# The Change in the Concentration Levels of Extracellular Vesicles and of Cytokines in the circulatory blood system in Response to different exercise protocols

**Ayman A Abu-Seer**

Thesis submitted in partial fulfilment of the requirements of the award of Doctor of Philosophy

Department of Sport and Health Sciences

Oxford Brookes University

# Abstract


## Introduction

There is little is known about the effects of different types of exercise and the release of extracellular vesicles (EVs) during exercise into the circulatory blood system. Also, there is a paucity of published data on the relationship between different modalities and types of exercise and the secretions of inflammatory cytokines into the circulatory blood system. Moreover, although it is becoming increasingly evident that a physically active lifestyle is associated with lower concentration levels of inflammatory markers, many studies have researched only leisure-time physical activity and have not investigated whether different modalities and eccentric exercise as compared to moderate intensity concentric cycling exercise may be associated with changes in inflammatory marker concentrations. Therefore, this study aims to measure the content of extracellular vesicles levels in resting healthy male individuals and then to use this information to investigate the systemic concentration levels of EVs and inflammatory cytokines in response to eccentric exercise in healthy individuals.

## Methods

Eleven healthy male individuals were eligible and participated in this study. In order to measure perceptual responses to exercise level and to provide a valuable and reliable indicator of exercise tolerance, Rate of Perceived Exertion (RPE) scale was used as the subjective measure in this study. The variation in the concentration levels of extracellular vesicles and also of inflammatory cytokines (IL-6, TNF-α, IL-10) were assessed during resting and in response to a single bout of eccentric and a single bout of moderate intensity cycling exercise. The study also isolated and

examined the variability and content of plasma EVs concentration levels within and between subjects.

EVs were prepared and isolated, and Nanoparticle Tracking Analysis (NTA) was then used to assess EV concentration levels and vesicle sizes. A mouse muscle cell line (C2C12) model was used to explore the effect of exercise on EVs concentration levels. As a measure of changes in differentiation of muscle cells the expression level of Myogenin in EV-treated C2C12 cells by plasma samples was determined by qPCR. The experimental conditions and cell model for the differentiation of C2C12 was standardised and the expression of muscle differentiation gene markers was analysed by quantitative real time Reverse Transcription-Polymerase Chain Reaction. The effect of plasma EVs on C2C12 myoblast differentiation was also explored.

**Results**

This study suggests that exercise triggers a release into the circulatory blood system of vesicles with the characteristic size of EVs. It was found that there is a significant difference between the average EV concentration levels during the first three hours of rest and after eccentric exercise $p<0.05$, and that there was a reduction in the average EV concentration levels after two hours following eccentric exercise $p<0.05$. There was a trend for a reduction in myogenin in all treated plasma samples at the time point t=27 h, but the difference was not significant $p> 0.05$. Contrary to our hypothesis our results suggest plasma EVs had no statistically significant effect on C2C12 myogenesis.

**Conclusion**

It is hypothesised that that the reduced EV after exercise may be because EVs are taken in by muscles and become involved in the recovery process from the damage caused by eccentric exercise. However, the mechanism of the secretion and uptake remains to be clarified. The consistent reduction in myogenin in all the samples may point to potential clinical significance. In order to more fully understand the role of EVs in muscle cell differentiation, further studies need to be conducted.

*Key Words; Extracellular vesicles, exosomes, cytokines, myokines, eccentric exercise, C2C12 mouse line, intercellular communication, immune modulation, identification of* diseases, *disease biomarkers, EVs concentration levels, cytokine concentration levels.*

# List of Abbreviations

| | |
|---|---|
| ANOVA | Analysis of variance |
| ATP | Adenosine triphosphate |
| BDNF | Brain Derived Neurotrophic Factor |
| [BLa-] | blood lactate concentration |
| BP | Blood pressure |
| BSA | Bovine serum albumin |
| C2C12 | A mouse myogenic cell line |
| CD | Cluster of differentiation |
| CDP | Cluster of differentiation protein |
| ºC | Celsius |
| cm | Centimetre |
| c-miRNAs | Circulating microRNAs |
| cDNA | Complementary DNA |
| CRP | C-Reactive Proteins |
| CV | Coefficient of variation |
| Δ | Delta |
| DDI | Distilled deionised water |

| | |
|---|---|
| DLS | Dynamic light scattering |
| DMEM | Dulbecco's modified Eagle's medium |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylenediamine tetraacetic acid |
| EGFR | Epidermal growth factor receptor |
| EVs | Extracellular vesicles |
| FCS | Foetal calf serum |
| FGF-21 | Fibroblast growth factor-21 |
| g or G | Relative centrifugal force (G-force) |
| HR | Heart rate |
| HSP60 | Heat shock protein 60 kDa (kilo Daltons) |
| HSP70 | Heat shock protein 70 kDa |
| HSP90 | Heat shock protein 90 kDa |
| ICC | Intraclass correlation coefficient/Intracluster correlation coefficient |
| IL-1β | Interleukin-1β |
| IL-6 | Interleukin-6 |
| IL-10 | Interleukin-10 |
| IL-15 | Interleukin-15 |

| | |
|---|---|
| kg | Kilogram |
| LIF | Leukaemia inhibitory factor |
| MIQE | Minimum information for publication of quantitative real-time PCR experiments |
| ml | Millilitre |
| min | Minutes |
| miRNA | Micro ribonucleic acid |
| mmol | Millimole |
| mRNA | Messenger ribonucleic acid |
| mtDNA | mitochondrial DNA |
| MVs | Microvesicles |
| MRF4 | Myogenic regulatory factor |
| N | Sample size in statistical analyses |
| nm | nanometres |
| NSCLC | Non-small cell lung carcinoma |
| NTA | NanoSight® tracking analysis |
| p | p value in statistical analyses |
| PFA | Paraformaldehyde reagent grade |
| PBS | Phosphate-buffered saline solution |

| | |
|---|---|
| PCR | Polymerase chain reaction |
| PPIA | Peptidylprolyl isomerase A |
| PVDF | Polyvinylidene difluoride |
| qPCR | Quantitative PCR/quantitative real-time PCR/real-time quantitative PCR |
| RIPA | Radioimmunoprecipitation assay |
| RNA | Ribonucleic acid |
| RPE | Rate of perceived exertion |
| RPM/rpm | revolutions per minute |
| RT-PCR | Reverse transcription polymerase chain reaction |
| -RT | Minus reverse transcription (-RT) |
| SD | Standard deviation |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SPARC | Secreted protein acidic and rich in cysteine |
| TEM | Transmission electron microscopy |
| TNF-α | Tumour necrosis factor-alpha |
| tris | Tris(hydroxymethyl)aminomethane |
| tRNA | Total RNA |
| µl | Microlitres |

| | |
|---|---|
| WHO | World Health Organisation |
| w/v | Weight per volume |
| y | Year |

# Contents

# List of Figures

# List of Tables

# Chapter One - Literature Review

## 1.1 Introduction

It has been reported that physical activity has an impact on physiological parameters such as heart rate, blood pressure, respiratory rate, lactate levels, and the inflammatory response of the secretion of cytokines and extracellular vesicles amongst other things (Walsh *et al.,* 2011). Regular exercise helps in the process of long-term adaptation in muscle metabolism and in the regulation of the cardiovascular system, and it also has a modulating effect on the immune system (Breitbach, Tug, and Simon 2012; Egan and Zierath, 2013). Extracellular vesicles (EVs) are membrane vesicles that are released by various cells into the extracellular environment, and they play an important role in intercellular communication between cells (Raposo and Stoorvogel, 2013). EVs (of 30-120nm in diameter) that are known to play many roles in human physiology and pathology (Théry *et al.,* 2002).

Cytokines and EVs are released into the circulatory blood system during exercise, and the effect of different exercise protocols on circulating cytokines and EVs has been reported in different studies (Frühbeis *et al.,* 2015). There is an association between exercise and the secretion of cytokines, and it is suggested that EVs play an important role in immune modulation (Zhang *et al.,* 2016) causing a reduced level of inflammation as one of the benefits of exercise. Randomised control studies of aerobic physical activity have concluded that regular aerobic exercise decreases chronic inflammation and decreases the expression of inflammatory markers in skeletal muscles (Campbell *et al.,* 2009; Fairey, 2005; Nicklas, 2008). However, although it is becoming increasingly evident that a physically active lifestyle is associated with the secretion of lower levels of inflammatory markers, many studies have examined the anti-inflammatory response only with regard to leisure-time physical activity (Friedenreich, 2011; Il'yasova *et al*., 2005), and have not investigated whether or not different modalities and intensities of physical activity may be associated with changes in inflammatory marker secretion levels. The level of exercise undertaken

is a key determinant of the benefit related to exercise, and it is thus important to understand the relationship of different types of exercise with the secretion of EVs into the circulatory blood system.

The intention of this study is to explore the content of the resting concentration levels of these EVs in human plasma (cf. Chapter 3), and then to explore the correlation between different types of exercise and their effect on cytokine and EV secretions (cf. Chapter 4). In this study, in the context of exercise and the systemic effect of EVs on muscle development, the discussion about the secretion of EVs in response to exercise will be explored. In addition, Chapter 1 will provide an overview of the published literature on the importance, benefit, and effect of exercise on the human body, and of the effect of exercise on EV secretion.

### 1.2 What is physical activity?

Physical activity has been defined in many ways. Generally, physical activity is considered to be any action or movement of the body that results in the working of the muscles, and the consumption of more energy than when compared to the resting (Westerterp, 2013). Some examples of physical activity include running, walking, yoga, swimming, and dancing. According to the World Health Organisation (in its publicity material on its website), physical activity is defined as any bodily movement produced by skeletal muscles that need energy expenditure, while exercise refers to a type of physical activity that is planned, structured, repetitive, and purposeful in the sense that the improvement or maintenance of one or more components of physical fitness is the objective. According to the available literature, the main recommendations of physical activity for health and wellbeing can be categorised as either aerobic or resistance exercise (Westerterp, 2013). Aerobic exercise is any activity that depends on oxygen to consume energy,

increases heart rate, and involves several large muscle movements, while resistance exercise relies on the use of weights, machines, or body resistance to train muscles.

The level and duration of physical activity are major factors in the determination of the expression profile of cytokines (Nieman and Pedersen, 1999). Work rate (or power) is defined as the function of force and velocity during exercise (Enoka, 2002) and it describes the amount of work achieved per unit of time. The level of the work rate can be manipulated by changing the amount of exercise performed within a specific time, or by altering the time to produce a specific volume of exercise. Exercise level and duration are inversely related: the higher the exercise level, the shorter the duration that the exercise can be sustained (Heyward and Gibson, 2014). The level of the work rate reflects the compliance and integrity of physiological, neurological and perceptive mechanisms that control human movement and performance. However, exercise level can be described in absolute terms such as heart rate (HR), power output (watts), and blood lactate levels (BLa), or as energy expenditure in terms of calories, and as a subjective measure such as the rating of perceived exertion (RPE) (Heyward and Gibson, 2014).

Measures of blood lactate concentration levels are generally used as a means of calculating metabolic responses to the level of exercise. The use of blood lactate concentration levels is based on the accumulation of lactate in muscles which have been stimulated by exercise. The increase in blood lactate concentrations can occur either due to an elevation in lactic acid production, or a reduction in lactic acid removal (Powers and Howley, 2004). However, there is considerable debate surrounding the precise mechanism that leads to an increase in blood lactate levels. Although lactate accumulation is not a causative factor of fatigue, blood lactate level is a useful tool for assessing the performance of exercise (Shimojo *et al.,* 1993), and the Lactate Pro® analyser has been shown to be a reliable method of measuring blood lactate concentration levels (Shimojo *et al.,* 1993).

The intensity of exercise is a key determinant of the benefit related to exercise. For example, an improvement in the health of patients with cardiovascular disease has been associated with low-intensity exercise training *viz.* exercise at less than 45% of maximum aerobic power (Blumenthal *et al.,* 1988). Warburton *et al. (*2006) clearly state that regular physical activity is effective in the secondary prevention of cardiovascular disease and in attenuating the risk of premature death.

The term high intensity exercise can include both eccentric and concentric activity , but Herzog *et al.* (2008) said that if a skeletal muscle contracts isometrically, then a force that is relative to the final isometric sarcomere length (SL) is produced. However, when a muscle is stretched during a contraction, i.e. eccentric contraction, the same final SL is achieved at a relatively higher force. This phenomenon is referred to as residual force enhancement. Herzog *et al.* (2015) added that muscle contraction was originally believed to be controlled exclusively by the contractile filaments, actin, and myosin. Eccentric contractions are associated with a force that was not assigned to just actin and myosin but associated to filamentous protein titin.

Eccentric exercise is considered to be an initiator of the stress physical response (Nehlsen-Cannarella, 1998), and experiments in senescent animals subjected to exercise training (Hammeren *et al.,* 1992), show increases in antioxidant mechanisms (Moran, 2004) and in hypertrophy induction (Narici *et al.,* 2004). Other researchers have highlighted the importance of different parameters of exercise depending on different muscular types and have reported either increases in oxidative capacity (Silbermann *et al.,* 1983), muscle weight (Wernig *et al.,* 1990), the number and size of mitochondria (Howald *et al.,* 1985), and an altered distribution of muscle fibre types (Kovanen and Suominen, 1987).

Excessive workouts are tantamount to physical stress that is equivalent to clinical physical stress caused by sepsis, burns, trauma, and surgery (Natale *et al.,* 2003). Pedersen and Hoffman-Goetz (2000) claim that exercise can cause a cascade of immunological responses and hormonal changes

that is similar to that caused by clinical physical stress, and that cytokines are usually expressed and released in response to infectivity, injuries, or inflammation of tissue. These cytokines facilitate an incursion of B cells, T cells, monocytes, and neutrophils that contribute to the clearing of the antigen and hence prepare for the healing of tissue (Pedersen and Hoffman-Goetz, 2000).

### 1.2.1 Why is physical activity important?

There are a number of benefits of a physically active lifestyle that are well described in the scientific literature. Importantly, regular physical activity reduces the risk related to coronary heart disease and stroke, diabetes, hypertension, colon cancer, breast cancer, and depression (WHO, 2010). Physical inactivity is recognised as the fourth major risk factor for global mortality with an estimated 3.2 million deaths globally (WHO, 2016), and it is thought to be responsible for 6% of the burden of disease (Lee *et al.,* 2012). The benefits of regular exercise include both preventative and therapeutic aspects of different diseases such as obesity, hypertension, dyslipidaemia, diabetes mellitus, cardiovascular disorders, muscle, bone and joint diseases, pulmonary diseases, cancer, and depression (Warburton *et al.,* 2006).

Physical activity is associated with a reduced risk of non-communicable diseases and many plausible mechanisms have been suggested to account for this risk reduction (Gleeson *et al.*, 2011; Tiernan *et al.*, 2008). The duration of exercise, frequency, and environmental conditions play a major role in the physiological benefits of exercise. Intense exercise results in chemical, mechanical, and thermal changes to meet the increased physical demands that result in amendments to metabolic, cardiovascular and ventilatory function (Burton *et al.,* 2004). The key role of red blood cells is to transport respiratory gases ($O_2$ and $CO_2$) from the lungs to body tissues and from tissues back to the lungs for expiration. The increase in $O_2$ demand during the exercise of skeletal muscles is matched by an increase in muscle blood flow caused by increasing cardiac

output, the modulation of blood flow among the vital and inactive organs, and by optimisation of microcirculation (Laughlin *et al.,* 2012). Exercise increases the total haemoglobin mass by stimulating erythropoiesis, and this increases the production of red blood cells, which in turn increases the amount of $O_2$ that can be transported by blood (Mairbäurl, 2007).

Eccentric exercise produces significant benefits (Gregg *et al.,* 2003; Brown *et al.,* 2002; Middleton *et al.,* 2007) and such physical activity ensures several long and short-term benefits both in childhood and in adolescence (Dias *et al.,* 2016). Physical activity not only reduces the risk factors for the development of hypertension (Leary *et al.,* 2008) and dyslipidaemia (Jago *et al.,* 2008), but extensive physical activity is accompanied by a reduced risk of the development of excess adipose tissue (Ness *et al.,* 2007). Brodersen *et al.* (2007) note that in Western societies physical activity is declining and sedentary lifestyles are becoming more common for all age groups, but especially in children aged 10-12. Their conclusion therefore was that, in order to stop the trend towards obesity and other poor health conditions resulting from a sedentary lifestyle, there needs to be an increase in physical activity by all the different age groups in a population.

Physical activity increases muscle mass and strength, as well as decreasing fatigability as a result of changes in muscle metabolism (Rimmer *et al.,* 2012). Physical activity enhances the ability of muscles to resist fatigue in people of all ages (Bishop *et al.,* 2011). Mechanical forces relating to physical activity (so-called 'impact' exercises) result in forces that lead to maintenance of or the gaining of bone mass (Vieira *et al.,* 2013; Moreira *et al.,* 2013; and Moreira *et al.,* 2014). In addition, a study conducted by Yaffe *et al.* (2001) on the correlation of physical activity and cognitive decline concluded that women with greater levels of baseline physical activity were less prone to developing cognitive disabilities, and those suffering from any diagnosed disability were able to improve their mental health and show greater resilience with an appropriate change in their life style.

There are several possible mechanisms that are directly influenced by physical activity, and these include, but are not limited to, the reduction of the risk of cardiovascular and cerebrovascular diseases (Blumenthal *et al.*, 1988), increasing blood flow to the brain (Basso and Suzuki, 2017), and stimulating neuronal growth (Van Praag *et al.,* 1999).

### 1.2.2 Cardiovascular, respiratory, and metabolic responses to exercise

The regulation and circulation of blood flow has been intensively investigated, and it is evident that the mechanisms controlling the heart play a role in (1) providing muscles that are being exercised with a sufficient level of blood and also ensuring adequate metabolic washout of end-products; and (2) regulating arterial blood pressure to provide sufficient perfusion in the main organs without increased pressure differences among them (Ichinose *et al*., 2014).

The association between the oxygen demand of exercising muscles and the beating heart is obvious because of the fact that during physical activity, output of the heart increases linearly to address $O_2$ uptake (Faulkner e*t al*., 1977). The increase in heart output is induced by an increase in heart rate and stroke volume, both of which act against the reduction in systemic vascular resistance through a flow-increment mechanism. Furthermore, exercise that targets large muscles, *e.g.* cycling and swimming, leads to significant vasodilatation in the muscle vasculature, thus decreasing systemic vascular resistance. This represents a big challenge for the heart because it would need lead to a decrease in blood pressure, inhibiting brain and muscle perfusion. Dynamic physical activity leads to a small increase in mean arterial pressure while isometric physical activity causes a sharp increase in mean arterial pressure. This small increment in mean arterial pressure during dynamic physical activity occurs despite the significant decrease in the systemic

vascular resistance due to the muscle vasodilatation (Lewis e*t al*., 1983). The skeletal muscle produces many putative vasodilators during contractions, including potassium, adenosine, hydrogen ions, carbon dioxide, and phosphates. Exercise induces sheer stress which triggers the endothelial function to produce Nitric Oxide which works as a vasodilator to deliver more blood from the heart to the muscle that is being exercised (Nakano e*t al*., 2000).

It is a well-known fact that cardiovascular responses are controlled by mechanical mechanisms (skeletal muscles and lung pumps), which drive blood towards the heart, thereby improving its preload and stroke volume, and by nervous mechanisms, which control the vagal tone and flow to satisfy the metabolic need of muscles during exercise.

The respiratory and skeletal muscle pumps lead to an increase in cardiac output and stroke volume during dynamic exercise. Compared with the respiratory pump, the skeletal muscle pump has the most significant role since the muscle rhythmic contractions happening during dynamic exercise lead to intramuscular oscillations which support blood flow to the cardiovascular apparatus and improve heart preload, thereby improving stroke volume via the Frank-Starling mechanism (Higginbotham *et al*., 1986).

Exercise triggers parasympathetic withdrawal and sympathetic activation, which depend on exercise level and the muscle mass exercising (Nakano e*t al*., 2000). The role of parasympathetic withdrawal is to increase heart rate, and the sympathetic activity works on increasing heart rate, at improving myocardial contractility to enhance stroke volume, at triggering venoconstriction to promote venous return, at increasing vascular resistance in the abdominal viscera and resting skeletal muscles, and at maintaining the available heart output for the perfusion of the muscle exercising, where metabolic-mediated vasodilation occurs.

Central and peripheral mechanisms are needed for normal physiological functions. In the central mechanism, the activation of parts of the brain trigger the heart control regions in the medulla.

Haemodynamic status is regulated by the nervous system during physical activity through signals initiated by the brain and the periphery (exercise pressor reflex, baroreflexes, and arterial chemoreflex) (Higginbotham *et al*., 1986).

### 1.2.3 The effect of exercise modality and level of eccentric exercise on the inflammatory response

Inflammation is associated with a number of non-communicable diseases such as rheumatoid arthritis, hypertension, heart diseases, and cancer. Inflammation is also related to increased risk of obesity in children (Fröhlich *et al*., 2000; Straczkowski *et al.,* 2001), and it has been suggested that both inflammation and hyper-coagulable status are related, probably through modifications in metabolic control, to the production of higher levels of reactive oxygen and the development of chronic but low-grade inflammation (Franceschi and Campisi, 2014).

Gleeson *et al.* (2011) and Friedenreich (2011) proposed that physical activity affects adipokines and inflammatory markers such as the viscosity of plasma, erythrocyte sedimentation rate which helps to detect the inflammation associated with diseases such as cancer, and C-Reactive Protein (CRP) which is secreted by the liver in response to cytokines, and Petersen and Pedersen *(*2005) state that chronic inflammation is characterised by elevated blood levels of proteins and cytokines including CRP, Tumour Necrosis Factor-α (TNF-α), and Interleukin-6 (IL-6).

Although it is becoming increasingly evident that a physically active lifestyle is associated with lower concentrations of inflammatory markers, many research papers have assessed only leisure-time physical activity (Friedenreich, 2011; Il'yasova *et al*., 2005), and have not investigated whether different modalities and levels of physical activity may be associated with changes in inflammatory marker concentration levels. According to the Encyclopaedia of Public Health,

leisure time physical activity refers to exercises or sports that are performed at the discretion of the person. Hence, there is a distinction between it and physical activity which is engaged in as part of gainful employment or an essential activity of daily living.

Intense eccentric exercise acutely impairs the cellular immune system and causes inflammation, and Pedersen and Toft (2000) state that strenuous exercise can increase the concentration levels of IL-6 by as much as 100-fold, and that TNF-α levels increase by a factor of 2 to 3. The concentration levels of IL-6 peak with the cessation of exercise, but the levels of IL-1ra do not rise until the exercise has ceased and peak about 2 hours after exercise has ceased. Strenuous exercises vigorous activities are normally performed by athletes, and they include swimming laps, running in marathons, and basketball.

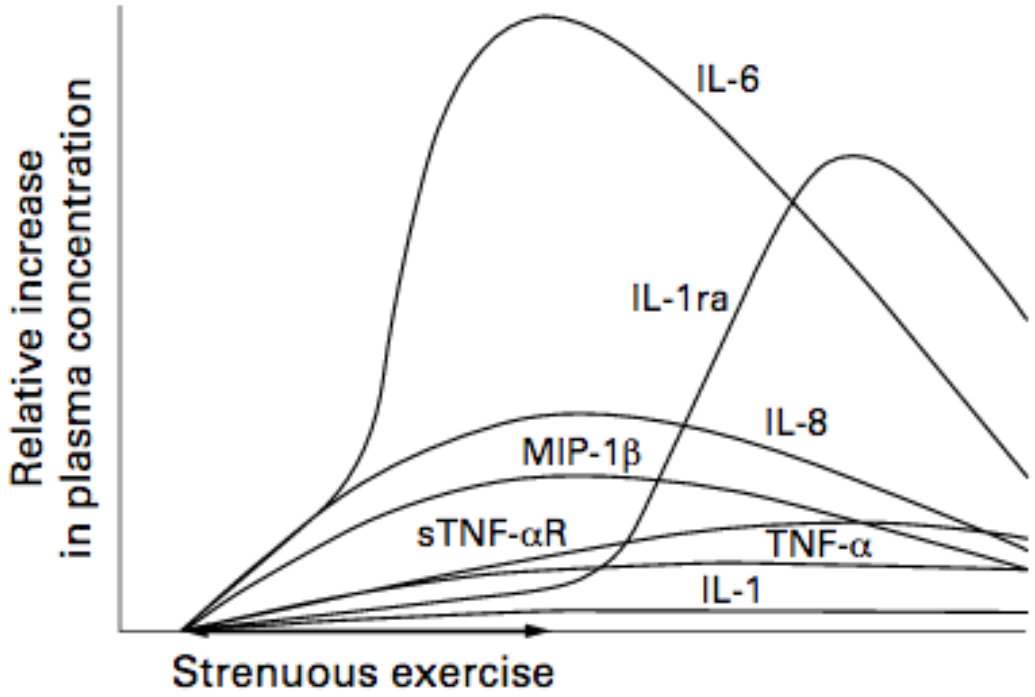



*Figure 1: The production of cytokines in response to strenuous exercise (from Pedersen and Toft, 2000). Interleukin-6 (IL-6), Interleukin-1 receptor antagonist (IL-1ra), Interleukin-8 (IL-8), Macrophage inflammatory protein 1β (MIP-1β), Soluble tumour necrosis factor-α receptor (sTNF-αR), Tumour necrosis factor R), Interleukin-1 (IL-1).*

Bruunsgaard *et al.* (1997) found a correlation between muscle damage and increased concentration levels of IL-6 and found IL-6 in the biopsies of skeletal muscles, whereas IL-1ra mRNA was expressed by blood mononuclear cells. They therefore concluded that the production of IL-6 is produced in response to tissue injury and to infections.

Findings from experimental studies designed to assess the effects of physical activity on inflammation and inflammation markers show less significant and consistent results than the results obtained from observational studies. This might be due to publication bias, differences in energy expenditure and differences in modalities (aerobic vs. resistance), level and duration of physical activities used in studies, and lack of appropriate control groups in randomised control trials. In the text that follows, a summary of some studies on the effect of different modalities and intensities of physical activity on inflammation is provided.

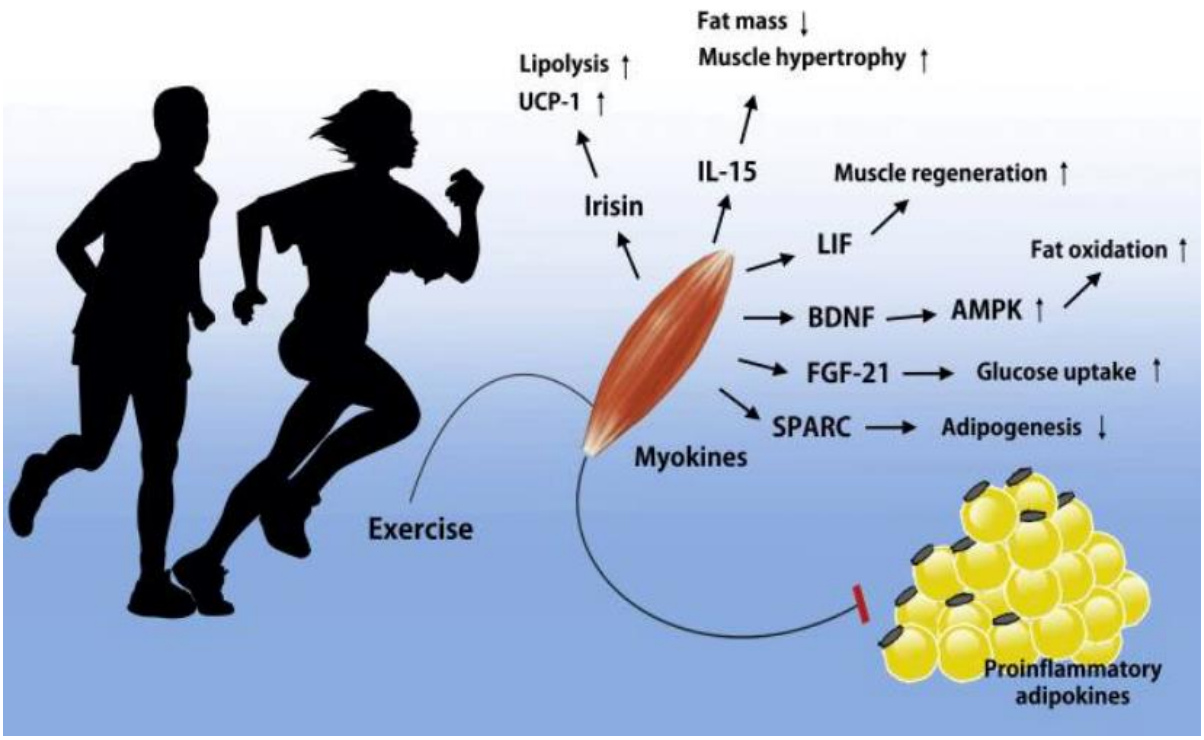



*Figure 2: The release of myokines by exercise-induced muscle contraction from So et al. (2014). Interleukin-15 (IL-15), Leukaemia Inhibitory factor (LIF), Brain-Derived Neurotrophic Factor (BDNF), Fibroblast Growth Factor-21 (FGF-21)*

### 1.2.3.1 The findings of Experimental Studies on the response to the inflammation system to exercise

Acute inflammation starts immediately after a specific injury, and usually lasts for a short period of time. It promotes the migration of neutrophils and macrophages to the area of inflammation and stimulates the production of a whole range of cytokines such as IL-1, TNF-α, IL-6, and IL-11, whereas chronic inflammation tends to stimulate IL-3, IL-4. IL-5, IL-7, IL-9 (Feghali and Wright, 1997). Randomised control studies of aerobic physical activity have concluded that regular aerobic exercise decreases chronic inflammation and decreases the expression of inflammatory markers in skeletal muscles (Campbell *et al.,* 2009; Fairey, 2005; Nicklas, 2008). For instance, Gielen *et al*. (2003) randomly selected and recruited twenty male patients with chronic heart disease to assess the effects of aerobic physical activity on inflammatory parameters. They found that regular aerobic physical activity was associated with diminished levels of microRNA, TNF-α, IL-1β, and IL-6 in skeletal muscles. To assess changes in circulating microRNA (miRNA) (non-coding RNA molecules of about 20-25 nucleotides in length) in response to acute and chronic aerobic exercise, eleven healthy young men participated in Aoi *et al.'s* (2013) study and performed a single bout of steady-state cycling (acute aerobic physical activity) for 60 min and cycling training (an aerobic physical activity) for three days a week for one month. Blood samples were collected before and after the exercises, and the levels of miRNAs were measured and it was found that miR-486 was significantly decreased by both acute and chronic cycling.

Although more studies have been conducted on the influence of aerobic physical activity on inflammation, some studies used resistance physical activity to assess inflammation response with most of the findings being negative. The first published study was conducted by Rall *et al.* in 1996, and they found that three months' worth of high-level eccentric exercise did not reduce IL-

1β, TNF-α, IL-6, or IL-2 production. Similarly, McFarlin *et al.* (2004) also reported that there was no significant association between the level of inflammatory cytokine expression (IL-6, TNF-α) and resistance training in women between 65 and 80 years of age, compared with untrained women (control group). However, the null effect of these two trials may be due to the small number of participants (6-8) in the intervention groups; but, in 2009, Levinger *et al.* conducted a larger randomised clinical trial in which 56 middle-aged women and men undertook resistance physical activity for almost three months, and their finding also was that this did not significantly decrease IL-6 or CRP expression.

However, in contrast to these negative results, Brooks *et al.* (2007) found that a four-month resistance physical activity trial reduced CRP and increased adiponectin expression among people with Type 2 diabetes. One year later, Lambert *et al.* (2008) asserted that the coupling of resistance physical activity with aerobic physical activity reduced concentrations of IL-6 and TNF-α. These results were similar to those of Brochu *et al.* (2009) who found that resistance physical activity alone did not improve inflammatory markers among participants in their study, but when combined with weight loss, significant improvements in markers were in fact noticed. Similarly, a noticeable decrease in TNF-α mRNA and protein levels in skeletal muscle were reported after resistance training among older men and women (Greiwe e*t al.,* 2001). In conclusion, there appears to be considerable evidence that aerobic physical activity interventions are useful for reducing levels of inflammatory markers, and cytokines, both locally in skeletal muscle and systemically in the circulatory blood system.

Many studies such as Moldoveanu *et al.* (2001) have concluded that acute periods of intense or prolonged physical activity can lead to biochemical changes that contribute to improvements in inflammatory response, but less is known about the impact of regular physical activity on the concentrations of inflammatory markers. Four trials (Mattusch *et al.,* 2000*;* Smith *et al.,* 1999;

Straczkowski *et al.,* 2001; Tsukui *et al.,* 2000) found that regular physical activity of various durations and intensities reduced levels of TNF-α and CRP in younger individuals. Moreover, many cross-sectional studies (Geffken *et al.,* 2001; Ford, 2002; Taaffe e*t al.,* 2000) concluded that the regular performance of physical activity or fitness is associated with lower blood concentrations of IL-6 and CRP in different age groups.

It has also been shown by Gleeson (2004) that nutrient intake alters immune response at rest and during exercise through direct or indirect mechanisms. In Gleeson's study, all the subjects were asked to perform eccentric exercise after an overnight fast. They were then asked to consume carbohydrates after the exercise, and it was shown that plasma IL-6 and cortisol concentrations then increased significantly after exercise during the fasting state. However, there was a reduction in IL-6 and cortisol concentration after the consumption of the carbohydrate.

The performance of eccentric exercises results in the secretion of IL-6 and has been associated with muscle damage (Petersen *et al.,* 2001). However, a study by Croisier *et al*. (1999) did not show any such association between exercise-induced muscle damage and IL-6 levels, leading to speculation that the release of myokine is associated mainly with contraction of muscle fibres in order to mobilize substrate from fuel depots so that glucose homeostasis is maintained during intense physical activity.

Croisier *et al.* (1999) concluded that there was a 6-fold increase in IL-6 concentration levels immediately after exercise and an 8-fold increase half an hour post-exercise which then returned to baseline levels a few days after the exercise. In their study, the authors used an exercise that involved greater use of musculature than in Petersen *et al.'s (* 2001) study because they used two-legged eccentrically based exercise of the knee flexors and extensors in their intervention. Pedersen *et al.* (2001) argued that the big and immediate increase in IL-6 levels after long-duration exercise is not related to muscle damage, but muscle damage itself is affected by repair

mechanisms such as the invasion of macrophages into the muscle leading to an increase in IL-6 levels. IL-6 generation in relation to muscle damage happens later and is of a smaller scale than IL-6 generation related to muscle contractions (Pedersen *et al.*, 2001).

Febbraio and Pedersen (2002) argued that increases in the level of IL-6 were found to be associated with muscle contraction. In support, Ostrowski *et al.* (1999) hypothesised that inflammatory cytokines were produced in skeletal muscles in response to long-term exercise such as a marathon race. Additionally, elevated IL-6 and IL-1Ra levels have also been recorded in rats (Jonsdottir, 2000). Cytokine production is therefore not concurrent with tissue damage. However, IL-6 is expressed after muscle exercise.

To conclude, IL-6 is the most expressed cytokine during exercise, and it is produced in skeletal muscles. It has been established that IL-6 is produced without muscle damage in eccentric exercises, and it has been reported that the level of that exercise is strongly correlated to the time and duration of the exercise (Rippe, 2011). Among participants who perform high- and low-level eccentric exercises, the level of IL-6 is greater with eccentric exercise than the level seen in low-level eccentric exercise of the same duration (Cullen *et al.,* 2016). Schäfer and Stein (2003) reported that it is still unclear whether low level eccentric exercise would produce a similar pattern of cytokines as eccentric exercise, and there is a lack of sufficient evidence on this in the published literature. Schäfer and Stein (2003) point out the need for more clinical trials to assess definitively the effects of different intensities and durations of exercise on inflammation markers.

### 1.3 Extracellular vesicles

Philip Stahl, in the introductory lecture that he gives on ‘Extracellular Vesicles’ at the University of California, Irvine says that the existence of membrane fragments and vesicles shed from the

surface of cells by selective blebbing and found in extracellular fluids has been known since the Second World War, and they are given the generic name of Extracellular Vesicles, this being a generic term including microvesicles, microparticles, ectosomes, oncosomes, prostasomes, and many other (entities). Extracellular vesicles (EVs) are membrane-contained vesicles released by prokaryotes, higher eukaryotes and plants (Figure 3); but, Doyle & Wang (2019) suggest that EVs are lipid bound vesicles secreted by cells into the extracellular space, and that the three main sub-types of EVs are micro-vesicles and apoptotic bodies, whilst Edgar (2016) said that extracellular vesicles are released from cells upon fusion of an intermediate endocytic compartment, the multivesicular body (MVB), with the plasma membrane.

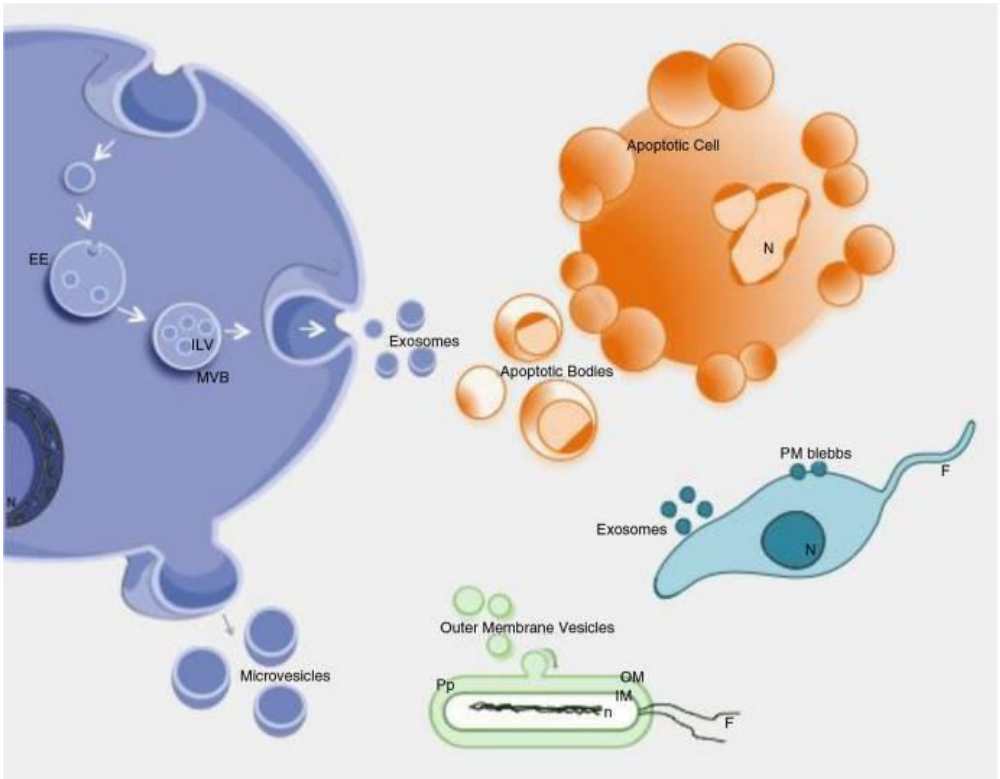



*Figure 3: Biogenesis and release of extracellular vesicles, adapted from Yáñez-Mó et al. (2015).*

EVs were first observed in 1946 by Chargaff and West (1946) and named as ‘platelet dust’ by Wolf (1967) because they were thought to contain nothing but biological debris (Kowal et al., 2014). EV observation occurred simultaneously in various physiological settings indicating that this form of function or communication is a universally shared cell biological property (Yáñez-Mó *et al.,* 2015).

EVs can be isolated by centrifugation to separate particles of large size, and then by ultracentrifugation at 100,000g for >1 hour (Whitham et al., 2018). EVs can also be isolated by other procedures such as gel filtration or by ultracentrifugation followed by size exclusion chromatography (Benedikter *et al*., 2017).

Every ‘discovery’ in science is preceded by advances made by earlier generations of scientists, and the pioneering work that led to formulation of the current thinking on EVs was done on membrane trafficking by the Nobel Laureate Christian de Duve in the 1950s who pioneered the work on lyosomes and intra-cellular digestion and on receptor-mediated endocytosis in the 1970s.

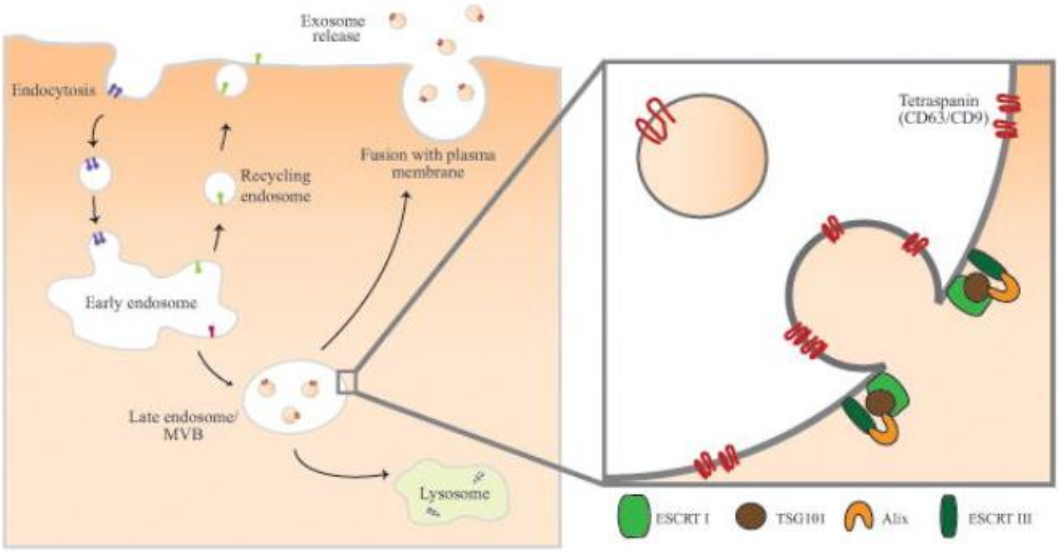



*Figure 4: the development of an endosome leading to the release of an EV with the support of Endosomal Sorting Complexes Required for Transport (ESCRT) viz. ESCRT I, and II (for membrane budding), and III (to complete the budding), and by Alg2-interacting protein X (ALIX) as described by Petiot et al. (2008), while the role of Tetraspanin-Enriched Microdomains (TEMs) has been described by Pols & Klumperman (2009).*

de Duve's work culminated in 1983 in two ground-breaking papers on the role of transferrin binding to clathrin coated membrane-surface pits, the first by Pan and Johnstone (Johnstone *et al*., 1983 and 1987) who, apparently in 1983, coined the term extracellular vesicles to describe secreted membrane vesicles derived from multivesicular bodies (MVBs), and the second by Harding, Heuser and Stahl (1983 and 2013). Less publicised, but also in 1983 is the work by Dautry-Varsat on human hepato-cellular carcinoma HepG2 cells (1983), and Klausner on the leukaemia cell line K562 (1983). Following that, work was done by many others, including Théry (1999) in the 1990s.

However, as is the case at the beginning of all sciences, the names given to EVs were initially not standardised, and the nomenclature included the liberal use of terms such as extracellular vesicles (EVs), endosomes, microvesicles, retrovirus like particles (RLPs), apoptotic bodies, etc; but, the

formation of the International Society for Extracellular Vesicles (ISEV) in 2011 was followed by detailed guidelines for the naming of EVs in MISEV 2014 which also gave guidance on separation, isolation, characterisation, and functional studies, and MISEV 2014 was subsequently updated in 2018.

In this century, there has a been an explosion in interest in what are now called extracellular vesicles, with the primary difference between EVs and exosomes being that of size. EVs are heterogenous in size and range from 50-1000nm in diameter, while exosomes are more homogenous in size and range from 50-100nm or 30-120nm in diameter (depending on different usages by researchers, and also on the mechanisms by which the exosomes were produced).

EVs are considered to be a mode of intercellular communication due to their ability to transfer cytosolic proteins, lipids, and ribonucleic acid (RNA) between cells and their membranes (Raposo and Stoorvogel, 2013). Gould and Raposo (2013) state that there are three sub-groups that form EVs: (a) apoptotic bodies (ABs), 800-5,000nm in diameter, (b) cellular microparticles /microvesicles/ectosomes (MVs), 50-1,000nm in diameter, and (c) exosomes, 30-120nm in diameter – although this suggestion has since been superseded by the directives in MISEV 2014 and 2018.

Théry *et al., (*2002) said that apoptotic bodies are secreted when plasma membrane blebbing happens during apoptosis, and that microvesicles (MVs) include vesicles of various sizes that pinch directly off the plasma membrane, and that extracellular vesicles are small membrane vesicles of endocytic origin that are secreted by most cells. However, for the purposes of clarity, it might be useful to make a distinction between

a) EVs as being nano-sized biovesicles released into surrounding body fluids upon *fusion* of multivesicular bodies and the plasma membrane

b) ectosomes/microvesicles that arise from the outward budding and then the *fission* of the plasma membrane where the phospholipid distribution of the bi-layer is governed by the amino-phospholipid translocases ‘flippase’ and ‘floppase’ (Muralidharan-Chari, et al., 2009)
c) Retro-Virus like Particles that are different fromEVs and microvesicles, in that whilst RLPs bud directly from the plasma membrane, the formation of RLPs involves the interaction with retroviral proteins such as Gag, Group Antigen Polyprotein (also known as p24) that forms the inner layer of the nucleocapsid, and
d) apoptosome vesicles that are created in the process of apoptosis (Elsmore, 2007).

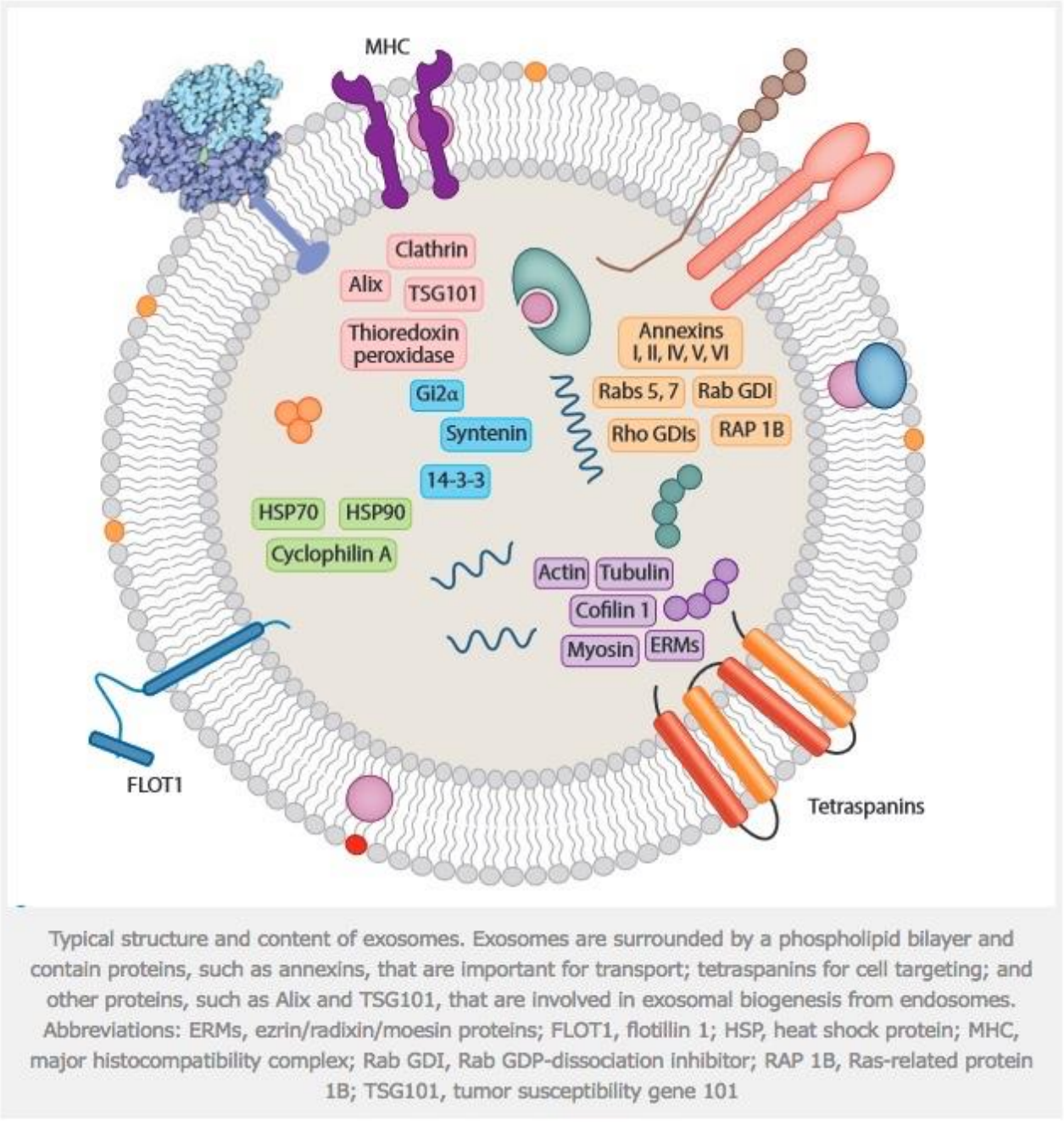
Goldstein (1961) proposed that ‘tissue crosstalk’ is a mechanism for the physical effects of exercise, and since then many studies have studied the effect of physical activity protocols on circulating EVs, and the concept of tissue crosstalk during exercise has focused upon uncovering novel proteins or peptides, classically secreted, that act in ligand-receptor-binding complexes (Whitham & Febraio, 2016). EVs have been shown to be secreted by many different organs and tissues in the body, ranging from the brain (Lancaster *et a*l., 2004) to ‘skeletal muscle’ (Moon *et al*., 2016), and it has been suggested that the levels of platelet-derived pro-coagulant proteins and monocyte-derived proteins increase when participants perform strength resistance exercises (Chaar *et al.,* 2011; Chen *et al.,* 2013; Maruyama *et al*., 2012). The concentrations of endothelial-derived MVs do not normally alter (Sossdorf e*t al.*, 2011; Wahl *et al.,* 2014), or else they increase after endurance exercise of moderate intensity (Sossdorf e*t al.*, 2011), and Whitham *et al.* ( 2018) say that proteins can change biological function in recipient cells are secreted in EVs during incremental exercise, *independently* of classic protein secretion. The emphasis in italics is mine because protein production accompanies a huge number of bodily functions, and in any case identifying certain proteins in the midst of the vast proteomic complexity of plasma is not trivial

(Whitham *et al*., 2018) – especially when 50% of protein presence by mass is accounted for by albumin alone.

