael Fish. Lecturer - Sport, Exercise & Nutritional Sciences. m.fish@hud.ac.uk. Office HW3/14

Appendix 7

Informed Consent form 'Aronia intervention'

University of HUDDERSFIELD
Inspiring tomorrow's professionals

PARTICIPANT ID

Investigation into Delayed Onset Muscle Soreness (DOMS).

CONSENT FORM

It is important that you read, understand and sign this consent form.
If you require any further details please ask or send an email to an address below.

I have been fully informed of the nature and aims of this research as outlined in the Information Sheet version.	YES ☐	NO ☐
I confirm that I have read and understood the exclusion criteria on the Information Sheet and I am eligible to take part.	YES ☐	NO ☐
I am aged 18 years or over.	YES ☐	NO ☐
I consent to taking part in this research.	YES ☐	NO ☐
I consent to having blood samples taken.	YES ☐	NO ☐
I consent to drinking a fruit drink.	YES ☐	NO ☐
I understand that I have the right to withdraw from the research procedures at any time without giving a reason.	YES ☐	NO ☐
I understand that the information collected will be kept under secure conditions. And that my identity will be protected by the use of a participant identification (ID) number and I will not be identified in any reports or publications.	YES ☐	NO ☐
I understand that no person other than the researchers will have access to the information provided.	YES ☐	NO ☐
I understand that this research will be published.	YES ☐	NO ☐

If you are satisfied that you understand the Information Sheet and are happy to take part in this project please put a tick in the box aligned to each sentence above and print and sign below.

DECLARATION

Name of participant _____ Contact telephone number _____

Signature of participant _____ Contact Email Address _____

Name of Researcher_____ Signature of Researcher_____

BLOOD SAMPLE COLLECTION

Date_____ Time _____

Signed _____ Print name_____

WHO CAN I CONTACT FOR FURTHER INFORMATION?

Katie Speirs BSc (Hons). Researcher.	Dr Michael Fish. Lecturer - Sport, Exercise & Nutritional Sciences.
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(one copy to be retained by Participant / one copy to be retained by Researcher)

Appendix 8

Smoothie ingredients sheet for participants.

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Investigation into
Delayed Onset Muscle Soreness (DOMS).

FRUIT SMOOTHIE RECIPE

Experimental drink
60g Fresh Banana
18.2g (2 tbls) Biojoy Aronia powder (425micron particle size)
120ml Sainsbury's Pineapple juice
0.5g Canderel Sweetener

Placebo drink
60g Fresh Banana
120ml Sainsbury's Pineapple juice
0.5g Canderel Sweetener

MIXING & SERVING
All ingredients (except 20ml of Pineapple juice) into Nutribullet and mix for 20 seconds. Pour into dome lid serving cup and add remaining pineapple juice to nutribullet cup. Shake nutribullet cup to mix remaining drink and add to serving cup. Stir and serve with 3 ice cubes and a straw.

Appendix 9

Recipe Experimental and Control Smoothie Drinks.

Experimental Smoothie Ingredients	Quantity	Calories
Sainsbury's pineapple juice (ambient)	120ml	60kcal
Fresh Banana	60g	57kcal
Biojoy Aronia Powder (425µm particle size)	18.2g	51kcal
Canderel granulated bulk Sweetener	1g	2kcal
	Total	170kcal

Control Smoothie Ingredients	Quantity	Calories
Sainsbury's pineapple juice (ambient)	222ml	111kcal
Fresh Banana	60g	57kcal
Canderel granulated bulk Sweetener	1g	2kcal
	Total	170kcal

Procedure

The ingredients for each drink were weighed into a Nutribullet cup using scales. The ingredients were then processed for 20 seconds in the Nutribullet 600w blender. Each smoothie was decanted into a domed lid smoothie cup and refrigerated for up to two hours. Three ice cubes and a straw were added before serving.

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