However, a limited number of studies have assessed the effect of physical activity on smaller EVs in the circulatory blood system. In their study, Frühbeis *et al.* (2015) analysed small (≤200nm) EVs detected in plasma during and after performing acute exercise. They found that the concentration levels of small EVs increased after cycling exercises and reduced during the early recovery phase. However, the reduction might be due to the difference in the dynamics of small EVs between these exercise protocols.

### 1.3.1 Extracellular vesicles

In normal cell biology, there is constant transport of materials across cellular membranes. This transport is thought to be due to an evolutionarily conserved mechanism and involves active and passive modes of transport along with trafficking through vesicles, including extracellular vesicles, produced by the cell (Azmi *et al.,* 2013). extracellular vesicles are small vesicles (30-120nm) released by a variety of cell types into the extracellular environment and contain nucleic acid and protein, and act as carriers of this cargo between diverse locations in the body (Vlassov *et al.,* 2012). Figure 5 illustrates the typical content of an extracellular vesicle.




Typical structure and content of exosomes. Exosomes are surrounded by a phospholipid bilayer and contain proteins, such as annexins, that are important for transport; tetraspanins for cell targeting; and other proteins, such as Alix and TSG101, that are involved in exosomal biogenesis from endosomes. Abbreviations: ERMs, ezrin/radixin/moesin proteins; FLOT1, flotillin 1; HSP, heat shock protein; MHC, major histocompatibility complex; Rab GDI, Rab GDP-dissociation inhibitor; RAP 1B, Ras-related protein 1B; TSG101, tumor susceptibility gene 101

*Figure 5:Illustration of an extracellular vesicle cross-section from Kourembanas (2014).*

Extracellular vesicles are nano-vesicles that play a role in the process of intercellular communications for transporting proteins and RNAs. Recent studies have shown that they play a role in many physiological and pathological processes in the immune system, cell differentiation,

and in infection (Zhang *et al.,* 2016). This has encouraged the attempt to use extracellular vesicles clinically as a new type of anti-cancer therapy in humans (Chaput and Théry, 2011).

It has been proposed that many types of extracellular vesicles, either secreted into culture media by cells or by different tissues into allied bodily fluids, play a role in communication between cells, resulting in either favouring or blocking physiological processes (Lakkaraju and Rodriguez-Boulan, 2008, Schorey and Bhatnagar 2008, van Niel *et al.,* 2006). Extracellular vesicles have been detected in several biological fluids such as plasma, saliva, nasal lavage, placenta, and in breast milk (Lässer *et al.*, 2012). The molecular content of extracellular vesicles acts as the fingerprint of the releasing cell type and of its status (Properzi *et al.,* 2013), and extracellular vesicles levels in plasma are clinically interesting in many applications *e.g.* the plasma levels in some cancer patients could be a potential tool for cancer screening (Logozzi *et al.,* 2009). In addition to immune cells, cancer cells secrete immunologically active extracellular vesicles that influence both the physiological and pathological processes of the disease (Greening *et al.,* 2015). Extracellular vesicles have been detected in blood as an immediate response to exercise by the immune system (Li *et al.,* 2006), and Witham *et al*., (2018) highlight a correlation between an increase in several classes of proteins associated with small vesicles and extracellular vesicles and robust exercise. Frühbeis *et al*. (2015) also show that physical activity is associated with the release of nano-sized EVs into circulation. Comparing the secretion of inflammatory cytokines alongside extracellular vesicles secretions by the immune system, before and after exercise in healthy participants, is important in order to identify early markers for reduced immune function.

Extracellular vesicles have been involved in the pathogenesis of some diseases such as neurodegenerative disease and cardiovascular disease (Stoorvogel 2012). It has been shown that extracellular vesicles are involved in the accumulation of β-amyloid protein which is associated with Alzheimer's disease (Ohno *et al.,* 2013), and there might be variation between subjects over

the course of one day and across several days. Circadian variations are often detected in human immune biomarkers, and these have been studied. For example, Ündar *et al*. (1999) reported a significant circadian variation in different blood biomarkers (IL-6), coagulation inhibitor proteins, and anti-thrombin factors in healthy men (Ündar *et al.,* 1999). However, the circadian rhythm of extracellular vesicles in healthy individuals remains unknown.

### 1.3.2 Extracellular vesicles in disease

The production of extracellular vesicles by cancer cells has a critical role in intercellular communication, enhancing signalling to neighbouring tumour cells as well as to distant sites through blood circulation or biological fluid transportation. One of the areas of research with regard to EVs has been the role of EVs in tumour progression and metastasis including fibroblasts, endothelial cells, and immune cells, and Khanna *et al.* (2021) say that evidence of EV presence in biological fluids has led to considerable efforts focused on identifying their cargo and determining their utility as a non-invasive diagnostic platform for cancer – but the role of EVs in many other fields such as neurodegenerative disease and cardiovascular disease (Stoorvogel 2012), Alzheimer's disease (Ohno *et al.,* 2013), ocular diseases (Zhang *et al*. (2020), prostate problems (Khanna *et al*., 2021), thrombosis (Zarà *et al*., 2019) is also being researched. However, although malignant tumours occur at all ages, cancer disproportionately strikes people at the age of 65 years or older (Yancik, 2005), and with an ageing population, cancer is going to become an increasing cause of mortality, and the role of EVs in identifying cancerous growths will become increasingly important (Kobayashi *et al.,* 2015).

In addition to their function in the normal development and physiology of the nervous system, extracellular vesicles also play a role in the development of many diseases. According to Ghidoni

*et al*. (2007), extracellular vesicles have been called the Trojan horses of Neurodegeneration due to their involvement in transporting toxic substances from unhealthy neurons to neighbouring cells.

As key players in communication between cells, extracellular vesicles enhance tumour progression and metastasis by mediating the communication between tumour and stromal cells in the human body. However, extracellular vesicles in tumours as well as in normal cells also seem to have systemic characteristics (Pucci *et al*., 2016; Thomou *et al*., 2017).

EVs were identified at least 30 years ago (Raposo and Stoorvogel, 2013) and, according to Valadi *et al*. (2007), extracellular vesicles consist of both mRNA and microRNA, that can be delivered by intracellular communication. Extracellular vesicles contain specific RNA transcripts that have great potential as disease biomarkers (Huang *et al.,* 2013). Emerging evidence reports that extracellular vesicles can arbitrate between different biological functions such as cell proliferation, angiogenesis, immune response, and antigen presentation by the transfer of proteins, mRNAs and non-coding RNAs to neighbouring or distant cells (Pegtel *et al.,* 2011, Huang *et al.,* 2013). However, the packaging of RNAs into extracellular vesicles is selective as the RNA profiles in EVs do not completely reflect the RNA profiles in the parent cells (Valadi *et al.,* 2007, Skog *et al.,* 2008).

It has been hypothesised that since extracellular vesicles carry a particular cellular content, they can be exploited as a potential biomedical tool to detect changes in the content of cells, such as in the initiation and progression of cancer or tumorous growths (Kobayashi *et al.,* 2015). The miRNA content of the extracellular vesicles corresponds only to particular cell types, and EVs are attributed with the characteristic of changing the phenotype of the progenitor cells by effectively transferring pro-oncogenic molecules in order to initiate the growth of cancers in these cells and hence give rise to a metastatic environment in the neighbouring cells (Kobayashi *et al.,* 2015). It

has been suggested that an increased level of extracellular vesicles can be detected in urine, serum, and in malignant effusions from cancer patients (Ohno *et al.,* 2013). Kharaziha *et al.* (2012) reported that cancer cells secrete higher amounts of extracellular vesicles than normal cells. Furthermore, Taylor and Gercel-Taylor (2008) found that cancer patients have excessive amounts of blood circulating EVs compared to healthy individuals.

The prevalence of EVs is therefore important, and this can be used as a marker in many diseases including cardiac diseases and cancer. Research studies have reported that EVs in healthy individuals are not the same as the extracellular vesicles in diseased individuals (Taylor and Gercel-Taylor 2008, Rabinowits *et al.,* 2009) *e.g.,* as in the plasma of patients with lung adenocarcinoma (Rabinowits, Gerçel-Taylor *et al.,* 2009) and prostate cancer (Tavoosidana *et al.,* 2011). It has also been suggested by Huang *et al*. (2013) that circulating extracellular vesicles could play a vital role as a tool for the non-invasive diagnosis and prognosis of human diseases. It is the case that the numbers of extracellular vesicles proteins such as CD91, CD317 and EGFR have been recognised as potential extracellular vesicles markers of non-small-cell lung carcinoma (Jakobsen, *et al*., 2015).

### 1.3.3 Muscle development and extracellular vesicles

Contracting skeletal muscles release cytokines and other peptides acting in a paracrine or endocrine manner – the so-called myokines (Pedersen, 2009). The term myokine (cytokines synthesised and released by myocytes during muscular contractions) was first introduced in 2003 by the Swedish Professor in exercise physiology, Bengt Saltin. Since myostatin was first introduced as a myokine in 1997, secretome-based analysis (analysis of all the secreted proteins of a cell, tissue or organism) of human myocyte culture medium has so far revealed over 600 myokines. These include myostatin, apelin, β-aminoisobutyric Acid (BAIBA), IL-15, IL-6, irisin,

SDF-1, decorin, SPARC, and VEGF-A expression, and Whitham *et al*. (2018) say that given the correlation between exercise and the production of EVs, that this might be a mechanism by which skeletal muscle can release myokines independent of the classic secretory pathway, but most of these mechanisms are currently still not sufficiently understood.

Human apelin was identified and isolated in 1998 as an endogenous ligand of the G-protein-coupled receptor AP (Tatemoto *et al.,* 1998), while BAIBA was first discovered in human urine in 1951, and is a no-protein myokine with a small molecular weight (Crumpler *er al.,* 1951). Interleukin 6 (IL-6) was identified in 2000 and it is currently the most studied myokine that there is (Steensberg et al., 2000).

With discoveries of myokines (~5–20 kDa in size) continuing to be made, it has been hypothesised that muscle cells can act as an endocrine organ (Pedersen, 2009). Besides the release of microRNAs, contracting muscle cells have been observed to release EVs containing humoral factors, also known as myokines, and these have profound effects on the biochemistry of muscle cells (Chan *et al.,* 2004, Febbraio *et al.,* 2004, Izumiya *et al.,* 2008, Ouchi *et al.,* 2008). The humoral factors, which are similar to hormones, can have an effect on the metabolism in many target tissues and organs (Pedersen and Febbraio, 2008). As they are released in response to exercise or intense muscle activity, they may play a plausible role in the adaptation of organs to such physical activity, and to contribute to maintaining homeostasis (Guescini *et al.,* 2010). It has also been proposed that muscle cells release cytokines such as IL-8 and IL-15, and these are important in adapting peripheral organs to physical activity, and to regulate the process of myogenesis (Nielsen *et al.,* 2007).

Some of the specific miRNAs are miR-1, miR-133a/b, miR-206 and miR-208b, each of which has been shown to have a potential role in skeletal muscle development (Chen *et al.,* 2006, Kim *et al.,* 2006, van Rooij *et al.,* 2009). From these, some miRNAs are released following exercise, and this regulates the adaptation of the muscle (Safdar *et al.,* 2009, Aoi *et al.,* 2010, Nielsen *et al.,* 2010). Other miRNAs are involved in the uptake of amino acids from dietary intake (Drummond *et al.,* 2009). These miRNAs are present in different fluids in the body and circulate through them to reach their target cells, and hence they are known as circulating miRNAs (c-miRNA). For instance, continuous training in intense cycling or rowing for 90 days elevates levels of several miRNAs including miR-21, miR-146, miR-221 and miR-222 levels (Makarova *et al*., 2014).

Choi *et al.,* 2005 concluded that the release of EVs from the mouse myoblast C2C12 cells in culture was accompanied by release of other factors, and this study has focused on the presence of mtDNA and several specific proteins in the content of C2C12 cells. The abundance of histone proteins in these vesicles is further confirmed by the occurrence of mtDNA. Guescini *et al*. (2010) also identified the presence of several heat shock proteins in the microvesicles released by cells, and these included HSP 25, 70, 75 and 90.

Mylona *et al*. (2006) performed proteomic analysis of C2C12 secreted EVs and hypothesised about their possible role in inter-cell communication. Figure 6 is adopted from Le Grand and Rudnicki (20074) who mentioned that quiescent skeletal muscle satellite cells could be activated by a stimulus originating from their associated fibre or from the micro-environment. They added that their proliferating progeny, the skeletal myoblasts, express the paired-box transcriptions factors Pax7 and Pax3, in addition to the myogenic regulatory factors Myf5 and MyoD.

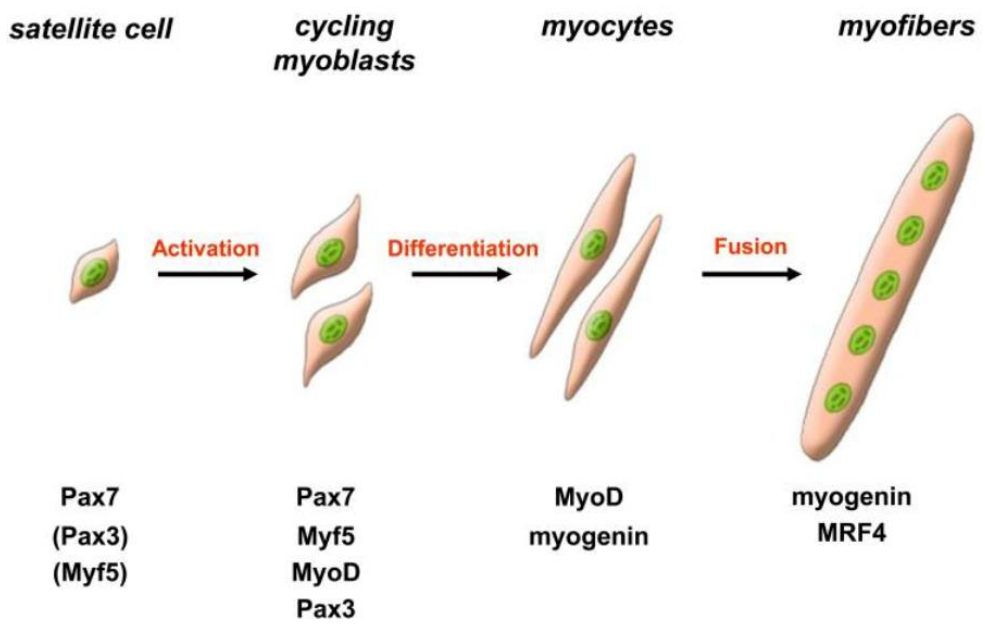



*Figure 6: Activation, Differentiation and Fusion of Satellite Cells*

The study also presented evidence that myotube-secreted EVs reduce myoblast proliferation and induce differentiation or promote the process of myogenesis. These EVs vesicles downregulate cyclin D1, a cell cycle control protein, and upregulate myogenin, shown to increase differentiation. It was found that the EVs secreted by C2C12 cells had specific cell-adhesion molecules on their surfaces, and these play a part in the recognition of one myoblast by another, allowing myoblasts to fuse with each other (Mylona *et al.,* 2006.). The study thus showed that the contents of EVs produced by muscle cells can coordinate myoblasts during differentiation and can also act as a medium of communication between myoblasts and myotubes (Forterre *et al.,* 2014). In addition, EV-like vesicles released by muscle cells contain proteins of the Guanine family involved in many different cellular processes, including myogenesis (Guescini *et al.,* 2010).

Furthermore, skeletal muscles undergo various molecular and structural changes during the process of exercise. These anatomical and morphological changes indicate the high plasticity of the skeletal muscles in response to the large amount of stress and load being placed on them

(Narici and Maganaris, 2006). This plasticity can be described as a feedback mechanism of the body in order to be prepared for the adequate development of its tissues to bear the load and strengthen the associated cells and organs to become resilient and hence not to get damaged due to high-load or exhaustive exercise activities (Hoppeler, 2016).

### 1.4 study aims and objectives

In response to the information that has been revealed by this Literature Review, the aims of this study are to measure the content of plasma EVs concentration levels in resting healthy male individuals and then to use this information to investigate the systemic concentration levels of EVs and inflammatory cytokines in response to high- and low-level eccentric exercise in healthy individuals.

The key objectives of this study are

1) To measure the content of EVs concentration in human male plasma.

2) To examine the inflammatory response of the human body to high- and low-level eccentric exercise by secretion of inflammatory cytokines and EVs into the circulatory blood system.

3) To measure the subjective response (muscle soreness) and metabolic response (lactate measures) to high- and low-level eccentric exercise.

4) To clarify the potential role of EVs in C2C12 (a mouse myogenic cell line) as a model for myogenesis.

In this study therefore, experimental designs were used to test the following hypotheses.

Hypothesis One: That the concentration levels of plasma EVs is stable, and that there is little day-to-day variability in the concentration levels of plasma EVs over different days of blood sampling. This hypothesis will be tested in Chapter Three.

Hypothesis Two: That there is a change in the concentration levels of plasma EVs and cytokines such as IL-6, IL-10 and TNF-α after low and high physical activity, and after eccentric exercise. This hypothesis will be tested in Chapter Four.

Hypothesis Three: That there is a change in the differentiation of C2C12 cells when treated with plasma EVs. This hypothesis will be tested in Chapter Five.

# Chapter Two - Methodology

## 2.1 The Participants in this study

### 2.1.1 Criteria for inclusion in the study

The study conformed to the latest revision of the Declaration of Helsinki, and all the procedures and potential risks associated with the study were fully explained to each participant. All the participants reported to the Human Performance Laboratory of Oxford Brookes University and completed screening questionnaires to determine their eligibility for participation in this study. Participants were eligible for inclusion in the study if they were healthy, male and between 20 and 40 years of age. Females were excluded because they have hormonal changes that may affect inflammation response and EVs concentration levels. In addition to this, women have greater adipose tissue than men, and this could be a confounding factor in the relationship between physical activity and inflammation.

Exclusion criteria included the presence of a chronic inflammatory condition (for example, diabetes mellitus, or a cardiovascular disease), or the recent consumption of anti-inflammatory medications (such as Ibuprofen) by a potential participant. Since the scope of the study was to investigate the variation found in the concentration levels of EVs in healthy individuals, the indication of any inflammatory condition would therefore suggest a natural increase in the concentration levels of EVs and of the proteins that are associated with an anti-inflammatory response. Subjects were therefore excluded if they had any pathology known to influence an immune response (*e.g.* auto-immune diseases, anaemia, heart diseases, orthopaedic limitations or a renal failure disorder), and also if they had a neurological/ neuromuscular disorder. Subjects who had experienced cold or flu symptoms in the previous 24 hours were also excluded from the study.

Because of the natural increase in the level of EVs in cases of inflammatory response, the subjects, who were all non-smokers, were excluded from the study if they had had any cold or flu symptoms

in the previous 24 hours. Subjects arrived at the laboratory after abstaining from caffeine (for 24 hours), alcohol (for 48 hours), food (for 8 hours) and exhaustive exercise (for 48 hours). They were allowed to consume only water. All experimental sessions were performed at a time of day that was kept constant for each subject, and the purpose, procedures and risks of each study were fully explained to each participant. A physical activity readiness questionnaire was completed to determine the suitability to participate in physical exercise, and a pre-screening health questionnaire was used to check the participants' medical history to determine (based on the answers to specific health-history questions) the safety or possible risk of exercise for each participant. Subjects with mental health problems were excluded from the study by asking them the question: Have you been treated for any mental health problem before? (cf. Appendix B). Subjects were then provided with a written informed consent form (cf. Appendix E) which they were required to sign in order to become a participant in the study.

### 2.1.2 Muscle soreness in the participants

The visual analogue scale uses verbal mainstays corresponding to numerical values which determine the relationship between physical intensity and perception of exertion (Borg, 1982). Subjects were asked to self-assess their muscle soreness using a visual analogue scale rating from 1 to 10, with '1' denoting 'no pain' and '10' denoting 'the worst pain ever'. To capture the inflammatory response to exercise, muscle soreness was assessed immediately after the exercise, then 24 hours later, and then again 48 hours after the experiment.

### 2.1.3 Rate of perceived exertion (RPE) scale

In order to measure perceptual responses to exercise level and to provide a valuable and reliable indicator of exercise tolerance, RPE was used as the subjective measure in this study. RPE is monitored during exercise tests, and it has been applied to assess and to understand performance (Starbuck and Eston, 2012). It is characterised by subjects' self-reports of their perceptual responses (Merikle, 1992). Responses to exercise level represent the complex interaction of various feedback signals from the heart, lungs, muscles, joints and skin in response to stress, effort and discomfort caused in the body during exercise (Williamson *et al.,* 2006). It was shown by Noble and Robertson (1996), that there is strong evidence that perception of exertion increases with increases in workload, force, oxygen consumption, blood lactate, and heart rate (HR) across different exercise conditions and modalities. Initially, the RPE scale was developed as a 15-point scale from 6-20, and this reflects the range of HR in young healthy subjects from 60 heart beats per minute at rest, to 200 beats per minute during maximal exercise on a cycle ergometer. This ratio scaling tool showed that the perception of exertion increased with the physical workload. It was later revised to the CR-10 scale, which is simpler to use and has shown reasonable validity, sensitivity and content (Borg, 1990; Neely *et al.,* 1992) because this is more applicable to physiological responses and physical functions (Borg 1990). The CR-10 scale is suitable for use alongside various physiological variables and it allows for inter-individual and inter-model comparisons. In this study, RPE was used during the eccentric exercise tests, and the CR10 scale was used as it is relatively simple to understand and it is suitable for recording measures related to the sensation of breathing and to the sensation relating to muscles in the legs (Borg, 1990; Neely *et al.,* 1992).

## 2.2 Methods used in the examination of EVs concentration levels in the blood samples

### 2.2.1 The preparation of EVs

For each investigation, three blood samples (of 4 ml each) were drawn consecutively from the antecubital vein and placed in EDTA vacutainers (Becton Dickinson; San José, California). The blood samples were then centrifuged at 1000g for 10 min at 4ºC to separate off unwanted white and red blood cells/platelets/and other 'debris' that contaminated the EVs. The plasma was then separated and divided into 500µl aliquots. The freshly prepared samples (designated as Samples A) were then ready for examination, while Samples B were stored at -80ºC ready for thawing and subsequent examination. The EVs were then processed as outlined below.

### 2.2.2 Preparation of samples A (the 'fresh' EVs) and Samples B (the frozen and then later to be thawed EVs).

Sample A EVs were isolated by differential ultracentrifugation, and by the basic EVs isolation protocols described by Théry *et al.* (2006). Plasma samples were diluted with an equal amount of phosphate-buffered saline (PBS) solution and gently agitated until a homogenous mixture was achieved. The mixture was then transferred to 50ml tubes, and centrifuged at 2,000g for 30 min and 4°C, followed by 12,000g for 45 min. The suspension was filtered through a 1% bovine serum albumin blocked 0.22µm filter, collected in a fresh ultracentrifuge tube, and centrifuged at 110,000g for 2 hours and 4°C in a Beckman LE-80K centrifuge swing out rotor (Beckman Coulter Optima, SW 32 Ti). The resulting pellets were then harvested and washed in PBS by placing them in a 14 ml tube with PBS, and then centrifuged for 70 minutes at 110,000g and 4°C.

The Sample B EVs were prepared in the same way once they had been thawed.

### 2.2.3 The isolation of EVs

The most used method of EVs isolation depends on a series of successive centrifugations (Lässer *et al*., 2012). Lobb *et al.* (2015) found that an ultrafiltration method based on centrifugation is better and less time-consuming than pressure-driven devices for the concentration of EVs samples. The requirement for methods with high specificity using a minimal amount of body fluid led to the development of other techniques based on ultrafiltration, immunoaffinity, liquid chromatography and polymeric precipitation (Yamashita *et al.,* 2016). Results from these alternative techniques were variable, and in the immunoaffinity-based method, the production of high purity EVs was expensive. Various trials have used total protein content based on Micro Bicinchoninic Acid (BCA) Protein Assay as a technique to quantify and normalise EVs preparations before conducting downstream analysis. However, the use of total protein content may lead to aggregation with different EVs, and lead to the rupture in vesicle membranes leading to false results and study bias.

Trials aimed at comparing purity and integrity of EVs using different isolation techniques are thus still needed. In 2013, Kalra *et al*. compared alternative methods for EVs isolation from human blood and concluded that the density gradient method was better than ultracentrifugation. Kalra *et al.* (2013) evaluated the stability of EVs at different temperatures and found that although all samples were stable after three months, there was an advantage in keeping EVs at −80 °C.

Among these techniques, differential ultracentrifugation is the standard protocol and the most used technique for EVs isolation (Théry *et al.,* 2006). Alternatively, EVs can be isolated using either sucrose density ‘cushions’ where there is a uniform concentration in the test-tube, or ‘gradients’ where there is varying concentration. Because the EVs have a relatively low-density, they float, and thus they can easily be retrieved from the supernatant (Cantin *et al.,* 2008, Lamparski *et al.,* 2002). Ultracentrifugation by itself produces protein contamination, and one of the advantages of

using sucrose density gradients is that this method reduces the contamination from large protein aggregates that co-sediment with EVs during ultracentrifugation (Tauro *et al.,* 2012). If ultracentrifugation is combined with sucrose gradients it can result in a higher concentration of EVs (Théry *et al.,* 2006), but the yield would be too low and unreliable for the purpose of this study.

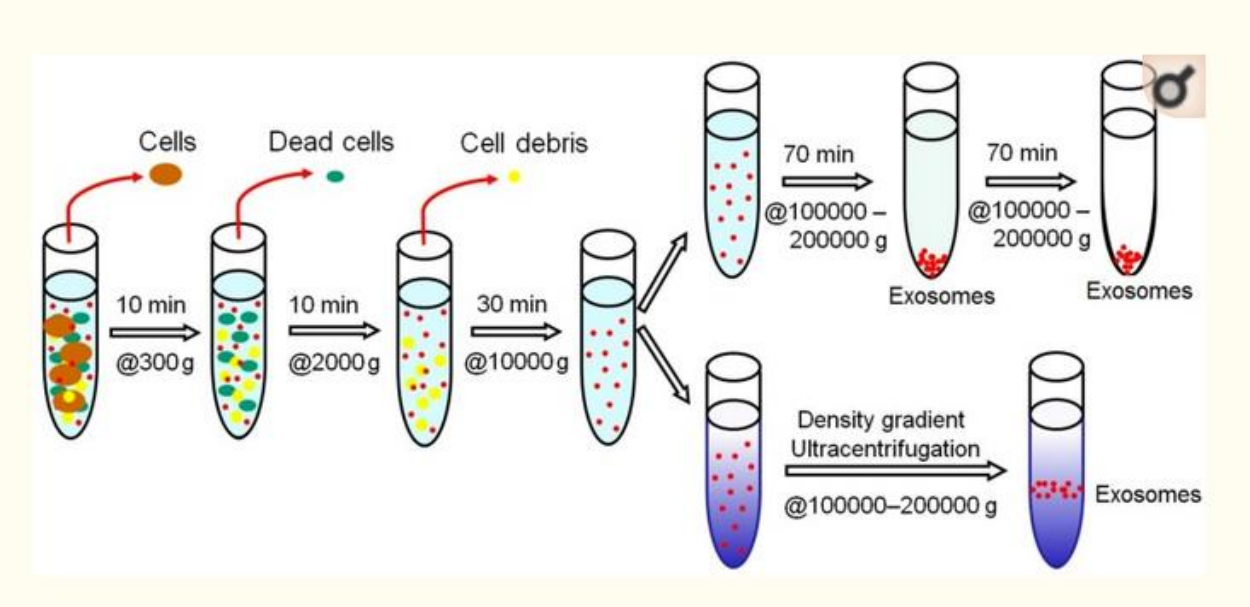



*Figure 7: Successive centrifugation at 4○C (adapted from Li et al., 2017).*

### 2.2.4 The use of ExoQuick™ precipitation to isolate the EVs from the plasma

The production of commercial kits such as ExoQuick® (System Biosciences) EVs precipitation for the isolation of EVs has thus increased rapidly in recent years (Andreu *et al.,* 2016), and the commercial EVs isolation kit that was used here was ExoQuick™ precipitation. The isolation was carried out according to the manufacturer's instructions and 500 microlitres (µl) of plasma was diluted to 5 millilitres (ml) in PBS and mixed with 1 ml of ExoQuick™ solution by inverting the

tube several times. The sample was incubated overnight at 4°C and then centrifuged at 1,500g for 30 minutes and then centrifuged again for five minutes respectively in order to remove the supernatant. The supernatant was discarded, and the pellet was re-suspended in 200ml of PBS.

### 2.2.5 The Characterisation of EVs

Challenges in EVs characterisation (according to their size/their surface proteins/their ability to bind to specific antibodies/etc) still remain to be clarified in EVs research (Théry *et al.,* 2006), and there are various technologies such as optical particle tracking/photon correlation spectroscopy/cryo-electron microscopy/etc that can be used to investigate EVs characterisation (Taylor and Shah, 2015), but there is no single gold standard method that has been agreed on by the experts in the field (Raposo and Stoorvogel, 2013). In this study, EVs have been characterised by (i) Nanoparticle Tracking Analysis (NTA), (ii) transmission electron microscopy (TEM), and (iii) fluorescent staining with confocal microscopy. These have been the most effective methods that have been used in differing EVs studies.

### 2.2.6 Nanoparticle Tracking Analysis (NTA) to ascertain EVs concentration levels and vesicle sizes

NTA employs direct, real-time visualisation and analysis of nanoparticles in liquids and resolves most of the problems that accompany the previously described methods (Gardiner *et al.,* 2013) (Figure 8). This technique utilises a laser beam that strikes the sample, and the visualisation of vesicles takes place by scattering the observed light through a light microscope. The tracking of the Brownian movement of individual vesicles is performed through the video taken by the NTA

software (Dragovic *et al.,* 2011), and the size of the vesicles and their total concentration is calculated by the diffusion coefficient of the particles (Dragovic *et al.,* 2011).

Sample suspensions containing EVs vesicles were first diluted and then the samples were kept on ice. Samples were analysed using a NanoSight® LM10, NS500 instrument (NanoSight®, Amesbury, UK). 90 second videos were used to track the Brownian movement of the EVs using Version 2.2 of the NanoSight® software to give mean and median vesicle size and oncentration. For every sample, this reading was conducted five times, with mixing in between, and then an average was taken to remove sample mixing bias.

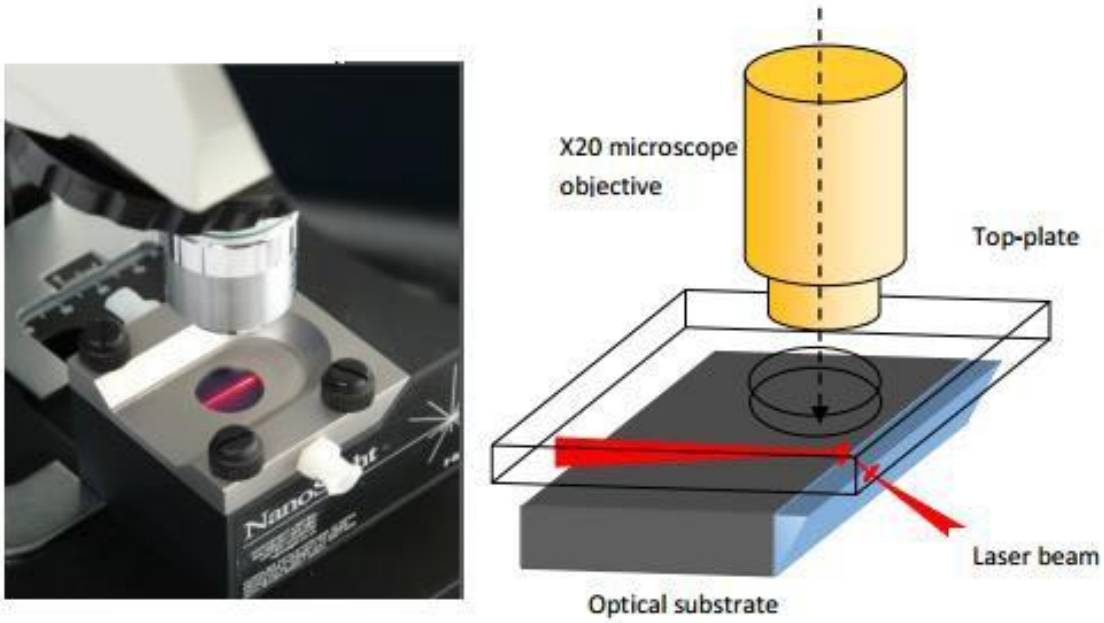



*Figure 8: Nanoparticle Tracking Analysis, adapted from Carr and Wright (2008).*

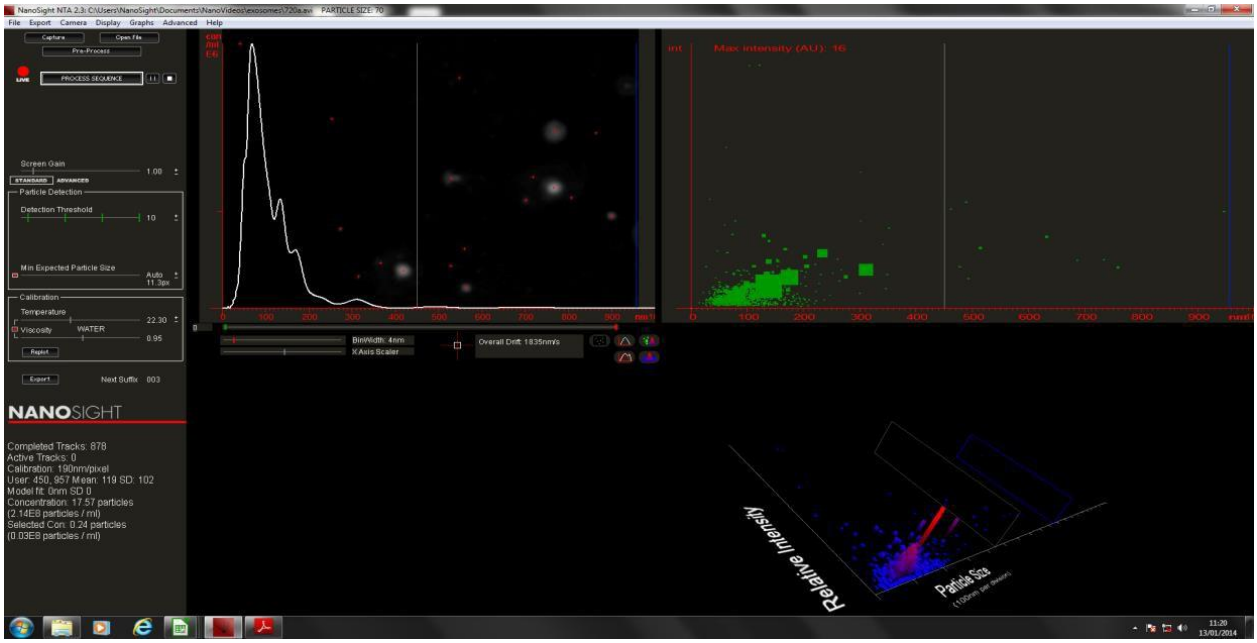



*Figure 9: Example of NTA measurement of particle size and concentration.*

The main advantage of NTA is that it successfully resolves one of the fundamental problems associated with other light scattering methods (*i.e.* the problem of dynamic light scattering). It does this by having the capability to resolve particles of different sizes at the same time whilst utilising the same solution (Dragovic *et al.,* 2011). In spite of its accuracy in measuring the size and concentration levels of vesicles, the main disadvantage of conventional NTA is that it cannot identify the phenotype nor the origin cell of vesicles.

### 2.2.7 Variability in technical EVs measurement

To date, a wide range of EVs physiological and pathological function studies have been conducted, but there is a need to understand better the variables that affect plasma EVs concentration levels and the mechanisms that control the production and mediation of EVs. Due to the pathological involvement of EVs, it is important to know the variability in measuring EVs

sizes and concentration levels. An understanding of the variability in size and concentration levels may help identify the variety of normal and pathological roles that EVs perform in the body. Considering the physiological and pathological importance of EVs as potential biomarkers for neurodegenerative diseases and multiple cancer types, there exists a need for measures of the content of the concentration levels of EVs in plasma, as this should lead to reliable and accurate prognosis of disease initiation and development. Batterham and George (2003) state that within subject variation, systemic change in the mean, and re-test correlations are the main measures of reliability. It is thus important to measure the bias that can occur in the data affecting measurement variability. This can occur in pre-analytical (specimen collection, handling and processing), analytical (imprecision and bias), and post-analytical (interpreting and reporting data) data sets (Picotte *et al.* 2009).

Gardiner *et al.* (2013) used NanoSight® Tracking Analysis (NTA) to examine the analytical measurement variation of the EVs concentration measurement processes. This is essentially a technique where a beam of laser light is scattered by particles in Brownian motion in suspension in the path of the beam, and a video camera captures the movement of these particles according to the prevalence of the different sized EVs. To increase the level of accuracy associated with the measurements involved, Gardiner *et al.* (2013) used silica microspheres of known size as standards by which to calibrate the measurements taken. The additive features of the technology are distributed across five distinct levels. This allows for the determination of a detection threshold which refers to the minimum value for the level of an image that can help distinguish a particle for further analysis. The technology used can help to determine the maximum distance that the software expects a particle to move from one frame of reference to another. This is directly proportional to the size of the particle, and larger particles take longer to move across the frames. The minimum expected particle size is automatically determined by the software. ‘Blur’ is another automatic setting built into the software and it is defined by the degree of smoothing of the images

received by the software after removing unrequired noise (and predominantly includes the diffraction rings found around the larger particles). However, a biological standard for EVs measurement has not been agreed, and the within-subject variations have not been addressed.

The NanoSight® equipment can be manually adjusted to cater for larger as well as smaller particle sizes. For a particle to be included in the size distribution plot, the equipment determines the minimum track length that a particle should cover based on its size. This plot is based on the Mean Square Displacement profile for all the particles that have been tracked for a minimum number of frames – this being determined by the machine automatically or set manually by the researcher. Higher values for minimum track length mean that the particles have been tracked for a greater length of time and this helps in the accurate sizing of all the particles under scrutiny. However, this does not imply that the particles that spend less time in crossing one frame of reference to another one is measured less accurately. It could mean that these particles are smaller in size and thus take less time to get across the threshold.

Lastly, background noise or pixel contamination can be easily removed from the extracted data sets or images to make the readings as accurate as possible for the size of the particles. In order to develop the importance of their molecular physiology in relation to the function that they perform, NTA can be successfully employed to determine the size of biological molecules such as EVs (Gardiner *et al.* 2013).

### 2.2.8 Same day content

In order to assess same day content, the same eleven individuals (N=11) participated in this study. In the visit, the first fasting blood (consisting of three successive blood samples) was drawn by venepuncture using a winged infusion device at the baseline of 0 hours. Then 4ml samples of

blood were drawn at hour 1, hour 2, hour 3, hour 4, hour 5, hour 6, hour 8, and hour 10. After the second hour and the sixth hour, the participants were offered a standard carbohydrate diet (of porridge, oat biscuits and brown toast). A carbohydrate diet is a low-fat diet that provides long-term energy for the participants and could be picked up in the samples. All the blood samples were processed as described in Chapter Two.

### 2.2.9 Content across several days

In order to examine the content of plasma EVs concentration levels across multiple days, the same eleven participants reported to the laboratory five times over five consecutive days. During each visit, each participant had 4ml blood samples drawn at the same time of each day. Each participant had a specific time of the day assigned to him.

### 2.2.10 Transmission Electron Microscopy (TEM) examination of EVs samples to characterise their morphology

An aliquot of the EVs vesicle sample was combined in a 1:1 ratio with 4% paraformaldehyde reagent grade (PFA) and cooled for 15 minutes on ice. A single drop of each sample was placed onto a strip of Parafilm® (produced by VWR™/Avantor). Carbon-formvar coated copper grids, 200mesh (F077, TAAB) were placed dull-side down onto the EVs/PFA droplet and left at room temperature for 45 minutes. The grids were then placed sample-side down onto three 30µl drops of 0.22µm filtered ultra-pure water for one minute each. To remove any excess liquid, the sides of the grids between each drop were gently touched with a filter paper. The grids were then placed sample-side down onto a 30µl drop of 2% uranyl acetate for two minutes. All the grids were touched with filter paper and left to dry, sample side up for one hour. After this, the grids were

stored in a box for analysis using a Hitachi H7650 Transmission Electron Microscope (TEM) at 120 kV.

### 2.2.11 Blood lactate

Lactate Pro is a portable blood analyser that uses an electrochemical method of measuring enzyme reactions on a small electrode by capillary action straight from a fingertip. Only a few drops (5µl) of blood are needed, and this is an advantage when multiple samples are required from each individual. In this study, blood lactate concentrations ([La-]) were determined from a fingertip capillary whole-blood sample using a portable analyser (Lactate Pro, Arkray, Japan). The fingertip area was sterilised with an alcohol wipe and then punctured using a lancet. The first drop of blood was removed to avoid sample contamination and the second drop was extracted for analysis.

### 2.2.12 Quantikine® enzyme linked immunosorbent assay (ELISA) for cytokine measurement

ELISA (R&D, D6050) was used for the quantitative determination of human Interleukin 6 (IL-6, IL-10, and TNF-α) in plasma. All reagents and samples were brought to room temperature before use. Working standards and reagents were prepared as directed in the (IL-6, or IL-10, or TNF-α) instruction catalogues. Microplate strips were removed from the foil pouch. A 100µl of assay diluent RD1W was added to each well, then 100µl of standards, sample or control were added to differing wells, covered with adhesive strip and incubated for two hours at room temperature. The solution was aspirated and washed from each well, and this process was repeated three times for a total of four washes. 200µl of (IL-6, or IL-10 or TNF-α) conjugate were then added and covered with new adhesive strips and incubated for two hours at room temperature. The aspiration/wash was repeated for a total of four washes. Then 200µl of substrate solution was added to each well

and incubated for 20 minutes at room temperature in a dark place. A 50µl of stop solution was added to each well. The colour in the wells changed from blue to yellow, and then the plate was measured using a microplate reader set at a wavelength of 450nm.

# Chapter Three - The variability/content of plasma EVs concentration levels in healthy male individuals

### 3.1 Introduction: aims and objectives

Different diseases affect the secretion of EVs into the blood stream, and the yield of EVs that can be isolated from plasma is influenced by many factors including the method of isolation (cf. Chapters 1 and 2) and the method of quantification *e.g.* NTA/DLS/Flow Cytometry/ELISA/etc. For example, Fernando (2017), using three different methodologies, found concentrations of 92.5 $\times 10^8$ particles/ml ranging up to 240 x $10^8$ particles/ml. However, yield can be an inverse 'trade-off' against purity, and Gudbergsson (2016) added that the inter-study reproducibility of EV analysis including yield is very limited because a standard methodology does not exist. .

The main aim of this part of this study is to explore the variability/content of plasma EVs concentration levels in healthy individuals, and there were two specific objectives in this part of the study.

1) To assess the content of plasma EVs concentration levels from the same individuals, from (a) multiple samples of blood taken at the same time point, and also from (b) multiple samples taken at different times of the day and (c) across different days.

2) To determine whether there was any statistical difference in the above measure between fresh and frozen-and-then-thawed plasma (hereinafter referred to as frozen/thawed plasma).

Although, circadian variations are often detected in human immune biomarkers, the first Hypothesis of this study is that the concentration levels of plasma EVs are stable within the sample, and that there is little day-to-day variability in the concentration levels of plasma EVs over the five days of blood sampling that was conducted in this study.

### 3.2 Statistical analysis

The data presented in this Chapter, which related to the participants' characteristics, are presented as mean ± standard deviation (SD). Statistical analyses were performed in SPSS for Windows version 21 (SPSS Inc., Chicago, IL, USA). The SPSS normality test showed a box-plot that was reasonably symmetrical and in which the Q2 and Q3 quartiles were of roughly the same order of magnitude, and there were no 'outliers'. The analysis also showed that (in accordance with the SPSS user manual recommendation) both the Skewness and Kurtosis were not more than twice the Standard Error of the Mean, and the assumption of Normality was thus not violated (Statistics in Psychology, British Psychological Society-Blackwell). In order to detect any differences over the course of one day (at time points 0 hours, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 8 hours and 10 hours), the variables were examined by one-way repeated measures ANOVA, with time as the independent variable, and in like manner across multiple days (days 1, 2, 3, 4 and 5).

By time of day (across all nine time points in one day and across the different days) the content was examined by means of a two-way mixed effect model interclass correlation coefficient (ICC 3,1 as defined by SPSS) for absolute agreement (Shrout and Fleiss, 1979). Tests for homoscedasticity showed that the variance in the data was homogeneous, and the ICC 95% confidence intervals (95% CI) were calculated. An ICC $\geq 0.7$ was accepted, with values of 0.60-0.74 being considered as good, and 0.75-1.00 as excellent (Cicchetti, 1994). The coefficient of variation (CV) was determined for each participant using the formula: SD/mean*100 to determine measurement variability, and same day CVs were calculated across the time points. Within the sample and across the five-day period, CVs were calculated across the five visits and with the group average presented. Significance was set at the level of $p < 0.05$.

### 3.3 Results

Twelve males volunteered to participate in this study, but it proved difficult to find a suitable vein from which to draw blood from one individual, and he therefore dropped out of the study. The sample size in this study was therefore eleven (N = 11).

Eleven healthy male participants (aged 27.6 ± 4.2 y, height, 177.0 ± 9.4cm, and of body weight, 80.3 ± 12kg) thus participated in this study which was approved by the Research Ethics Committee of Oxford Brookes University (Appendix A - Ethics Approval Number 130712).

*Table 1: Age/Height/Weight/Blood Pressure/and Heart Rate of the eleven participants in this study (Mean ± Standard Deviation (SD).*

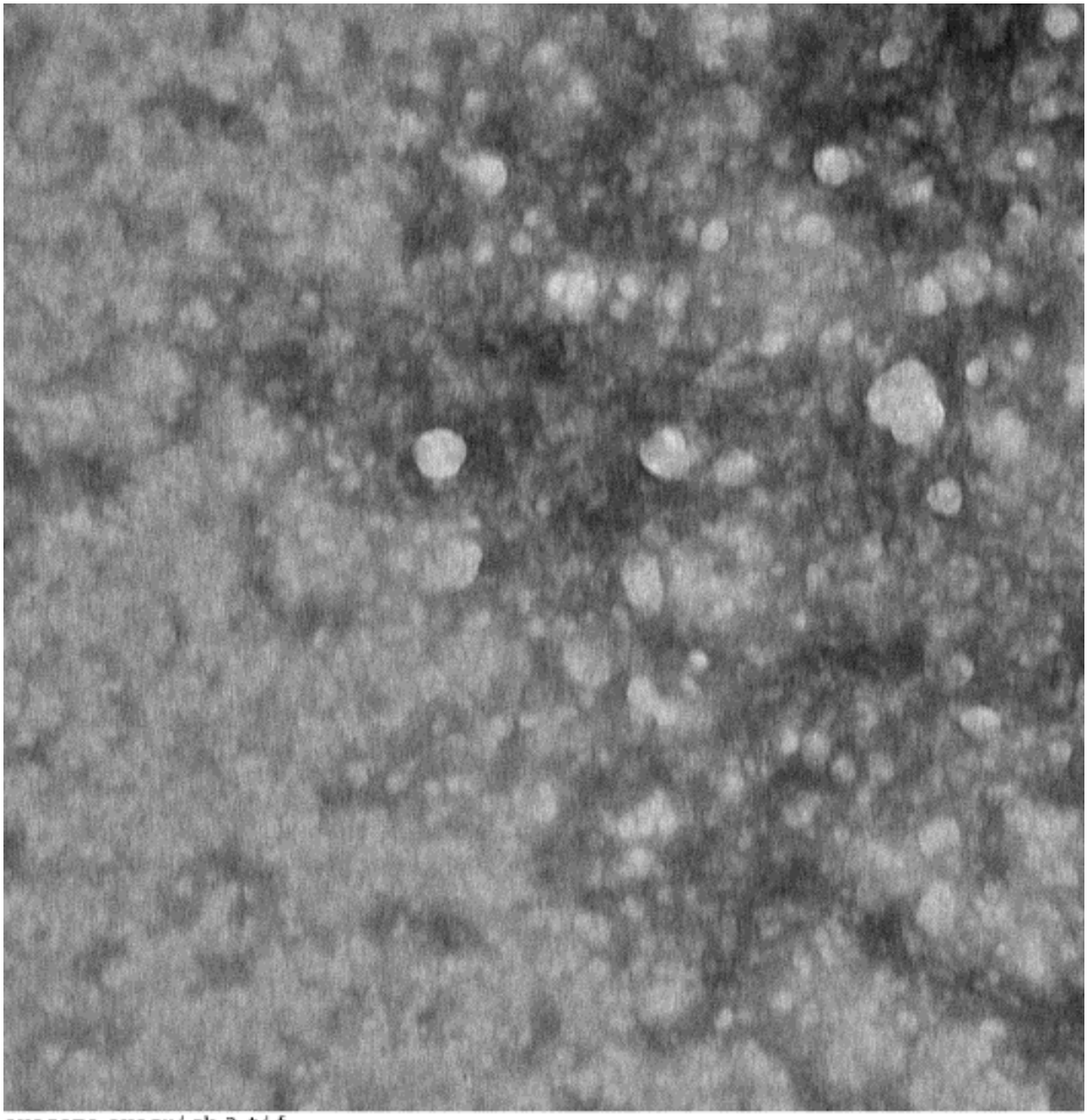
| Characteristics of the 11 subjects in this study | Mean ± SD |
|---|---|
| **Age (years)** | 27.6 ± 4.2 |
| **Height (cm)** | 177.2 ± 9.4 |
| **Weight (kg)** | 80.3 ± 12 |
| **Blood pressure (mm Hg)** | 130/73 ± 7.37 |
| **Heart rate (beat/min)** | 90 ± 30 |

*Data is reported as mean ± standard deviation (SD)

#### 3.3.1 Stability of plasma EVs concentration levels

In order to assess the stability of plasma EVs under different conditions, fresh and frozen/ thawed plasma EVs were isolated by differential ultracentrifugation as described in Chapters 1 and 2.

They were analysed by NTA and showed a particle size ranging from 30 to 300nm in diameter, with an average of 120 ± 70nm. The concentration levels of fresh and frozen/thawed plasma EVs were stable (fresh samples 3.68 ± 3.1, $E*10^8$/ml, frozen/thawed samples 4.36 ± 2.8, $E*10^8$/ml), and there was no significant difference between fresh and frozen/thawed plasma EVs concentration levels ($p=0.072$) in all the eleven samples that were analysed by NTA. However, given our small sample size, we cannot definitely conclude that there is no significant difference between fresh and frozen plasma EVs concentration levels. Future studies would benefit from exploring this further with larger sample sizes. Under the electron microscope, EVs appeared as spherical vesicles with a cup shape and their diameters ranged from 30 to 120nm (Figure 10 next page).



exosome exoquick 3.tif
Cal: 0.750751 nm/pix
10:25:11 a 10/10/12
TEM Mode: Imaging

100 nm
HV=100.0kV
Direct Mag: 20000x
Oxford Brookes

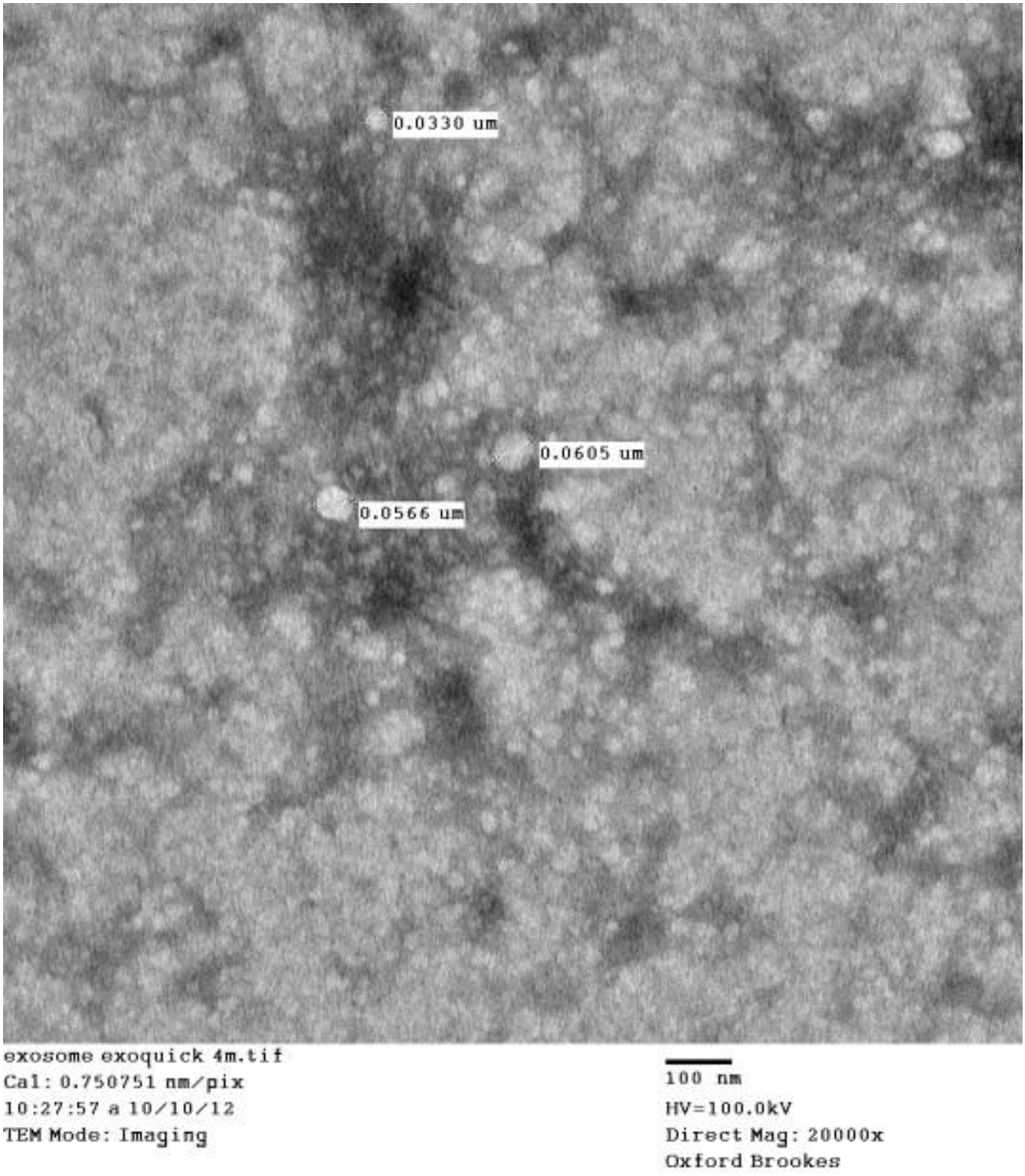



*Figure 10: Example of extracellular vesicles characterisation isolated from human blood plasma. Observed by electron microscopy they appeared as round cup shaped particles ranging in size between 30 and 120nm.*

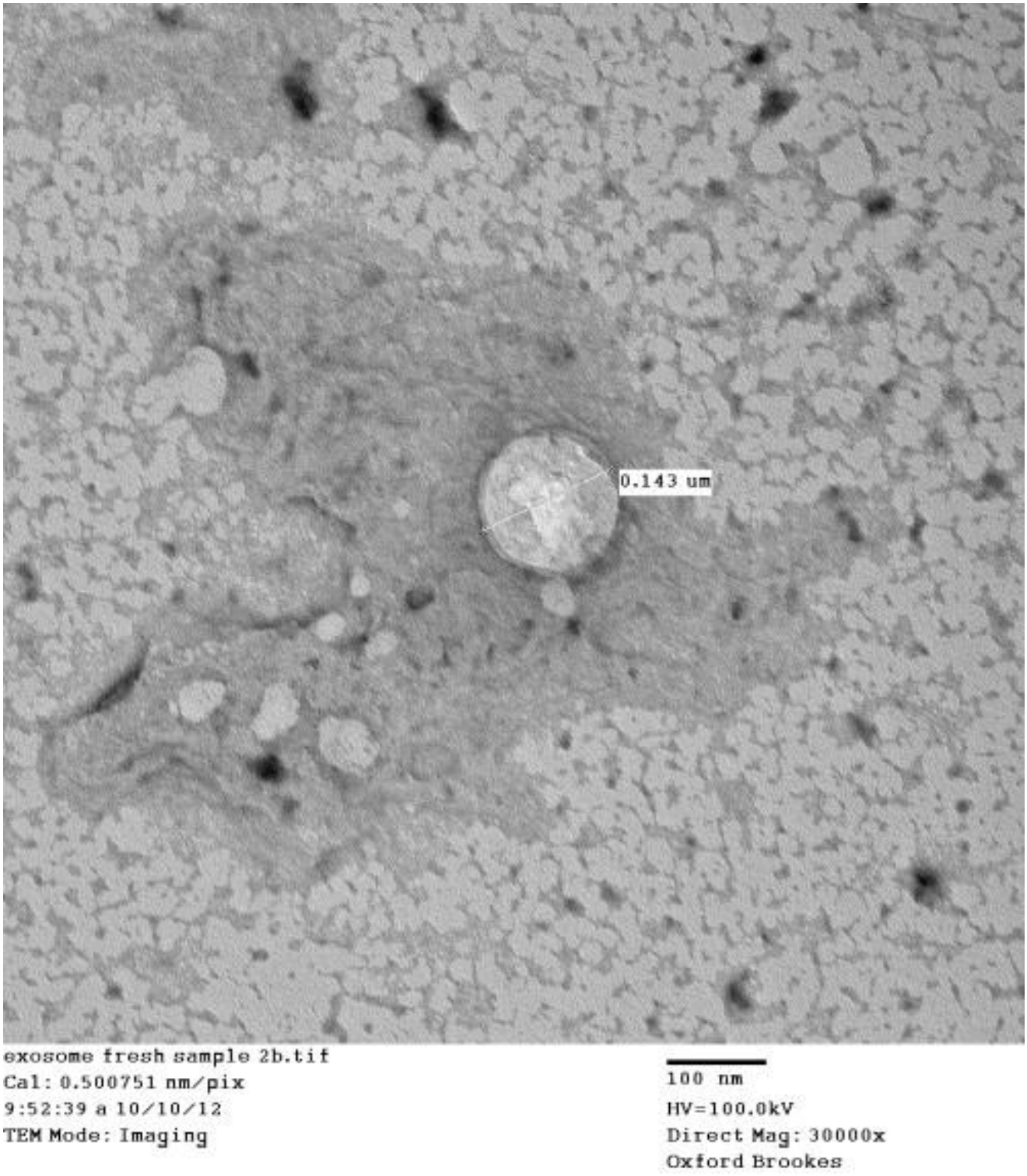



*Figure 11: Plate A: scanning electron micrographs of plasma EVs extracted from fresh blood samples of healthy individual shown by electron microscopy.*

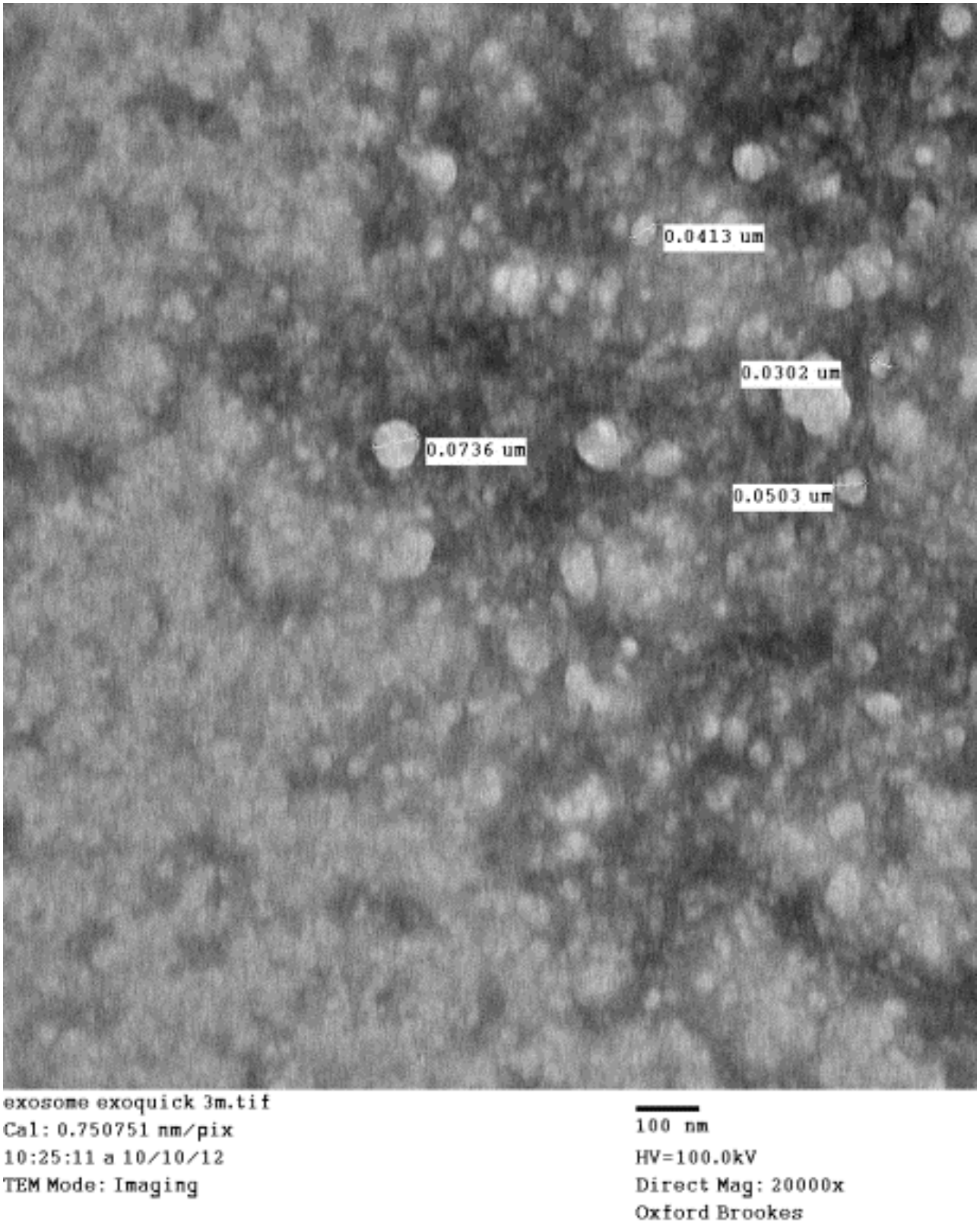



*Figure 12: Plate B: scanning electron micrographs of frozen/thawed plasma EVs of healthy individuals shown by electron microscopy.*

The total plasma EVs concentration was obtained by NTA. Based on particle size, data were divided into two categories: vesicles sized 30-100nm termed EVs, and vesicles sized 101-1000nm termed 'microvesicles' (Fig 13, and 14 below). There was no change in the number of both extracellular vesicles. There was also no significant change in the extracellular vesicles size within sample.

### 3.3.2 Content/variability of plasma EVs concentration levels within sample, within day, and between days

This study was conducted to assess the content of plasma EVs concentration levels in healthy individuals over the course of one day and over the course of several days. The EVs concentration levels of eleven healthy men were assessed for content over the same sampling period which consisted of ten hours in a single day over the course of five consecutive days. Table 2 shows the descriptive characteristics of all the eleven subjects who completed the study. No participant had reported any illness in the 24 hours' period prior to blood being drawn. All the subjects were non-smokers.

*Table 2: Average number of three EVs samples which were measured by NTA under two different conditions (fresh, and frozen/thawed samples). Error bars represent Standard Deviation (SD).*

| Number of subjects | 11 |
|---|---|
| **Age (years)** | 27.6 ± 4.2 |
| **Height (cm)** | 177.2 ± 9.4 |
| **Weight (kg)** | 80.3 ± 12 |
| **Blood pressure (mm Hg)** | 130/73 ± 7.37 |
| **Heart rate (beat/min)** | 90 ± 30 |

*Data is reported as mean ± standard deviation (SD)

### 3.3.3 Content/variability of plasma EVs concentration levels within the same subject at a single time point

To assess the sample variability of blood plasma EVs concentration levels, three different samples that had been drawn consecutively from each subject were measured using the NanoSight® equipment. The results from a single level (the sample-variation repeated measures ANOVA test performed in this study) showed that there was no significant difference between the concentration levels of EVs from the three repetitions of blood collected from everyone (P = 0.676). The sample plasma EVs concentration level measures showed excellent content (Cicchetti, 1994) within sample (ICCs ≥ 0.88) and minimal variability (Coefficient of variation CV= 42) (Table 3). The results therefore show consistent results regarding the concentration levels of plasma EVs within

consecutive samples taken at the same time point. Toebe et al. (2018) asserted that larger sizes did not decrease CV levels but decreases the variability of the estimate of the same, which increases the precision in CV estimation.

*Table 3: Within sample measures of plasma EVs concentration levels taken on a single day and across five days.*

| | ANOVA *P VALUE* | *Degrees of freedom DF n-1* | ICC | 95% CONFIDENCE INTERVALS (CI) | Coefficient of variation (CV) % |
|---|---|---|---|---|---|
| **Within Sample** | 0.67 | 20 | 0.88 | 0.24 - 0.9 | 42 |
| **Single day** | 0.42 | 10 | 0.84 | 0.827- 0.926 | 34 |
| **Five days** | 0.95 | 10 | 0.75 | 0.457- 0.924 | 30 |

CV showed as SD/Mean *100

### 3.3.4 Content/variability of plasma EVs concentration levels and size within the same subject at multiple time points during the same day

To assess the same day content of the concentration levels of EVs from each subject, plasma vesicles were measured from the samples taken over the 10 hours during the day. The size distribution of the EVs was also measured over the course of a day at different time points and across five consecutive days in eleven healthy individuals.

A single level repeated measures ANOVA test was performed here. The results show that there were no significant differences at any time point (P = 0.95). The average EVs concentration level values were 4.8 x$10^8$/ml for all time points in all the subjects (represented in Figure 13). The same day plasma EVs concentration levels showed excellent (Cicchetti, 1994) content (ICCs ≥ 0.75, CV = 30). For size distribution, the results show that there was no statistically significant difference at any time point of the day (P=0.14).

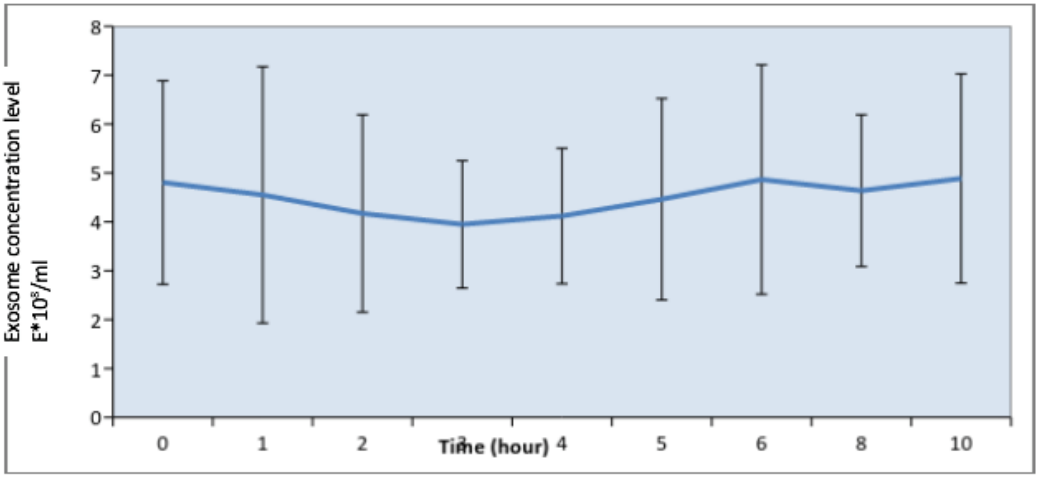



*Figure 13: Plasma EVs concentration levels of nine samples taken hourly over many hours within one day. The average was that of all participant samples (N= 11), each replicate was measured five times on NanoSight® equipment (represented as mean ± standard deviation. Time was measured per hour.*

### 3.3.5 Content and variability of plasma EVs concentration levels and size over multiple days

The variability of the plasma EVs measures demonstrates consistent measurements for plasma EVs concentration levels (ICCs ≥ 0.84, and CV = 34%). Over five days the average EVs concentration value of each sample was 1.8 ×$10^8$/ml. There was no significant difference in the concentration levels over five days (P = 0.50). One-way analysis of variance (ANOVA) of the

EVs data also showed no significant difference across several days (represented by the average value for each of the five days) as shown in Figure 14. Moreover, there was no change in the average distribution of the EVs concentration levels of all eleven subjects over the five consecutive days.

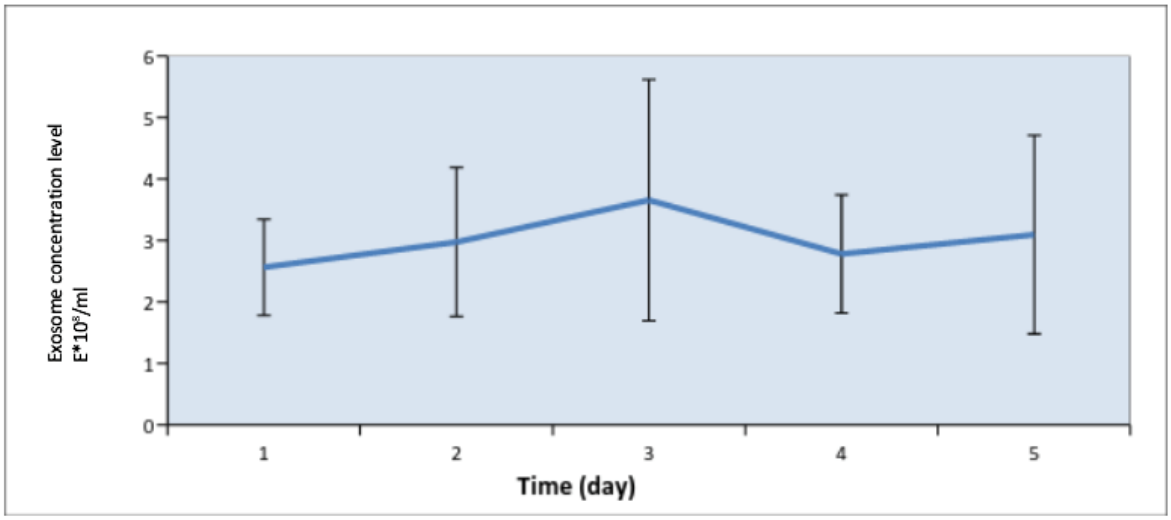



*Figure 14: Plasma EVs concentration levels taken from the average of all participant samples (N= 11) over five consecutive days (time was measured per day). Each replicate was measured five times on the NanoSight® equipment (represented as mean ± standard deviation).*

### 3.4 Summary

This study assessed the content of plasma EVs concentration levels in healthy male individuals within the sample, by time of day and across several days. It has been shown that the concentration levels of EVs isolated from blood plasma are consistent, and thus can be a good indicator to use in the search for vesicular biomarkers. It was also shown that freezing has no effect on the size and concentration levels of plasma EVs. Hypothesis One (that the concentration levels of plasma EVs are stable within the sample, and that there is little day-to-day variability in the concentration

levels of plasma EVs over the five days of blood sampling that was conducted in this study) was therefore accepted.

# Chapter Four - The role of EVs and inflammatory cytokines in response to a single bout of eccentric exercise and moderate in healthy individuals

## 4.1 Introduction: aims and objectives of this part of the study

The main aim of this part of the study is to look at the natural variation of inflammatory cytokines (IL-6, TNF-α, IL-10) alongside EVs secretion during a normal day, and secretion in response to a single bout of eccentric exercise and moderate cycling exercise. The objectives of this part of the study are to explore the extent of the systemic release of EVs following different types of exercise, and to examine the extent of the correlation between EVs and inflammatory cytokines. We hypothesize that there is a change in the concentration levels of cytokines and EVs in plasma after eccentric and moderate cycling exercise, the protocol of High *et al*., 1989 was applied into this study because we chose an exercise model that was a stressor to evoke the inflammatory response. To test this hypothesis, the following investigation was performed.

## 4.2 Sample size

The size of the sample chosen was based on data from a review done by Ploeger *et al.* (2009), comparing the measurement of cytokines (TNF-α, IL-6, IFN-γ) between two healthy groups. With an effect size of 0.55, the power of this study was set at 80%, and the level of significance was set at 5% (p=0.05 in a two tailed analysis). The size of the sample in this study (N=11) was based on a study by Aoi *et al.* (2013) (cf. Chapter One) which looked at changes in muscle RNA in response to different exercise protocols.

## 4.3 Design of this part of the study

Cytokines and EVs and especially EVs are released into the circulatory blood system during exercise, and the effect of different exercise protocols on circulating cytokines and EVs has been reported in different studies (Frühbeis *et al.,* 2015). There is an association between exercise and

the secretion of cytokines, and it is suggested that EVs play an important role in immune modulation (Zhang *et al.,* 2016). However, although it is becoming increasingly evident that a physically active lifestyle is associated with the secretion of lower levels of inflammatory markers, many studies have examined the anti-inflammatory response only to leisure-time physical activity (Friedenreich, 2011; Il'yasova *et al*., 2005), and have not investigated whether or not different modalities and intensities of physical activity may be associated with changes in inflammatory marker secretion levels. The level of exercise is a key determinant of the benefit related to exercise, and it is thus important to understand the relationship of different types of exercise with the secretion of cytokines and EVs into the circulatory blood system.

This is an experimental crossover randomised trial with control and experimental groups. The study was designed to explore the inflammatory response in healthy individuals in relation to two different types of exercise intensities, (i) eccentric exercise, and (ii) moderate cycling exercise. The participants were monitored over a period of ten hours (with hourly monitoring). Blood samples collected over that time were kept on ice and then spun for plasma. All plasma samples were aliquoted and stored at – 80 °C until needed.

### 4.4 Experimental procedures in this part of the study

Extracellular vesicles (EVs) are present in all bodily fluids, and physical activity is associated with the release of EVs into the blood circulatory system (Frühbeis *et al*., 2015). However, little is known about the effects of different types of exercise level and the secretions of EVs into the circulatory blood system, and there is a paucity of published data on the relationship between different modalities of exercise level and the secretions of inflammatory cytokines into the circulatory blood system. Moreover, although it is becoming increasingly evident that a physically active lifestyle is associated with lower concentrations of inflammatory markers, many studies

have assessed only leisure-time physical activity (Friedenreich, 2011; Il'yasova *et al.*, 2005), and have not investigated whether or not different modalities and intensities of physical activity may be associated with changes in inflammatory marker concentrations. This part of this study therefore investigates the EVs response to specific modalities of exercise.

The following procedure was followed with all the participants attending the study on Day One and then attending on Day Two and Day Three.

Day One (REST): the natural variation of EVs concentration levels over the course of a day was tested. Participants were asked to first fill in three questionnaires (as shown in the Appendices), to determine eligibility. Results showed all respondents were eligible to participate. A 4ml blood sample was drawn by venepuncture using a winged infusion device (BD vacutainer, BD VS367288) at the baseline (0 hours), and the needle was sealed with hyper-allergic tape. A blood sample was collected every hour for the first four hours by drawing blood from the cannula or the butterfly needle. Following this, blood samples were collected at eight hours and at ten hours by venepuncture (Figure 17).

Day Two (eccentric exercise): The study followed the standard high-level eccentric step-up protocol (High *et al.* (1989) that is recognised as causing muscle damage (Newham *et al.,* 1983). The eccentric exercise protocol began by each participant placing one leg on a 50cm-high step at a hip angle of 90°, and then stepping up and down 20 times while holding a weight equal to 25% of his total body weight. A two-minute rest was then given between each set of exercises, and the participants were asked to complete five sets of 20 steps-and-rest cycles. The duration of the test was approximately 15 minutes. A post-exercise 4ml blood sample was collected immediately after the exercise by venepuncture using a winged infusion device under the same conditions as on Day One.

Day Three (moderate cycling): Subjects were seated on a friction-braked Monark Ergo-medic 824E cycle ergometer (Monark AG, Sweden) in an upright posture with the handlebars adjusted for comfort. Saddle heights were adjusted to accommodate partial flexion of the knee between 170° and 175°, with 180° denoting a straight leg position. The Participants' feet were firmly supported by toe clips reinforced with straps, and the heel was held away from the pedal crank (Mulcare and Jackson, 2004).

The participants were asked to engage in a low-level eccentric cycling exercise (Mangione *et al.,* 1999) by performing an incremental cycle ergometer exercise test. Subjects were asked to pedal at a cadence of 60 rpm, and every two minutes the workload was increased by 25 watts until it reached 50% of their maximum HR (this was calculated prior to the test based on the participants' physiological parameter measurement from the previous study). Every two minutes, physiological parameters (HR, RPE and blood pressure using an arm cuff) were monitored during the exercise test period of a total of ten minutes of cycling. Resting and post-exercise 4ml blood samples were collected by venepuncture before and immediately after the exercise. On day 3, the EVs and cytokine analyses were based only the blood samples of the first three hours. This was done based on the results of the previous two days, which showed no changes after three hours. Resting and post exercise blood lactate measures were also recorded.

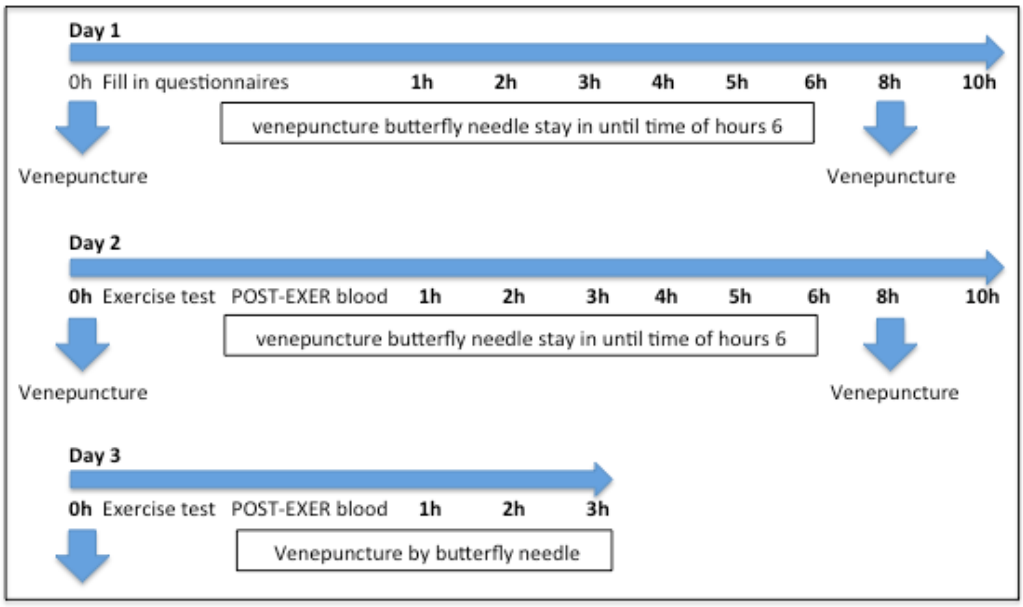



*Figure 15: Protocol of eccentric (Day 2) and moderate cycling exercise (Day 3). The venepuncture needle stayed in for 6 hours. Subsequently blood was collected by venepuncture.*

### 4.5 Plasma preparation in this part of the study

All plasma collection and preparation protocols, as described in previous Chapters, were adhered to and for this part of the study the University Ethics Committee approval number was 120661 (Appendix A). Participant Information Sheet is in Appendix B.

### 4.6 Statistical analysis

Data are presented as mean ± SD in the tables and text and mean ± SD in the figures. All eccebtric and moderate cycling exercise and EVs data were analysed, and statistical analyses were performed using SPSS v. 21 (SPSS Inc., USA) and tested for Normal distribution by the Shapiro-Wilk test. Within-exercise measurements were analysed to detect differences between levels of

exercise (eccentric and moderate cycling) with two-way (level × time) repeated-measures ANOVAs for the cytokines and EVs and paired-samples t-tests at control and exercise. The correlation data was tested by Pearson's correlation test and SPSS was used to test for homoscedasticity.

## 4.7 Results

### 4.7.1 Subject characteristics

The participants' age, height, weight and blood pressure is illustrated in Table 4. Data obtained from the eleven subjects in the training intervention were analysed. The table below shows descriptive statistics (viz. age, height, weight, blood pressure) of the eleven participants.

*Table 4: Subject descriptive characteristics based on subject's average age, height, weight and blood pressure values.*

| **Age in years** | **27.5 ± 3.9** |
|---|---|
| **Height, cm** | 179.6 ± 12.6 |
| **Weight, kg** | 76.6 ± 16.8 |
| **Systolic Blood Pressure (mm Hg)** | 126 ±4.4 |
| **Diastolic Blood Pressure (mm Hg)** | 71.3 ± 6.1 |

### 4.7.2 Delayed onset muscle soreness and lactate measure

As measured by the RPE, the participants experienced low levels of pain immediately after the exercise (rating of 1.7± 2.5), but the participants reported greater pain 24 hours after the exercise (rating of 3.3 ± 2.5) p = 0.01, and the peak of the pain that they experienced was 48 hours after the exercise day (rating of 5 ± 2.1). Subject variation was evident on day two ranging from almost no pain (a rating of 2), through moderate pain (a rating of 5) and then on to severe pain (a rating of 9).

For the lactate measure, the average lactate level after eccentric exercise was 7.68 ± 1.1 millimole per litre (mmol/l), and the average lactate concentration after moderate cycling exercise was 0.88 ± 0.4 mmol/l.

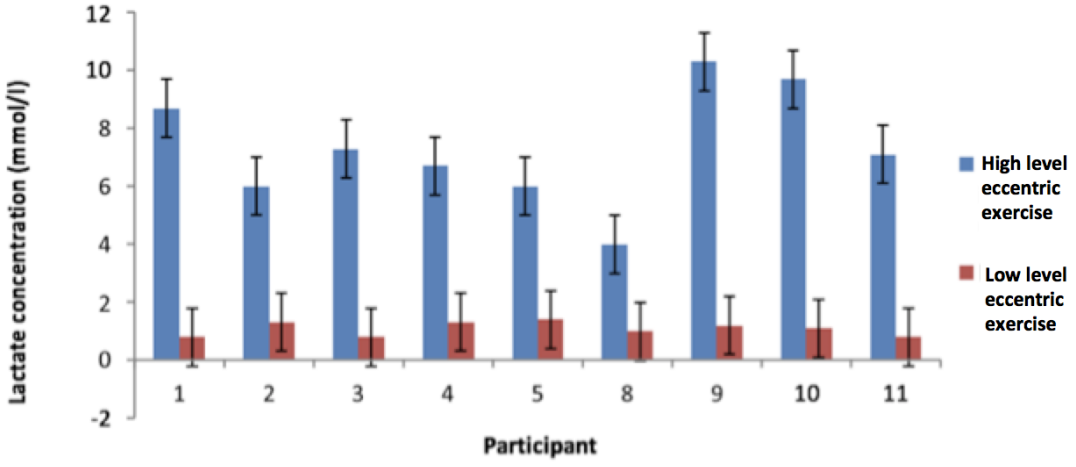



*Figure 16: Lactate concentration following eccentric and moderate cycling exercise measured in the eleven participants immediately after the exercise.*

### 4.7.3 IL-6 plasma measurements during rest and in response to eccentric exercise.

There was a significant difference in IL-6 concentration levels between resting and after eccentric exercise *(P* < 0.02). IL-6 values are presented as means ± SD in Table 5. It was found that IL-6 concentration levels peaked twice during the exercise experiment day. The first peak was observed two hours after the exercise, whereas the most substantial increases in plasma IL-6 levels were observed 6-8 hours after the exercise. In contrast, IL-6 concentration levels were undetectable on the rest day between hours one to four, with a gradual increase from hour five and a peak at hour six on the rest day.

*Table 5: Age/Height/Weight/Blood Pressure/and Heart Rate of the eleven participants in this study (Mean ± Standard Deviation (SD).*

| **Characteristics of the 11 subjects in this study** | **Mean ± SD** |
|---|---|
| **Age (years)** | 27.6 ± 4.2 |
| **Height (cm)** | 177.2 ± 9.4 |
| **Weight (kg)** | 80.3 ± 12 |
| **Blood pressure (mm Hg)** | 130/73 ± 7.37 |
| **Heart rate (beat/min)** | 90 ± 30 |
| **Rate of Perceived Exertion (Immediately after the exercise)** | 1.7 ± 2.5 |
| **Rate of Perceived Exertion (24 hours after the exercise)** | 3.3 ± 2.5 |
| **Rate of Perceived Exertion (48 hours after the exercise)** | 5.1 ± 2.1 |

### 4.7.4 TNF-α concentration levels in response to the exercise modality

From Figure 17 there was no significant difference in TNF-α average concentration levels between rest and eccentric exercise (*P*= 0.391). The effect of the differing time points on rest and exercise did not show any significant difference between subjects (*P= 0.739*).

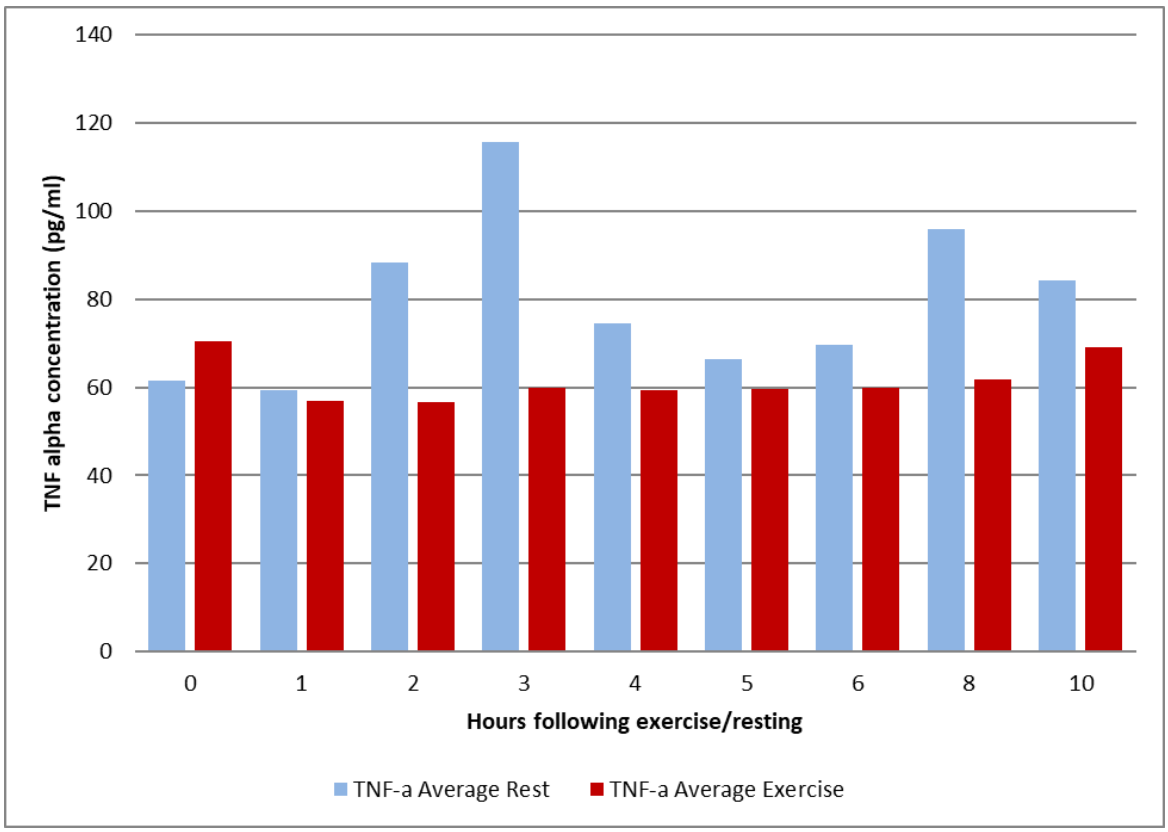



*Figure 17: TNF-α average concentration levels at rest and after eccentric exercise.*

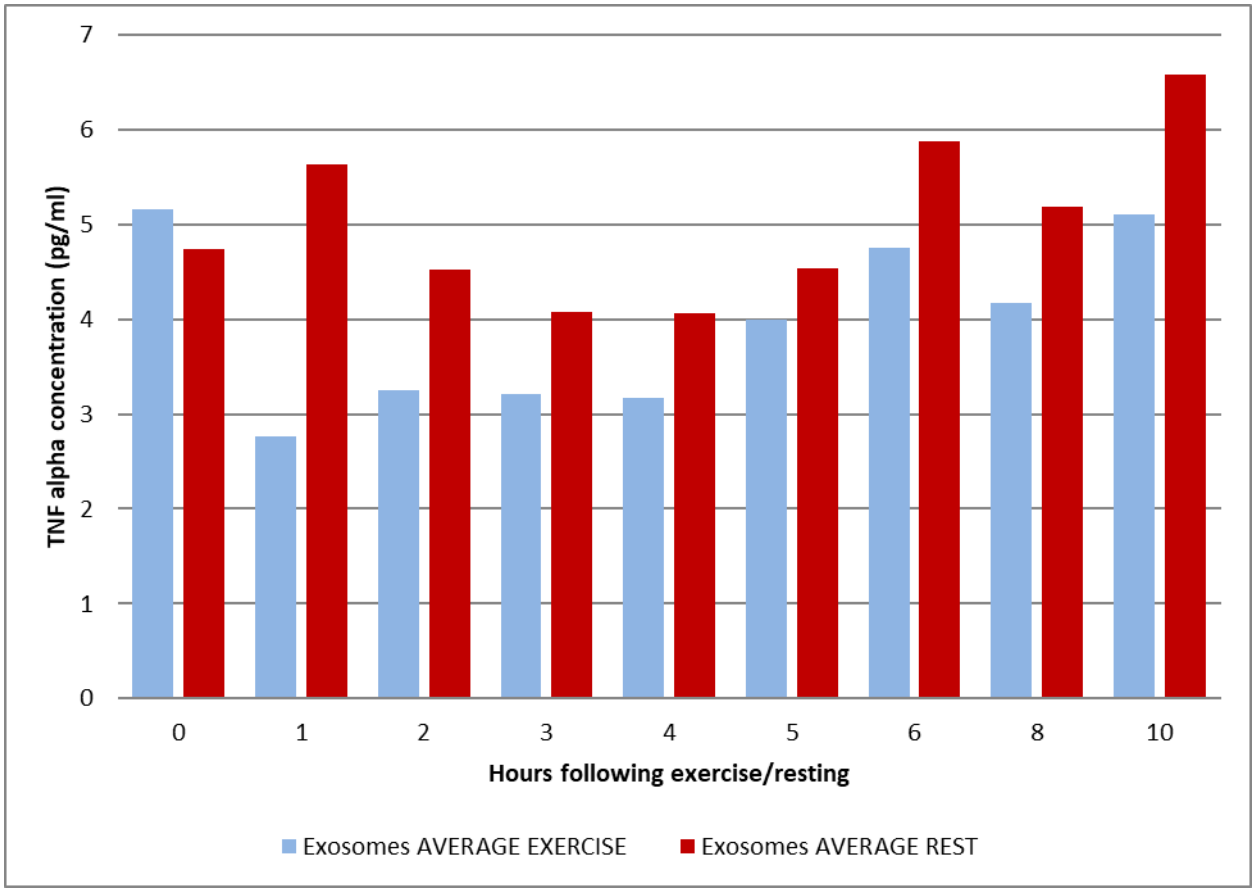



*Figure 18: EVs average concentration levels at rest and after eccentric exercise.*

### 4.7.5 EVs analysis (EVs concentration level measurements between rest and eccentric exercise)

In this part of the study, it was found that the average EVs concentration levels fluctuated during the rest day. In contrast, there was a reduction in the average two hours' EVs concentration levels in response to the eccentric exercise day. There was a significant difference between the measurement of the first three hours' average of EVs concentration levels at rest and after eccentric exercise ($P = 0.001$) (Figure 29).

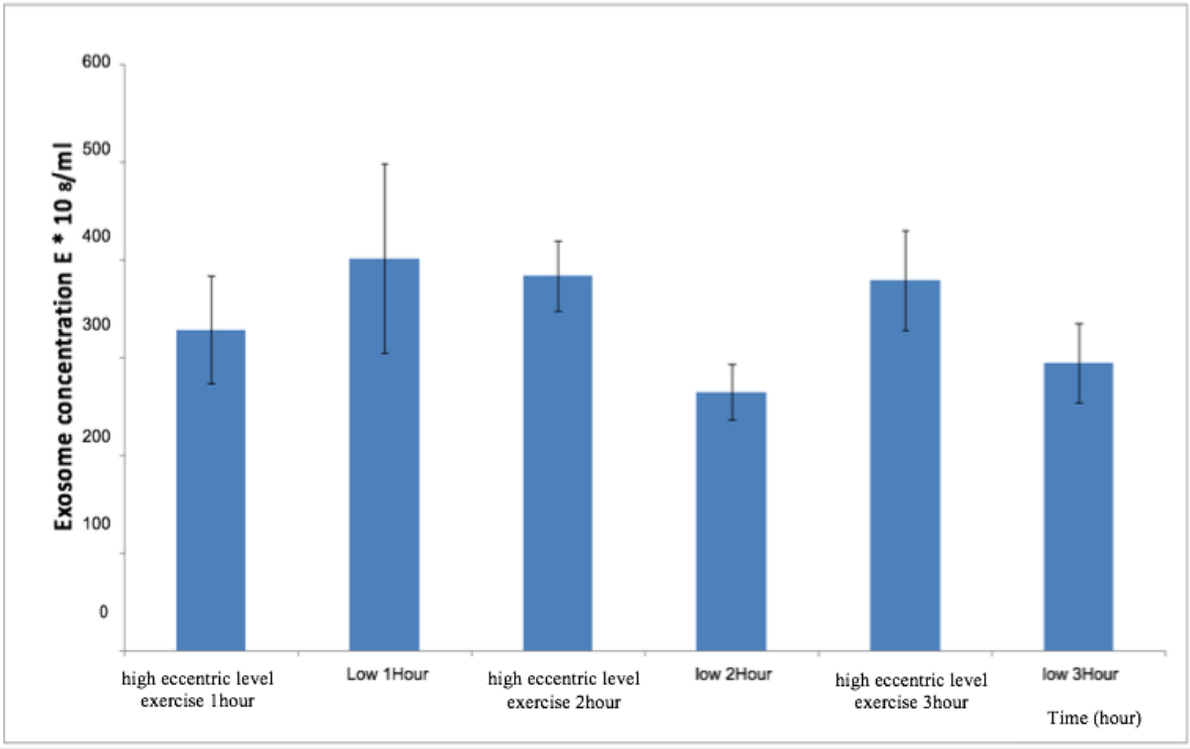
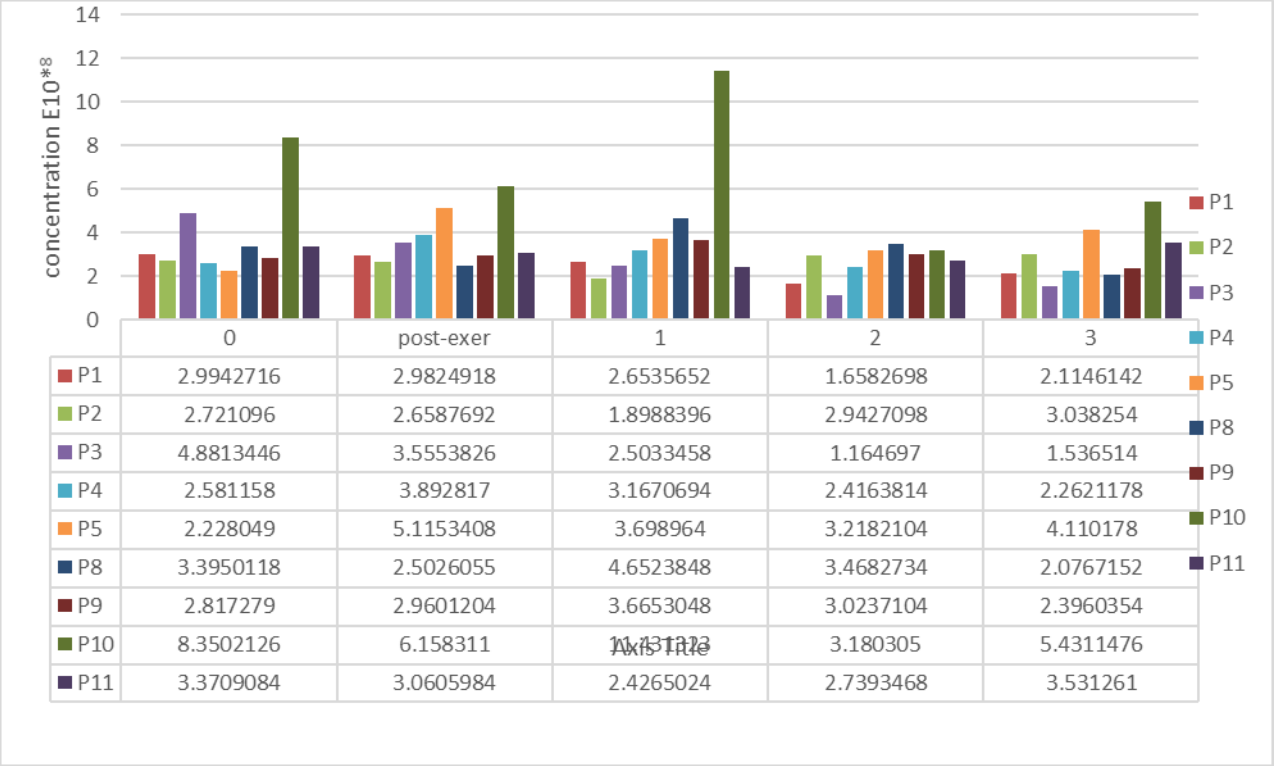



*Figure 19: Comparing of average EVs concentration in eccentric and moderate cycling exercise during hours one, two, and three*

For the moderate cycling exercise, the EVs concentration levels are shown for three hours only, because no perceived changes were observed thereafter. The average EVs concentration levels fluctuated during a normal day (Figure 20), and there was no significant difference between the measurement of EVs concentration levels at rest and the moderate cycling exercise measurement ($P > 0.05$).




| | 0 | post-exer | 1 | 2 | 3 |
|---|---|---|---|---|---|
| P1 | 2.9942716 | 2.9824918 | 2.6535652 | 1.6582698 | 2.1146142 |
| P2 | 2.721096 | 2.6587692 | 1.8988396 | 2.9427098 | 3.038254 |
| P3 | 4.8813446 | 3.5553826 | 2.5033458 | 1.164697 | 1.536514 |
| P4 | 2.581158 | 3.892817 | 3.1670694 | 2.4163814 | 2.2621178 |
| P5 | 2.228049 | 5.1153408 | 3.698964 | 3.2182104 | 4.110178 |
| P8 | 3.3950118 | 2.5026055 | 4.6523848 | 3.4682734 | 2.0767152 |
| P9 | 2.817279 | 2.9601204 | 3.6653048 | 3.0237104 | 2.3960354 |
| P10 | 8.3502126 | 6.158311 | 11.131373 | 3.180305 | 5.4311476 |
| P11 | 3.3709084 | 3.0605984 | 2.4265024 | 2.7393468 | 3.531261 |

*Figure 20: Average EVs concentration levels at each time point measured in nine healthy subjects following moderate cycling exercise. Error bars are presented as SD.*

### 4.7.6 EVs and IL-6, IL-10 and TNF-α concentration levels in response to eccentric exercise

There were no significant differences in the eccentric exercise EVs concentration levels and cytokine concentrations at any of the time points. Means and standard deviations for eccentric EVs and cytokine concentration level measurements are shown in Table 6. There was no significant correlation between EVs concentration levels and those of any of the inflammatory cytokines. The total plasma EVs concentration was obtained from NTA. Based on particle size, data were divided into two categories. Vesicles between 30-100 nm in size are termed as EVs, while vesicles between 101-1000 nm in size are termed as microvescicles. There was no change in the number of both extracellular vesicles. There was also no significant change in the extracellular vesicle size within sample.

*Table 6: shows values represented as means ± standard deviation of EVs and cytokine concentration levels during the first three hours following eccentric exercise.*

| Measures | Mean of concentration |
|---|---|
| Exo (E*$10^8$ /ml) | 4.043 |
| IL-6 (pg/ml) | 127.565105.2 |
| IL-10 (pg/ml) | 25 |
| TNF-α (pg/ml) | 63.808 |

### 4.7.7 EVs and IL-6, IL-10 and TNF-α concentration levels in response to moderate cycling

No significant correlation (*P > 0.05)* was reported between EVs concentration levels and cytokine measurements. Means and standard deviations for moderate cycling EVs and cytokine concentration levels are shown in Table 7.

*Table 7: Values represented as Mean ± Standard deviation of EVs and cytokine concentration levels.*

| Measures | Mean | Std. Error |
|---|---|---|
| Exo (E* $10^8$/ml) | 332.926 | 48.548 |
| IL-6 (pg/ml) | 15.594 | 0.541 |
| IL-10 (pg/ml) | 66.974 | 5.264 |
| TNF-α (pg/ml) | 97.651 | 14.630 |

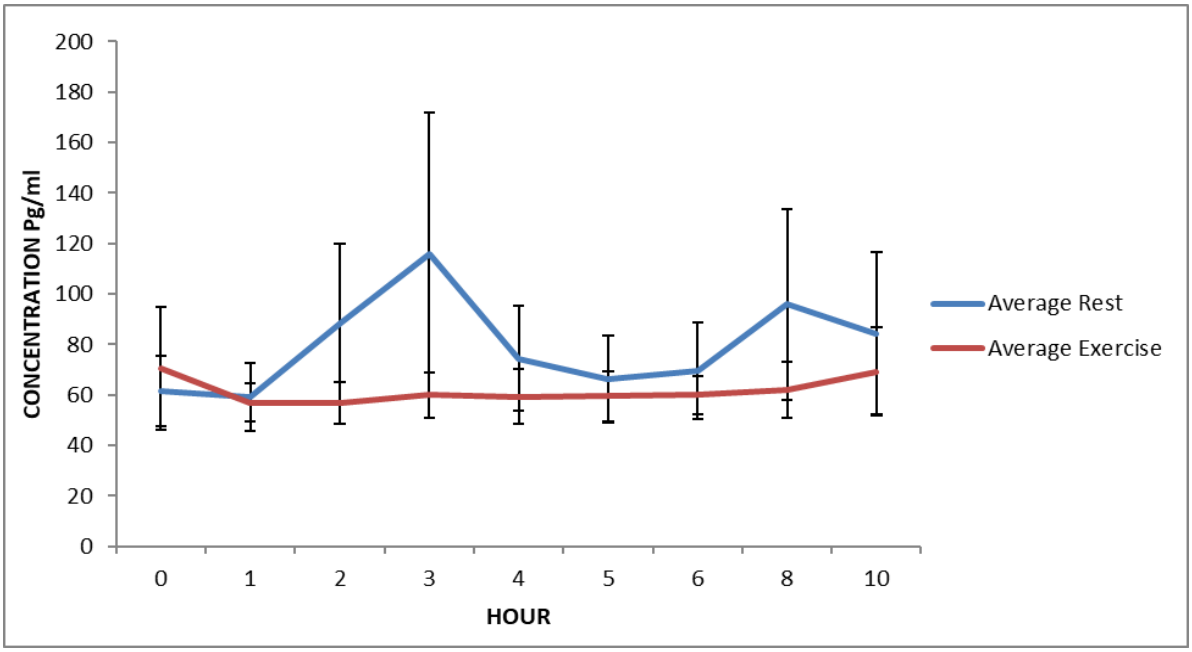



*Figure 21: TNF-α concentration (pg/ml) measured in plasma sample (triplicate) of all participants (N= 11) at rest (blue line) and following eccentric exercise (red line). Time was measured per days.*

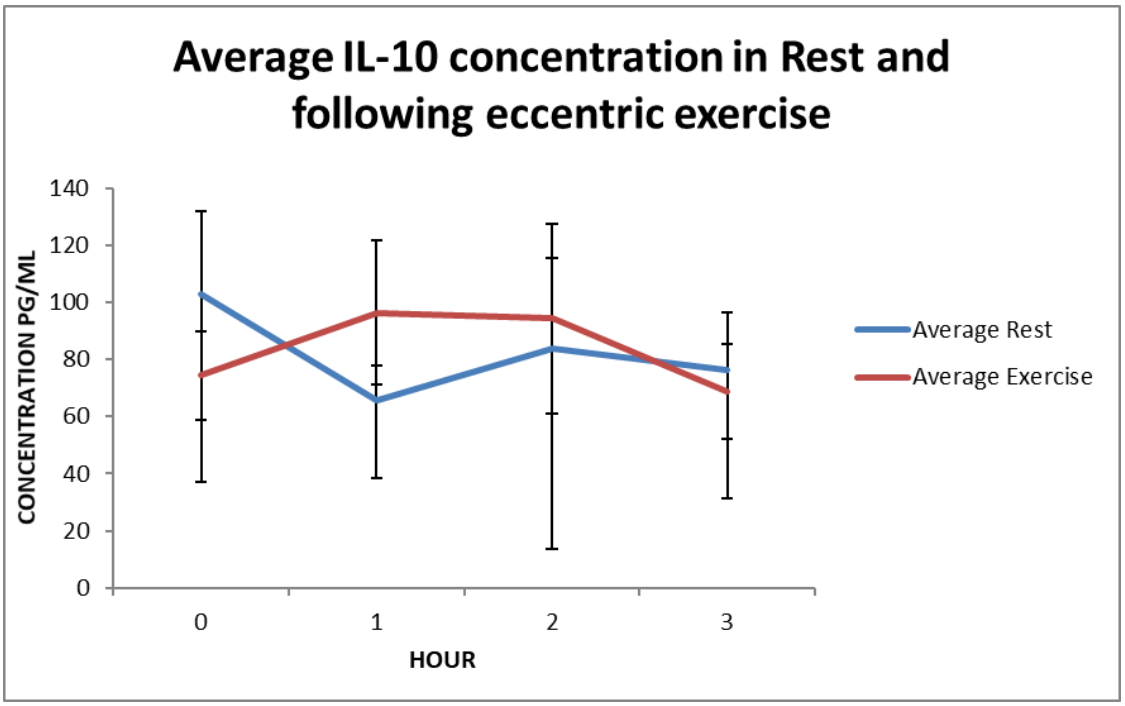



*Figure 22: Average IL-10 concentrations (pg/ml) were measured for three hours (Time) in rest and after eccentric exercise among all participants (N= 11). Each sample was measured in triplicate.*

### 4.7.8 Correlation of EVs concentration levels following eccentric exercise and lactate measurements

The results show that there was an apparent weak correlation between the EVs concentration levels after two hours and the concentration of lactate after eccentric exercise. Figure 23 shows that when the level of the exercise rose, EVs concentration levels declined.

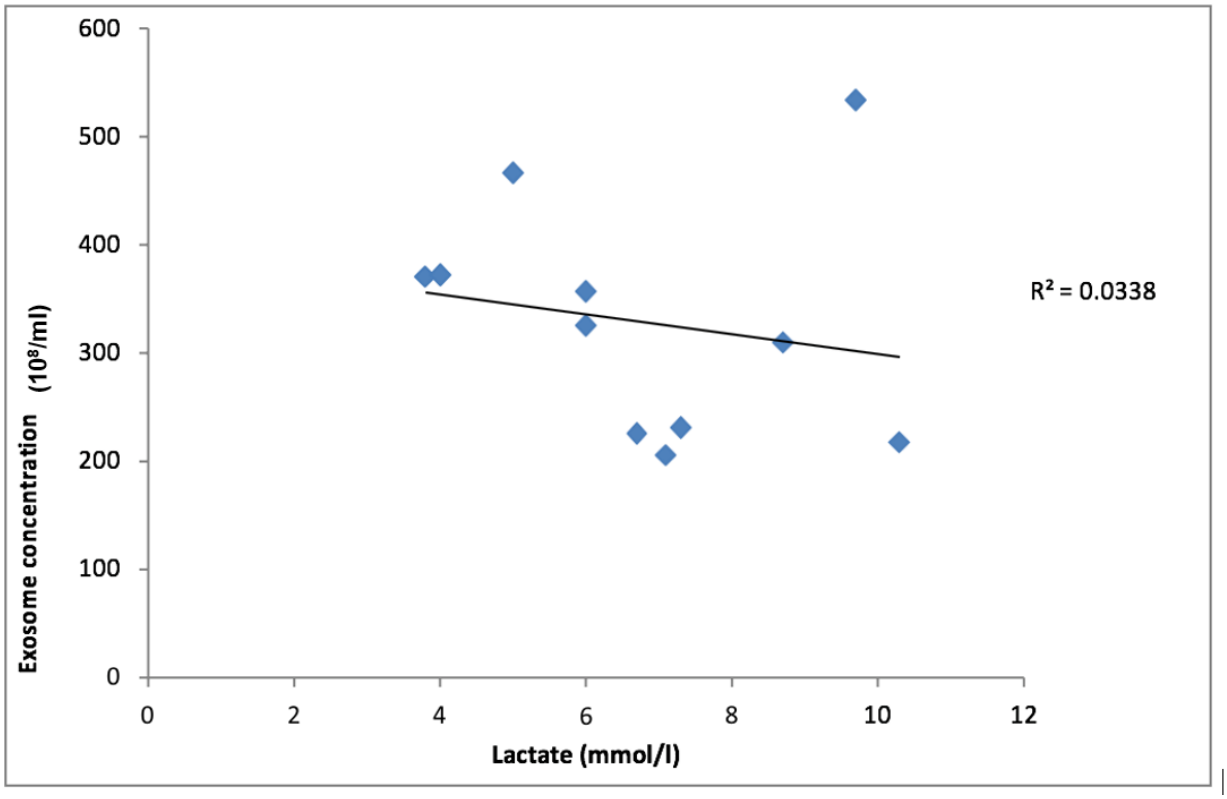



*Figure 23: Correlation of EVs concentration levels measured two hours after eccentric exercise and lactate concentration straight after eccentric exercise.*

## 4.8 Summary

It was found that there was a reduction in the average EVs concentration levels after two hours following the eccentric exercise. However, a significant difference was observed between the average of the EVs concentration levels during the first three hours of rest and the eccentric exercise. The hypothesis for this part of study (that there is a change in the concentration levels of plasma after moderate physically intense activity, and after eccentric exercise) was therefore accepted.

# Chapter Five - C2C12 myoblasts as a model of differentiation: the effect of exercise EVs on myogenesis

## 5.1 Introduction: Aim of this part of the study

Choi *et al.* (2016) found that in vivo studies have demonstrated that EVs from differentiating human skeletal myoblasts increased the number of regenerated myofibers in an injury site but decreased the fibrotic area leading to an improvement of skeletal muscle regeneration. However, in adult life satellite cells undergo little or no mitosis, but the stress that is caused by eccentric exercise can activate the signals for and the mechanisms of muscle cell regeneration of the damaged fibres of skeletal muscles (Chargé and Rudnicki, 2004).

For the purpose of muscle regeneration, myogenic differentiation is triggered in response to muscle damage, and the main aim of this part of this study is to establish the extent of the secretion of EVs in a C2C12 mouse myogenic cell line as a model for myogenesis. The main aim of this part of this study was to clarify the potential role of EVs in C2C12 (a mouse myogenic cell line) as a model for myogenesis. In order to do so, the experimental conditions and cell model for the differentiation of C2C12 was standardised and the expression of muscle differentiation gene markers was analysed by quantitative real time Reverse Transcription-Polymerase Chain Reaction. The effect of plasma EVs on C2C12 myoblast differentiation was also explored.

## 5.2 Methods

### 5.2.1 C2C12 cell culture

C2C12 myoblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf-serum (FCS), and 1% penicillin-streptomycin. The cells were maintained at 37°C in 5% $CO_2$ and myotube formation (differentiation of C2C12 cells) was initiated upon reaching confluence (85-90%) by replacing the growth medium with medium supplemented with 2% heat-inactivated horse serum, rather than calf serum.

### 5.2.2 Detachment/dissociation and myotube formation/differentiation of the C2C12 cells

To achieve detachment, the C2C12 cells were trypsinised at 75-80% confluence, and the cells were washed once with 9ml phosphate-buffered saline solution, PBS (Fisher).

5ml of 0.05% trypsin (Fisher, VX15400054) in PBS was then added to the flask to enable the process of cell detachment, and the flask was then put back into the incubator for five minutes. After the cells became detached, 5ml of whole cell media was added to the trypsin-cell PBS solution and centrifuged at 400g for 5 minutes to pellet the cells. The suspension was taken off and the cells were re-suspended in whole media and seeded into flasks at the required dilution. The cells were then stored in an incubator at 37°C and 5% $CO_2$. For these experiments the cells were seeded at a concentration of 50,000 cells/cm² in 6-well plates. A 0.5ml of plasma EVs were added to the muscle cells (Number of EVs was the amount enriched from 0.5ml of plasma). The variable is the number of EVs.

The C2C12 cells were differentiated for seven days after being seeded at a concentration of 50,000 cells/cm², and after the cells had reached 85-90% confluence, the growth medium was changed and replaced with serum differentiation medium (DMEM, 2% horse serum and 1% Pen/Strep antibiotics). PBS was then added to the cells and allowed to differentiate for seven days. In order to establish the progress of the differentiation, protein, RNA extraction and slide fixation in 75% methanol was carried out every day for seven days, and an immunocyto-fluorescent examination was performed using Hoechst nuclear counterstain and anti-myosin heavy chain antibody (MF20).

### 5.2.3 Protein and RNA extraction

For protein extraction, old media was pipetted off and washed with 1ml of cold PBS. 0.5ml of radioimmunoprecipitation assay (RIPA) buffer was added and left for 3-5 minutes. The flask was placed at an angle for 30 seconds to allow cell lysis, then pipetted into a labelled tube, and stored at -20ºC until it was required for analysis.

For RNA extraction, cells were scraped from the slide, spun down and snap frozen in liquid nitrogen and then stored at -80°C until needed for RNA extraction. In order to avoid RNase contamination, RNase-free reagents were used in all of the steps of the process. Cell pellets were resuspended directly in a lysis buffer and the RNA extracted. An miRCURYTM RNA Isolation Kit (Cell and Plant, Exiqon 300110) was used for all RNA extractions. Cell pellets were resuspended in 350µl lysis solution and vortex-mixed for 15 seconds. 200µl 95% ethanol was then added and the sample vortex-mixed for 10 seconds. A new column was placed in a new collection tube and the lysed cell solution was added to the column, and centrifuged at 14,000g for one minute. DNase I (Sigma, AMPD1) treatments were performed, according to the manufacturer's instructions, on the RNA whilst it was on the column. The flow-through was retained for protein analysis and the column was washed three times with 400µl wash solution for one minute at 14,000g and the flow-through was discarded. The column containing the washed RNA was centrifuged for two minutes at 14,000g to ensure that the column was dry, and it was then placed in an RNase-free Eppendorf tube. For elution from the column, 50µl of nuclease free water (Ambion, AM9937) was added directly to the column and centrifuged at 200g for two minutes, then spun for one minute at 14,000g. The RNA was then measured by NanoDropTM equipment and a 45µl aliquot was stored at -80°C for further use.

### 5.2.4 Quantitative reverse transcription polymerase chain reaction PCR (qPCR)

qPCR of RNA samples was used here for validation. Reverse transcriptase from tDNA or mRNA can be used to transcribe RNA into primer cDNA, and the cDNA can then be used as the template for qPCR.

Prior to qPCR, cDNA was therefore prepared from cell and extracellular vesicle RNA samples that had been quantified using the NanoDrop equipment. For each cellular RNA sample, 2µg of RNA was DNase 1 treated (Sigma, AMPD1) according to the manufacturer's instructions with 2 units of DNase 1 in a total reaction volume of 10µl. Following DNase 1 treatment, the RNA was re-quantified using NanoDrop equipment. For each individual sample, RNA was converted into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814) in a total reaction volume of 20µl, cDNA was also developed for a minus reverse transcription (-RT) control sample. Samples were stored at -20°C for future use.

Real-time qPCR was performed using Absolute QPCR SYBR Green ROX Mix (Abgene, Courtaboeuf,France) with a Rotor- Gene 6000 system. Results were normalized with the gene P21 used as the reference. PCR primer sequences were MYOG CATCCAGTACATTGAGCGCCTA, MYOG (myogenin) AS- GAGCAAATGATCTCCTGGGTTG, MYOD ATGATGACCCGTGTTTCGACT, AS-CACCGCAGTAGGGAAGTGT, MYF5 GACAGGGCTGTTACATTCAGG, AS-TGAGGGAACAGGTGGAGAAC, MRF4 GGAGTTTGCGTTCCTCTGAA, AS-AGGGGCCTCGTGATAACTG.

### 5.2.5 Protein quantification (Bio-Rad protein assay)

Dye reagent was prepared by a dilution of 1 in 4 (one part of dye reagent concentrate with four parts of distilled deionised water). The diluted reagent was filtered through 0.22µm filter (Millipore, Rev 10/11) to remove contaminants. Five dilutions of protein standard concentrations of 0.05-0.5mg/ml were prepared to protein solution. 100µl of each standard and sample solution were pipetted into a clean tube and 0.5ml of diluted dye reagent was added and vortex spun. All standards and samples were incubated at room temperature for five minutes and then measured.

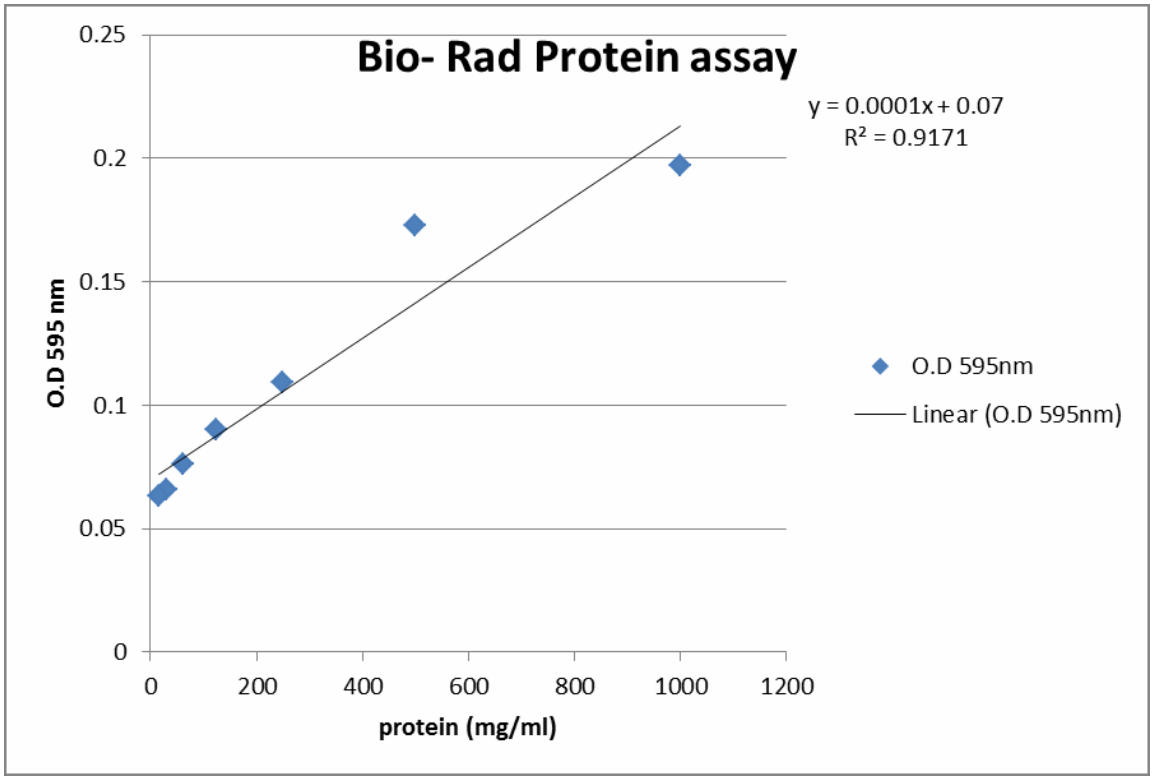



*Figure 24: Protein assay standard curves using the micro assay procedure with BSA using Bio-Rad*

### 5.2.6 Western blotting/Immunoblotting to identify the muscle differentiation gene markers

Proteins from C2C12 cells were extracted at different time points and migrated on SDS-PAGE gels (30µg). Following electrophoresis, proteins were transferred onto Polyvinylidene Difluoride (PVDF) membrane and blocked at room temperature with 5% BSA in tris-buffered saline solution. The primary Ab was MF20 was diluted in 1/1000 in 1% BSA. They were then incubated overnight at 4°C on a roller (to eliminate air bubbles between the blot and the blot holder) with anti-β-actin (diluted in1/5000) as a loading control. The signal was detected by using a horseradish peroxidase (HRP) conjugated secondary antibody (diluted in 1/5000). Finally, membranes were scanned on a Bio-Rad ChemiDoc™ MP system.

### 5.2.7 Fixing the Cover Slips

Media from each well was taken off with a Pasteur pipette, thus avoiding the touching of the cover slip in each well. Each well was washed twice with 2ml of phosphate-buffered saline (PBS) solution by adding and removing the PBS with a Pasteur pipette, and then the PBS was gently swilled over the well surface before removal. Cover slips were transferred with forceps sterilised in ethanol (to kill any mould spores) into the new wells containing methanol to fix them, and then each cover slip was gripped by the outside to transfer to the liquid methanol. Cover slides were rinsed and left in a refrigerator for 10 min. The forceps were sterilised between the touching of each plate and cover slip so as to avoid cross-contamination. After 10 minutes the well was removed from the refrigerator and the methanol was taken off the cover slips. Every well was washed twice with 2ml cold PBS (using a Pasteur pipette). 3ml of PBS were added to each well and then stored at 4˚C to prevent bacterial and mould-spore growth before immunocytochemistry.

### 5.2.8 Immunocytochemistry of C2C12 cells

Forceps cleaned in 100% ethanol were then used to remove the cover slips from the plate, and the cover slips were then placed on an empty dish. 200µl of PBS were added to each cover slip and placed on a rocker for five minutes. The PBS was then removed and 200µl of block 1% w/v bovine serum albumin (BSA) in PBS was added and incubated on a rocker for one hour. The BSA solution was removed, 200µl of mouse monoclonal antibody MF20 (Abcam, ab55152, mouse monoclonal to MHC Class II) was added to each cover slip in the ratio of 1:500 in 1% BSA and incubated on a rocker for one hour at room temperature. The cover slips were then washed three times for five minutes each time in PBS on the rocker. 200µl of goat anti-mouse immunoglobulin-G (IgG) antibody (Abcam, ab150113, Goat polyclonal Secondary Antibody to Mouse IgG - H&L) was added to each coverslip and incubated on a rocker for 30 minutes in the dark at room temperature. The secondary antibody was removed, and 50µl of diluted Hoechst stain (sigma B2261-25MG) was added for one minute. The coverslips then underwent three five-minute washes on the rocker. The PBS was removed while the cover slips were kept in the dark. 5µl of Fluromount™ was then added to the centre of each of the labelled slides. The coverslips were removed using tweezers and any excess liquid drained off onto tissue, and then the cover slips were placed face-down onto the slide. The edges of the cover slips were sealed with nail varnish and left to dry in the dark at 4ºC until needed. For myoblast fusion measurement, myoblast fusion index (MFI) was calculated. The MFI is determined by immunostaining day 0,1,2,3, and 4 differentiated cells (DM) with myosin heavy chain (MHC), counting the number of nuclei in each MHC positive cell, and then comparing the number of multinucleated cells to the overall number of MHC positive cells. Myotubes were considered partially fused if they contained 2–3 nuclei, and fully fused if they contained 4 or more nuclei.

### 5.3 Statistical analysis

The analyses were performed using SPSS 21 software. All the results were expressed in mean ± standard deviation (SD). SPSS was used to carry out tests for Normality and Homoscedasticity, and Gosset's parametric 'student' t-test was used to compare the means, and a 'p' value of 0.05 was considered significant. A one-way ANOVA was performed for the comparison of one variable in different groups. The results were analysed using the ΔΔCt method to quantify the relative abundance of mRNA and plotted as a fold change relative to one of the 'housekeeping' genes. qPCR data was shown by calculating the fold difference. Cycle threshold (Ct) is the number of PCR cycles at which the fluorescence signal increases above the threshold value. The data was analysed by comparing ΔΔCt results of C2C12 cells treated with EVs from exercised and rested participants respectively.

$$\text{Fold Difference} = 2^{\Delta Ct}$$

$$\Delta Ct = Ct_{\text{gene of interest}} - Ct_{\text{HKG}}$$

$$\Delta\Delta Ct = \Delta Ct_{\text{Day0 cell differentiation}} - \Delta Ct_{\text{sample x}}$$

P values less than 0.05 were considered statistically significant.

#### 5.3.1 Determination of the fusion index

Pictures were captured with a Leica camera in five-by-five tiled pictures using Zeiss fluorescence microscopy. The images were then counted, and the fusion index was calculated as the percentage of myotube cells which contained two or more nuclei versus the total number of nuclei. The number of nuclei in myotubes and the total number of nuclei in the cells were counted by using (ImageJ 1.44) (64-bit) software. The fusion index was calculated as the percentage of myotube cells which contained 2 or more nuclei versus the total number of nuclei.

## 5.4 Results

### 5.4.1 Treating differentiated C2C12 cells with PBS

In order to show that C2C12 differentiation had taken place, images of C2C12 cell differentiation were taken by fluorescence microscopy. A significant difference of the fusion index was detected between Day 0 vs. Day 1 ($P = 0.023$), Day 1 vs. Day 2 ($P = 0.0005$), Day 2 vs. Day 3 ($P = 0.0009$), and Day 3 vs. Day 4 ($P = 0.044$). This significant increase reflects C2C12 myogenesis over the course of differentiation.

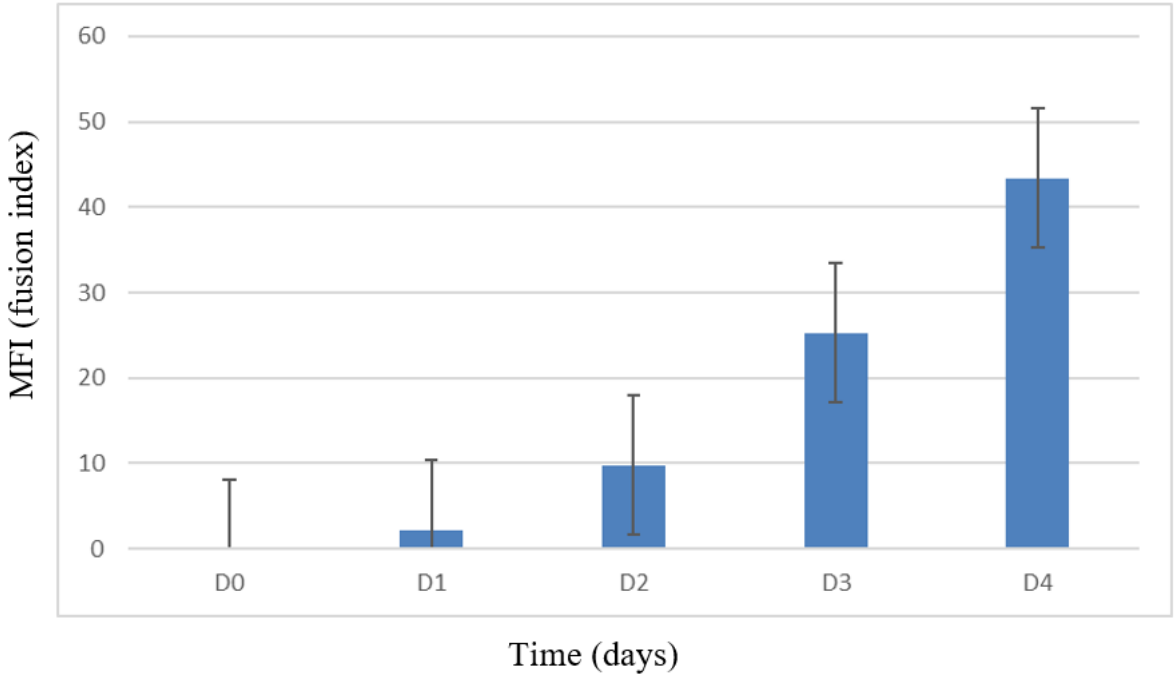



*Figure 25: This is the fusion index following differentiation of C2C12. C2C12 myoblasts were seeded at a concentration of 50,000 cells per well and allowed to differentiate for the times indicated. Error bars represent SDs. All the values were significant ($p<0.05$) in comparison to the previous day's reading. D indicates the time in days.*

### 5.4.2 The expression of Myogenic regulatory markers during C2C12 cell differentiation

Over the course of C2C12 differentiation, expression levels of myogenic markers (Myogenin and MyoD1) were determined by qPCR at different time points following the induction of C2C12 differentiation. The MyoD1 level was slightly altered around t= 48h and decreased beyond t= 72 while the MyoG was up-regulated around t= 72h (Figure 26).

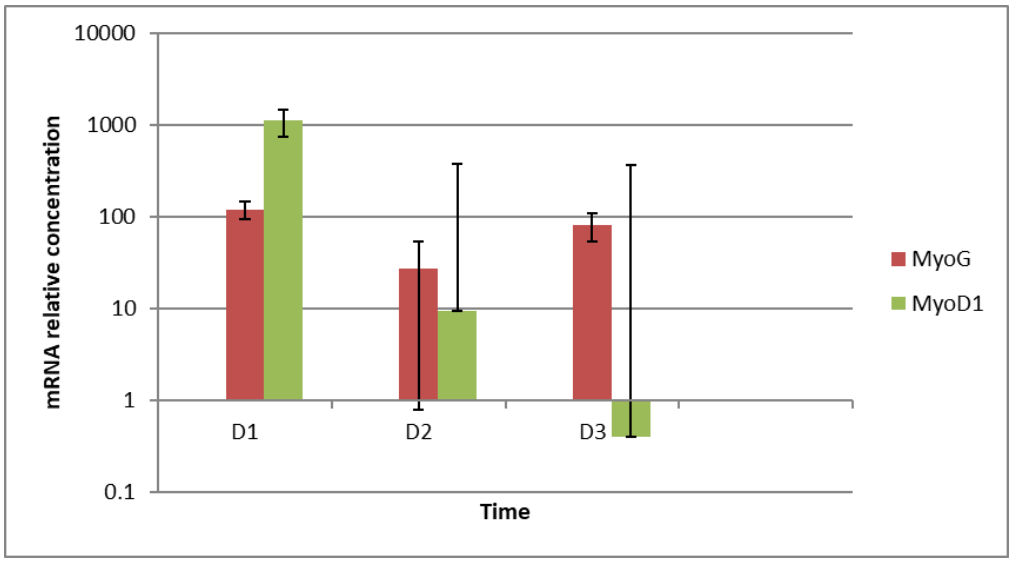



*Figure 26: Relative mRNA quantification concentration by qPCR from the myogenic markers Myogenin and MyoD1 compared to PPIA housekeeping gene. This is an average of three repeat plasma samples. Error bars represent SDs.*

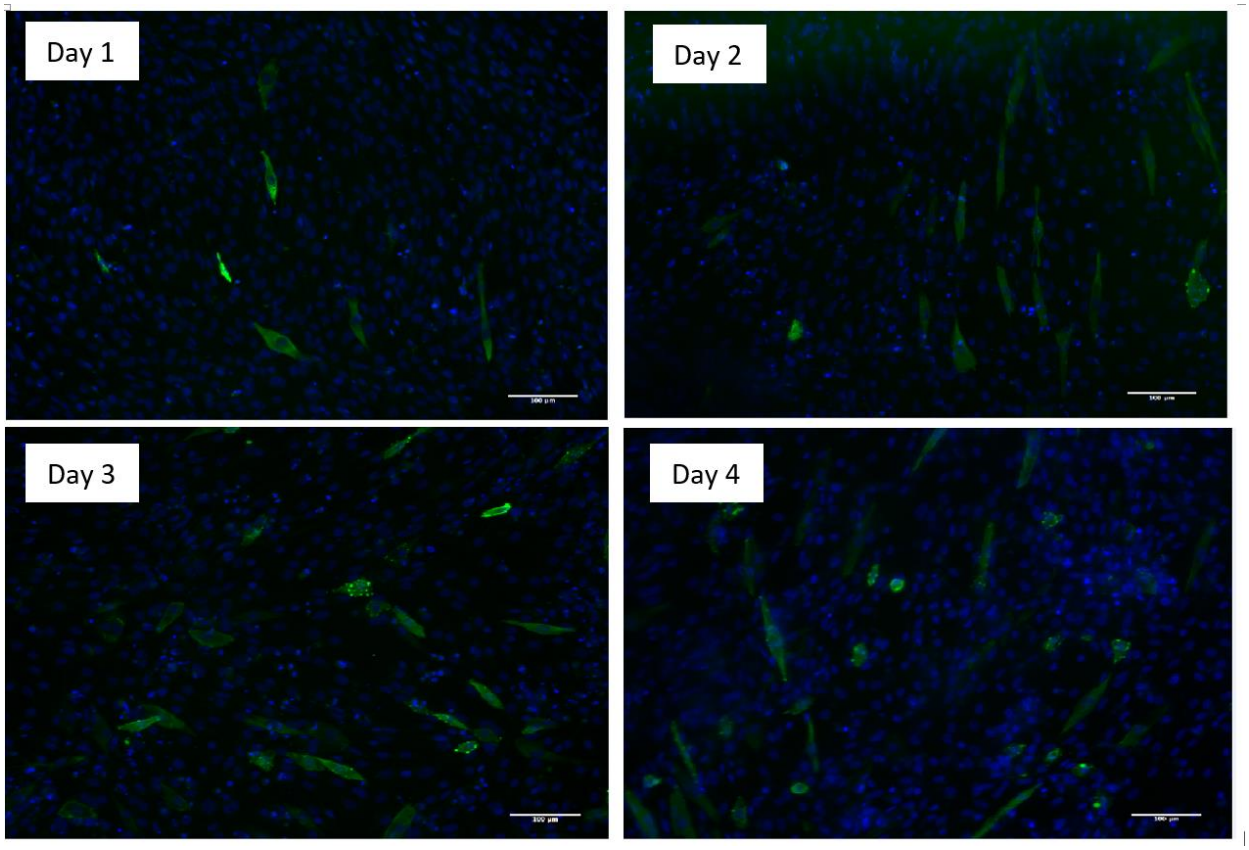



*Figure 27: C2C12 myoblasts induced to differentiate by culture for the indicated times in differentiation medium, and subjected to immunofluorescence analysis with anti-MHC Class II antibody, and nuclear counter-stained by Hoechst sigma B2261-25MG. Images of C2C12 cells differentiation taken by fluorescence microscopy.*

### 5.4.3 Treating differentiated C2C12 cells with plasma EVs

In order to explore the effects of EVs on C2C12 differentiation, the experiments in this study were repeated in which C2C12 cells were seeded at the concentration of 50,000 cells per well. After the cells had reached 85-90 % confluence, the growth medium was changed and replaced by serum differentiation medium (DMEM, 2% horse serum and 1% PBS). Plasma EVs that had previously been extracted from six healthy participants who had undergone two hours of eccentric exercise and then rested, were then added to the cells and allowed to differentiate for three days

(Figure 28). Protein, RNA extraction and slide fixation in 75% methanol were done every day for seven days.

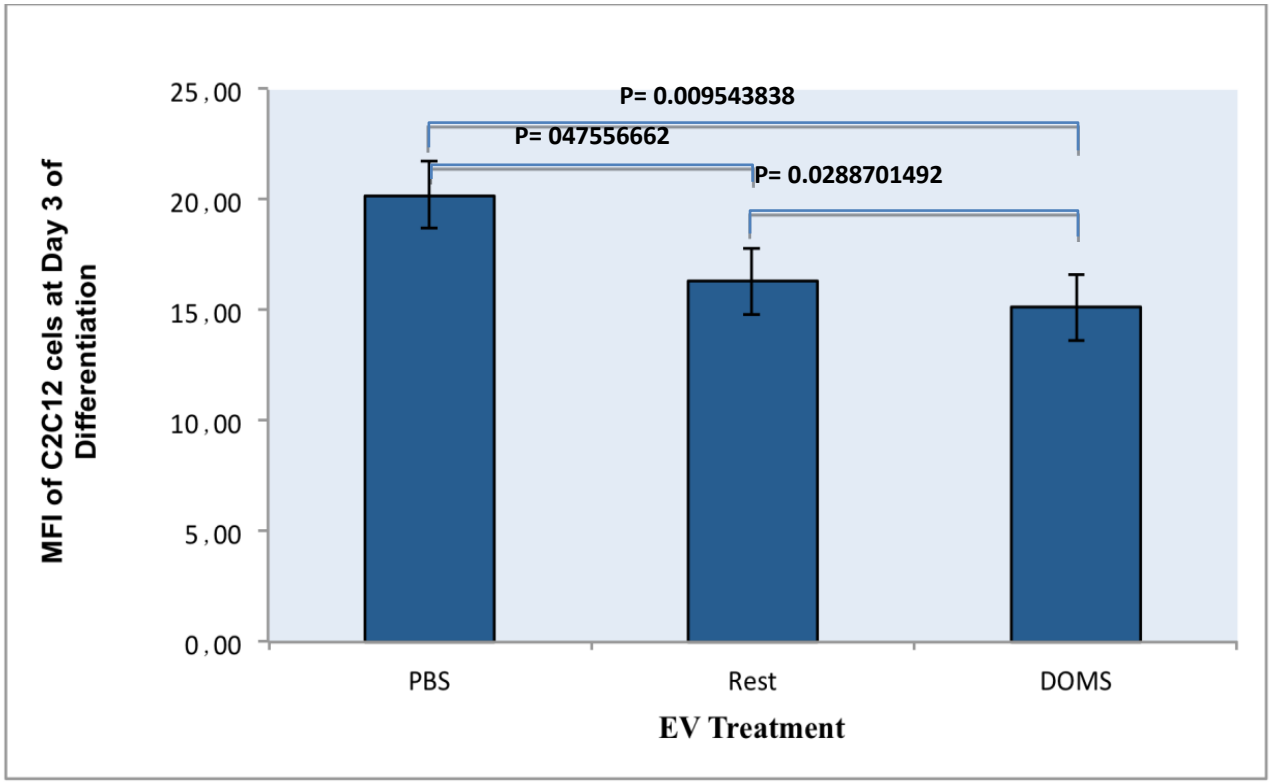



*Figure 28: MFI of C2C12 at Day three of differentiation after treated by EVs previously extracted from resting and* eccentric *exercise plasma sampling. Error bars represent standard deviation (SD).*

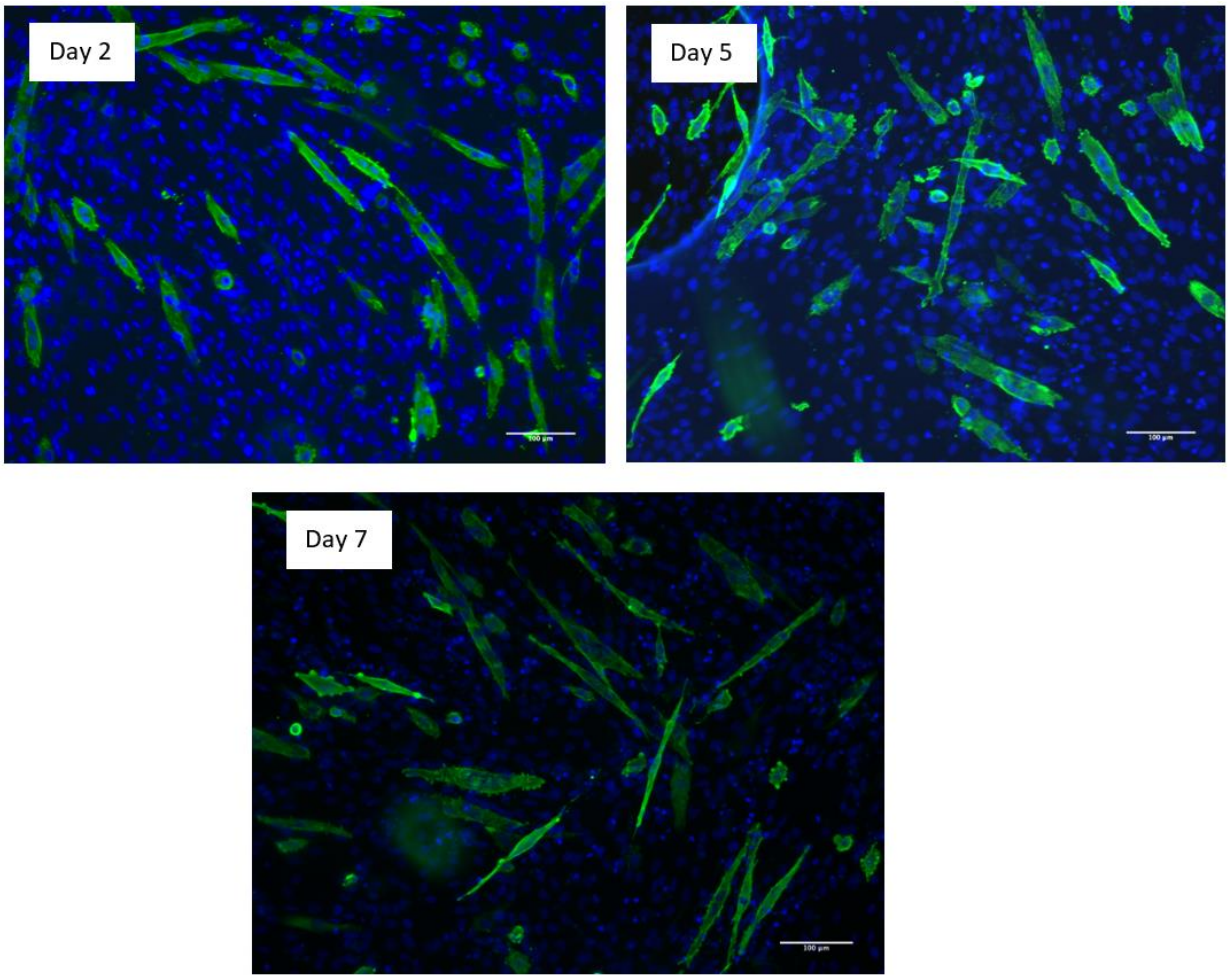



*Figure 29: shows C2c12 at different days of differentiation after treatment*
*In day two of differentiation, C2c12 was treated by EVs previously extracted from resting plasma sampling. In day three, C2c12 was treated by EVs previously extracted from eccentric exercise plasma sampling. While in day seven, C2c12 was treated with PBS cl (control).*

### 5.4.4 Quantification of Myogenin by Western Blot analysis

There was a problem with loading control in myogenesis ( beta- actin) not getting normalized and myogenin band was not detected. Western blots required further optimisation (Figure 30).

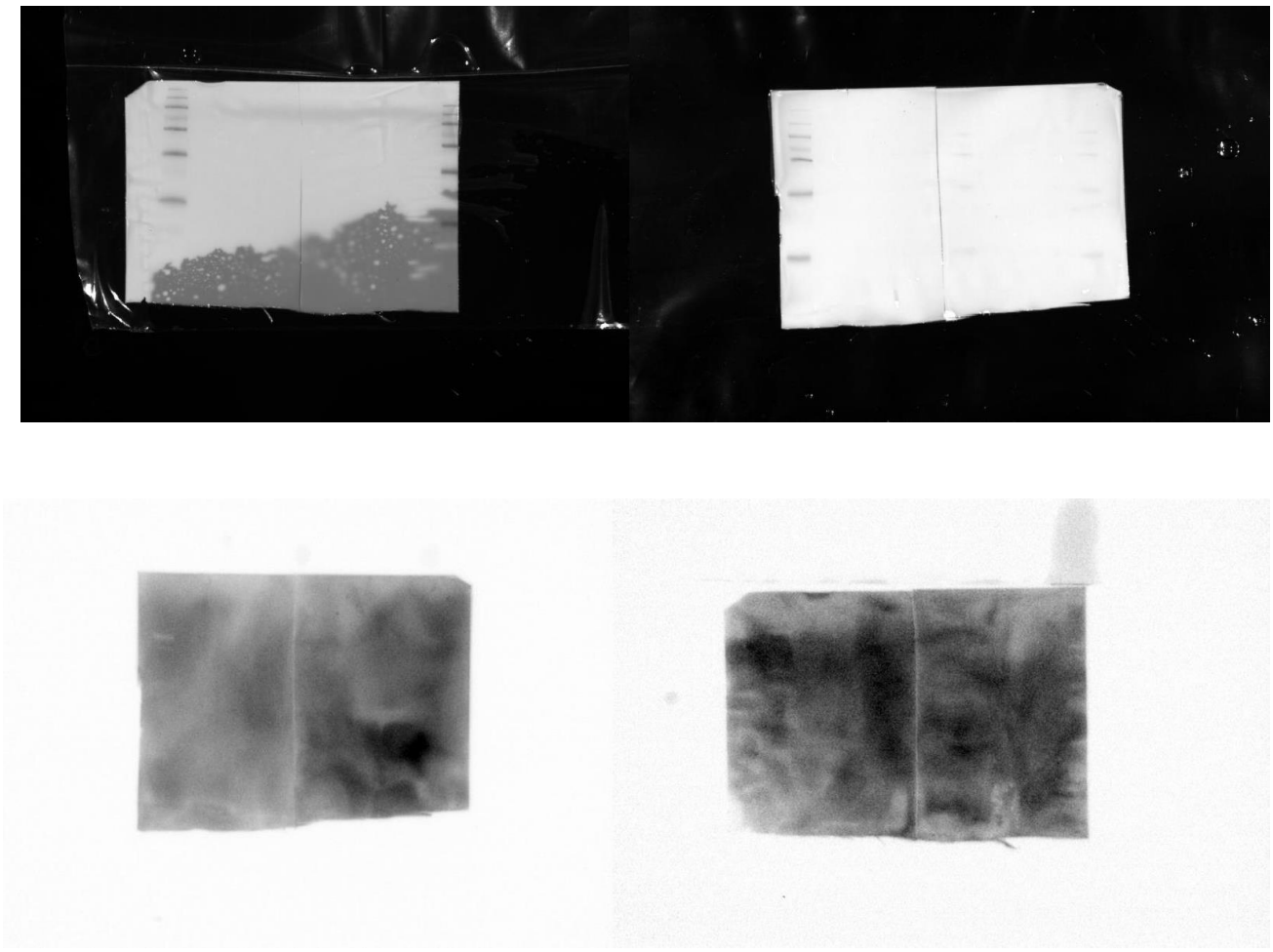
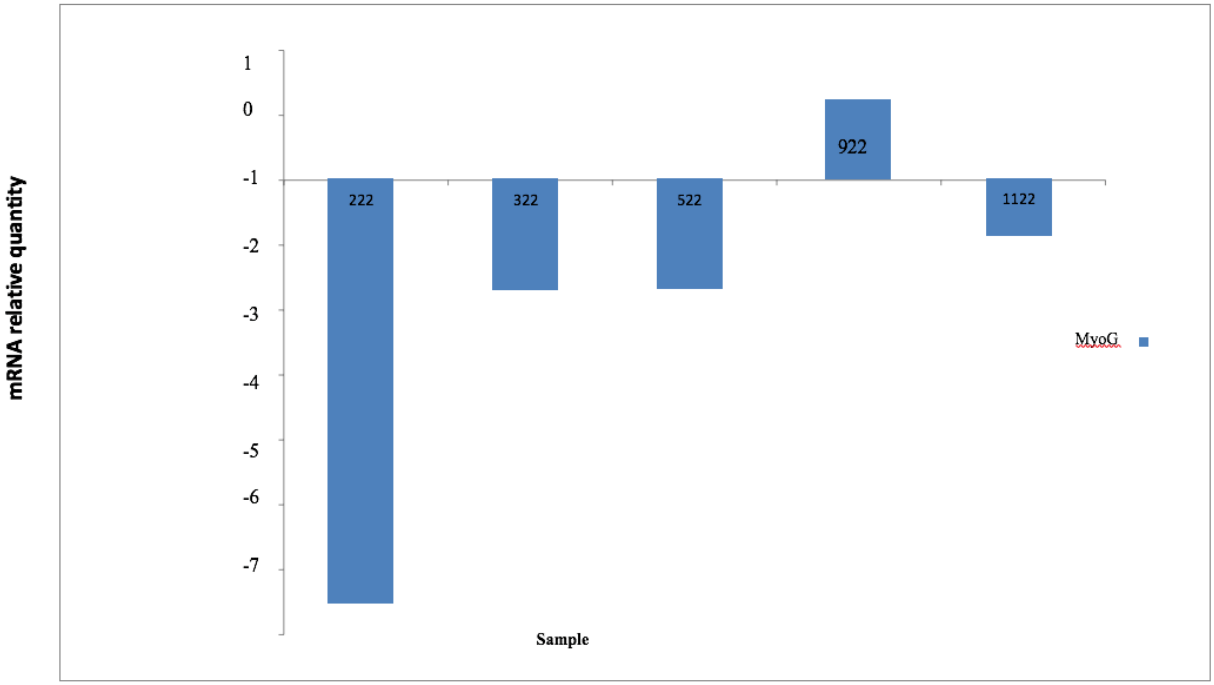


*Figure 30: shows figures of myogenin quantification by western blots.*
*Western blot analysis of myogenin( EV's marker) was enriched and expressed in EV's plasma samples.*

### 5.4.5 The expression of Myogenin change in EV-treated C2C12 cells

The expression level of Myogenin in EV-treated C2C12 cells by plasma samples was determined by qPCR. It shows reduced Myogenin in all treated plasma samples at the time point t= 27h, but the difference was not significant $p > 0.05$ (Figure 31).



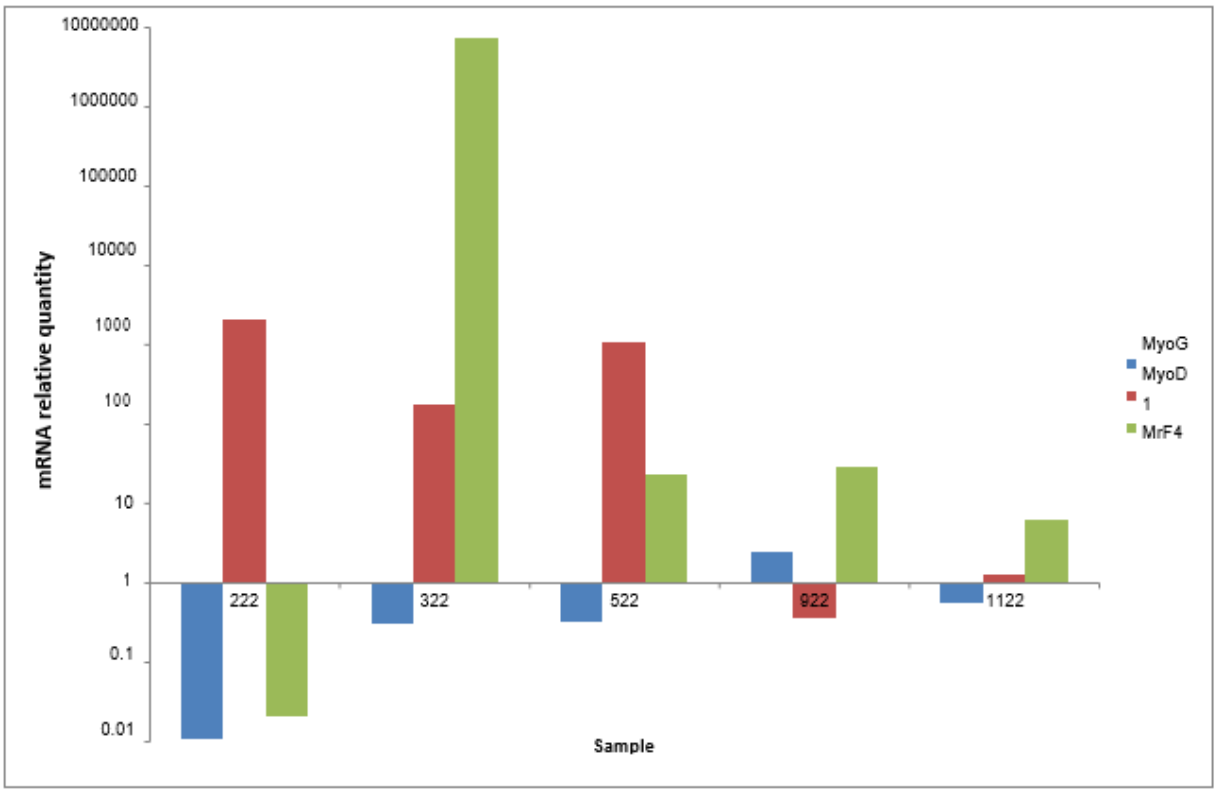




*Figure 31: The expression of Myogenin change in EV-treated C2C12 cells mRNA from the myogenic markes Myogenin, MyoD and Mrf4. Transcription levels are expressed as relative quantities compared to the intiation of differentiation ( t=0h). 222, 322,522,922 and 1122 refer to EV's samples previously extracted from eccentric exercise plasma sampling from participants no 2,3,5,9 and 11.*

## 5.5 Summary

Our study may provide a basis for future research. Our overall results suggest that physical exercise induces a release of small extracellular vesicles called EVs into the blood circulatory system (Frühbeis *et al*., 2015), and the effect of exercise protocols on the secretion of circulating EVs has been reported by different studies. However, our research had several limitations that future studies can address.

One of limitation of this study is C2C12 a secondary muscle model which does not reflect muscle myogensis in human, however C2C12 is more versatile, more ethical and reflect muscle model very well.

In future study, I would prefer to repeat this experiment on exercise mice though primary cell line is more expensive and ethical hard to get it.

Another limitation is EV's counting as in this study Ev's were measured by NTA, in which there is an argue that NTA is not the gold standard and there is other method such as I Zone tend to be more precise though the main issue with it is blocking up and contamination.

In future study, I would do EV's measure by counting their number by hand or set up a computer programme which can count the number of these vesicles from electron microscopy images.

Also, this study was conducted only in young males which could not be generalising to reflect the whole population. In future study, I would do four groups of young males/young females, old males/old females to give a wider reflection of the population.

# Chapter Six - Discussion

### 6.1 Hypothesis One

**"The concentration levels of plasma** EVs **are stable, and that there is little day-to-day variability in these concentration levels over different days of blood sampling".**

Chapter Three of this study examined the effect that different conditions have on the stability of EVs concentration levels, and it was shown that there is no significant difference between the biologic activity of EVs derived from frozen-and-then-thawed plasma and that of EVs derived from fresh plasma. The blood plasma was then pooled and prepared for EVs isolation. These findings are consistent with Kalra *et al*.'s (2013) study, which reported that freezing had no effect on the size and concentration levels of plasma EVs. Kalra *et al*. (2013) also assessed the stability of EVs in plasma under various storage conditions, and over the course of 90 days from frozen and then thawed plasma samples and also from fresh samples obtained from five healthy donors. The authors found that, western blotting analysis revealed that EVs are stable for 90 days. They also found that the isolated EVs were able to fuse with target cells revealing that they were indeed biologically active (Kalra *et al*., 2013). Kalra *et al*. (2013) concluded that the density gradient centrifugation allows separation of vesicles based on their buoyant densities and isolation of pure population of EVs from blood plasma. However, density gradient centrifugation method cannot be employed effectively in clinical experiments. Therefore, robust and efficient methods to isolate EVs need to be developed.

A study conducted by Lässer (2012) discussed some methods used for isolating EVs and their RNA cargo, however these methods could impact the purity and quality of the isolated RNA. Furthermore, this study found that processing of body fluids could be more problematic and complex than cell culture media, as the body fluids is viscous and difficult to retrieve. This may result in lower EVs yields. Therefore, a well-established method for EVs isolation is needed. Moreover, he importance of correct characterization is also needed, as cells release other

extracellular vesicle in addition to EVs such as microvesicles and apoptotic bodies which could contaminate the EVs fraction. Therefore, validation by different identification and detection methods can improve quality of the results. In 2016, Lässer *et al*. have conducted another study that described the importance of having well-established protocols for EVs' isolation and characterization by combination of different methods including electron microscopy, NTA, flow-cytometry and western blot to get high-quality results.

This study has also examined the content of plasma EVs concentration levels within-and between-subjects and it was shown that EVs are stable and reliable and that they can be used as a good indicator for vesicle biomarkers. This study first examined the within-sample content of three different samples drawn from each individual at the same time and found that there was no significant difference between the three different repetitions of blood collection at the 5% alpha-level. Since the results showed good content, this suggests that there were no technical issues that needed attention with regard to taking blood samples from the same participant at any one particular point in time during a day. This study then examined the same day content of plasma EVs concentration levels over different time points (10 hours of monitoring) during a given day and found that there was no significant difference at any of the different time points. This suggests that, if nothing unusual happens during the day, EVs concentration levels are stable over the course of any one day. There were also no significant differences in the measures taken across five consecutive days.

The results demonstrate that plasma EVs concentration levels are stable in healthy subjects. The intraclass/intracluster correlation coefficients (ICCs) were $\geq 0.8$ for within-sample measures taken across five days and for same day, and Cicchetti (1994) regards coefficients between 0.75 and 1.00 as 'excellent'.

The study examined the stability of plasma under different conditions. Plasma EVs concentration levels were also analysed with regard to particle size. The data were divided into two categories, vesicles 30-100nm in size which are known as 'exosomes', and microvescicles where the size range is 101-1000nm. There was no change in the concentration levels of both these types of extracellular vesicles. There was also no significant change in EVs concentration levels within the same subject at multiple time points of the day and over multiple days.

### 6.1.1 Limitations

There were several limitations in the study that could have contributed to increased variability in the measurement of plasma EVs concentration levels but did not do so. Firstly, there was no dietary control nor a dietary record for the participants *e.g.* it was not known what the subjects had eaten the previous day. However, the study attempted to control this, both by asking all the participants to fast and also by collecting blood samples at the same time of day over the five consecutive days. Secondly, physical stress is related to various immune changes, and this could be one of the physiological factors that fluctuates from day to day (Herbert and Cohen, 1993). It is therefore suggested that stress could be a possible cause for the variability in EVs concentration levels over a period of several days. However, the study attempted to minimise this by asking the participants to report to the laboratory at the same time every morning to ensure that the blood collection was conducted while the participants were in a rested state. Thirdly, the small sample size could have been one of the study's limitations, but the results showed good to excellent content with regard to the ICC coefficients. However, since this study was confined to male subjects, further research would need to be conducted among women to confirm the content of plasma EVs concentration levels in human blood in general. Additionally, results cannot be definitively confirmed given the small sample size. Statistically insignificant results, however,

may still bear clinical significance, highlighting the need for future studies with larger sample sizes.

### 6.2 Hypothesis two

**"There is a change in the concentration levels of plasma** EVs **and cytokines after low- and high-level physical activity and after eccentric exercise".**

Many research papers have assessed only leisure-time physical activity (Friedenreich, 2011; Il'yasova *et al*., 2005) and have not investigated whether or not different modalities and levels of physical activity may be associated with changes in inflammatory marker concentration levels.

This study found that the incremental cycle ergometer test was well tolerated by the subjects, and all the physiological parameters and perceptual markers indicated that the subjects were achieving 50% of their maximum heartbeat. In this study, no significant difference in EVs and cytokine concentration levels were recorded after low level physical activity, and there were no significant differences in lactate measures after the exercise. However, EVs concentration levels markedly decreased after eccentric exercise compared to the resting state. This study thus found no evidence that inflammation and the prevalence of EVs is linked. In contrast, there was a negative correlation between EVs concentration levels two hours after eccentric exercise and lactate concentration levels. Lactate is an aerobic metabolite, consumed by skeletal muscles and by the heart when oxygen levels are sufficient, and it may contribute to acetyl-CoA production (Chatham, 2002). A major amount of muscle-derived lactate is transported to the liver to be used in glucose synthesis through gluconeogenesis; glucose is then transferred to the blood circulation to the muscles to be consumed as a metabolite in glycolysis (Cori's cycle) (Pérez et al., 2010).

In response to the eccentric exercise, no significant correlation was reported between the prevalence of EVs and any of the inflammatory cytokines IL-6, IL-10, and TNF-α. There was a reduction in EVs concentration levels after eccentric exercise but there was an increase in IL-6 concentration levels, and several mechanisms could be responsible for this difference. One could be that eccentric exercise could affect the protein and miRNA content of EVs. Another possible reason for a reduction in EVs concentration levels could be that EVs are taken in by contracted muscles and are involved in the recovery process which helps to repair the damage caused by eccentric exercise. However, the most likely reason for secretion and uptake still remains to be determined. Further research is required to measure muscle-enriched microRNA and protein changes before and after eccentric exercise.

In this study IL-6 concentration levels were below the sensitivity of the enzyme linked immunosorbent assay (ELISA) in the first few hours of the rest day, but there was a gradual elevation in IL-6 concentrations after the fourth hour and a peak at six hours on the same day. Furthermore, it was found that IL-6 concentration levels peaked twice during the exercise experiment day. The first peak of IL-6 was observed one hour after the eccentric exercise, whereas substantial increases in plasma IL-6 levels were observed between six and eight hours after the exercise. This variation in IL-6 plasma concentrations could be due to the effect of nutrient intake on the immune system which was noted in the study conducted by Gleeson (2004) (cf. Chapter One). Nehlsen-Cannarella *et al.* (1997) state that the consumption of carbohydrates during strenuous exercise attenuates the stress hormone increase of cortisol and epinephrine along with pro-inflammatory cytokines. This study therefore tried to control food intake by choosing light meals containing only carbohydrates. Participants were given food after two and after six hours after starting the study, and a gradual elevation in IL-6 concentration levels was found after the fourth hour and a peak at the sixth hour.

The concentration of TNF-α did not significantly change between rest and after eccentric exercise. This might be due to the fact that muscle damage during eccentric exercise may result in pro-inflammatory cytokine accumulation (Scott *et al.,* 2013). This conflicts with the evidence that TNF-α does not accumulate in, nor is it released by contracting muscles during eccentric exercise (Scott *et al.,* 2013).

### 6.2.1 Limitations

A sample size of eleven subjects in this study was based on the calculation discussed in Chapter Four in Section 4.2. This small sample size may not be sufficiently robust to reflect the characteristics of the total population, but it is sufficient to show significant molecular changes in human beings. Furthermore, the difference in exercise intensities between subjects might have been caused by the difference in fitness between subjects (athletes and non-athletes). This could have been measured by $VO_2$ max, but that was not the focus of this study. Each one of the tests took place over a period of approximately ten minutes. Additionally, control over the weight load was set at about 25% of total body weight. How difficult the activity was to perform and how tired the muscles felt was determined by measuring the heart rate and the lactate level.

## 6.3 Hypothesis Three

**"There is a change in the differentiation of C2C12 cells when treated with plasma EVs".**

Chapter Five of this study examined the effects of eccentric exercise EVs on C2C12 muscle cells as a model of differentiation. The experimental conditions were first standardised for the C2C12 model, and then the expression of differentiation markers (Myogenin, MyoD1) that are known for

their involvement in differentiation was analysed. The expression levels of Myogenin and MyoD1 were slightly increased after the start of differentiation. At around 48 hours, the Myogenin gene was up-regulated more than two-fold, while beyond t = 72 hours it was up-regulated ~100-fold. For MyoD1, the expression level was up-regulated beyond t = 48 hours, while its expression more than halved beyond t = 72 hours. For the myogenic regulatory factor MRF4, transcripts were not detected during C2C12 differentiation. The study also looked at the myogenin expression in EV-treated C2C12 cells by plasma sample, but no significant reduction in myogenin expression level was found. This may be because a larger plasma sample was needed because qPCR is a sensitive assay that needs more samples. (However, the MIQE guidelines for qPCR analysis recommends triplicates).

The expression of muscle differentiation genes (Myogenin, MyoD1) was consistent with the expression pattern described in the literature (Janot *et al.,* 2009, Delgado *et al.,* 2003)**.** In Janot *et al*.'s (2009) study, 95 exhibited altered mRNA expression among the 276 genes expressed when C2C12 cells differentiated and 37 displayed more than 4-fold up- or down-regulations. These genes were clustered in three main groups. The first cluster had genes with gradually reducing quantities of transcripts where transcript levels reached a maximum at 24-48 h of differentiation in the second set of genes and then diminished. The thirst set of clustered genes increased throughout differentiation. The authors concluded that the myoblast cell membrane and ECM could be modified for cell fusion during C2C12 differentiation.

This study provided evidence that C2C12 as a model of differentiation shows a gradual increase in C2C12 myogenesis over 7 days of differentiation, even when treated with the EVs experimental carrier PBS. Hoechst staining of the nuclei shows that there are multinucleated myotubes from the myoblasts. Cells were imaged and analysed to obtain the fusion index as the percentage of myotube cells which contained 2 or more nuclei versus the total number of nuclei as a reflection

of cell differentiation, and the findings of the study are consistent with those of other studies (Janot *et al.,* 2009). Once the model had been established, this study then examined the effect of treating differentiating C2C12 cells with plasma EVs previously extracted from six healthy participants in rest periods and also two hours after the participants had performed eccentric exercise. On the first day of the C2C12 differentiation process, eccentric EVs samples were added and allowed to differentiate for 7 days. It was found that eccentric exercise EVs resulted in decreased C2C12 differentiation (formed fewer multinucleated structures) at Day 3 of differentiation than did C2C12 treated with rest EVs from the same participant or the PBS control treatment. This reduction suggests that stress induced by eccentric exercise has an effect on muscle differentiation, and it may be that the reduction of systemic EVs indicates that they have already been re-absorbed back into damaged tissue. This could explain the reduction (found in this study) in C2C12 differentiation when plasma EVs extracted from healthy participants after performing a eccentric exercise were added. However, further in-vivo testing is required.

A study conducted by Fabbri et al. (2012) showed that some miRNAs secreted by tumor cells in EVs can bind to TLR8 (and TLR7) and activate these receptors in immune cells, leading to activation and secretion of prometastatic inflammatory cytokines. This could explain EV's secretion and stimulation could be induced because of the binding of toll-like receptor to miRNA expressed on EVs' vesicles surface.

Other studies (Valadi *et al.* 2007, Kosaka *et al*. 2010, Mittelbrunn *et al*. 2011) have shown that exercise may increase the uptake of miRNAs from the blood circulatory system into other recipient cells, and specifically EVs might be taken into muscle cells (Aoi *et al*. 2010).

Another study conducted by Coenen-Stass *et al.* (2016) demonstrated that the same extracellular microRNAs shown to be secreted during myogenic differentiation also increased systemically after exercise induced injury. This could explain what this study has shown viz. that EVs

concentration levels were reduced after eccentric exercise, and it is to be expected that this would cause delayed onset muscle soreness. The findings of this study were therefore consistent with the findings of other studies. However, the exact mechanism of secretion and uptake remains to be discovered. Further research is required to understand the impact of exercise induced muscle damage on EVs secretion into the circulation systems of human beings.

Since EVs play a potential role in myogenesis, then this role can be further elaborated by more research on the contents of EVs released by muscle cells.

# Chapter Seven - Conclusion

## 7.1 Conclusion

It has been reported that physical exercise has an impact on physiological biomolecules and is associated with a number of immediate physiological responses along with long-range homeostatic processes (Yáñez-Mó *et al.,* 2015). Furthermore, it has been reported that physical exercise induces a release of small extracellular vesicles into the blood circulatory system (Frühbeis *et al.,* 2015), and the effect of exercise protocols on the secretion of circulating EVs has been reported by different studies (Maruyama *et al.,* 2012, Chen *et al.,* 2013). In addition to the interest in EVs as potential biomarkers, their physiological roles have been the main focus of recent research into the pathways relating to intracellular and intercellular communications (Nolte *et al.,* 2016).

In recent years a wide range of EVs physiological and pathological function studies have been conducted (Zhang *et al.,* 2016, Ailawadi *et al.,* 2015, De Toro *et al.,* 2015). Despite the increasing number of studies examining plasma EVs, there is a need to understand better the variables that affect EVs concentration levels and the possible variation in between-subjects over a day and within a day.

A study by Gardiner *et al.* (2013) showed data from several repeat measurements of known concentration of EVs. However, a biological standard for EVs measurement has not been agreed and the within-subject variations have not been addressed. This study has endeavoured to explore the content of plasma EVs concentration levels in healthy individuals. In order to determine whether the method of extracting EVs was reproducible, three blood samples were drawn consecutively from the same subjects into EDTA vacutainers. The study tried to assess within-day content by taking multiple blood samples over a period of 10 hours, and plasma EVs content between multiple days was also assessed by taking blood samples from the same subjects five

times over five consecutive days. The study showed that EVs concentration levels isolated from blood plasma are consistent and can be used as a good indicator to search for vesicular biomarkers.

EVs have been detected in blood as an immediate response of the immune system (Ji *et al.,* 2006). It is also known that physical exercise induces cytokine secretion into the circulatory blood system that begins 3 hours after exercise and lasts until 12 hours after the exercise (Petersen and Pedersen, 2005). Although far less is known about the effects of eccentric exercise on EVs secretion in the circulation system**,** the effects of eccentric exercise on the immune system are well documented. The pro-inflammatory cytokines, TNF-α and IL-1, are the first two cytokines to appear in the cytokine cascade (Petersen and Pedersen, 2005), therefore a comparison of inflammatory cytokine secretion alongside EVs secretion as a response before and after physical exercise in healthy subjects is important in order to identify early markers for reduced immune function. This study therefore looked at the natural variation of inflammatory cytokines (IL-6, TNF-α, IL-10) alongside EVs secretion during a normal day and in response to a single bout of either eccentric exercise in eleven healthy subjects. The study found no significant correlation between the concentration levels of EVs and any of the inflammatory cytokines (IL-6, IL-10, and TNF-α) in response to eccentric exercise and moderate exercise.

However, it was found that eccentric exercise evokes a significant IL-6 response. There is a significant difference between the concentration levels of resting IL-6 and eccentric exercise IL-6, but the study did not find that the concentration of TNF-α altered significantly between rest and post eccentric exercise. This result is similar to the findings of Rall *et al.* (1996) and McFarlin *et al.* (2004) who found that eccentric physical exercise did not reduce the levels of TNF-α. This may be due to the fact that muscle damage during eccentric exercise can result in pro-inflammatory cytokine accumulation (Scott *et al.,* 2013). However, this conflicts with the evidence that TNF-α does not accumulate in, nor is it released by contracting muscles during

eccentric exercise (Scott *et al.,* 2013). For IL-10 there was no significant difference between rest and post-exercise concentrations, and for cytokine measurements/results, the findings of this study were consistent with the study by Petersen and Pedersen (2005).

The findings of this study suggest that there is a reduction in the average EVs concentration levels two hours the eccentric exercise day, and a significant difference between the measurement of the first three hours' averages of EVs concentration levels in rest and after eccentric exercise was observed.

This study measured the extent of the subjective response (muscle soreness) and metabolic response (lactate measures) to eccentric exercise for all subjects and found a positive correlation between intense exercise and EVs concentration levels. The study also made a comparison between RPE and EVs and cytokine concentrations and found that there was no significant change, but this could be a result of the size of the sample. A potential reason for why no response was observed is that our sample was comprised primary of young, healthy adults.

This study suggests that, in response to exercise, the skeletal muscles induce a rapid release of extracellular vesicles with the characteristic size of EVs into the blood circulatory system. The findings are consistent with those of Aoi *et al.* (2013) in which EVs released during physical exercise may participate in cell communication during exercise-mediated adaptation processes.

Another possible mechanism could be that eccentric exercise destroys EVs and leads to the degradation of the body's own miRNA. A further reason for a reduction in EVs concentration levels could be that EVs are taken in by contracted muscles and are involved in the recovery process from damage caused by eccentric exercise. According to Aoi *et al*. (2013), regular exercise can improve skeletal muscle function along with adaptation effects in all other tissues in the body. Furthermore, skeletal muscles undertake several molecular and structural changes

during exercise. It has been shown that EVs play a potential role in myogenesis and this could be further studied by conducting more research on EVs released by muscle cells. In order to study the development and differentiation of myocytes, this study was set up to observe C2C12 cells in culture, these being commonly used as a model to study skeletal muscle cell differentiation (Burattini *et al.*, 2004).

Chapter Five of this study examined the effects of exercise EVs on C2C12 muscle cells as a model of differentiation. The experimental conditions for the C2C12 model of differentiation were first standardised, and the study then used qPCR to analyse the expression of myogenic markers (Myogenin, MyoD1) that are known for their involvement in myogenic differentiation. The findings showed that the expression of myogenic regulatory markers (Myogenin, MyoD1) was consistent with the expression pattern described in the literature (Janot *et al.,* 2009, Delgado *et al.,* 2003). The study also provided evidence that C2C12 as a model of differentiation was standardised from immunocytochemistry, and this showed a gradual increase in C2C12 myogenesis over 7 days of differentiation after treatment with PBS.

The study then examined the effect of treating differentiating C2C12 cells with plasma EVs that were previously extracted from six healthy participants both at rest and two hours after the performance of high-level eccentric exercise. On the first day of the C2C12 differentiation process, plasma samplings were added and allowed to differentiate for 7 days. It was found that eccentric EVs resulted in decreased C2C12 differentiation that expressed less myosin and formed fewer multinucleated structures at Day 3 of differentiation than C2C12 treated with rest EVs from the same participant or from the PBS control treatment. This reduction suggests that stress induced by eccentric exercise may destroy EVs vesicles. According to Aoi *et al.* (2013), acute exercise induces reductions in circulating miRNA, and this could be associated with changes in metabolic processes during exercise or reduction in EVs secretion from muscle cells to the blood circulatory

system. This is particularly relevant for miR-486 which is one of the muscle-enriched miRNAs that decreases in circulation in response to acute exercise. The exact mechanism of secretion and uptake remains to be elucidated. Further research is required to investigate the exact physiological role of EVs in response to exercise level and to identify the effects of different exercise intensities on the secretion of EVs into the blood circulatory system.

# References


Ailawadi, S., Wang, X., Gu, H., and Fan, G.C. (2015) 'Pathologic function and therapeutic potential of exosomes in cardiovascular disease', *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease,* 1852(1), pp.1-11.

American College of Sports Medicine. (2014) 'ACSM's Guidelines for Exercise Testing and Prescription'. 9th Ed. pp.162-179. Philadelphia, USA: Lippincott Williams & Wilkins.

American College of Sports Medicine and Ehrman, J.K. (2010) '*ACSM's Resource Manual for Guidelines for Exercise Testing and Prescription'*. Philadelphia, USA: Wolters Kluwer Health/Lippincott Williams & Wilkins.

An, T., Qin, S., Xu, Y., Tang, Y., Huang, Y., Situ, B., Inal, J. M. and Zheng, L. (2015) 'Exosomes serve as tumour markers for personalized diagnostics owing to their important role in cancer metastasis', *Journal of Extracellular Vesicles,* 19(4), 27522. doi: 10.3402/jev.v4.27522.

Andreu, Z., Rivas, E., Sanguino-Pascual, A., Lamana, A., Marazuela, M., González-Alvaro, I., Sánchez-Madrid, F., de la Fuente, H. and Yáñez-Mó, M. (2016) 'Comparative analysis of EV isolation procedures for miRNAs detection in serum samples', *Journal of Extracellular Vesicles, 5*(5): 10.3402/jev.v5.31655.

Aoi, W., Ichikawa, H., Mune, K., Tanimura, Y., Mizushima, K., Naito, Y. and Yoshikawa, T. (2013) 'Muscle-enriched microRNA miR-486 decreases in circulation in response to exercise in young men', *Frontiers in Physiology,* 11(4), 80. doi: 10.3389/fphys.2013.00080.

Aoi, W., Naito, Y., Mizushima, K., Takanami, Y., Kawai, Y., Ichikawa, H. and Yoshikawa, T. (2010) 'The microRNA miR-696 regulates PGC-1α in mouse skeletal muscle in response to physical activity', *American Journal of Physiology-Endocrinology and Metabolism,* 298(4), pp.E799-E806. doi: 10.1152/ajpendo.00448.2009.

Arend, W.P. (2002) 'The mode of action of cytokine inhibitors'. *The Journal of Rheumatology, 65*, pp.16-21.

Armstrong, R.B. (1984) 'Mechanisms of exercise-induced delayed onset muscular soreness: a brief review'. *Medicine and Science in Sports and Exercise*, 16(6), pp.529-538.

Azmi, A. S., Bao, B. and Sarkar, F. H. (2013) 'Exosomes in cancer development, metastasis, and drug resistance: a comprehensive review', *Cancer and Metastasis Reviews,* 32(3-4), pp.623-642.

Bala, S., Petrasek, J., Mundkur, S., Catalano, D., Levin, I., Ward, J., Alao, H., Kodys, K. and Szabo, G. (2012) 'Circulating microRNAs in exosomes indicate hepatocyte injury and inflammation in alcoholic, drug-induced, and inflammatory liver diseases', *Hepatology*, 56(5), pp.1946-1957.

Basso, J. C., & Suzuki, W. A. (2017). The Effects of Acute Exercise on Mood, Cognition, Neurophysiology, and Neurochemical Pathways: A Review. *Brain plasticity*, 2(2), pp.127–152.

Batterham, A. M. and George, K. P. (2003) 'Consistency in evidence-based clinical practice: a primer for allied health professionals', *Physical Therapy in Sport,* 4(3), pp.122-128.

Bellingham, S. A., Coleman, B. M. and Hill, A. F. (2012) 'Small RNA deep sequencing reveals a distinct miRNA signature released in exosomes from prion-infected neuronal cells', *Nucleic Acids Research,* 40(21):10937-49. doi: 10.1093/nar/gks832.

Benedikter, B. J., Bouwman, F. G., Vajen, T., Heinzmann, A. C. A., Grauls, G., Mariman, E. C., Wouters, E. F. M., Savelkoul, P. H., Lopez-Iglesias, C., Koenen, R. R., Rohde, G. G. U. & Stassen, F. R. M. (2017) 'Ultrafiltration combined with size exclusion chromatography efficiently isolates extracellular vesicles from cell culture media for compositional and functional studies', Scientific Reports, 7(1):15297.

Bentzinger, C.F., Wang, Y.X. and Rudnicki, M.A., (2012) 'Building muscle: molecular regulation of myogenesis'. *Cold Spring Harbor Perspectives in Biology,* 4(2), pii: a008342. doi: 10.1101/cshperspect.a008342.

Bishop, D., Girard, O. and Mendez-Villanueva, A., (2011) 'Repeated-sprint ability: Part II. *Sports Medicine*, 41(9), pp.741-756.

Blumenthal, J. A., Rejeski, W. J., Walsh-Riddle, M., Emery, C. F., Miller, H., Roark, S., Ribisl, P. M., Morris, P. B., Brubaker, P. and Williams, R. S. (1988) 'Comparison of high- and low-intensity exercise training early after acute myocardial infarction', *The American Journal of Cardiology*, 61(1), pp.26-30.

Bobrie, A., Colombo, M., Raposo, G. and Théry, C. (2011) 'Exosome secretion: molecular mechanisms and roles in immune responses', *Traffic,* 12(12), pp.1659-1668.

Brodersen, N. H., Steptoe, A., Boniface, S. R., and Wardle, J. (2007) 'Trends in physical activity and sedentary behaviour in adolescence: ethnic and socioeconomic differences. *Br J Sports Med.,* 41(3), pp. 140–144.

Brown, J.P., Josse, R.G. and Scientific Advisory Council of the Osteoporosis Society of Canada, (2002) '2002 clinical practice guidelines for the diagnosis and management of osteoporosis in Canada'. *Canadian Medical Association Journal,* 167(10 suppl), pp.S1-S34.

Bogdanis, G. C. (2012) 'Effects of physical activity and inactivity on muscle fatigue', *Frontiers in Physiology,* 3, 142, https://doi.org/10.3389/fphys.2012.00142.

Booth, F. W., Ruegsegger, G. N., Toedebusch, R. G. and Yan, Z. (2015) 'Chapter Six- Endurance Exercise and the Regulation of Skeletal Muscle Metabolism', *Progress in Molecular Biology and Translational Science,* 135, pp.129-151.

Borg, G. (1982) 'Ratings of perceived exertion and heart rates during short-term cycle exercise and their use in a new cycling strength test', *International Journal of Sports Medicine,* 3(03), pp.153-158.

Borg, G. (1990) 'Psychophysical scaling with applications in physical work and the perception of exertion', *Scandinavian Journal of Work, Environment & Health,* 16(1), pp.55-58.

Bradley, J. (2008) 'TNF-mediated inflammatory disease', *The Journal of Pathology,* 214(2), pp.149-160.

Breitbach, S., Tug, S. and Simon, P. (2012) 'Circulating cell-free DNA: an up-coming molecular marker in exercise physiology', *Sports Medicine,* 42(7), pp. 565-586.

Brochu M., Malita M.F., Messier V., Doucet E., Strychar I., Lavoie J.M., Prud'homme, D., and Rabasa-Lhoret, R. (2009) 'Resistance training does not contribute to improving the metabolic profile after a 6-month weight loss program in overweight and obese postmenopausal women'. *J Clin Endocrinol Metab,* 94(9), pp.3226–3233.

Brodersen, N. H., Steptoe, A., Boniface, D. R. and Wardle, J. (2007) 'Trends in physical activity and sedentary behaviour in adolescence: ethnic and socioeconomic differences', *British Journal of Sports Medicine,* 41(3), pp.140-144.

Brooks, N., Layne, J., Gordon, P., Roubenoff, R., Nelson, M., Castaneda-Sceppa, C. (2007) Strength training improves muscle quality, insulin sensitivity in Hispanic older adults with type 2 diabetes. *International Journal of Medical Sciences*, 4, pp.19–27.

Bruunsgaard, H., Galbo, H., Pedersen BK, et al. (1997) Exercise-induced increase in interleukin-6 is related to muscle damage. *J Physiol (Lond)*, 499, pp.833–41.

Buchan, D.S., Ollis, S., Young, J.D., Cooper, S.M., Shield, J.P. and Baker, J.S., (2013) 'High intensity interval running enhances measures of physical fitness but not metabolic measures of cardiovascular disease risk in healthy adolescents. *BMC Public Health*, 13, pp.498. doi: 10.1186/1471-2458-13-498.

Burattini, S., Ferri, P., Battistelli, M., Curci, R., Luchetti, F. and Falcieri, E. (2004) 'C2C12 murine myoblasts as a model of skeletal muscle development: morpho-functional characterization', *European Journal of Histochemistry: EJH,* 48(3), pp.223-233.

Burton, D.A., Stokes, K. and Hall, G.M., (2004) 'Physiological effects of exercise'. *Continuing Education in Anaesthesia, Critical Care & Pain*, 4(6), pp.185-188.

Buschow, S. I., Liefhebber, J. M., Wubbolts, R. and Stoorvogel, W. (2005) 'Exosomes contain ubiquitinated proteins', *Blood Cells, Molecules, and Diseases,* 35(3), pp.398-403.

Campbell, P.T., Campbell, K.L., Wener, M.H. Wood, B.L., Potter, J.D., McTiernan, A., and Ulrich, C.M. (2009) 'A yearlong exercise intervention decreases CRP among obese postmenopausal women'. *Med Sci Sports Exerc*, 41(8), pp.1533–1539.

Cantin, R., Diou, J., Bélanger, D., Tremblay, A. M. and Gilbert, C. (2008) 'Discrimination between exosomes and HIV-1: purification of both vesicles from cell-free supernatants', *Journal of Immunological Methods,* 338(1), pp.21-30.

Carr, B. and Wright, M. (2008) 'Nanoparticle tracking analysis', *Innovations in Pharmaceutical Technology,* 26, pp.38-40.

Caspersen, C. J., Powell, K. E. and Christenson, G. M. (1985) 'Physical activity, exercise, and physical fitness: definitions and distinctions for health-related research', *Public Health Reports,* 100(2), pp.126.

Chaar V., Romana M., Tripette J., Broquere C., Huisse M.G., Hue O., Hardy-Dessources, M.D. and Connes, P. (2011) 'Effect of strenuous physical exercise on circulating cell-derived microparticles'. *Clin Hemorheol Microcirc*, 47(1), pp.15–25.

Chatham, J.C. (2002) Lactate—The forgotten fuel! *J. Physiol*, *542* (Pt 2), pp. 333.

Centres for Disease Control and Prevention. (2015) *'Physical Activity Basics'*, [online], available at https://www.cdc.gov/physicalactivity/basics/ (accessed 20th August 2018)

Chan, M. S., Carey, A. L., Watt, M. J. and Febbraio, M. A. (2004) 'Cytokine gene expression in human skeletal muscle during concentric contraction: evidence that IL-8, like IL-6, is influenced by glycogen availability', *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology,* 287(2), pp.R322-R327.

Chaput, N. and Théry, C. (2011) 'Exosomes: immune properties and potential clinical implementations'. *Semin Immunopathol*., 33(5), pp419-40. doi: 10.1007/s00281-010-0233-9.

Chargaff, E. and West, R. (1946) 'The biological significance of the thromboplastic protein of Wood', *Journal of Biological Chemistry,* 166, pp.189-197.

Charlton, C., Mohler, W. and Blau, H. (2000) 'Neural cell adhesion molecule (NCAM) and myoblast fusion', *Developmental Biology,* 221(1), pp.112-119.

Chen, J.-F., Mandel, E. M., Thomson, J. M., Wu, Q., Callis, T. E., Hammond, S. M., Conlon, F. L. and Wang, D.-Z. (2006) 'The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation', *Nature Genetics,* 38(2), pp.228-233.

Chen, L., Wang, Y., Pan, Y., Zhang, L., Shen, C., Qin, G., Ashraf, M., Weintraub, N., Ma, G. and Tang, Y. (2013) 'Cardiac progenitor-derived exosomes protect ischemic myocardium from acute ischemia/reperfusion injury', *Biochemical and Biophysical Research Communications,* 431(3), pp.566-571.

Cicchetti, D.V. (1994) Guidelines, criteria, and rules of thumb for evaluating normed and standardized assessment instruments in psychology. *Psychological Assessment,* 6(4), pp.284–290.

Chargé, S. B. and Rudnicki, M. A. (2004) Cellular and molecular regulation of muscle regeneration. *Physiol Rev.,*84, pp 209-238.

Chief Medical Officers of England S, Wales, and Northern Ireland (2013) 'Start Active, Stay Active. A report on physical activity for health from the four home countries' *Chief Medical Officer.* London: Department of Health.

Choi, J. S., Yoon, H. I., Lee, K. S., Choi, Y. C., Yang, S. H., Kim, I-S. and Cho, Y. W. (2016) 'Exosomes from differentiating human skeletal muscle cells trigger myogenesis of stem cells and provide biochemical cues for skeletal muscle regeneration.' *Journal of Controlled Release,* 222, pp.107-115.

Choi, Y. S., Ryu, B. K., Min, H. K., Lee, S. W. and Pak, Y. K. (2005) 'Analysis of Proteome Bound to D-Loop Region of Mitochondrial DNA by DNA-Linked Affinity Chromatography and Reverse-Phase Liquid Chromatography/Tandem Mass Spectrometry', *Annals of the New York Academy of Sciences,* 1042(1), pp.88-100.

Coenen-Stass, A. M., Betts, C. A., Lee, Y. F., Mäger, I., Turunen, M. P., Andaloussi, S. E., Morgan, J. E., Wood, M. J. and Roberts, T. C. (2016) 'Selective release of muscle-specific, extracellular microRNAs during myogenic differentiation', *Human Molecular Genetics*, 25(18):3960-3974. doi: 10.1093/hmg/ddw237.

Corrado, C., Raimondo, S., Chiesi, A., Ciccia, F., De Leo, G. and Alessandro, R. (2013) 'Exosomes as intercellular signaling organelles involved in health and disease: basic science and clinical applications', *International Journal of Molecular Sciences,* 14(3), pp.5338-5366.

Croci, D. O., Cerliani, J. P., Pinto, N. A., Morosi, L. G. and Rabinovich, G. A. (2014) 'Regulatory role of glycans in the control of hypoxia-driven angiogenesis and sensitivity to anti-angiogenic treatment', *Glycobiology*, 24(12), pp1283-1290.

Croisier, J. L., Camus, G., Venneman, I., Deby-Dupont, G., Juchmes-Ferir, A., Lamy, M., Crielaard, J. M., Deby, C. and Duchateau, J. (1999) 'Effects of training on exercise-induced muscle damage and interleukin 6 production', *Muscle & Nerve*, 22(2), pp.208-212.

Crumpler, H.R., Dent, C.E., Harris, H., Westall, R.G. (1951) 'β-Aminoisobutyric acid (α-methyl-β-alanine); a new amino-acid obtained from human urine.' *Nature,* 167, pp.307–308.

Cullen, T., Thomas, A. W., Webb, R. and Hughes, M. G. (2016) 'Interleukin-6 and associated cytokine responses to an acute bout of high intensity interval exercise: the effect of exercise intensity and volume', *Applied Physiology, Nutrition, and Metabolism,* 41(8), pp 803-808.

Davies, K. E. and Nowak, K. J. (2006) 'Molecular mechanisms of muscular dystrophies: old and new players', *Nature Reviews Molecular Cell Biology,* 7(10), pp.762-773.

De Toro, J., Herschlik, L., Waldner, C. and Mongini, C. (2015) 'Emerging roles of exosomes in normal and pathological conditions: new insights for diagnosis and therapeutic applications'. *Frontiers in Immunology,* 6(203), doi: 10.3389/fimmu.2015.00203.

Delgado, I., Huang, X., Jones, S., Zhang, L., Hatcher, R., Gao, B. and Zhang, P. (2003) 'Dynamic gene expression during the onset of myoblast differentiation in vitro', *Genomics,* 82(2), pp.109-121.

Dias, K.A., Coombes, J.S., Green, D.J., Gomersall, S.R., Keating, S.E., Tjonna, A.E., Hollekim-Strand, S.M., Hosseini, M.S., Ro, T.B., Haram, M., Huuse, E.M., Davies, P. S., Cain, P. A., Leong, G. M. and Ingul, C. B. (2016) 'Effects of exercise intensity and nutrition advice on

myocardial function in obese children and adolescents: a multicentre randomised controlled trial study protocol'. *BMJ Open*, 6(4), e010929. doi: 10.1136/bmjopen-2015-010929.

Ding, G., Zhou, L., Qian, Y., Fu, M., Chen, J., Chen, J., Xiang, J., Wu, Z., Jiang, G., Cao, L. (2015) Pancreatic cancer-derived exosomes transfer miRNAs to dendritic cells and inhibit rfxap expression via miR-212-3p. *Oncotarget,* 6, pp.29877–29888.

Dishman, R. K., Berthoud, H. R., Booth, F. W., Cotman, C. W., Edgerton, V. R., Fleshner, M. R., Gandevia, S. C., Gomez-Pinilla, F., Greenwood, B. N. and Hillman, C. H. (2006) 'Neurobiology of exercise', *Obesity,* 14(3), pp.345-356.

Doyle, L. and Wang, M.Z. (2019) 'Overview of Extracellular Vesicles, Their Origin, Composition, Purpose, and Methods for Exosome Isolation and Analysis', *Cells.,* 8(7), 727.

Dragovic, R. A., Gardiner, C., Brooks, A. S., Tannetta, D. S., Ferguson, D. J., Hole, P., Carr, B., Redman, C. W., Harris, A. L. and Dobson, P. J. (2011) 'Sizing and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis', *Nanomedicine: Nanotechnology, Biology and Medicine,* 7(6), pp.780-788.

Drummond, M. J., Glynn, E. L., Fry, C. S., Dhanani, S., Volpi, E. and Rasmussen, B. B. (2009) 'Essential amino acids increase microRNA-499,-208b, and-23a and downregulate myostatin and myocyte enhancer factor 2C mRNA expression in human skeletal muscle', *The Journal of Nutrition,* 139(12), pp.2279-2284.

Edgar, J.R. (2016) 'What are exosomes exactly?' *BMC Biol*., 14, 46.

Egan, B. and Zierath, J. R. (2013) 'Exercise metabolism and the molecular regulation of skeletal muscle adaptation', *Cell Metabolism,* 17(2), pp.162-184.

Ellis, R. J. and Van der Vies, S. M. (1991) 'Molecular chaperones', *Annual Review of Biochemistry,* 60(1), pp.321-347.

Elmore, S. (2007) 'Apoptosis: a review of programmed cell death', *Toxicol Pathol*., 35(4), pp.495–516.

Enoka, R.M. (2002) Neuromechanics of human movement. Champaign, Ill: Human Kinetics.

Enwere, E.K., LaCasse, E.C., Adam, N.J. and Korneluk, R.G., (2014) 'Role of the TWEAK-Fn14-cIAP1-NF-κB signalling axis in the regulation of myogenesis and muscle homeostasis'. *Frontiers in Immunology,* 5(34). doi: 10.3389/fimmu.2014.00034.

Erikssen, G., Liestøl, K., Bjørnholt, J., Thaulow, E., Sandvik, L. and Erikssen, J. (1998) 'Changes in physical fitness and changes in mortality', *The Lancet,* 352(9130), pp.759-762.

Escrevente, C., Keller, S., Altevogt, P. and Costa, J. (2011) 'Interaction and uptake of exosomes by ovarian cancer cells', *BMC Cancer,* 11, pp.108. doi: 10.1186/1471-2407-11-108.

Fabbri, M., Paone, A., Calore, F., Galli, R., Gaudio, E., Santhanam, R., Lovat, F., Fadda, P., Mao, C., Nuovo, G. J., Zanesi, N., Crawford, M., Ozer, G. H., Wernicke, D., Alder, H., Caligiuri, M. A., Nana-Sinkam, P., Perrotti, D., Croce, C. M. (2012) MicroRNAs bind to Toll-like

receptors to induce prometastatic inflammatory response. *Proceedings of the National Academy of Sciences of the United States of America,* 109(31), pp. E2110-6.

Faulkner, J.A., Heigenhauser, G.F., and Schork, M.A. (1977) 'The cardiac output-oxygen uptake relationship of men during graded bicycle ergometry', *Medicine and Science in Sports and Exercise,* 9(3), pp. 148–154.

Fairey, A.S., Courneya, K.S., Field, C.J., Bell, G. J., Jones, L. W., Martin, B. S., and MacKey, J. R. (2005) 'Effect of exercise training on C-reactive protein in postmenopausal breast cancer survivors: a randomisedcontrolled trial'. *Brain Behav Immun.*19, pp. 381–388.

Febbraio, M. A., Hiscock, N., Sacchetti, M., Fischer, C. P. and Pedersen, B. K. (2004) 'Interleukin-6 is a novel factor mediating glucose homeostasis during skeletal muscle contraction', *Diabetes,* 53(7), pp.1643-1648.

Febbraio, M. A. and Pedersen, B. K. (2002) 'Muscle-derived interleukin-6: mechanisms for activation and possible biological roles', *The FASEB Journal,* 16(11), pp.1335-1347.

Feghali, C .A. and Wright, T. M. (1997) 'Cytokines in acute and chronic inflammation'. *Frontiers in Bioscience,* 2, pp. 12–26.

Fernando, M. R., Jiang, C., Krzyzanowski, G D. and Ryan, W. L. (2017) 'New Evidence that a large proportion of human blood plasma cell-free DNA is localized in exosomes. *PLOS ONE*, https://doi.org/10.1371/journal.pone.0183915.

Ford, E. (2002) 'Does exercise reduce inflammation? Physical activity and C-reactive protein among US adults'. *Epidemiology,* 13(5), pp.561–568.

Forterre, A., Jalabert, A., Berger, E., Baudet, M., Chikh, K., Errazuriz, E., De Larichaudy, J., Chanon, S., Weiss-Gayet, M. and Hesse, A.-M. (2014) 'Proteomic analysis of C2C12 myoblast and myotube exosome-like vesicles: a new paradigm for myoblast-myotube cross talk?', *PLoS ONE,* 9(1), e84153.

Foulds, H. J., Bredin, S. S., Charlesworth, S. A., Ivey, A. C. and Warburton, D. E. (2014) 'Exercise volume and intensity: a dose–response relationship with health benefits', *European Journal of Applied Physiology,* 114(8), pp.1563-1571.

Franceschi, C. and Campisi, J. (2014) 'Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases', *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences,* 69(Suppl 1), pp.S4-S9.

Friedenreich, C.M. (2011) 'Physical activity and breast cancer: review of the epidemiologic evidence and biologic mechanisms'. *Recent Results Cancer Res*, 188, pp. 125-39.

Fröhlich, M., Imhof, A., Berg, G., Hutchinson, W.L., Pepys, M.B., Boeing, H., Muche, R., Brenner, H., and Koenig, W. (2000) 'Association between C-reactive protein and features of the metabolic syndrome'. A population-based study. *Diabetes Care*, 23(12), pp. 1835-1839.

Frontera, W. R. and Ochala, J. (2015) 'Skeletal muscle: a brief review of structure and function', *Calcified Tissue International,* 96(3), pp.183-195.

Frühbeis, C., Helmig, S., Tug, S., Simon, P. and Krämer-Albers, E.-M. (2015) 'Physical exercise induces rapid release of small extracellular vesicles into the circulation', *Journal of Extracellular Vesicles,* 4, 10.3402/jev.v4.28239.

Gabay, C. (2006) 'Interleukin-6 and chronic inflammation', *Arthritis Research & Therapy, 8*(Suppl.2): S3, https://doi.org/10.1186/ar1917.

Garber, C. E., Blissmer, B., Deschenes, M. R., Franklin, B. A., Lamonte, M. J., Lee, I.-M., Nieman, D. C. and Swain, D. P. (2011) 'American College of Sports Medicine position stand. Quantity and quality of exercise for developing and maintaining cardiorespiratory, musculoskeletal, and neuromotor fitness in apparently healthy adults: guidance for prescribing exercise', *Medicine and Science in Sports and Exercise,* 43(7), pp.1334-1359.

Gardiner, C., Ferreira, Y. J., Dragovic, R. A., Redman, C. W. and Sargent, I. L. (2013) 'Extracellular vesicle sizing and enumeration by nanoparticle tracking analysis', *Journal of Extracellular Vesicles,* 2(2): 10.3402/jev.v2i0.19671.

Gebel, K., Ding, D., Chey, T., Stamatakis, E., Brown, W. J. and Bauman, A. E. (2015) 'Effect of moderate to vigorous physical activity on all-cause mortality in middle-aged and older Australians', *JAMA Internal Medicine,* 175(6), pp.970-977.

Geffken, D.F., Cushman, M., Burke, G.L., Polak, J.F., Sakkinen, P.A., and Tracy, R.P. (2001) 'Association between physical activity and markers of inflammation in a healthy elderly population'. *Am J Epidemiol,* 153(3), pp.242–250.

Ghidoni, R., Benussi, L., Binetti, G. (2007) Exosomes: the Trojan horses of neurodegeneration. *Med Hypotheses,* 70, pp.1226–710.

Gidlow, C., Johnston, L. H., Crone, D., Ellis, N. and James, D. (2006) 'A systematic review of the relationship between socio-economic position and physical activity', *Health Education Journal,* 65(4), pp.338-367.

Gielen, S., Adams, V., Mobius-Winkler, S., Yu, J., Niebauer, J., Jiang, H., Fiehn, E., and Schuler, G. (2003) 'Anti-inflammatory effects of exercise training in the skeletal muscle of patients with chronic heart failure'. *J Am Coll Cardiol,* 42(5), pp. 861–868.

Gleeson, M. (2004) 'Immune function and exercise', *European Journal of Sport Science,* 4(3), pp.52-66.

Gleeson, M., Bishop, N.C., Stensel, D.J., Lindley, M.R., Mastana, S.S., and Nimmo, M.A.. (2011) 'The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease'. *Nature Reviews Immunology,* 11(9), pp. 607-15.

Gleeson, M. and Bishop, N.C., (2000) 'Special feature for the Olympics: effects of exercise on the immune system: modification of immune responses to exercise by carbohydrate, glutamine and anti-oxidant supplements'. *Immunology and Cell Biology,* 78(5), pp.554-561.

Gomez-Cabrera, M. C., Borrás, C., Pallardó, F. V., Sastre, J., Ji, L. L. and Viña, J. (2005) 'Decreasing xanthine oxidase-mediated oxidative stress prevents useful cellular adaptations to exercise in rats', *The Journal of Physiology,* 567(1), pp.113-120.

Gould, S. J. and Raposo, G. (2013) 'As we wait: coping with an imperfect nomenclature for extracellular vesicles', *Journal of Extracellular Vesicles,* 2: doi: 10.3402/jev.v2i0.20389.

Grabowska, I., Szeliga, A., Moraczewski, J., Czaplicka, I. and Brzóska, E. (2011) 'Comparison of satellite cell-derived myoblasts and C2C12 differentiation in two-and three-dimensional cultures: changes in adhesion protein expression', *Cell Biology International,* 35(2), pp.125-133.

Greening, D.W., Gopal, S.K., Xu, R., Simpson, R.J. and Chen, W., (2015) 'Exosomes and their roles in immune regulation and cancer'. *Seminars in Cell & Developmental Biology,* 40, pp.72-81.

Greiwe, J.S., Cheng, B., Rubin, D.C., Yarasheski, K.E. and Semenkovich, C.F. (2001) 'Resistance exercise decreases skeletal muscle tumor necrosis factor alpha in frail elderly humans'. *FASEB J.*, 15(2), pp.475–482.

Gregg, E.W., Gerzoff, R.B., Caspersen, C.J., Williamson, D.F. and Narayan, K.V., (2003) 'Relationship of walking to mortality among US adults with diabetes'. *Archives of Internal Medicine,* 163(12), pp.1440-1447.

Gudbergsson, J. M., Johnsen, K. B. Skov, M. N. and Duroux, M (2016) 'Systematic review of factors influencing extracellular vesicle yield from cell cultures'. *Cytotechnology*, 68(4), pp 579-592.

Guescini, M., Maggio, S., Ceccaroli, P., Battistelli, M., Annibalini, G., Piccoli, G., Sestili, P. and Vilberto Stocchi, V. (2017) 'Extracellular Vesicles Released by Oxidatively Injured or Intact C2C12 Myotubes Promote Distinct Responses Converging toward Myogenesis'. *Int J Mol Sci,* 18(11), p.2488. doi: 10.3390/ijms18112488

Guescini, M., Guidolin, D., Vallorani, L., Casadei, L., Gioacchini, A., Tibollo, P., Battistelli, M., Falcieri, E., Battistin, L. and Agnati, L. (2010) 'C2C12 myoblasts release micro-vesicles containing mtDNA and proteins involved in signal transduction', *Experimental Cell Research,* 316(12), pp.1977-1984.

Halova, I. and Draber, P. (2016) 'Tetraspanins and Transmembrane Adaptor Proteins As Plasma Membrane Organizers – Mast Cell Case', *Frontiers in Cell and Developmental Biology,* 4, https://doi.org/10.3389/fcell.2016.00043

Hammeren, J., Powers, S., Lawler, J., Criswell, D., Martin, D., Lowenthal, D. and Pollock, M. (1992) 'Exercise training-induced alterations in skeletal muscle oxidative and antioxidant enzyme activity in senescent rats', *International Journal of Sports Medicine,* 13(05), pp.412-416.

Harding, C., Heuser, J., and Stahl, P. (1983) 'Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes' *J. Cell Biol*., 97, pp.329–339.

Haskell, W. L., Lee, I.-M., Pate, R. R., Powell, K. E., Blair, S. N., Franklin, B. A., Macera, C. A., Heath, G. W., Thompson, P. D. and Bauman, A. (2007) 'Physical activity and public health: updated recommendation for adults from the American College of Sports Medicine and the American Heart Association', *Med Sci Sports Exerc.,* 39(8):pp.1423-1434.

Hemler, M. E. (2005) 'Tetraspanin functions and associated microdomains', *Nature Reviews Molecular Cell Biology,* 6(10), pp.801-811.

Herbert, T.B. and Cohen, S., (1993) 'Stress and immunity in humans: a meta-analytic review'. *Psychosomatic Medicine,* 55(4), pp.364-379.

Herzog, W., Leonard, T. R., Joumaa, V. and Mehta, A. (2008) 'Mysteries of muscle contraction', *J Appl Biomech.,*24(1), pp.1–13.

Herzog, W., Powers, K., Johnston, K. and Duvall, M. (2015) 'A new paradigm for muscle contraction'. *Front. Physiol.,* 6, p.174. doi: 10.3389/fphys.2015.00174

Heyward, V. H. and Gibson, A. (2014) '*Advanced fitness assessment and exercise prescription', 7th edition.* Leeds, UK: Human Kinetics Europe Ltd, pp. 1-122.

Higginbotham, M. B., Morris, K. G., Williams, S., McHale, P. A., Coleman, R. E., and Cobb, F.R. (1986) 'Regulation of stroke volume during submaximal and maximal upright exercise in normal man', *Circulation Research,* 58(2), pp. 281–291.

High, D. M., Howley, E. T. and Franks, B. D. (1989) 'The effects of static stretching and warm-up on prevention of delayed-onset muscle soreness', *Research Quarterly for Exercise and Sport,* 60(4), pp.357-361.

Hikida, R. S. (2011) 'Aging changes in satellite cells and their functions', *Current Aging Science,* 4(3), pp.279-297.

Hoffman-Goetz, L. and Pedersen, B. K. (1994) 'Exercise and the immune system: a model of the stress response?', *Immunology Today,* 15(8), pp.382-387.

Hopkin, K. (2016) 'Core Concept: Extracellular vesicles garner interest from academia and biotech', *Proceedings of the National Academy of Sciences,* 113(33), pp.9126-9128.

Hopkins, W. G. (2000) 'Measures of consistency in sports medicine and science', *Sports Medicine,* 30(1), pp.1-15.

Hoppeler, H. (2016) 'Molecular networks in skeletal muscle plasticity', *Journal of Experimental Biology,* 219(2), pp.205-213.

Howald, H., Hoppeler, H., Claassen, H., Mathieu, O. and Straub, R. (1985) 'Influences of endurance training on the ultrastructural composition of the different muscle fiber types in humans', *Pflügers Archiv,* 403(4), pp.369-376.

Huang, X., Yuan, T., Tschannen, M., Sun, Z., Jacob, H., Du, M., Liang, M., Dittmar, R. L., Liu,Y. and Liang, M. (2013) 'Characterization of human plasma-derived exosomal RNAs by deep sequencing', *BMC Genomics,* 14(1), pp.1.

Ichinose, M., Maeda, S., Kondo, N., and Nishiyasu, T. (2014) 'Blood pressure regulation II: what happens when one system must serve two masters: oxygen delivery and pressure regulation?' *European Journal of Applied Physiology and Occupational Physiology,* 114 (3), pp. 451–465.

Il'yasova, D., Colbert, L.H., Harris, T.B., Newman, A.B., Bauer, D.C., Satterfield, S., and Kritchevsky, S.B. (2005) 'Circulating levels of inflammatory markers and cancer risk in the health aging and body composition cohort'. *Cancer Epidemiol Biomarkers Prev,* 14(10), pp.2413-2418.

Izumiya, Y., Bina, H. A., Ouchi, N., Akasaki, Y., Kharitonenkov, A. and Walsh, K. (2008) 'FGF21 is an Akt-regulated myokine', *FEBS letters,* 582(27), pp.3805-3810.

Jakobsen, K.R., Paulsen, B.S., Bæk, R., Varming, K., Sorensen, B.S. and Jørgensen, M.M., (2015) 'Exosomal proteins as potential diagnostic markers in advanced non-small cell lung carcinoma'. *Journal of Extracellular Vesicles,*4(26659). doi: 10.3402/jev.v4.26659.

Jago, R., Wedderkopp, N., Kristensen, P. L., Møller, N. C., Andersen, L. B., Cooper, A. R. and Froberg, K. (2008) 'Six-year change in youth physical activity and effect on fasting insulin and HOMA-IR', *American Journal of Preventive Medicine,* 35(6), pp.554-560.

Jain, S., Gautam, V. and Naseem, S. (2011) 'Acute-phase proteins: as diagnostic tool', *Journal of Pharmacy and Bioallied Sciences,* 3(1), pp.118-127.

Janot, M., Audfray, A., Loriol, C., Germot, A., Maftah, A. and Dupuy, F. (2009) 'Glycogenome expression dynamics during mouse C2C12 myoblast differentiation suggests a sequential reorganization of membrane glycoconjugates', *BMC Genomics,* 10:483. doi: 10.1186/1471-2164-10-483.

Ji, L. L., Gomez-Cabrera, M. C. and Vina, J. (2006) 'Exercise and hormesis', *Annals of the New York Academy of Sciences,* 1067(1), pp.425-435.

Johnstone, R. M., Adam, M., Hammond, J., Orr, L. and Turbide, C. (1987) 'Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes)', *Journal of Biological Chemistry,* 262(19), pp.9412-9420.

Jonsdottir, I. H., Schjerling, P., Ostrowski, K., Asp, S., Richter, E. A. and Pedersen, B. (2000) 'Muscle contractions induce interleukin-6 mRNA production in rat skeletal muscles', *The Journal of Physiology,* 528(1), pp.157-163.

Jurkat-Rott, K. and Lehmann-Horn, F. (2005) 'Muscle channelopathies and critical points in functional and genetic studies', *The Journal of Clinical Investigation,* 115(8), pp.2000-2009.

Kalra, H., Adda, C. G., Liem, M., Ang, C. S., Mechler, A., Simpson, R. J., Hulett, M. D. and Mathivanan, S. (2013) 'Comparative proteomics evaluation of plasma exosome isolation techniques and assessment of the stability of exosomes in normal human blood plasma', *Proteomics, 13*(22), pp.3354-3364.

Khan, S., Jutzy, J. M., Valenzuela, M. M. A., Turay, D., Aspe, J. R., Ashok, A., Mirshahidi, S., Mercola, D., Lilly, M. B. and Wall, N. R. (2012) 'Plasma-derived exosomal survivin, a plausible biomarker for early detection of prostate cancer', *PLoS ONE,* 7(10), pp.e46737.

Khanna, K., Salmond, N., Lynn, K.S., Leong, H.S., Williams, K.C. (2021) 'Clinical significance of STEAP1 extracellular vesicles in prostate cancer', Prostate Cancer and Prostatic Dieases, doi: 10.1038/s41391-021-00319-2. Online ahead of print.

Kharaziha, P., Ceder, S., Li, Q. and Panaretakis, T. (2012) 'Tumor cell-derived exosomes: a message in a bottle', *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer,* 1826(1), pp.103-111.

Kim, H. K., Lee, Y. S., Sivaprasad, U., Malhotra, A. and Dutta, A. (2006) 'Muscle-specific microRNA miR-206 promotes muscle differentiation', *The Journal of Cell Biology,* 174(5), pp.677-687.

Kislinger, T., Gramolini, A. O., Pan, Y., Rahman, K., MacLennan, D. H. and Emili, A. (2005) 'Proteome dynamics during C2C12 myoblast differentiation', *Molecular & Cellular Proteomics,* 4(7), pp.887-901.

Kobayashi, M., Kawase, T., Okuda, K., Wolff, L. F. and Yoshie, H. (2015) 'In vitro immunological and biological evaluations of the angiogenic potential of platelet-rich fibrin preparations: a standardized comparison with PRP preparations', *International Journal of Implant Dentistry,* 1(1), p.31. doi:10.1186/s40729-015-0032-0.

Kosaka, N., Iguchi, H., Yoshioka, Y., Takeshita, F., Matsuki, Y. and Ochiya, T. (2010) 'Secretory mechanisms and intercellular transfer of microRNAs in living cells', *Journal of Biological Chemistry,* 285(23), pp.17442-17452.

Kourembanas S. (2014) 'Exosomes: Vehicles of Intercellular Signaling, Biomarkers, and Vectors of Cell Therapy'**.** *Annu Rev Physiol* , 77, pp.13-27. doi: 10.1146/annurev-physiol-021014-071641.

Kovanen, V. and Suominen, H. (1987) Effects of age and life-time physical training on fibre composition of slow and fast skeletal muscle in rats', *Pflügers Archiv,* 408(6), pp.543-551.

Kowal, J., Tkach, M., Théry, C., (2014) 'Biogenesis and secretion of exosomes', Curr Opin Cell Biol., 29, pp.116-25.

Kristjánsson, Á. and Campana, G. (2010) Where perception meets memory: A review of repetition priming in visual search tasks', *Attention, Perception, & Psychophysics,* 72(1), pp.5-18.

Lakkaraju, A. and Rodriguez-Boulan, E. (2008) Itinerant exosomes: emerging roles in cell and tissue polarity, *Trends in Cell Biology,* 18(5), pp.199-209.

Lambert, C.P., Wright, N.R., Finck, B.N., and Villareal, D.T. (2008) 'Exercise but not diet induced weight loss decreases skeletal muscle inflammatory gene expression in frail obese elderly'. *J Appl Physiol*,105(2), pp.473-478.

Lamparski, H. G., Metha-Damani, A., Yao, J.-Y., Patel, S., Hsu, D.-H., Ruegg, C. and Le Pecq, J.-B. (2002) 'Production and characterization of clinical grade exosomes derived from dendritic cells', *Journal of Immunological Methods,* 270(2), pp.211-226.

Lässer, C., Eldh, M. and Lötvall, J. (2012) 'Isolation and characterization of RNA-containing exosomes', *JoVE (Journal of Visualized Experiments),* (59), e3037. doi: 10.3791/3037.

Lässer, C., O'Neil, S. E., Shelke, G. V., Sihlbom, C., Hansson, S. F., Gho, Y. S., Lundbäck, B., & Lötvall, J. (2016) Exosomes in the nose induce immune cell trafficking and harbour an altered protein cargo in chronic airway inflammation. *Journal of translational medicine*, 14(1), pp.181.

Laughlin, M.H., Davis, M.J., Secher, N.H., Lieshout, J.J., Arce-Esquivel, A.A., Simmons, G.H., Bender, S.B., Padilla, J., Bache, R.J., Merkus, D. and Duncker, D.J., (2012) 'Peripheral circulation'. *Comprehensive Physiology*, 2, pp.321–447.

Leandro, C. G., Castro, R., Nascimento, E., Pithon-Curi, T. C. and Curi, R. (2007) 'Mecanismos adaptativos do sistema imunológico em resposta ao treinamento físico', *Rev Bras Med Esporte,* 13(5), pp.343-348.

Leary, S. D., Ness, A. R., Smith, G. D., Mattocks, C., Deere, K., Blair, S. N. and Riddoch, C. (2008) 'Physical Activity and Blood Pressure in Childhood Findings from a Population-Based study', *Hypertension,* 51(1), pp.92-98.

Lee, I.M. and Skerrett, P.J., (2001) 'Physical activity and all-cause mortality: what is the dose-response relation?'. *Medicine and science in sports and exercise*, 33(6; SUPP), pp.S459-S471.

Lee, I.M., Shiroma, E.J., Lobelo, F., Puska, P., Blair, S.N., Katzmarzyk, P.T. and Lancet Physical Activity Series Working Group (2012) 'Effect of physical inactivity on major non-communicable diseases worldwide: an analysis of burden of disease and life expectancy'. *The Lancet*, 380(9838), pp.219-229.

Le Grand, F. and Rudnicki, M. A. (2007) 'Skeletal muscle satellite cells and adult myogenesis'. *Curr Opin Cell Biol,* 19(6): pp.628–633.

Levinger I., Goodman C., Hare D.L., Jerums G., Toia D., Selig S.(2009) The reliability of the 1RM strength test for untrained middle-aged individuals. *Journal of Science and Medicine in Sport* 12 (3), pp.310-316.

Lewis, S. F., Taylor, W.F., Graham, R.M., Pettinger, W.A., Schutte, J.E. and Blomqvist, C. G. (1983) 'Cardiovascular responses to exercise as functions of absolute and relative work load', *Journal of Applied Physiology Respiratory Environmental and Exercise Physiology,* 54(5), pp. 1314–1323.

Li, P., Kaslan, M., Lee, S .H. Yao, J. and Zhiqiang Gao z. (2017) 'Progress in Exosome Isolation Techniques'. *Theranostics.,* 7(3): pp. 89-804.

Lobb, R.J., Becker, M., Wen, S.W., Wong, C.S.F., Wiegmans, A.P., Leimgruber, A., and Möller, A. (2015) 'Optimized exosome isolation protocol for cell culture supernatant and human plasma'. *J. Extracell. Vesicles*, 4:27031. doi: 10.3402/jev.v4.27031.

Logozzi, M., De Milito, A., Lugini, L., Borghi, M., Calabro, L., Spada, M., Perdicchio, M., Marino, M. L., Federici, C. and Iessi, E. (2009) 'High levels of exosomes expressing CD63 and caveolin-1 in plasma of melanoma patients', *PLoS ONE,* 4(4), e5219. doi: 10.1371/journal.pone.0005219.

Mairbäurl, H.,( 2007) 'Red blood cells in sports: effects of exercise and training on oxygen supply by red blood cells'. *Frontiers in Physiology,* https://doi.org/10.3389/fphys.2013.00332

Malatesta, D., Werlen, C., Bulfaro, S., Chenevière, X. and Borrani, F. (2009) 'Effect of high-intensity interval exercise on lipid oxidation during postexercise recovery', *Medicine and Science in Sports and Exercise,* 41(2), pp.364-374.

Mangione, K. K., McCully, K., Gloviak, A., Lefebvre, I., Hofmann, M. and Craik, R. (1999) 'The effects of high-intensity and low-intensity cycle ergometry in older adults with knee osteoarthritis', *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences,* 54(4), pp.M184-M190.

Marcos, A., Nova, E. and Montero, A. (2003) 'Changes in the immune system are conditioned by nutrition', *European Journal of Clinical Nutrition,* 57(Suppl.1), pp.S66-S69.

Martín-Cordero, L., J Garcia, J. and Ortega, E. (2013) 'Noradrenaline-mediated inhibition of inflammatory cytokines is altered in macrophages from obese Zucker rats: effect of habitual exercise', *Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune, Endocrine & Metabolic Disorders),* 13(3), pp.234-239.

Maruyama, K., Morishita, E., Sekiya, A., Omote, M., Kadono, T., Asakura, H., Hashimoto, M., Kobayashi, M., Nakatsumi, Y. and Takada, S. (2012) 'Plasma levels of platelet-derived microparticles in patients with obstructive sleep apnea syndrome', *Journal of Atherosclerosis and Thrombosis,* 19(1), 98-104.

Mathew, A., Bell, A. and Johnstone, R. (1995) 'Hsp-70 is closely associated with the transferrin receptor in exosomes from maturing reticulocytes', *Biochemical Journal,* 308(3), 823-830.

Mattusch, F., Dufaux, B., Heine, O. Mertens, I. and Rost, R. (2000) 'Reduction in the plasma concentration of C-reactive protein following nine months of endurance training'. *Int J Sports Med*, 21(1), pp. 21–24.

Makarova, J.A., Maltseva, D.V., Galatenko, V.V., Abbasi, A., Maximenko, D.G., Grigoriev, A.I., Tonevitsky, A.G. and Northoff, H. (2014) 'Exercise immunology meets MiRNAs'. *Exerc Immunol Rev*, 20, pp.135-164.

McCarthy, J. J., Esser, K. A. and Andrade, F. H. (2007) 'MicroRNA-206 is overexpressed in the diaphragm but not the hindlimb muscle of mdx mouse', *American Journal of Physiology-Cell Physiology,* 293(1), C451-C457.

McFarlin, B. K., Flynn, M. G., Campbell, W W, Stewart, L. K. and Timmerman, K. L. (2004) 'TLR4 is lower in resistance-trained older women and related to inflammatory cytokines'. *Med Sci Sports Exerc*, 36, pp.1876–1883.

Mendelson, M., Jinwala, K., Wuyam, B., Levy, P. and Flore, P. (2012) 'Can crossover and maximal fat oxidation rate points be used equally for ergocycling and walking/running on a track?', *Diabetes & metabolism,* 38(3), pp.264-270.

Merikle, P. M. (1992) 'Perception without awareness: Critical issues', *American Psychologist,* 47(6), pp.792.

Middleton, N., Shave, R., George, K., Whyte, G., Hart, E., Oxborough, D., Forster, J. and Gaze, D., (2007) 'Altered left ventricular diastolic filling following a marathon is a reproducible phenomenon'. *International Journal of Cardiology,* 122(1), pp.87-89.

Miles, M. P. (2008) 'How do we solve the puzzle of unintended consequences of inflammation? Systematically', *Journal of Applied Physiology,* 105(4), pp.1023-1025.

Mittelbrunn, M., Gutiérrez-Vázquez, C., Villarroya-Beltri, C., González, S., Sánchez-Cabo, F., González, M. Á., Bernad, A. and Sánchez-Madrid, F. (2011) 'Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells', *Nature Communications,* 2, 282. doi: 10.1038/ncomms1285.

MIQE & qPCR iBook (2016) 'How to apply the MIQE guidelines - a visual, interactive and practical qPCR guide'. 2nd Edition (version 2.5), Eds: Afif M. Abdel Nour & Michael W. Pfaffl. ISBN 9783000488061.

Moldoveanu, A. I., Shephard, R. J., and Shek, P. N. (2001) 'The cytokine response to physical activity and training'. *Sports Med,* 31(2), pp.115–144.

Moon, H.Y., Becke, A., Berron, D., Becker, B., Sah, N., Benoni, G., Janke, E., Lubejko, S.T., Greig, N.H., Mattison, J.A., Duzel, E., van Praag, H. (2016) 'Running-Induced Systemic Cathepsin B Secretion Is Associated with Memory Function', Cell Metab., 24(2), pp.332-40.

Moran, A. (2004) '*Sport and exercise psychology: A critical introduction'.* Abingdon, UK: Routledge.

Moreira, L.D.F., Oliveira, M.L.D., Lirani-Galvão, A.P., Marin-Mio, R.V., Santos, R.N.D. and Lazaretti-Castro, M. (2014) 'Physical exercise and osteoporosis: effects of different types of exercises on bone and physical function of postmenopausal women'. *Arquivos Brasileiros de Endocrinologia & Metabologia,* 58(5), pp.514-522.

Moreira L, Fronza FC, dos Santos RN, Teixeira LR, Kruel LF, Lazaretti-Castro M (2013) 'High-intensity aquatic exercises (HydrOS) improve physical function and reduce falls among postmenopausal women'. *Menopause*; 20(10), pp.1012-1019.

Mulcare, J. A. and Jackson, K. G. (2004) Multiple Sclerosis. In: LEMURA, L. & VON DUVILLARD, S. (eds.) *Clinical Exercise Physiology; application and physiological principals*. Philadelphia PA: Lippincott, Williams and Wilkins.

Muralidharan-Chari, V., Clancy, J., Plou, C., Romao, M., Chavrier, P., Raposo, G., D'Souza-Schorey, C. (2009) 'ARF6-regulated shedding of tumor cell-derived plasma membrane microvesicles', Curr Biol., 19(22), pp.1875-85.

Muroya, S., Ogasawara, H. and Hojito, M. (2015) 'Grazing affects exosomal circulating MicroRNAs in cattle', *PLoS ONE,* 10(8): e0136475. https://doi.org/10.1371/journal.pone.0136475

Musumeci, G. (2015) 'Effects of exercise on physical limitations and fatigue in rheumatic diseases', *World Journal of Orthopedics,* 6(10), pp.762-769.

Myers, J., Prakash, M., Froelicher, V., Do, D., Partington, S. and Atwood, J. E. (2002) 'Exercise capacity and mortality among men referred for exercise testing', *New England Journal of Medicine,* 346(11), pp.793-801.

Mylona, E., Jones, K. A., Mills, S. T. and Pavlath, G. K. (2006) 'CD44 regulates myoblast migration and differentiation', *Journal of Cellular Physiology,* 209(2), pp.314-321.

Nakano, T., Tominaga, R., Nagano, I., Okabe, H., and Yasui, H. (2000) 'Pulsatile flow enhances endothelium-derived nitric oxide release in the peripheral vasculature', *The American Journal of Physiology—Heart and Circulatory Physiology,* 278(4), pp. H1098–H1104.

Narici, M. V., Reeves, N. D., Morse, C. I. and Maganaris, C. N. (2004) 'Muscular adaptations to resistance exercise in the elderly'. *J Musculoskelet. Neuronal Interact*., 4(2): pp.161-164.

Narici, M.V. and Maganaris, C.N.,( 2006) 'Adaptability of elderly human muscles and tendons to increased loading'. *Journal of Anatomy*, 208(4), pp.433-443.

Natale, V. M., Brenner, I. K., Moldoveanu, A. I., Vasiliou, P., Shek, P. and Shephard, R. J. (2003) 'Effects of three different types of exercise on blood leukocyte count during and following exercise', *Sao Paulo Medical Journal,* 121(1), pp.9-14.

Neely, G., Ljunggren, G., Sylven, C. and Borg, G. (1992) 'Comparison between the Visual Analogue Scale (VAS) and the Category Ratio Scale (CR-10) for the evaluation of leg exertion', *International Journal of Sports Medicine,* 13(02), pp.133-136.

Nehlsen-Cannarella, S.L., (1998) 'Cellular responses to moderate and heavy exercise'. *Canadian Journal of Physiology and Pharmacology,* 76(5), pp.485-489.

Nehlsen-Cannarella, S., Fagoaga, O., Nieman, D., Henson, D., Butterworth, D., Schmitt, R., Bailey, E., Warren, B., Utter, A. and Davis, J. (1997) 'Carbohydrate and the cytokine response to 2.5 h of running', *Journal of Applied Physiology,* 82(5), pp.1662-1667.

Ness, A. R., Leary, S. D., Mattocks, C., Blair, S. N., Reilly, J. J., Wells, J., Ingle, S., Tilling, K., Smith, G. D. and Riddoch, C. (2007) 'Objectively measured physical activity and fat mass in a large cohort of children', *PLoS Med,* 4(3), e97. https://doi.org/10.1371/journal.pmed.0040097

Newham, D., Mills, K., Quigley, B. and Edwards, R. (1983) 'Pain and fatigue after concentric and eccentric muscle contractions', *Clinical Science (Lond.),* 64(1), pp.55- 62.

Nicklas BJ, Hsu FC, Brinkley T.J. Church, T., Goodpaster, B.H., Kritchevsky, S.B., and Pahor, M. (2008) 'Exercise training and plasma C-reactive protein and interleukin-6 in elderly people'. *J Am Geriatr Soc*, 56(11), pp.2045–2052.

Nielsen, A. R., Mounier, R., Plomgaard, P., Mortensen, O. H., Penkowa, M., Speerschneider, T., Pilegaard, H. and Pedersen, B. K. (2007) 'Expression of interleukin-15 in human skeletal muscle–effect of exercise and muscle fibre type composition', *The Journal of Physiology,* 584(1), pp.305-312.

Nielsen, S., Scheele, C., Yfanti, C., Åkerström, T., Nielsen, A. R., Pedersen, B. K. and Laye, M. (2010) 'Muscle specific microRNAs are regulated by endurance exercise in human skeletal muscle', *The Journal of Physiology,* 588(20), pp.4029-4037.

Nieman, D. C. and Pedersen, B. K. (1999) 'Exercise and immune function', *Sports Medicine,* 27(2), pp.73-80.

Nishikawa, K. (2016) 'Eccentric contraction: unraveling mechanisms of force enhancement and energy conservation', *Journal of Experimental Biology*, 219(2), pp.189-196.

Noble, B. J. and Robertson, R. J. (1996) '*Perceived Exertion'.* Illinois, USA: Human Kinetics.

Nolte, E., Cremer, T., Gallo, R. C. and Margolis, L. B. (2016) 'Extracellular vesicles and viruses: Are they close relatives?', *Proceedings of the National Academy of Sciences*, 113(33): 9155–9161.

Oh, S., Tanaka, K., Warabi, E. and Shoda, J. (2013) 'Exercise reduces inflammation and oxidative stress in obesity-related liver diseases', *Medicine and Science in Sports and Exercise,* 45(12), pp.2214-2222.

Ohno, S.-i., Ishikawa, A. and Kuroda, M. (2013) 'Roles of exosomes and microvesicles in disease pathogenesis', *Advanced Drug Delivery Reviews,* 65(3), 398-401.

Ostrovidov, S., Hosseini, V., Ahadian, S., Fujie, T., Parthiban, S.P., Ramalingam, M., Bae, H., Kaji, H. and Khademhosseini, A., (2014) 'Skeletal muscle tissue engineering: methods to form skeletal myotubes and their applications'. *Tissue Engineering Part B: Reviews*, 20(5), pp.403-436.

Ostrowski, K., Rohde, T., Asp, S., Schjerling, P. and Pedersen, B. K. (1999) 'Pro-and anti-inflammatory cytokine balance in strenuous exercise in humans', *The Journal of Physiology,* 515(1), pp.287-291.

Ouchi, N., Oshima, Y., Ohashi, K., Higuchi, A., Ikegami, C., Izumiya, Y. and Walsh, K. (2008) 'Follistatin-like 1, a secreted muscle protein, promotes endothelial cell function and revascularization in ischemic tissue through a nitric-oxide synthase-dependent mechanism', *Journal of Biological Chemistry,* 283(47), pp.32802-32811.

Pan, B-T., and Johnstone, R. M. (1983) 'Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor'. *Cell,* 33(3):967-78.

Park, S.-J., Ahmad, F., Philp, A., Baar, K., Williams, T., Luo, H., Ke, H., Rehmann, H., Taussig, R. and Brown, A. L. (2012) 'Resveratrol ameliorates aging-related metabolic phenotypes by inhibiting cAMP phosphodiesterases', *Cell,* 148(3), pp.421-433.

Pedersen, B. K. (2009) 'Edward F. Adolph distinguished lecture: muscle as an endocrine organ: IL-6 and other myokines', *Journal of Applied Physiology,* 107(4), pp.1006-1014.

Pedersen, B. K. and Febbraio, M. A. (2008) 'Muscle as an endocrine organ: focus on muscle-derived interleukin-6', *Physiological Reviews,* 88(4), pp.1379-1406.

Pedersen, B. K. and Hoffman-Goetz, L. (2000) 'Exercise and the immune system: regulation, integration, and adaptation', *Physiological Reviews,* 80(3), pp.1055-1081.

Pedersen, B. K., Ostrowski, K., Rohde, T. and Bruunsgaard, H. (1998) 'The cytokine response to strenuous exercise', *Canadian journal of Physiology and Pharmacology,* 76(5), pp.505-511.

Pedersen, B. K., Steensberg, A., & Schjerling, P. (2001) Muscle-derived interleukin-6: possible biological effects. *The Journal of Physiology*, 536(Pt 2), pp 329–337.

Pedersen, B. K. and Toft, A. D. (2000) 'Effect of Exercise on Lymphocytes and Cytokines'. *British Journal of Sports Medicine,* 34, pp 246-251.

Pegtel, D. M., van de Garde, M. D. and Middeldorp, J. M. (2011) 'Viral miRNAs exploiting the endosomal–exosomal pathway for intercellular cross-talk and immune evasion', *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms,* 1809(11), pp.715-721.

Petersen, A. M. W. and Pedersen, B. K. (2005) 'The anti-inflammatory effect of exercise', *Journal of Applied Physiology,* 98(4), pp.1154-1162.

Petersen, E. W., Ostrowski, K., Ibfelt, T., Richelle, M., Offord, E., Halkjær-Kristensen, J. and Pedersen, B. K. (2001) 'Effect of vitamin supplementation on cytokine response and on muscle damage after strenuous exercise', *American Journal of Physiology-Cell Physiology,* 280(6), pp.C1570-C1575.

Petiot, A., Strappazzon, F., Chatellard-Causse, C., Blot, B., Torch, S., Verna, J.M., and Sadoul. R. (2008) 'Alix differs from ESCRT proteins in the control of autophagy', Biochem Biophys Res Commun., 375(1), pp.63-8.

Pette, D. (1998) 'Training effects on the contractile apparatus', *Acta Physiologica Scandinavica,* 162(3), pp.367-376.

Pérez de Heredia, F.; Wood, I.S. (210) Trayhurn, P. Hypoxia stimulates lactate release and modulates monocarboxylate transporter (MCT1, MCT2, and MCT4) expression in human adipocytes. *Pflugers Arch,* 459, pp. 509–518

Picotte, M., Campbell, C. G. and Thorland, W. G. (2009) 'Day-to-day variation in plasma interleukin-6 concentrations in older adults', *Cytokine,* 47(3), pp.162-165.

Ploeger, H. E., Takken, T., De Greef, M. and Timmons, B. W. (2009) 'The effects of acute and chronic exercise on inflammatory markers in children and adults with a chronic inflammatory disease: a systematic review', *Exerc Immunol Rev,* 15(1), pp.6-41.

Pollock, M. L., Franklin, B. A., Balady, G. J., Chaitman, B. L., Fleg, J. L., Fletcher, B., Limacher, M., Piña, I. L., Stein, R. A. and Williams, M. (2000) 'Resistance exercise in individuals with and without cardiovascular disease benefits, rationale, safety, and prescription an advisory from the committee on exercise, rehabilitation, and prevention, council on clinical cardiology, American Heart Association', *Circulation,* 101(7), pp.828-833.

Pols, M. S. and Klumperman, J. (2009) 'Trafficking and function of the tetraspanin CD63', Exp Cell Res., 315(9), pp.1584-92.

Powers, S. K. and Howley, E. T. (2004) *Exercise physiology: Theory and application to fitness and performance,* New York: McGraw-Hill.

Properzi, F., Logozzi, M. and Fais, S. (2013) 'Exosomes: the future of biomarkers in medicine'. *Biomark Med,* 7(5), pp.769-78. doi: 10.2217/bmm.13.63.

Pucci, F., Garris, C., Lai, C. P., Newton, A., Pfirschke, C., Engblom, C., ... Pittet, M. J. (2016) SCS macrophages suppress melanoma by restricting tumor-derived vesicle-B cell interactions. *Science,* 352, pp.242–246.

Pusic, A. D., Pusic, K. M. and Kraig, R. P. (2014) 'What are exosomes and how can they be used in multiple sclerosis therapy?', *Expert Review of Neurotherapeutics,* 14(4), pp.353-355.

Rabinowits, G., Gerçel-Taylor, C., Day, J. M., Taylor, D. D. and Kloecker, G. H. (2009) 'Exosomal microRNA: a diagnostic marker for lung cancer', *Clinical Lung Cancer,* 10(1), pp.42-46.

Radák, Z. (2005) *Exercise and Diseases: Prevention Through Training,* Meyer & Meyer Verlag.

Rall, L. C., Roubenoff, R., Cannon, J. G., Abad, L. W., Dinarello, C. A., and Meydani, S. N., (1996) 'Effects of progressive resistance training on immune response in aging and chronic inflammation'. *Med Sci Sports Exerc*, 28(11), pp. 1356–1365.

Rani, S. (2014) 'MicroRNA profiling of exosomes isolated from biofluids and conditioned media', *Methods in Molecular Biology*, 1182, pp.131-144.

Raposo, G. and Stoorvogel, W. (2013) 'Extracellular vesicles: exosomes, microvesicles, and friends', *The Journal of Cell Biology,* 200(4), pp.373-383.

Ren, Y., Yang, J., Xie, R., Gao, L., Yang, Y., Fan, H. and Qian, K. (2011) 'Exosomal-like vesicles with immune-modulatory features are present in human plasma and can induce CD4+ T-cell apoptosis in vitro', *Transfusion,* 51(5), pp.1002-1011.

Riddoch, C. J., Mattocks, C., Deere, K., Saunders, J., Kirkby, J., Tilling, K., Leary, S. D., Blair, S. N. and Ness, A. R. (2007) 'Objective measurement of levels and patterns of physical activity', *Archives of Disease in Childhood,* 92(11), pp.963-969.

Rimmer, J.H., Schiller, W. and Chen, M.D. (2012) 'Effects of disability-associated low energy expenditure deconditioning syndrome. *Exercise and Sport Sciences Reviews*, 40(1), pp.22-29.

Rippe, J. M. (2011) *Encyclopedia of lifestyle medicine and health.* New York, USA: Sage Publications.

Robbins, P.D. and Morelli, A.E., (2014) 'Regulation of immune responses by extracellular vesicles'. *Nature Reviews Immunology,* 14(3), pp.195-208.

Roberts, T. J. (2016) 'Contribution of elastic tissues to the mechanics and energetics of muscle function during movement', *Journal of Experimental Biology,* 219(2), pp.266-275.

Romain, A., Carayol, M., Desplan, M., Fedou, C., Ninot, G., Mercier, J., Avignon, A. and Brun, J. (2012) 'Physical activity targeted at maximal lipid oxidation: a meta-analysis', *Journal of Nutrition and Metabolism, Article ID 285395, https://doi.org/10.1155/2012/285395.*

Ross, M. (1999) 'Delayed-onset muscle soreness: work out now, pay later?' *The Physician and Sportsmedicine*, 27(1), pp.107-108.

Rubinstein, E. (2011) 'The complexity of tetraspanins', *Biochemical Society Transactions,* 39(2), pp.501-505.

Sadallah, S., Eken, C. and Schifferli, J. (2011) 'Ectosomes as modulators of inflammation and immunity', *Clinical & Experimental Immunology,* 163(1), pp.26-32.

Safdar, A., Abadi, A., Akhtar, M., Hettinga, B. P. and Tarnopolsky, M. A. (2009) 'miRNA in the regulation of skeletal muscle adaptation to acute endurance exercise in C57Bl/6J male mice', *PloS ONE,* 4(5), https://doi.org/10.1371/journal.pone.0005610

Salamon, M., Millino, C., Raffaello, A., Mongillo, M., Sandri, C., Bean, C., Negrisolo, E., Pallavicini, A., Valle, G. and Zaccolo, M. (2003) 'Human MYO18B, a novel unconventional myosin heavy chain expressed in striated muscles moves into the myonuclei upon differentiation', *Journal of Molecular Biology,* 326(1), pp.137-149.

Sanger, J. W., Chowrashi, P., Shaner, N. C., Spalthoff, S., Wang, J., Freeman, N. L. and Sanger, J. M. (2002) 'Myofibrillogenesis in skeletal muscle cells', *Clinical Orthopaedics and Related Research*, (403 Suppl.), pp.S153-S162.

Sasaki, T. and Takai, Y. (1998) 'The Rho small G protein family-Rho GDI system as a temporal and spatial determinant for cytoskeletal control', *Biochemical and Biophysical Research Communications,* 245(3), pp.641-645.

Schäfer, M. and Stein, C. (2003) '*Mind Over Matter-Regulation of Peripheral Inflammation by the CNS'.* Berlin, Germany: Springer Science & Business Media.

Scheller, J., Chalaris, A., Schmidt-Arras, D. and Rose-John, S. (2011) 'The pro-and anti-inflammatory properties of the cytokine interleukin-6', *Biochimica et Biophysica Acta (BBA)- Molecular Cell Research,* 1813(5), pp.878-888.

Schöneich, C., Dremina, E., Galeva, N. and Sharov, V. (2014) 'Apoptosis in differentiating C2C12 muscle cells selectively targets Bcl-2-deficient myotubes', *Apoptosis,* 19(1), pp.42-57.

Schorey, J. S. and Bhatnagar, S. (2008) 'Exosome function: from tumor immunology to pathogen biology', *Traffic,* 9(6), pp.871-881.

Schwander, M., Leu, M., Stumm, M., Dorchies, O. M., Ruegg, U. T., Schittny, J. and Müller, U. (2003) 'β1 integrins regulate myoblast fusion and sarcomere assembly', *Developmental Cell,* 4(5), pp.673-685.

Scott, J. P., Sale, C., Greeves, J. P., Casey, A., Dutton, J. and Fraser, W. D. (2013) 'Cytokine response to acute running in recreationally-active and endurance-trained men', *European Journal of Applied Physiology,* 113(7), pp.1871-1882.

Stahl, P. Introductory Course lecture: 'Basics of Extracellular Vesicles'. University of California, Irvine. https://www.coursera.org/lecture/extracellular-vesicles/history-of-exosomes-and-extracellular-vesicles-kC13C.

Starbuck, C., Eston R.G. (2011) Exercise-induced muscle damage and the repeated bout effect: evidence for cross transfer. *Eur J Appl Physiol*, 112(3), pp.1005-13.

Straczkowski, M., Kowalska, I., Dzienis-Straczkowska. S., Stepién, A., Skibińska, E., Szelachowska, M., and Kinalska, I. (2001) 'Changes in tumor necrosis factor-alpha system and insulin sensitivity during an exercise training program in obese women with normal and impaired glucose tolerance'. *Eur J Endocrinol*, 145(3), pp. 273–280.

Sharma, H. S., Cervos-Navarro, J. and Dey, P. K. (1991) 'Increased blood-brain barrier permeability following acute short-term swimming exercise in conscious normotensive young rats', *Neuroscience Research,* 10(3), pp.211-221.

Shimojo, N., Naka, K., Uenoyama, H., Hamamoto, K., Yoshioka, K. and Okuda, K. (1993) 'Electrochemical assay system with single-use electrode strip for measuring lactate in whole blood', *Clinical Chemistry,* 39(11), pp.2312-2314.

Shrout, P. E. and Fleiss, J. L. (1979) 'Intraclass correlations: uses in assessing rater consistency', *Psychol Bull,* 86(2), pp.420-8.

Silbermann, M., Finkelbrand, S., Weiss, A., Gershon, D. and Reznick, A. (1983) 'Morphometric analysis of aging skeletal muscle following endurance training', *Muscle & Nerve,* 6(2), pp.136-142.

Sivananthan, S., Sherry, E., Warnke, P. and Miller, M. D. (2012) '*Mercer's Textbook of Orthopaedics and Trauma Tenth edition'.* Boca Raton, USA: CRC Press.

Skog, J., Würdinger, T., van Rijn, S., Meijer, D. H., Gainche, L., Curry, W. T., Carter, B. S., Krichevsky, A. M. and Breakefield, X. O. (2008) 'Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers', *Nature Cell Biology,* 10(12), pp.1470-1476.

Smith, J., Dykes, R., Douglas, J.E., Krishnaswamy, G., Berk, S. (1999) 'Long-term exercise and atherogenic activity of blood mononuclear cells in persons at risk of developing ischemic heart disease'. *JAMA*, 281(18), pp. 1722–1727.

So, B., Kim, H-J., Kim, J. and Song, W. (2014) 'Exercise-induced myokines in health and metabolic diseases. *Integr Med Res.,* 3(4), pp.172–179.

Sossdorf, M., Otto, G.P., Claus, R.A., Gabriel, H.H., and Losche, W. (2011) 'Cell-derived microparticles promote coagulation after moderate exercise'. *Med Sci Sports Exerc*, 43, pp.1169–1176.

SPSS "Display. Available at: Statistics"https://www.ibm.com/support/knowledgecenter/en/SS3RA7_15.0.0/com.ibm.spss.modeler.help/dataaudit_displaystatistics.htm (accessed 22nd September 2018)

Stamatoyannopoulos, J. A., Snyder, M., Hardison, R., Ren, B., Gingeras, T., Gilbert, D. M., Groudine, M., Bender, M., Kaul, R. and Canfield, T. (2012) 'An encyclopedia of mouse DNA elements (Mouse ENCODE)', *Genome Biology,* 13(8), pp.1.

Steensberg, A., van Hall, G., Osada, T., Sacchetti, M., Saltin, B., and Klarlund Pedersen, B. (2000) 'Production of interleukin-6 in contracting human skeletal muscles can account for the exercise-induced increase in plasma interleukin-6.' *J. Physiol.* 529(Pt 1), pp.237–242.

Stoorvogel, W. (2012) 'Functional transfer of microRNA by exosomes', *Blood,* 119(3), pp.646-648.

Suzuki, K., Nakaji, S., Yamada, M., Totsuka, M., Sato, K. and Sugawara, K. (2002) 'Systemic inflammatory response to exhaustive exercise: cytokine kinetics', *Exerc Immunol Rev,* 8(6), pp.6-48.

Taaffe, D.R., Harris, T.B., Ferrucci, L., Rowe, J., Seeman, T.E. (2000) Cross-sectional and prospective relationships of interleukin-6 and C-reactive protein with physical performance in elderly persons: MacArthur studies of successful aging. *J Gerontol A Biol Sci Med Sci*, 55(12), pp.M709-15.

Tachibana, I. and Hemler, M. E. (1999) 'Role of transmembrane 4 superfamily (TM4SF) proteins CD9 and CD81 in muscle cell fusion and myotube maintenance', *The Journal of Cell Biology,* 146(4), pp.893-904.

Takano, H., Komuro, I., Oka, T., Shiojima, I., Hiroi, Y., Mizuno, T. and Yazaki, Y. (1998) 'The Rho family G proteins play a critical role in muscle differentiation', *Molecular and Cellular Biology,* 18(3), pp.1580-1589.

Tatemoto, K., Hosoya, M., Habata, Y., Fujii, R., Kakegawa, T., Zou, M.X., Kawamata, Y., Fukusumi, S., Hinuma, S., Kitada, C., et al. (1998) 'Isolation and characterization of a novel

endogenous peptide ligand for the human APJ receptor.' *Biochem. Biophys. Res. Commun.*, 251, pp.471–476.

Tauro, B. J., Greening, D. W., Mathias, R. A., Ji, H., Mathivanan, S., Scott, A. M. and Simpson, R. J. (2012) 'Comparison of ultracentrifugation, density gradient separation, and immunoaffinity capture methods for isolating human colon cancer cell line LIM1863-derived exosomes', *Methods,* 56(2), pp.293-304.

Tavaria, M., Gabriele, T., Anderson, R. L., Mirault, M.-E., Baker, E., Sutherland, G. and Kola, I. (1995) 'Localization of the gene encoding the human heat shock cognate protein, HSP73, to chromosome 11', *Genomics,* 29(1), pp.266-268.

Tavoosidana, G., Ronquist, G., Darmanis, S., Yan, J., Carlsson, L., Wu, D., Conze, T., Ek, P., Semjonow, A., Eltze, E., Larsson, A., Landegren, U. D., & Kamali-Moghaddam, M. (2011) Multiple recognition assay reveals prostasomes as promising plasma biomarkers for prostate cancer. *Proceedings of the National Academy of Sciences of the United States of America,* 108(21), pp.8809–8814.

Taylor, D. D. and Gercel-Taylor, C. (2008) 'MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer', *Gynecologic Oncology,* 110(1), pp.13-21.

Taylor, D. D. and Shah, S. (2015) 'Methods of isolating extracellular vesicles impact down- stream analyses of their cargoes', *Methods,* 87, pp.3-10.

Testoni, D., Hornik, C. P., Smith, P. B., Benjamin Jr, D. K. and McKinney Jr, R. E. (2013) 'Sports medicine and ethics', *The American Journal of Bioethics,* 13(10), pp.4-12.

Théry, C. (2011) 'Exosomes: secreted vesicles and intercellular communications', *F1000 Biol Rep,* 3(15), pp.130.

Théry, C., Amigorena, S., Raposo, G. and Clayton, A. (2006) 'Isolation and characterization of exosomes from cell culture supernatants and biological fluids', *Current Protocols in Cell Biology*, Chapter 3, Unit 3.22. doi: 10.1002/0471143030.cb0322s30.

Théry, C., Regnault, A., Garin, J., Wolfers, J., Zitvogel, L., Ricciardi-Castagnoli, P., Raposo, G., & Amigorena, S. (1999) Molecular characterization of dendritic cell-derived exosomes. Selective accumulation of the heat shock protein hsc73. *The Journal of cell biology*, 147(3), pp.599–610.

Théry, C., Zitvogel, L. and Amigorena, S. (2002) 'Exosomes: composition, biogenesis and function', *Nature Reviews Immunology,* 2(8), pp.569-579.

Thomou, T., Mori, M. A., Dreyfuss, J. M., Konishi, M., Sakaguchi, M.,Wolfrum, C., Kahn, C. R. (2017) Adipose-derived circulating miRNAs regulate gene expression in other tissues. *Nature,* 542, pp.450–455.

Tiernan, A. (2008) 'Mechanisms linking physical activity with cancer'. *Nat Rev Cancer,* 8(3), pp. 205-11.

Toebe, M., Machado, L.N., Tartaglia, F.L., Carvalho, J.O., Bandeira, C.T., Cargnelutti, Filho A. (2018) Sample size for estimating mean and coefficient of variation in species of crotalarias. *An Acad Bras Cienc,* 90(2).

Tolfrey, K., Jeukendrup, A. E. and Batterham, A. M. (2010) 'Group-and individual-level coincidence of the 'Fatmax'and lactate accumulation in adolescents', *European Journal of Applied Physiology,* 109(6), pp.1145-1153.

Tskhovrebova, L. and Trinick, J. (2003) 'Titin: properties and family relationships', *Nature Reviews Molecular Cell Biology,* 4(9), pp.679-689.

Tsukui, S, Kanda, T., Nara, M., Nishino, M., Kondo, T., and Kobayashi, I. (2000) 'Moderate-intensity regular exercise decreases serum tumor necrosis factor-a and HbA1c levels in healthy women'. *Int J Obes,* 24(9), pp. 1207–1211.

Turner, N.J. and Badylak, S.F., (2012) 'Regeneration of skeletal muscle'. *Cell and Tissue Research,* 347(3), pp.759-774.

Ündar, L., Ertugùrul, C., Altunbas, H. and Akça, S. (1999) 'Circadian variations in natural coagulation inhibitors protein C, protein S and antithrombin in healthy men: a possible association with interleukin-6', *Thrombosis and Haemostasis,* 81(4), pp.571-575.

Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J. J. and Lötvall, J. O. (2007) 'Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells', *Nature Cell Biology*, 9(6), pp.654-659.

van Niel, G., Porto-Carreiro, I., Simoes, S. and Raposo, G. (2006) 'Exosomes: a common pathway for a specialized function', *Journal of Biochemistry,* 140(1), pp.13-21.

Van Praag, H., Kempermann, G. and Gage, F. H. (1999) 'Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus', *Nature Neuroscience,* 2(3), pp.266-270.

van Rooij, E., Quiat, D., Johnson, B. A., Sutherland, L. B., Qi, X., Richardson, J. A., Kelm, R. and Olson, E. N. (2009) 'A family of microRNAs encoded by myosin genes governs myosin expression and muscle performance', *Developmental Cell,* 17(5), pp.662-673.

Vieira, S. S., Lemes, B., Silva Jr, J.A., Bocalini, D. S., Suzuki, F.S., Albertini, R., Caetano, A.S., de Carvalho, P.D.T.C., Arsa, G. and Serra, A.J. (2013) 'Different land-based exercise training programs to improve bone health in postmenopausal women'. *Med Sci Tech*, 54, pp.158-63.

Vlassov, A. V., Magdaleno, S., Setterquist, R. and Conrad, R. (2012) 'Exosomes: current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials', *Biochimica et Biophysica Acta (BBA)-General Subjects,* 1820(7), pp.940-948.

Wahl, P., Jansen, F., Achtzehn, S., Schmitz, T., Bloch, W., Mester, J., and Werner, N. (2014) 'Effects of high intensity training and high volume training on endothelial microparticles and angiogenic growth factors'. *PLoS ONE*, 9(4), pp. e96024.

Walsh, N.P., Gleeson, M., Rogers, C.J., Northoff, H., Abbasi, A., Simon, P., Shephard, R.J., Gleeson, M., Woods, J.A., Bishop, N.C. and Fleshner, M. (2011) *P'osition statement part one: immune function and exercise*. NOVA: The University of Newcastle's Digital Repository.

Warburton, D.E., Nicol, C.W. and Bredin, S.S., (2006) 'Health benefits of physical activity: the evidence'. *Canadian Medical Association Journal*,174(6), pp.801-809.

Westerterp K. R. (2013) 'Physical activity and physical activity induced energy expenditure in humans: measurement, determinants, and effects', *Frontiers in physiology*, 4(90)

Wernig, A., Irintchev, A. and Weisshaupt, P. (1990) 'Muscle injury, cross-sectional area and fibre type distribution in mouse soleus after intermittent wheel-running', *The Journal of Physiology,* pp.428, 639.

Whitham, M., Febbraio, M. (2016) 'The ever-expanding myokinome: discovery challenges and therapeutic implications', Nat Rev Drug Discov., 15, pp.719–729.

Wikimedia Commons (2016) *File:1008 Skeletal Muscle Contraction.jpg.* Available at: https://commons.wikimedia.org/wiki/File:1008_Skeletal_Muscle_Contraction.jpg (Accessed 20th August 2018)

Williamson, J., Fadel, P. and Mitchell, J. (2006) 'New insights into central cardiovascular control during exercise in humans: a central command update', *Experimental Physiology,* 91(1), pp.51-58.

Wolfe, R. R. (2006) 'The underappreciated role of muscle in health and disease', *The American Journal of Clinical Nutrition,* 84(3), pp.475-482.

World Health Organisation (2010) Global recommendations on physical activity for health. [online]. Available at: http://apps.who.int/iris/bitstream/10665/44399/1/9789241599979_eng.pdf. (Accessed: 10th September 2018).

World Health Organisation (2016) *Global Strategy on Diet, Physical Activity and Health*. [online]. Available at: http://www.who.int/dietphysicalactivity/pa/en/. (Accessed: 15th September 2018).

Yaffe, K., Barnes, D., Nevitt, M., Lui, L.-Y. and Covinsky, K. (2001) 'A prospective study of physical activity and cognitive decline in elderly women: women who walk', *Archives of Internal Medicine,* 161(14), pp.1703-1708.

Yamashita, T., Takahashi, Y., Nishikawa, M., and Takakura, Y. (2016) 'Effect of Exosome isolation methods on physicochemical properties of exosomes and clearance of exosomes from the blood circulation'. *Eur. J. Pharm. Biopharm,* 98, pp. 1-8.

Yancik, R. (2005) 'Population aging and cancer: a cross-national concern. Cancer J., 11(6), pp.437-41.

Yáñez-Mó, M., Siljander, P. R.-M., Andreu, Z., Zavec, A. B., Borràs, F. E., Buzas, E. I., Buzas, K., Casal, E., Cappello, F. and Carvalho, J. et al. (2015) 'Biological properties of

extracellular vesicles and their physiological functions', *Journal of Extracellular Vesicles,* 4:27066. doi: 10.3402/jev.v4.27066.

Yoshiko, Y., Hirao, K. and Maeda, N. (2002) 'Differentiation in C2C12 myoblasts depends on the expression of endogenous IGFs and not serum depletion', *American Journal of Physiology-Cell Physiology,* 283(4), pp.C1278-C1286.

Young, J. A., Symons, C. M., Pain, M. D., Harvey, J. T., Eime, R. M., Craike, M. J. and Payne, W. R. (2015) 'Role models of Australian female adolescents A longitudinal study to inform programmes designed to increase physical activity and sport participation', *European Physical Education Review,* 21(4), pp.451-466.

Yin, H., Price, F. and Rudnicki, M.A., (2013) 'Satellite cells and the muscle stem cell niche'. *Physiological Reviews,* 93(1), pp.23-67.

Zarà, M., Guidetti, G. F., Camera, M., Canobbio, I., Amadio, P., Torti, M., Tremoli, E., & Barbieri, S. S. (2019) 'Biology and Role of Extracellular Vesicles (EVs) in the Pathogenesis of Thrombosis', International journal of molecular sciences, 20(11), pp.2840.

Zhang, J.M. and An, J. (2007) 'Cytokines, inflammation and pain', *International Anesthesiology Clinics,* 45(2), pp.27.

Zhang, T., Hu, L., Ma, H., Ni, F., Liu, F., and Chen, H. (2020) 'Detection of Tear Components Using Matrix-Assisted Laser Desorption Ionization/Time-of-Flight Mass Spectrometry for Rapid Dry Eye Diagnosis', J Proteome Res., 19(9), pp.3644-3651.

Zhang, Y., Yu, M. and Tian, W. (2016) 'Physiological and pathological impact of exosomes of adipose tissue', *Cell Proliferation,* 49(1), pp.3-13.

Zhou, X., Fragala, M. S., McElhaney, J. E. and Kuchel, G. A. (2010) 'Conceptual and methodological issues relevant to cytokine and inflammatory marker measurements in clinical research', *Current Opinion in Clinical Nutrition and Metabolic Care,* 13(5), pp.541.

.

# Appendices

## Appendix A: Ethical Approval

Professor Helen Dawes
Director of Studies
Department of Sport and Health Sciences
Faculty of Health and Life Sciences
Oxford Brookes University
Gipsy Lane
Headington

12 March 2013

Dear Professor Dawes

**UREC Registration No: 130712**
**The influence of strenuous exercise on the inflammatory process in healthy participants**

Thank you for submitting the application to the University Research Ethics Committee on behalf of your research student Eman Abu-Seer. The Committee reviewed the application at its meeting on the 27 February 2013, and have agreed approval subject to meeting the following conditions:

1. It is unclear why the SF-36 and PASE questionnaires are repeated on days 1 & 2 of testing. Please would you explain the rationale for this?

The validated questionnaires on Day 1 are:
General health status questionnaire (SF-36)
Physical activity level questionnaire (PASE ), this was adapted and validated for use in young adults and this questionnaire is appropriate and valid for Multiple Sclerosis (MS) which i will conduct the same study on them after healthy participants.
Fatigue severity scale questionnaire (FSS).

Day 2
Fitness test questionnaire (CRF)
Visual Analogue Scale (VAS), to measure changes in muscle soreness which caused by strenuous exercise.
Vitality Scale ( Individual Difference Level Version ,State Level Version ), this scale is considered as an aspect of eudemonic well-being, as being vital and energetic is part of what it means to be fully functioning and psychologically well. (Ryan & Deci, 2001)
So SF-36 and PASE on Day 1 only and are not repeated on Day2.

2. It is proposed that participants would be drawn only from the movement science group (2.2 and 2.3). However, reference is later made to the potential for dependent relationships between researchers and students (2.5). Please clarify exactly who would be invited to take part, how and the steps that would be taken to avoid any unintentional coercion of peers / colleagues. It is inappropriate to approach individuals directly as it is difficult to decline to be involved if invited by word of mouth – so a less direct method should be used.

Participants will be recruited through students or staff contacts and word of mouth. Individuals who show interest will be given a brief explanation of the study, either orally or by email depending on how they make initial contact. If potential participants are interested in getting involved, a participant's information sheet will be sent to consider their decision by returning a reply slip wishing to participate in the study after given of at least 24 hours to think and decide to take part in the study. Furthermore, potential volunteers of the study will

be explicitly told that participation in the study is completely voluntary and participation or withdrawal from the study will not influence their work or academic progress. All data will be de-identified and only the researchers will have access to this.

3. With reference to the inclusion criteria for the study, it would seem sensible to exclude those who are known to be anaemic, given the volume of blood required. Please would you also explain when and by whom a medical history would be taken and how 'mental health problems' would be assessed as an exclusion criterion? It is also not clear whether those who score high on the Fatigue Severity Scale would be excluded from taking part in the study. Please would you clarify this point?

We will recruit 15 healthy controls who are between 20-60 years old. With no pathology known to influence immune response ( e.g. Auto immune diseases, anaemia, heart diseases, orthopaedic limitations or renal failure disease) by using the Physical Activity Readiness-Questionnaire PAR-Q, pre-screening health questionnaire and medical history to determine the safety or possible risk of exercising for an individual based upon their answers to specific health history questions. We will exclude participants with a mental health problems by asking them (Are you been treated for any mental health problem before?). Furthermore, participants who show high score on the Fatigue Severity Scale will be excluded.

4. Please justify the need to collect 9 ml blood on each occasion. This seems excessive to measure cytokine and exosome concentrations in plasma, particularly as this analysis is confined to exosome variation only on day 1.

   Day 1 is a monitoring day of natural variation of exosomes during a day. Firstly, a blood sample (6mls need to be collected as after remove all cells we end up with approxiamtely 2,5 mls of plasma ), In which we will use that amount of plasma for exosomes analysis.

5. The burden on research participants is high i.e. the study is time consuming and requires frequent blood samples totalling approximately 200 ml. Given this, it would seem necessary to offer an incentive / reward. Failing to do so may adversely affect recruitment.

   Participants in this study will receive a £10 book voucher at the end of the study, also they will be offered lunch and refreshments during the study.

6. Please would you clarify how participants would be occupied during the 10-hour experiment? Would they receive lunch and refreshments and, if so, who will provide this? Yes they will receive lunch and refreshments.

7. Please clarify whether resuscitation equipment is available in the lab where the exercise will take place.

   However, resuscitation equipment will be available in the lab where the exercise will take place. In addition, participants will be screened before starting exercise experiment and they will be made aware that they are free to withdraw from the study or stop any activity at any point without giving a reason. Moreover, participants will be advised not to donate blood for at least 7 days from the end of this study.

8. It is unclear why a questionnaire entitled 'Physical activity *in the elderly*' is being used for data collection in a population aged 20-60 years. If the validity of the data collection instrument is questionable, it is unethical to ask participants to complete this. Similarly, the relevance of the visual analogue scale and the vitality scales to the research question is unclear. Please would you clarify to justify their inclusion in the study?

Physical activity level questionnaire (PASE ), this was adapted and validated for use in young adults and this questionnaire is appropriate and valid for Multiple Sclerosis (MS) which i will conduct the same study on them after healthy participants. Also, Visual Analogue Scale (VAS), to measure changes in muscle soreness which caused by strenuous exercise.

9. It is not clear whether the research data would be retained for 20 years (as per E2U) or for 10 years (as stated in the participant information sheet).

Study data will be kept according to MRC guidelines and stored for 20 years.

10. A number of amendments are required to the participant information sheet:

a. Individuals have been invited to take part because they are members of staff / or are students within the Movement Science Group, not because they meet specific eligibility criteria.

You have been invited to participate because you are a healthy member of staff/ or a student with no history of autoimmune disease, heart attack, renal failure, or mental health problems, anaemia and you are over the age of 18 years.

b. It is unclear whether participants are required to fast and abstain from drinking alcohol and exercise prior to taking part (as stated in the PAR-Q). If so, this should be clearly stated in the information sheet.

You will be required to fast and abstain from drinking alcohol and any strenuous exercise prior to taking part in the study.

c. The information appears incorrect regarding the number of questionnaires for completion on day 2.

In the second session (which will be one week after the first session), we will collect a blood sample from your arm vein (9 mls), then you will be asked to fill in 3 questionnaires that should take about 5 mins.

d. Please explain the intended course of action if the researcher is unable to obtain blood.

e. Over the course of the study, 20 blood samples [not 10] would be taken.

If there is any difficulties obtaining blood or if we had two missing blood draw, we would stop at that point, and we will not ask them to return for day 2. If blood obtained at time 0, then is most likely blood can be drawn up until the end of first 6 hours. If fails at the 2 extra venepuncture (at hours 8 and hours10), we will not ask the participant to come back for any more blood draw.

f. It would seem unnecessary to state that a risk of taking part is infection. Proper aseptic precautions during phlebotomy should remove this risk.

Taking blood samples can cause local bruising or fainting in certain individuals. Every precaution will be taken to reduce the chances of these side effects – samples will be taken by a fully qualified individual using a sterile syringe and needle. You will be asked to sit or lie down during the procedure to reduce the chance of fainting.

g. The ‘step up and weight protocol’ should be clearly explained. The information sheet contains no reference to the use of weights.

After that we will ask you to undertake an exercise test (a step up and down test for approximately 15 minutes). During the exercise test, you will ask to perform 20 up and down steps for each leg with a weight of 10Kg for each hand, in which this will be the first set. A 2 mins rest between each set for a total of 5 sets, you will be asked to wear a heart rate monitor. Every two minutes we will ask you how you feel. At this point you will be completed the exercise test for this study.

h. Explain that those who take part would be ineligible to donate blood for at least a week beyond the end of the study.

Moreover, participants will be advised not to donate blood for at least 7 days from the end of this study.

i. As the legal limitations to data confidentiality do apply, please explain that confidentiality can only be protected within the limitations of the law. .
All data collected will be de-identified and confidentiality will be protected within the limitations of the law.

j. The information sheet contains numerous typographic errors requiring correction. This should be checked by the Supervisory team.

11. Consent form – Please clarify the wording of clauses 5 and 6. As currently presented they could be interpreted as contradictory.

I have been informed that all samples collected will be destroyed and not used for further research.
I agree for the anonymised data obtained from samples may be stored and used in other research Y NO

I attach all modified documents to include the E2U, participant information sheets and consent form,

Many thanks

Eman Abu Seer

Hazel Abbott
Chair of the University Research Ethics Committee

cc Dave Carter and Ryan Pink, Supervisory Team
Eman Abu-Seer, Research Student
Dido Green, Research Ethics Officer
Jill Organ, Graduate Office
Louise Wood, UREC Administrator

**UNIVERSITY RESEARCH ETHICS COMMITTEE, FACULTY OF HEALTH AND LIFE SCIENCES**

Headington Campus Gipsy Lane
Oxford OX3 0BP UK

Tel: 01865 482639
heabbott@brookes.ac.uk

Mrs Hazel Abbott
Chair of the University Research Ethics Committee
University Research Ethics Committee
Marston Road Campus
Oxford Brookes University

Dear Mrs Abbott,
**Ethics Amendment UREC 130712; The influence of strenuous exercise on the inflammatory process in healthy participants**

I respectively request an amendment to the original ethical approval application for the above stated study.

Since starting this study, we have found that there may be significant variation in exosomes levels and other inflammatory markers levels in response to strenuous exercise. Whereas exosomes and cytokines levels did not appear to markedly change over a normal day. In order to confirm that these findings, we not due to day- to-day variation.
We would like to add in additional collection of bloods over five consecutive days. Only one blood sample (4ml) will be drawn from the participants in the morning over five consecutive days.
This information is vital to understand the natural day- to- day variation of exosomes secretion and attribute an exercise response. We aim to explore day to day variability within the same person by measuring exosomes levels same time of the day over five consecutive days.

Additionally, I have added the aim of this amendment into Participant Information Sheet (3. What is the purpose of the study?). I also have made reference to the approved study in the consent form. I have clarified the amendment by considering the two different phases of the study 1) inflammatory response to exercise 2) natural variation of exosome ???,
In the event that individuals have already completed Phase 1, recruitment to Phase 2 will follow the same process but previous participants would only undertake Phase 2. They will be asked to sign informed consent (eg. provided with the updated PIS and had time for questions) for the amended protocol.
Changes have been highlighted in yellow.

I hope that this information is sufficient to grant us a minor amendment to the study.

I look forward to hearing from you.
Regards
Eman Abu-Seer

Appendix B: Participant Information Sheet

# Participant Information Sheet

# (Control)

OXFORD BROOKES UNIVERSITY

**Ethics approval number:**

**1. Study Title**

The influence of strenuous activity on the inflammatory process in healthy participants.

**2. Invitation paragraph**

You are being invited to take part in a research study. Before you decide whether to take part it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish.

- This information sheet, tells you the purpose of this study and what will happen to you if you take part.

Please ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

**3. What is the purpose of the study?**

The aim of this study is to understand how the immune system responds to exercise. As several studies have reported that exercise is good for health and the wellbeing, we aims to explore the immune system and mechanism of inflammation by looking at small vesicles in the blood. In order to do this work we need to obtain 20 blood samples over a certain time period which would be in this study approximately 20 hours from healthy volunteers before and after short but strenuous exercise.

**4. Why have I been invited?**
You have been invited to participate because you are a healthy member of staff/ or a student with no history of autoimmune disease, heart attack, renal failure, or mental health problems, anaemia and you are over the age of 18 years.

**5. What will happen to me if I take part?**
If you decide to take part you will be asked to sign a consent form for this study. A suitable time for you will be arranged to come to the movement science lab at Oxford Brookes University. You will be required to fast and abstain from drinking alcohol and any strenuous exercise prior to taking part in the study. Then you will be asked to attend two sessions. In the first session, we will ask you about your medical history, and we will ask you to complete a pre-screening health questionnaire (PAR-Q) to ensure your eligibility for the study. After that, we will collect a blood sample from your arm vein (6 mls) and we will keep the cannula in your arm and stabilize it by a medical tape. Afterwards, we will ask you to complete 3 questionnaires that should take about 10 minutes. Following this, we will collect blood samples from the butterfly cannula every one for the first six hours. After that we will remove the cannula from your arm and we will collect another two blood samples (one sample at hours 8.00 and one sample at hours 10.00) by venepuncture. A total of 60 mls blood will be taken from you over 10 hours.

In the second session (which will be one week after the first session), we will collect a blood sample from your arm vein (9 mls), then you will be asked to fill in 3 questionnaires that should take about 5 mins. After that we will ask you to undertake an exercise test (a step up and down test for approximately 15 minutes). During the exercise test, you will ask to perform 20 up and down steps for each leg with a weight of 10Kg for each hand, in which this will be the first set. A 2 mins rest between each set for a total of 5 sets, you will be asked to wear a heart rate monitor. Every two minutes we will ask you how you feel. After completed the exercise test, we will collect a post exercise blood sample (9 mls) from your arm vein by a butterfly cannula and we will keep it in your arm and stabilize it by a medical tape. We will collect blood samples by drawing blood from the cannula every one hour for the first six hours. After that we will remove the cannula from your arm and we will collect another two blood samples (one sample at hours 8.00 and one sample at hours 10.00) by venepuncture. A total of 100 mls blood will be taken from you over 11 hours.

At this point your involvement in the study will be completed. All data collected will be de-identified and confidentiality will be protected within the limitations of the law. If you are a student or staff, your decision to take part or not will not affect your program of studies or employment, respectively. You are free to withdraw from the study at any point or stop an activity without giving a reason.

**6. What are the side effects of taking part?**
Taking blood samples can cause local bruising or fainting in certain individuals. Every precaution will be taken to reduce the chances of these side effects – samples will be taken by a fully qualified individual using a sterile syringe and needle. You will be asked to sit or lie down during the procedure to reduce the chance of fainting.

**7. What are the other possible disadvantages and risks of taking part?**
Taking part in the exercise may cause you to feel out of breath or some muscle discomfort. However, we will check that you are healthy to participate by asking you to fill in four

questionnaires; we also will take your blood pressure before stating the exercise test. Moreover, there will be qualified individuals with first aid qualification on site at all the times.

**8. What are the possible benefits of taking part?**
There is no direct benefit to you, but we hope this study will improve our understanding of the response of immune system to inflammation to acute/strenuous exercise.

**9. What will happen to the information/results collected in the project?**
All the information about your participation in this study will be kept confidential. All data will be de-identified. All blood samples will be processed to remove cellular tissue within 24 hours in our facility labs at Oxford Brookes University and samples destroyed after processing. Protein samples will not have your name or any personal data attached so that you cannot be identified from the samples or the data produced from it. Only members of this research project will have access to any samples collected. No samples will be transferred outside of the UK and samples will not be used for any future research.
All information that is collected about you during the course of this research will be kept strictly confidential. All the samples will be analysed anonymously. All procedures for handling, processing, storage and destruction of your data are compliant with the Data Protection Act 1998. Furthermore, all data generated by the study must be retained in accordance with the University's policy on Academic Integrity, and that it must be kept securely in paper or electric form for a period of 20 years after the completion of a research project.

**10. What will happen to the results of the research study?**
Data generated from this study may be used in scientific publications and is part of a PhD project. If you wish, we can inform you of any publications that are generated in the course of this study. Also, a summary of the study which will be made available on the movement science group website in December 2013.

**11. Type of study?**
A PhD research project.

**12. Who is funding the research?**
This study would be funded by the Royal Embassy of Saudi Arabia.

**13. Ethical approval**

**14. What if there is a problem?**
If you have any concerns about the conduct of the research, you could contact the Chair of University Research Ethics Committee on ethics@brookes.ac.uk.

**15. Contact Details:**
**Name of researcher: Eman Abu-Seer**
Mobile: 07787344427

Email: 11103599@brookes.ac.uk
Address: Movement Science group, School of Life Sciences, Oxford Brookes University, Gipsy Lane Campus, Headington, OX3 0BP

**Name of supervisor: Prof Helen Dawes**
Telephone: 01865 483272
Email: hdawes@brookes.ac.uk
Address: S306j, School of Life Sciences, Oxford Brookes University, Gipsy Lane Campus, Headington, OX3 0BP

**16. Who else can I contact regarding this study?**

Prof Helen Dawes

Telephone: 01865 483272

Email: hdawes@brookes.ac.uk

Dr Dave Carter

Telephone: 01865484216

Email: dcarter@brookes.ac.uk

Dr Ryan Pink

Telephone: 01865 484187

Email: rpink@brookes.ac.uk

Dr Johnny Collett

Telephone: 01865 483630

Email: jcollett@brookes.ac.uk

This project has been reviewed and approved by the University Research Ethics Committee

---

If you would like to take part, please complete the tear-off reply slip below:

I would like to take part in this study. You could contact me on ……..

Signature:……. Date:……..

**Appendix C: pre-screening health questionnaire**

Age:……..yrs

Weight:_____kg Height:_____m____cm

**Blood pressure:**

Systolic_____mmHg_____mmHg_____mmHg Diastolic

_____mmHg_____mm Hg_____mmHg

**Prescribed medication use:**

| **Name drug** | **Dosage** | **Time of day** | **Date of change** |
|---|---|---|---|
| | | | |
| | | | |

study 1 (Participant 1)

| Resting BP<br><br>Sys/dia | Lactate | RPE<br><br>breath | RPE<br><br>leg | BLOOD<br><br>SAMPLE | VAS |
|---|---|---|---|---|---|

| | | | | | |
|---|---|---|---|---|---|
| | | | | | |

| Questionnaire | FSS | PAR-Q | FASE | SF-36 |
|---|---|---|---|---|
| | | | | |

**Visual Analogue Scale**

0 10

No pain Worst pain ever

Please indicate on this line how sore you feel

**End Test**

| Blood Sample | Lactate | BP<br>Sys/dia | VAS | Separate and store serum at (-80°) |
|---|---|---|---|---|
| Baseline (0 hour) | | | | |
| Immediately after the exercise | | | | |
| 1 hour | | | | |

| | | | | |
|---|---|---|---|---|
| 2 hours | | | | |
| 3 hours | | | | |
| 4 hours | | | | |
| 5 hours | | | | |
| 6 hours | | | | |
| 8 hours | | | | |
| 10 hours | | | | |
| 12 hours | | | | |

**Visual Analogue Scale (After exercise)**

0 10

No pain Worst pain ever

Please indicate on this line how sore you feel

LAB TEST (ELISA)

| TEST | TNF-α | IL-6 | IL-10 |
|---|---|---|---|
| | | | |

| EXOSOME | EXO EXTRACTION | EXO GRID PREPRATION FOR EM | EXO PREPRATION FOR NANOSIGHT |
|---|---|---|---|
| | | | |

**Physical Activity Readiness-Questionnaire (PAR-Q)**

Please read the following carefully and answer as accurately as possible by ticking the appropriate box for each question.

| | Yes | No |
|---|---|---|
| 1. Has a doctor ever said you have heart trouble? | ☐ | ☐ |
| 2. Do you ever suffer frequently from chest pains? | ☐ | ☐ |
| 3. Do you often feel faint or have spells of dizziness? | ☐ | ☐ |
| 4. Has a doctor ever said you have epilepsy? | ☐ | ☐ |
| 5. Has a doctor ever said you have high blood pressure? | ☐ | ☐ |
| 6. Has a doctor ever said you have diabetes? | ☐ | ☐ |
| 7. Has a doctor ever said you have asthma? | ☐ | ☐ |
| 8. Do you have a bone, joint or muscular problem which may be aggravated by exercise? | ☐ | ☐ |
| 9. Do you have any form of injury? | ☐ | ☐ |
| 10. Are you currently taking any prescription medications? | ☐ | ☐ |
| 11. Have you suffered from a viral illness in the last two weeks? | ☐ | ☐ |

| | Yes | No |
|---|---|---|
| Have you eaten anything within the ***last hour***? | | |
| Have you consumed alcohol within the ***last 24 hours***? | | |
| Have you performed exhaustive exercise within the ***last 48 hours***? | | |

If you have answered **YES** to any of the above questions, or know of any possible reason (physical or psychological) that might affect the safety or accuracy of the tests - please inform a member of staff.

Anything else you feel that we should know about:

**General Health Status Questionnaire (SF-36)**

This survey asks for your views about your health. This information will help keep track of how you feel and how well you are able to do your usual activities.

Please answer these questions by "check-marking" your choice. Please select only one choice for each item.

1. In general, would you say your health is:

☐ 1. Excellent ☐ 2. Very good ☐ 3. Good ☐ 4. Fair ☐ 5. Poor

2. Compared to ONE YEAR AGO, how would you rate your health in general NOW?

☐ 1. MUCH BETTER than one year ago.

☐ 2. Somewhat BETTER now than one year ago.

☐ 3. About the SAME as one year ago.

☐ 4. Somewhat WORSE now than one year ago.

☐ 5. MUCH WORSE now than one year ago.

3. The following items are about activities you might do during a typical day. **Does your health now limit you** in these activities? If so, how much?

| Activities | **1. Yes, Limited A Lot** | **2. Yes, Limited A Little** | **3. No, Not Limited At All** |
|---|---|---|---|
| a) **Vigorous activities,** such as running, lifting heavy objects, participating in strenuous sports? | 1. Yes, Limited A Lot | 2. Yes, Limited A Little | 3. No, Not Limited At All |
| b) **Moderate activities,** such as moving a table, pushing a vacuum cleaner, bowling, or playing golf? | 1. Yes, Limited A Lot | 2. Yes, Limited A Little | 3. No, Not Limited At All |
| c) Lifting or carrying groceries? | 1. Yes, Limited A Lot | 2. Yes, Limited A Little | 3. No, Not Limited At All |
| d) Climbing **several flights** of stairs? | 1. Yes, Limited A Lot | 2. Yes, Limited A Little | 3. No, Not Limited At All |
| e) Climbing **one** flight of stairs? | 1. Yes, Limited A Lot | 2. Yes, Limited A Little | 3. No, Not Limited At All |
| f) Bending, kneeing or stooping? | 1. Yes, Limited A Lot | 2. Yes, Limited A Little | 3. No, Not Limited At All |
| g) Walking **more than a mile**? | 1. Yes, Limited A Lot | 2. Yes, Limited A Little | 3. No, Not Limited At All |

| | | | |
|---|---|---|---|
| h) Walking **several** blocks? | 1. 1. Yes, Limited A Lot | 2. 2. Yes, Limited A Little | 3. No, Not Limited At All |
| i) Walking **one** block? | 1. 1. Yes, Limited A Lot | 2. 2. Yes, Limited A Little | 3. No, Not Limited At All |
| j) Bathing or dressing yourself? | 1. 1. Yes, Limited A Lot | 2. 2. Yes, Limited A Little | 3. No, Not Limited At All |

4- During the **past 4 weeks**, have you had any of the following problems with your work or other regular activities *as a result of your physical health*?

| | | |
|---|---|---|
| a) Cut down on the **amount of time** you spent on work or other activities? | 1. yes | 2. No |
| b) **Accomplished less** than you would like? | 1. yes | 2. No |
| c) Were limited in the **kind** of work or other activities? | 1. yes | 2. No |
| d) Had **difficulty** performing the work or other activities (for example it took extra effort)? | 1. yes | 2. No |

5. During the **past 4 weeks**, have you had any of the following problems with your work or other regular daily activities **as a result of any emotional problems** (such as feeling depressed or anxious)?

| | | |
|---|---|---|
| a) Cut down on the **amount of time** you spent on work or other activities? | 1. yes | 2. No |
| b) **Accomplished less** than you would like? | 1. yes | 2. No |
| c) Didn't do work or other activities as **carefully** as usual? | 1. yes | 2. No |

6. During the **past 4 weeks**, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbours, or groups?

☐ 1. Not at all

☐ 2. Slightly

☐ 3. Moderately

☐ 4. Quite a bit

☐ 5. Extremely

7. How much **bodily pain** have you had during the **past 4 weeks**?

⌋ 1. None

⌋ Very mild

⌋ 3. Mild

⌋ 4. Moderate

⌋ 5. Severe

⌋ 6. Very severe

8. During the **past 4 weeks**, how much did **pain** interfere with your normal work (including both work outside the home and housework)?

⌋ 1. Not at all

⌋ 2. A little bit

⌋ 3. Moderately

⌋ 4. Quite a bit

⌋ 5. Extremely

9. These questions are about how you feel and how things have been with you **during the past 4 weeks**. For each question, please give the one answer that comes closest to the way you have been feeling. How much of the time during the **past 4 week**s

| | 1. All of the time | 2. Most of the time | 3. A good bit of the time | 4. Some of the time | 5. A little of the time | 6. None of the time |
|---|---|---|---|---|---|---|
| a) Did you feel full of pep? | 1. All of the time | 2. Most of the time | 3. A good bit of the time | 4. Some of the time | 5. A little of the time | 6. None of the time |
| b) Have you been a very nervous person? | 1. All of the time | 2. Most of the time | 3. A good bit of the time | 4. Some of the time | 5. A little of the time | 6. None of the time |
| c) Have you felt so down in the dumps that nothing could cheer you up? | 1. All of the time | 2. Most of the time | 3. A good bit of the time | 4. Some of the time | 5. A little of the time | 6. None of the time |
| d) Have you felt calm and peaceful? | 1. All of the time | 2. Most of the time | 3. A good bit of the time | 4. Some of the time | 5. A little of the time | 6. None of the time |
| e) Did you have a lot of energy? | 1. All of the time | 2. Most of the time | 3. A good bit of the time | 4. Some of the time | 5. A little of the time | 6. None of the time |
| f) Have you felt downhearted and blue? | 1. All of the time | 2. Most of the time | 3. A good bit of the time | 4. Some of the time | 5. A little of the time | 6. None of the time |

| | | | | | | |
|---|---|---|---|---|---|---|
| g) Do you feel worn out? | 1. All of the time | 2. Most of the time | 3. A good bit of the time | 4. Some of the time | 5. A little of the time | 6. None of the time |
| h) Have you been a happy person? | 1. All of the time | 2. Most of the time | 3. A good bit of the time | 4. Some of the time | 5. A little of the time | 6. None of the time |
| i) Did you feel tired? | 1. All of the time | 2. Most of the time | 3. A good bit of the time | 4. Some of the time | 5. A little of the time | 6. None of the time |

10. During the **past 4 weeks**, how much of the time has your **physical health or emotional problems** interfered with your social activities (like visiting with friends, relatives, etc.)?

⌋ 1. All of the time

⌋ Most of the time.

⌋ 3. Some of the time

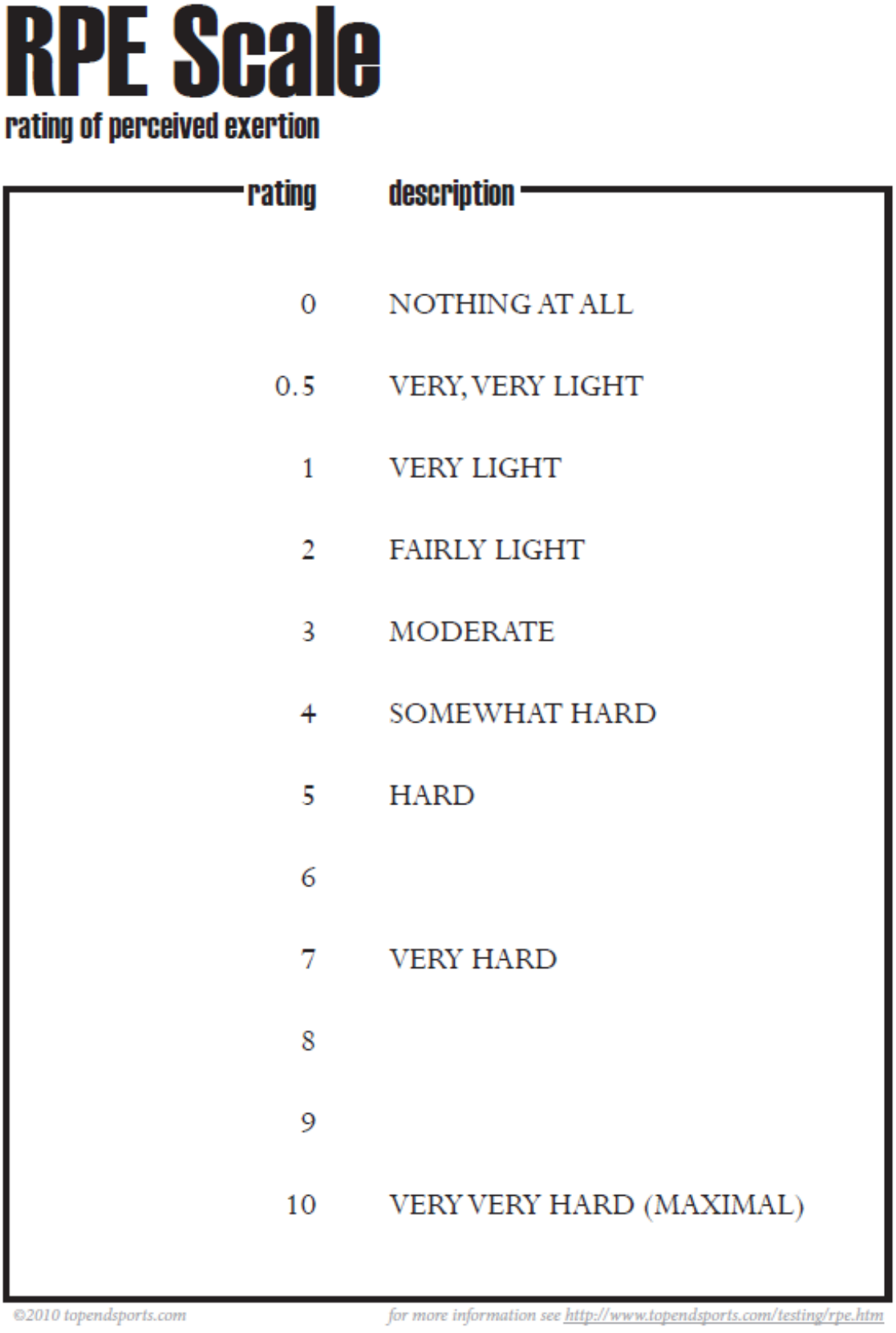
⌋ 4. A little of the time.

⌋ 5. None of the time.

11. How TRUE or FALSE is **each** of the following statements for you?

| | 1. Definitely true | 2. Mostly true | 3. Don't know | 4. Mostly false | 5. Definitely false |
|---|---|---|---|---|---|
| a) I seem to get sick a little easier than other people? | ⌋ 1. Definitely true | ⌋ 2. Mostly true | ⌋ 3. Don't know | ⌋ 4. Mostly false | ⌋ 5. Definitely false |
| b) I am as healthy as anybody I know? | ⌋ 1. Definitely true | ⌋ 2. Mostly true | ⌋ 3. Don't know | ⌋ 4. Mostly false | ⌋ 5. Definitely false |
| c) I expect my health to get worse? | ⌋ 1. Definitely true | ⌋ 2. Mostly true | ⌋ 3. Don't know | ⌋ 4. Mostly false | ⌋ 5. Definitely false |
| d) My health is excellent? | ⌋ 1. Definitely true | ⌋ 2. Mostly true | ⌋ 3. Don't know | ⌋ 4. Mostly false | ⌋ 5. Definitely false |

**Appendix D: RPE Scale**



# RPE Scale

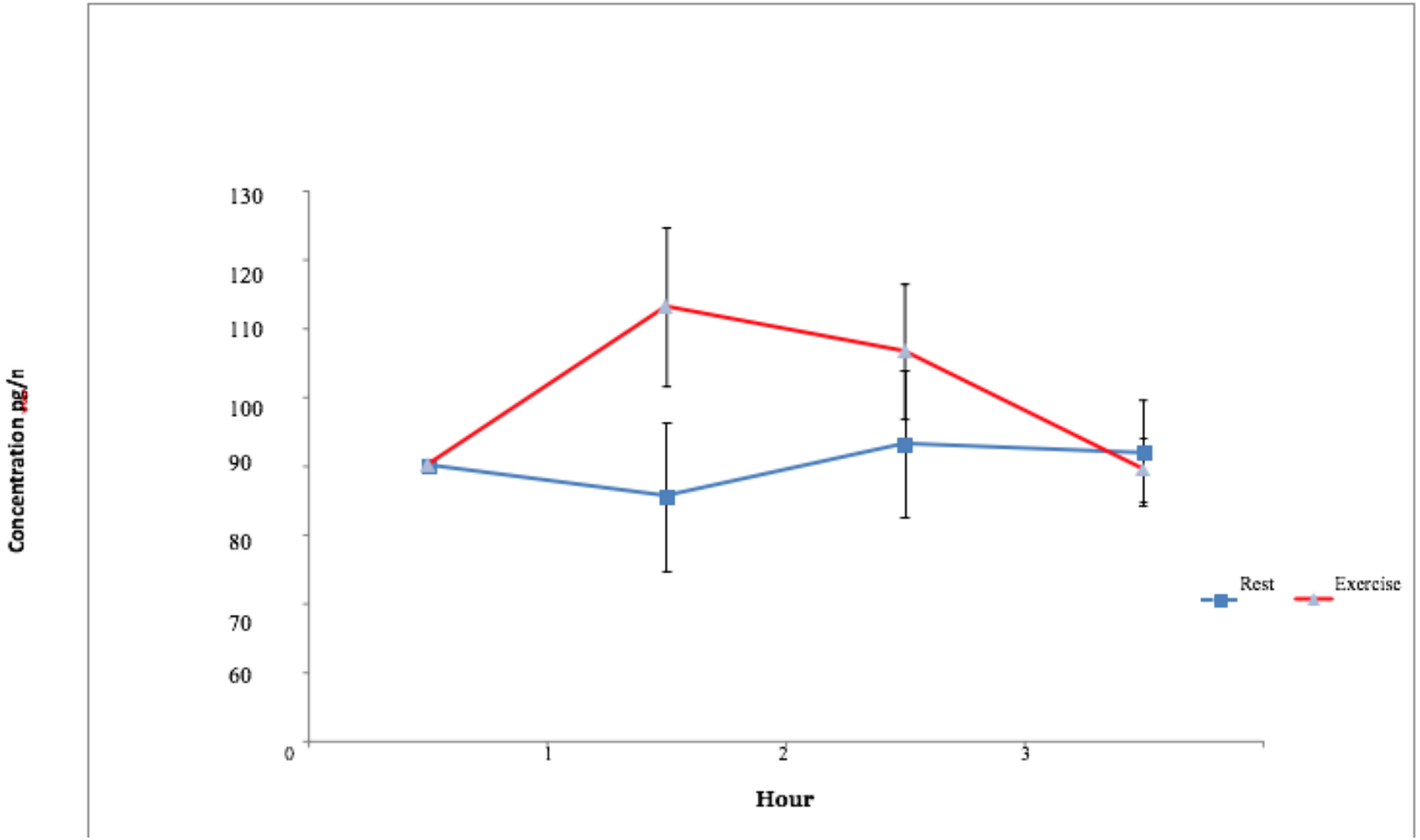
rating of perceived exertion

| rating | description |
|---|---|
| 0 | NOTHING AT ALL |
| 0.5 | VERY, VERY LIGHT |
| 1 | VERY LIGHT |
| 2 | FAIRLY LIGHT |
| 3 | MODERATE |
| 4 | SOMEWHAT HARD |
| 5 | HARD |
| 6 | |
| 7 | VERY HARD |
| 8 | |
| 9 | |
| 10 | VERY VERY HARD (MAXIMAL) |

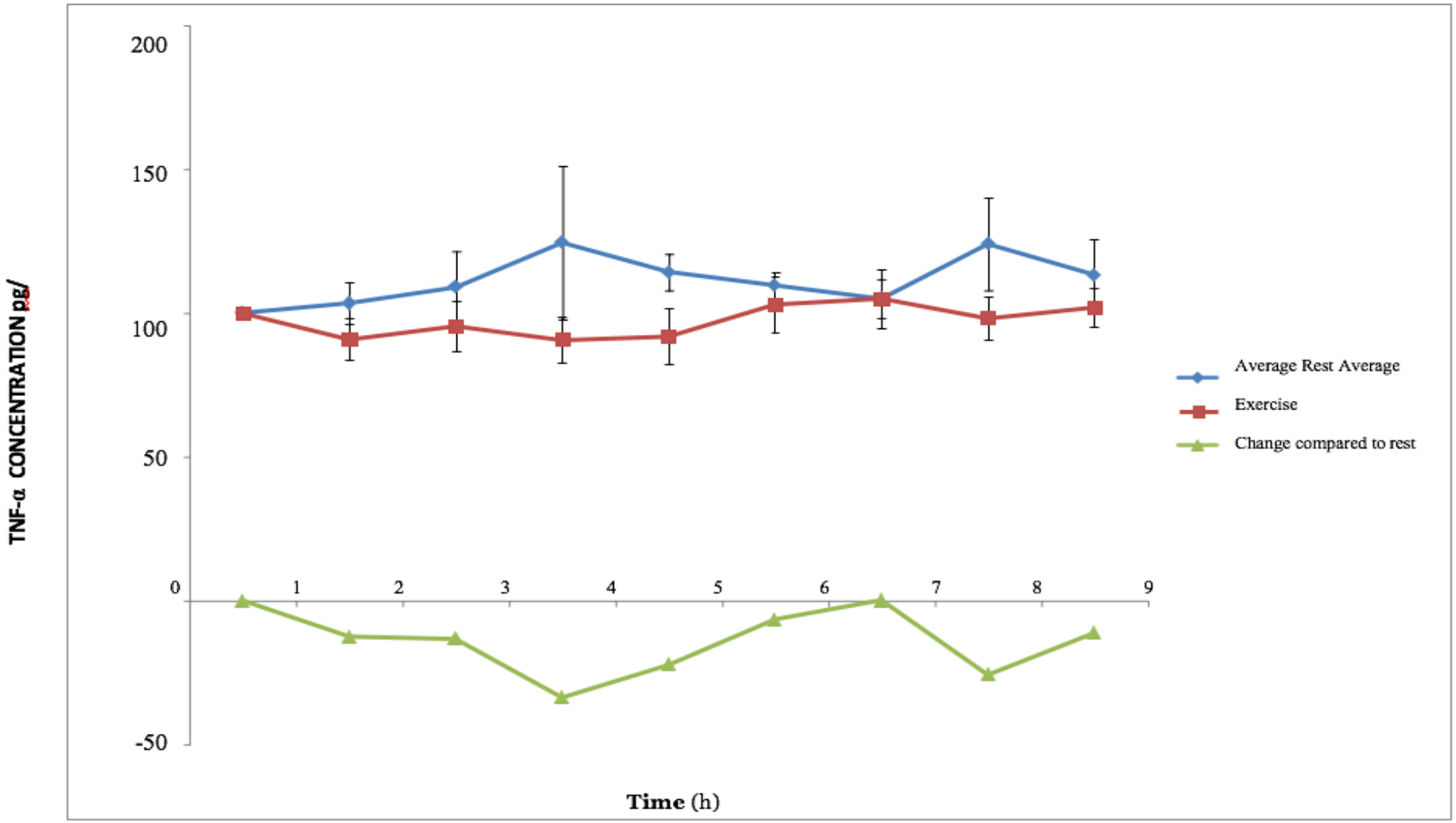


for more information see http://www.topendsports.com/testing/rpe.htm

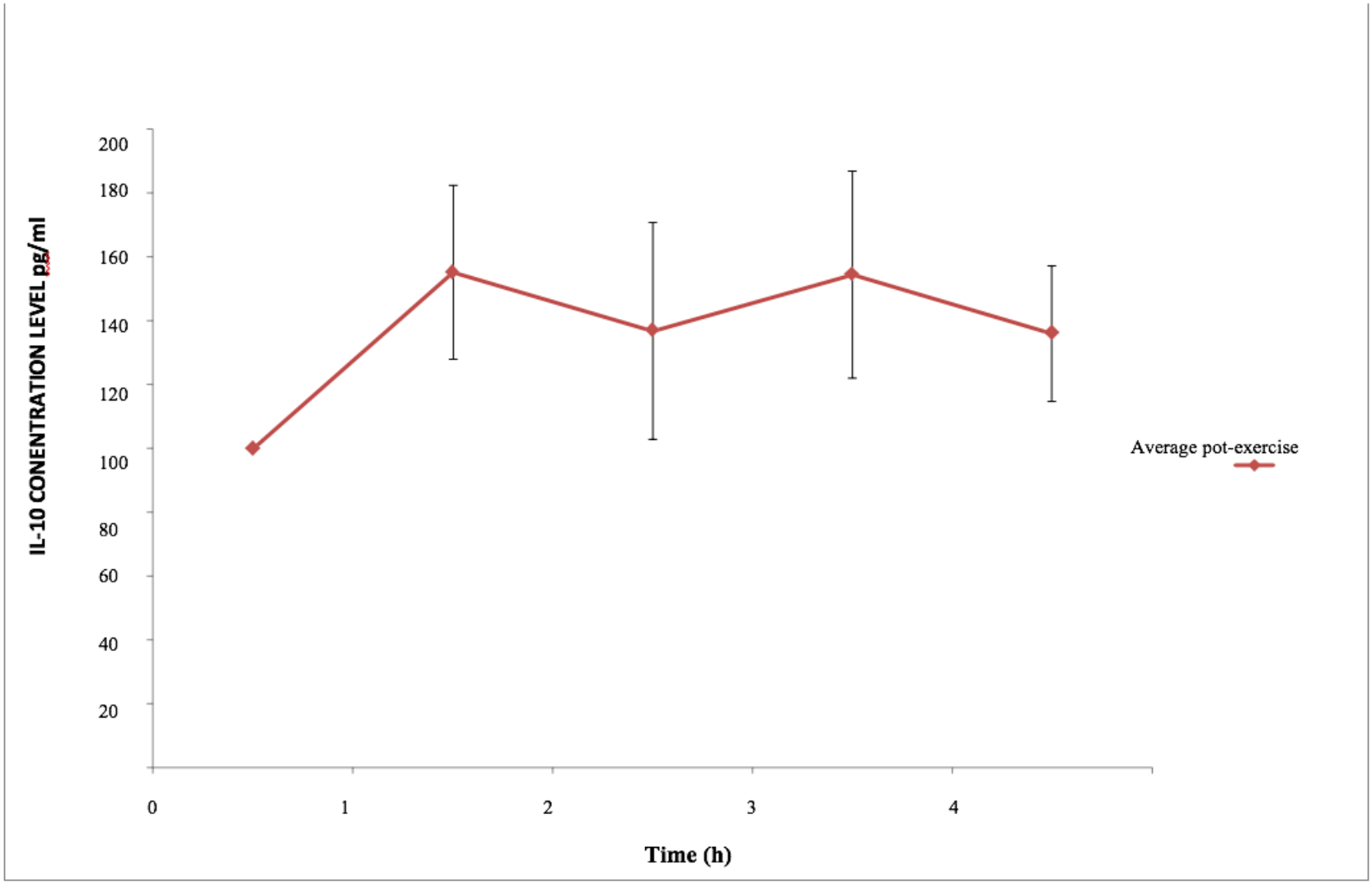
## Average IL-10 REST and following eccentric exercise



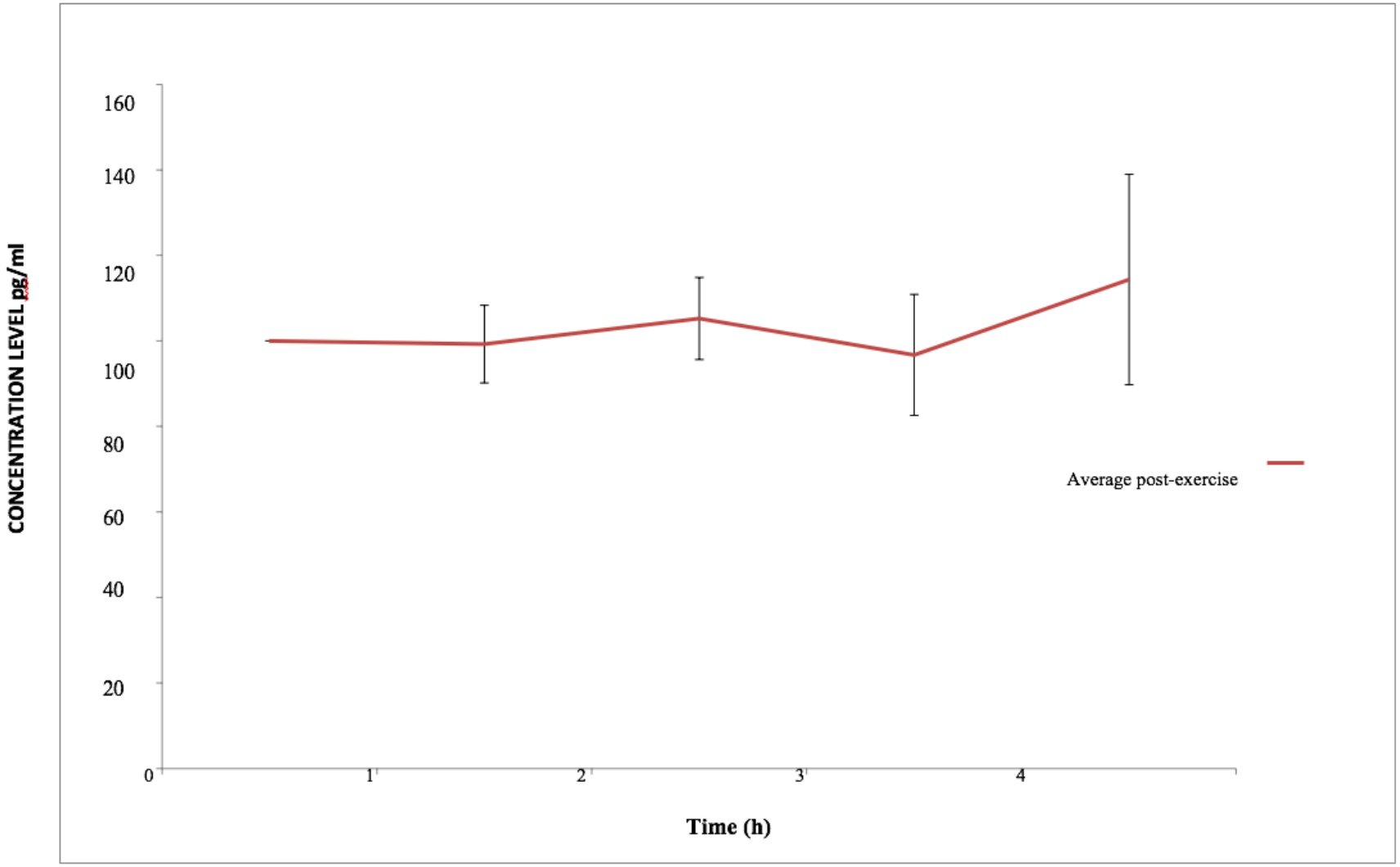
## Average TNF-α concentration level in rest and after eccentric exercise



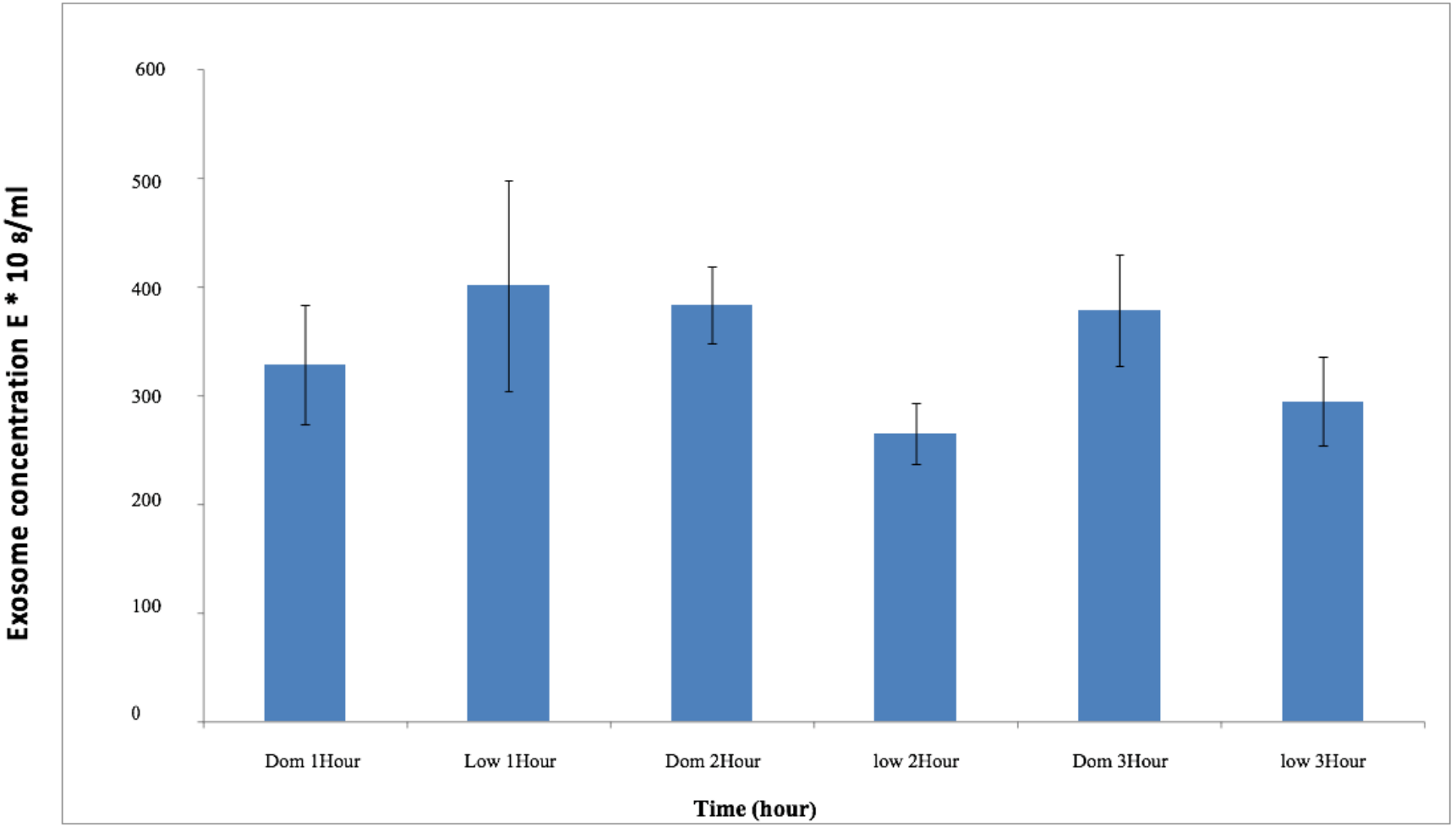
## Average IL-10 concentration after moderate cycling exercise



# Average TNF- a concentration following moderate cycling exercise



## Appendix E: Comparing of average exosome concentration in eccentric and moderate cycling exercise during hours one, two, and three



# Appendix F: Publication

Journal of Health Science 5 (2017) 81- 94
doi: 10.17265/2328-7136/2017.02.004

PUBLISHING

# The Reliability of Plasma Exosome Concentrations in Healthy Male Individuals

Eman Abu-Seer
*Oxford Brookes University, Gipsy Lane, Oxford, OX3 0BP, UK*

**Abstract:** Exosomes are nanometer sized vesicles (30-100 nm), released from cells following the fusion of multivesicular bodies with the plasma membrane. Exosomes have a major role in intracellular communication, immune response modulation, physiological and pathological functions, and an important use in different drug therapies. Moreover, they have been identified as potential biomarkers and are involved in cancer and many other disease processes. Despite the developments in this area, no studies exist that assess the variability of plasma exosomes measures within and between subjects. The purpose of this study was to assess within and between day variability of plasma exosomes concentration in healthy individuals. Also, the stability of exosomes during freeze/thawing cycles was measured in this study. Eleven healthy men were assessed for reliability of exosome concentrations taken over the same sampling period, over 10 hours of a day and over five days. Exosomes were isolated by differential ultracentrifugation and characterized by their size distribution and morphology by electron microscopy and using the Nanosight tracking analysis respectively. Mean plasma exosome values were1.505-2.245 × 10^8/mL). Within day variability was not significantly different ($P$ = 0.95), and between day variability was not significantly different ($P$ = 0.42). The interclass correlation coefficients (ICC) of 0.80 for within sample showed good reliability; ICC of 0.84 for between day plasma exosomes concentrations showed good reliability, and the within day ICC of 0.70 showed a moderate reliability. There was no significant difference observed in using fresh or frozen plasma in exosome quantification or exosome concentration. The statistics show that there is little significant variability in plasma exosome concentrations taken over a single sampling period, over a day or between days, which is vital for plasma exosome studies.

**Key words:**

**Corresponding author:** Eman Abu-Seer, research field: clinical trials.

## 1. Introduction

### *1.1 Exosomes*

In normal cell's physiology, there is constant transport of biological materials across the cellular membranes. This transport works according to an evolutionary conserved mechanism and involves active and passive modes of transport along with the trafficking through micro particles, vesicles produced by the cell, including exosomes [1]. Exosomes are under the scientific spotlight as important methods of extracellular excretions and are nanometer sized vesicles (30-100 nm), released by cells, both normal and cancerous, into the extracellular space by inward budding of endosomal membrane into structures called multi-vesicular bodies (MVBs), followed by fusing of MVBs with the plasma membrane and release their internal component (exosomes) extracellularly [2]. They are composed of nucleic acid and protein and have been largely attributed to be the carriers of these constituents to various parts of the body without any discrimination. However they are also widely known to carry intercellular messages, effects of and signalling macromolecules between highly specific set of cells [2]. Exosomes were

first reported by Johnstone and his colleagues in 1987, when they found that exosomes are secreted from reticulocytes during maturation [3]. Exosomes can be released by many cells, such as T-cells, dendritic cells, and oligodendrocytes cells and non-hematopoietic cells such as neuroglial cells, tumour cells and intestinal epithelial cells [4]. They have been detected in several biological fluids as plasma, saliva, nasal lavage, placenta, and in breast milk [5]. Exosomes levels in plasma are clinically

interesting in many applications for example the plasma levels in some cancer patient could be a potential tool for cancer screening [6].

*1.2 Exosomes as Potential Biomarkers in Cancer Identification and Development*

It has been largely hypothesized that since the exosomes carry a particular cellular content, they can be exploited as potential biomedical tools to detect the changes in the content of the cells, as in the initiation and progression of cancer or tumorous growths. The micro ribonucleic acid content of the exosomes corresponds to only particular cell types and are attributed to changing the phenotype of the progenitor cells by effectively transferring pro-oncogenic molecules in order to initiate the growth of cancers in these cells and hence give rise to metastatic environment in the neighbouring cells [7]. It has been suggested that increased level of exosomes can be detected in urine, serum, and in malignant effusions from cancer patients [8]. Kharaziha, Ceder [9] reported that cancer cells secrets higher amounts of exosomes than normal cells. Furthermore, Taylor and Gercel-Taylor [10]found that cancer patients have excessive amounts of blood circulating exosomes compared to healthy individuals. Therefore, the number of exosomes is important and can be used as markers in many diseases including cancer. Logozzi, De Milito [6] study shows high levels of exosomes that expressed CD63, housekeeping protein present in all human beings nonetheless, and Caveolin-1, tumour-associated biomarker, in the plasma of cancer patients. ELISA was used for quantifying the levels of exosomoes present in the plasma of the cancer patients that expressed the two proteins and it was found that the levels for CD63 and Caveolin-1 were significantly increased in melanoma patients as compared to the healthy individuals who donated plasma. Therefore, the analysis of exosomes for specific tumour-biomarkers can help in the early detection of cancer before it develops into a full-grown metastasized tumour and causes mortality [6]. Furthermore, Ren and colleagues were able to show that exosomes present in human plasma displayed a variety of shapes and exhibit the exosome marker protein CD81 besides CD63, along with other members of MHC Class II including CD86, Wnt3a, Wnt5a and FasL which are normally known as the cell signal transduction molecules [11]. Plasma exosomes were also shown to participate in the inhibition of CD4+ T cells involved in the immune system. They possess the ability to induce suppression, in dose-dependent manner, of the active CD4+ T cells and their proliferation in the plasma, leading to their natural apoptosis in the body [11]. Moreover, Salma Khan and colleagues researched on the levels of Exosomal Survivin in patients with prostate cancer and in control patients, derived from their plasma. Survivin is expressed in the plasma of patients diagnosed with prostate cancer and it is important for making the PCa cells sensitive to the chemotherapeutic agents. Its downregulation causes the effective eradication of the cancerous cells at the end of chemotherapy. The recent research in the exosomes has shown that the exosomes derived from the tumour, much like the ones derived in this experiment, help drastically in the initiation and development of cancer in the cells affect as well as help it metastasize to other parts of the body. Exosomes were purified from serum and plasma samples from PC patients as well control population

using the experimental techniques of ultracentrifugation and ELISA and Western blotting were used to quantify the amount of exosomes present in the samples isolated. It was found to be present in elevated levels in the tumour-derived samples as compared to the serum and plasma exosome levels in the control patients. The patients who had failed to respond to chemotherapies also projected increased levels for exosome-derived Survivin. Therefore, the researchers were successful in showing that Survivin exists in the plasma and serum-derived exosomes in

elevated amounts and can be used as a potential biomarker in early detection of cancer [12].

*1.3 Additional Functions of Exosomes*

In addition to the interest in exosomes as potential biomarkers, their physiological roles are also important which basically deal with the pathways related to intracellular and intercellular communications. They play a crucial role in cell to cell communication when they interact with other cells by carrying proteins, lipids, and genetic information [13], whether the cells present locally or at a distance. They have been found to be involved in immune regulation and act as antigen-presenting vesicles, the behaviour commonly found in the B and T immunity cells, in order to stimulate the immune response and induce tolerogenic effects [13-15]. Also exosomes have been involved in
some diseases' pathogenesis such as neurodegenerative disease and cardiovascular disease
[16]. It has been shown that exosomes are involved in the accumulation of β-amyloid protein which is associated with Alzheimer's' disease [8].

Recently a wide range of exosomes physiological and pathological function studies have been conducted. Although the number of studies examining plasma exosomes is increasing, however, there is a need to better understand the variables that affect exosomes levels and the mechanisms that control the production and mediation of exosome levels. Also there might be variation in between subjects over a day and within a day. Although, circadian variations are often detected on human immune biomarkers and have been studied. For example, the study conducted by Ündar, Ertugùrul
[17] reported a significant circadian variation in different blood biomarkers (IL-6, coagulation inhibitors proteins, and anti-thrombin factors in healthy men [17]. However, exosomes' circadian rhythm in healthy individuals is still unknown.

It is important to know the variability in measuring exosome numbers and size. The variability can help point towards the variety of roles that exosomes perform in the various parts of the human body in their multiple morphological orientations as demmed appropriate and essential based on the biochemical environment inside and outside the cells. Further which measurement bias in the data affects the measurement variability, which can occur in pre-analytical (specimen collection, handling and processing), analytical (imprecision and bias), and post-analytical (interpreting and reporting data) data sets [18]. Gardiner, Ferreira [19] examined the analytical measurement variation of exosomes measures processes by Nano sight Tracking Analysis (NTA). It is essentially a technique where the light is scattered by the subjects in focus which leads to the specification of their size and quantity present in a sample. They used silica microspheres in order to calibrate the measurements taken by the technique as standardized method for extra-vesicles (exosomes) measurements, in order to decrease the level of inaccuracy associated with the measurements. Gardiner, Ferreira [19] showed data from several repeat measurements of known concentration. However, a biological standard for exosomes measurement has not been studied and the within-subject variations have not been addressed. The additive features of the technology are distributed across five distinct levels. Primarily, it allows for determining the detection threshold which refers to the minimum value for the intensity of an image that can help distinguishing a particle for further analysis. Furthermore, the technology can help in determining the maximum distance that the software expects a particle to move from one frame of reference to another. This is directly proportional to the size of the particle and therefore, the larger particles would take longer to move across the frames. This minimum expected particle size is automatically determined by the software. Moving on, blur which defines the degree of smoothing of the images received by the software after removing the unrequired noise, including predominantly the diffraction rings found around the

larger particles, is another automatic setting built into the software. It can be manually adjusted to increase for bigger particles and decrease for the smaller ones. Moreover, it also determines the minimum track length that a particle should cover based on its size for it to be included in the size distribution plot. This plot is entirely based on the Mean Square Displacement (MSD) profile for all the particles and which have been tracked for a minimum number of frames as determined by the machine automatically or set manually by the scientific researcher. Higher values for minimum track length mean that the particles have been tracked for a greater length of time which helps in accurate sizing of all the particles under scrutiny. However, it does not imply that the particles that spend less time in crossing one frame of reference to another are measured inaccurately. It can also mean that these particles are smaller in size and hence, take less time to get across the threshold. Lastly, the background noise or pixel contamination can be easily removed from the extracted data sets or images in order to make the readings as accurate as possible for the size of the particles. Therefore, Nanoparticle Tracking Analysis can be successfully employed to determine size of the biological molecules such as exosomes to develop the importance of their molecular physiology to the function they perform [19].

Considering physiological and pathological importance of exosomes as potential biomarkers for neurodegenerative diseases and multiple cancer types, there exists a further need for reliability measures of plasma exosomes concentration in plasma, thereby leading to reliable and accurate prognosis of disease initiation and development. In conjunction to their need, the day-to-day variation in the readings should also be well accounted for and measured accurately to understand their association with changing biochemical environment in the body and how the exosomes can help as effective tools in marking these changes at early stages. This will help to determine whether a single fasted blood sample is sufficient or if consecutive day blood samples are needed to present an individual's exosomes status. Therefore, the purpose of this study is to assess within and between day reliability of plasma exosome concentration. We hypothesized that plasma exosomes concentration should be stable within sample, although there might be little within day variability, however the overall plasma exosome concentration is found to be stable over the period of five days of blood sampling in a week.

### *1.4 Aims and Objectives of the Study*

The main aim of this study is to explore reliability of plasma exosomes in healthy individuals. There are other objectives of the study:

- To assess within sample, within day and between days variability of plasma exosome concentrations
- To predict the size distribution for a given population of extracellular vesicles within a day at different time points and also between days over five consecutive days in healthy individuals.
- To determine the stability of exosomes during freeze/fresh plasma.

Although, circadian variations are often detected on human immune biomarkers. We hypothesized that plasma exosomes concentration should be stable within sample; there would be little day to day variability in plasma exosomes concentration over the five days of blood sampling.

## 2. Methods

### *2.1 Participants*

Eleven healthy male participants (age 27.6 ± 4.2 y, height, 177.0 ± 9.4 cm, and body weight, 80.3 ± 12 kg) volunteered to participate in the study which was approved by the University Research Ethics Committee. This study conformed with the latest revision of the Declaration of Helsinki. All procedures and potential risks associated with the study were fully explained to each participant. All participants reported to the human performance laboratory and answered

screening questionnaires to determine their eligibility for the participation in the study. Participants were eligible for inclusion if they were healthy male and between 20 and 60 years of age. Exclusion criteria included diagnosis of a chronic inflammatory condition (e.g diabetes mellitus and cardiovascular diseases or recent consumption of anti-inflammatory medications (e.g ibuprofen). Since the scope of the study is to study the variation found in the levels of exosomes or their concentration in healthy individuals, therefore the indication of any inflammatory condition refers to the natural increase in the concentration of exosomes and any proteins that are associated with anti-inflammatory response [20]. Moreover, subjects were excluded if they had any pathology known to influence immune response (e.g. Auto immune diseases, anaemia, heart diseases, orthopaedic limitations or renal failure disease), or neurological, neuromuscular disorder. Subjects who had any cold or flu symptoms in the previous 24 h were excluded from the study based on the same explanation as provided earlier about the natural increase in the level of exosomes in cases of inflammatory response.

2.1.1 Sample Size

This is part of another study (exosome and inflammatory markers (TNF-a, IL-6, IL-10) in healthy individuals before and after high/low intensity exercise), in which the same sample size calculation was also used in this study. The sample size calculation is based on data from a review which has been done by [21], based on cytokines (TNF-a, IL-6, IFN-γ) measurement between healthy and healthy group. With an effect size of 0.55, the power of this study is set at 80%, and the level of significance is set at 5% (0.05, two tailed-analyses). Also we used sample size of 11 individuals based on a previous study by [22], which looked at changes in muscle RNAs in response to acute and chronic aerobic exercise.

2.1.2 Within Sample

For all studies, during each visit individuals were asked to fast for 8 to 10 hours before blood draws and refrain from alcohol for 24 h, and an exhaustive exercise for 48 h before each visit. They were allowed to drink water only. In order to determine whether the method of extracting exosomes were reproducible during each visit, three blood samples (4 mL each) were drawn consecutively from the antecubital vein into EDTA vacutainers (Becton Dickinson; San Jose, California). Then blood sample was centrifuged at 1,000 g for 10 min at 4 ºC, plasma was separated, divided into 500µ aliquots and subsequently stored at -80 ºC. The exosomes were then processes as below.

2.1.3 Within Day Reliability

In order to assess within day reliability, the same eleven individuals were participated in this study. In this visit, the first fasted blood sample was drawn by venepuncture using a winged infusion device at the baseline 0 h. Then blood sample (4 mL) was drawn at 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 8 h, and at 10 h. Participants were offered a standardized diet after the second hour and the sixth hour from the beginning of the study. All blood samples were collected, and processed as stated above.

2.1.4 Between Day Reliability

In order to examine plasma exosomes reliability between multiple days, same eleven participants reported to the laboratory five times over five consecutive days. During each visit blood sample (4 mL) was drawn at the same time from every participant. All blood sample were processed same as stated above.

*2.2 Study Design*

2.2.1 Preparation of Plasma Exosomes

Exosomes were isolated by differential ultracentrifugation. Basic exosomes isolation protocol was followed as described by [23]. Plasma sample was separated from blood and diluted with an equal amount of PBS and gently mixed until homogenous. Then the mixture was transferred in 50-mL tubes, centrifuged for 30 min at 2,000× g, 4 °C followed by at 12,000 g for 45 min. The suspension was filtered

through a 1% BSA blocked 0.22-µm filter, collected in a fresh ultracentrifuge tube, and centrifuged for 2 h at 110,000× g, 4 °C in a Beckman LE-80K centrifuge, swing out rotor (Beckman Coulter Optima, SW 32 Ti). Subsequently the pellet was washed in PBS by filling up a 14 mL tube with PBS and then centrifuged at 110,000× g, 4 °C for 70 min. After, the pellets were re-suspended in 50 µ PBS, and stored at -80 °C.

Exosomes were immediately isolated from the first aliquted samples (A) by differential ultracentrifugation and then examined morphologically by electron microscopy. The number of exosomes were analysed by Nanosight tracking analysis. The second plasma samples (B) were

*2.3 Exosomes Analysis*

2.3.1 Stability of Plasma Exosomes

To determine the stability of exosomes during freeze/fresh plasma (5 mL) from eleven healthy individuals were collected and divided into two 2.5 mL samples. Then exosomes were immediately isolated from the first aliquted samples (A) by differential ultracentrifugation and then examined morphologically by electron microscopy. The number of exosomes were analysed by Nanosight tracking analysis. The second plasma samples (B) were aliquted and stored at - 80 ºC for three months. Exosomes were isolated, morphologically tested and analysed by NTA as prescribed below (preparation of plasma exosomes, and NTA).

2.3.2 Preparation of Plasma Exosomes (Isolation and Extraction)

Exosomes were isolated by differential ultracentrifugation. Basic exosome isolation protocols were followed as described [24]. A plasma sample was separated from blood by spinning whole blood at 1,000 g for 10 min in Biofuge primo ( Heraeus instrument). Then plasma was diluted with an equal amount of PBS (Sigma 764) and gently mixed until homogenous. Then the mixture was transferred in 1,5 mL Eppendrof tubes, centrifuged for 30 min at 2,000× g, 4 °C (Biofuge fresco, Heraeus instrument). Then centrifuged at 12,000 g for 45 min in a Beckman LE-80K centrifuge (Beckman Coulter Optima, swinging bucket rotors). The suspension was filtered through a 0.22-µm filter (pre-block with 1% BSA), collected in a fresh ultracentrifuge tube, and centrifuged for 2 h at 110,000× g, 4 °C in a Beckman LE-80K centrifuge (Beckman Coulter Optima, swinging bucket rotors). Subsequently the pellet was washed in PBS by filling the tube up to the top with PBS, and then centrifuged at 110,000× g, 4 °C for 70 min (Beckman Coulter Optima, swinging bucket rotors). After, the pellets were re-suspended in 50 µ PBS, and store d at -80 °C.

2.3.3 ExoQuickTM Precipitation

Exosome isolation from plasma ExoQuickTM precipitation was carried out according to manufacturer's instructions (System Biosciences). Briefly, 500 mL of clarified CCM was diluted to 5 mL in PBS and mixed with 1 mL of ExoQuick-TCTM solution by inverting the tube several times. The sample was incubated overnight at 4 °C then centrifuged twice at 1,500 g for 30 and 5 min, respectively, in order to remove the supernatant. The supernatant was discarded, and the pellet was resuspended in 200 mL of PBS.

2.3.4 Transmission Electron Microscopy (TEM) of Exosomes Samples

An aliquot of exosomes vesicle sample was combined 1:1 with 4% PFA (Sigma, 158127) and cooled for 15 min on ice. Then a single drop of each sample was placed onto a strip of Para film (VWR, 52858). Carbon-formvar coated copper grids, 200 mesh (F077, TAAB) were placed dull-side down onto the exosomes vesicles/PFA drop and left at room temperature for 30-45 min. Grids were then placed sample down onto three 30 µl drops of 0.22 µm filtered ultrapure water for one minute each. the side of the grids were gently touched to a filter paper between each drop to remove excess solution. Then grids were placed sample-side down onto a 30 µL drop of 2% uranyl acetate (aq) for two minutes. All grids were

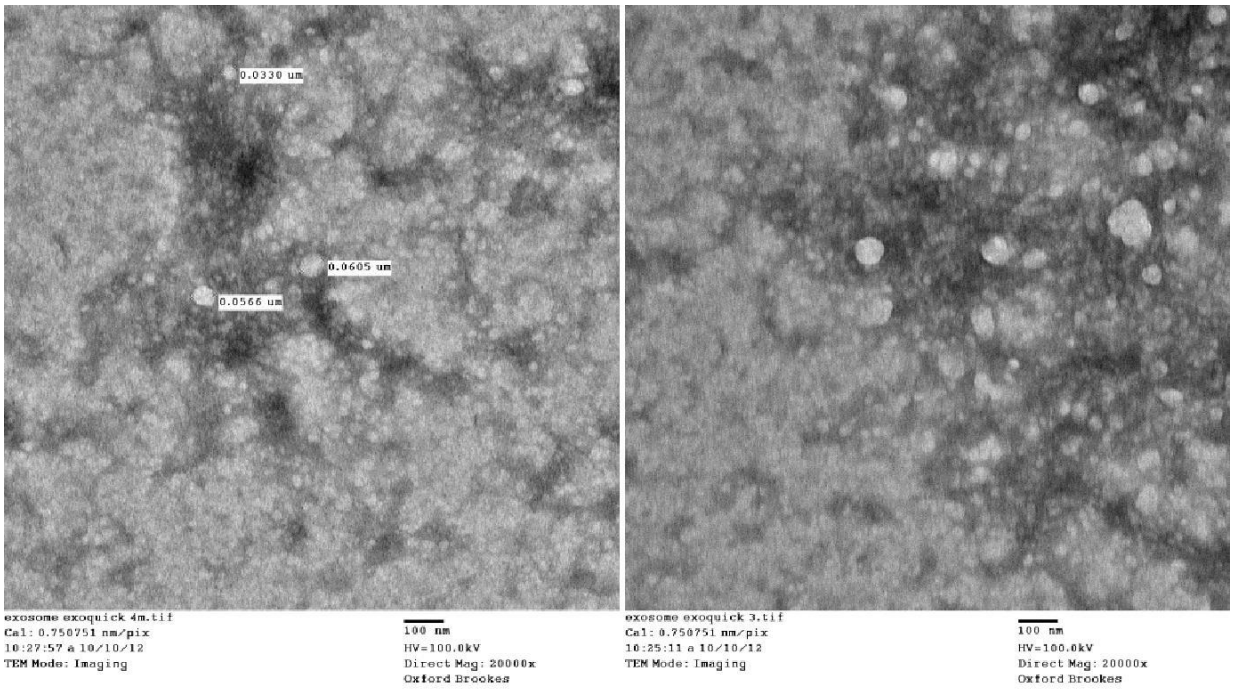



**Fig. 1**

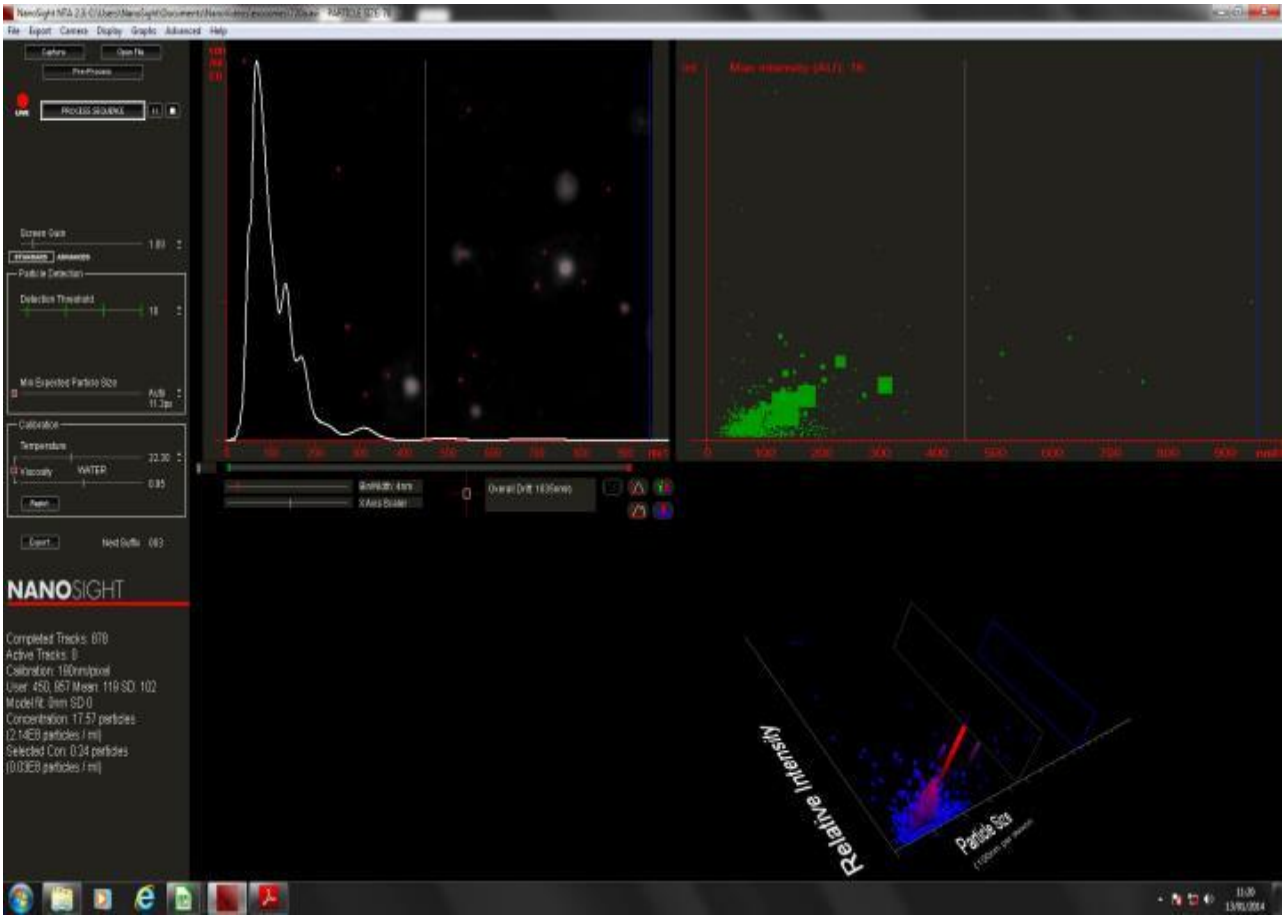
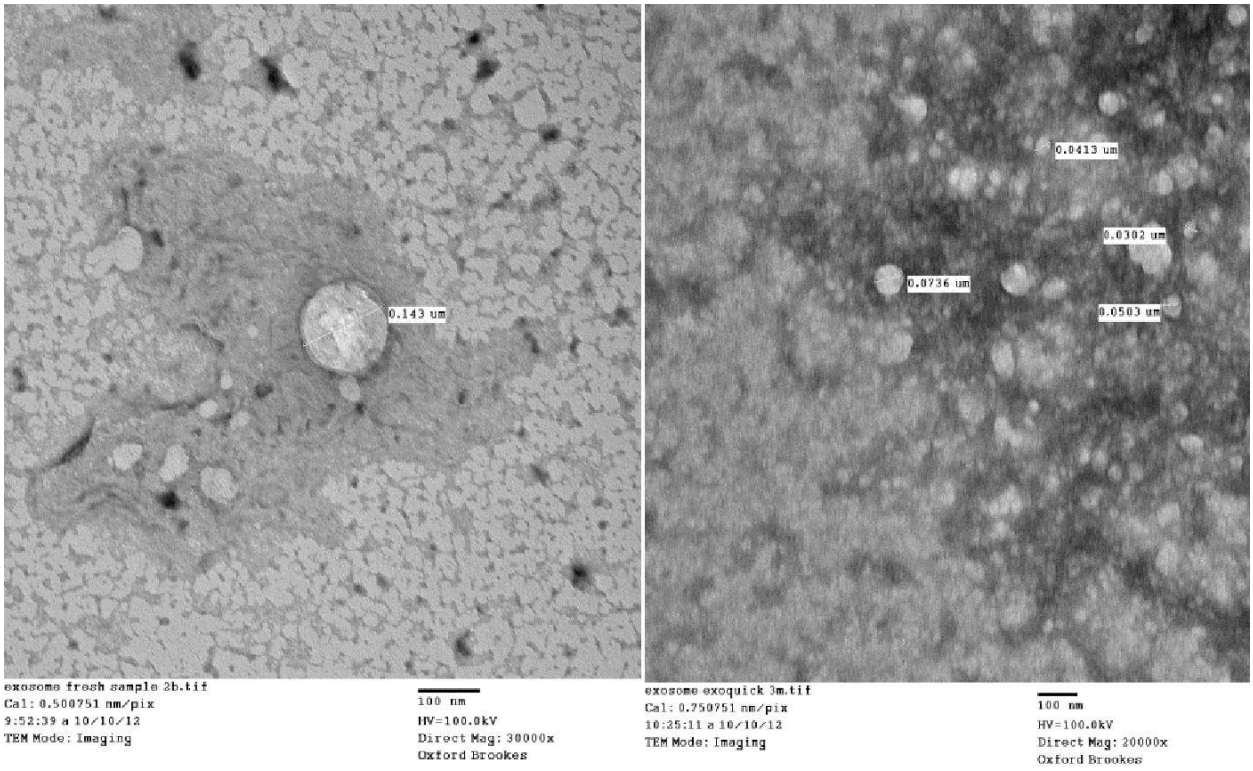



**Fig. 2 NTA measurement of particle size and concentration.**




(a) (b)

**Fig. 3 A. fresh exosomes shown by electron microscopy, B. Frozen exosomes.**

touched to the filter paper and left to dry, in which sample side up for one hour. After that, grids were stored in a box for analysis by using a Hitachi H7650 Transmission Electron Microscope (TEM) at 120 kV.

2.3.5 Nanoparticle Tracking Analysis (NTA)

Sample suspensions containing exosome vesicles were first diluted 1:500 PBS, kept on ice, then analysed using a Nanosight LM10, NS500 instrument (NanoSight, Amesbury, UK). For this, 90 second videos were used to track the Brownian movement using Version 2.2 Nanosight software to give mean and median vesicle size and concentration (Fig. 2). For every sample this reading was done five times with mixing in between and then an average taken to remove sample mixing bias.

*2.4 Statistical Analysis*

Participant's characteristics data are presented as mean ± standard deviation (SD). Statistical analyses were completed in SPSS for windows version 21 (SPSS Inc., Chicago, IL, USA). All variables were examined with one-way repeated measures ANOVA with time as the independent variable to detect any differences within day (time points 0 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 8 h and 10 h) or between days (day 1, 2, 3, 4 and 5). Within day (across all nine time points; 0 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 8 h and 10 h) and between day (across all five visits) reliability were examined with a two-way mixed effect models interclass correlation coefficient ($ICC_{3,1}$) for absolute agreement [25]. The ICC 95% confidence intervals (95% CI) were also calculated. An ICC ≥ 0.7 was accepted as moderate reliable as 0.6-0.8 considered as moderate reliability and ≥ 0.8 as good reliability. Coefficient of variation (CV) was determined for each participant using the formula: SD/mean × 100 to determine measurement variability. Within day CVs were calculated across the time points. Within sample and between day CVs were calculated across the five visits and with the group average presented. Significance was set at the level of $P < 0.05$.

## 3. Results

### *3.1 Stability of Plasma Exosomes*

In order to assess the stability of plasma exosomes under different conditions, fresh and frozen plasma exosomes were isolated by differential ultracentrifugation as described above. Then they were analysed by NTA showed a particle size ranging from 30 to 300 nm in diameter with an average of 120 ± 70 nm. The number of fresh and frozen plasma exosome was stable (fresh samples 3.68 ± 3.1, frozen samples 4.36 ± 2.8), there was no significant change between fresh and frozen plasma exosomes ($P = 0.072$) in all eleven samples which also analysed by NTA (Fig. 2). Under the electron microscopy, exosomes were appeared as spherical vesicles with a cup shape, also their diameters ranging from 30 to 120 nm (Fig. 3).

Reliability of plasma exosomes measures within sample, within day, and between days

This study was conducted to assess within and between day reliability of plasma exosomes concentration in healthy individuals. Eleven healthy men were assessed for reliability of exosome concentrations taken over the same sampling period, over 10 hours of a day and over five days. Table 1 shows the descriptive characteristics of all the eleven subjects who completed the study. No one had reported of any illness in the 24 h prior to the blood draw. All subjects were non-smokers.

Reliability and variability of plasma exosome concentration within the same subject at a single time point

Within sample variability of blood plasma exosomes concentration in three different samples withdrawn consecutively from each subject was measured using the nanosight.

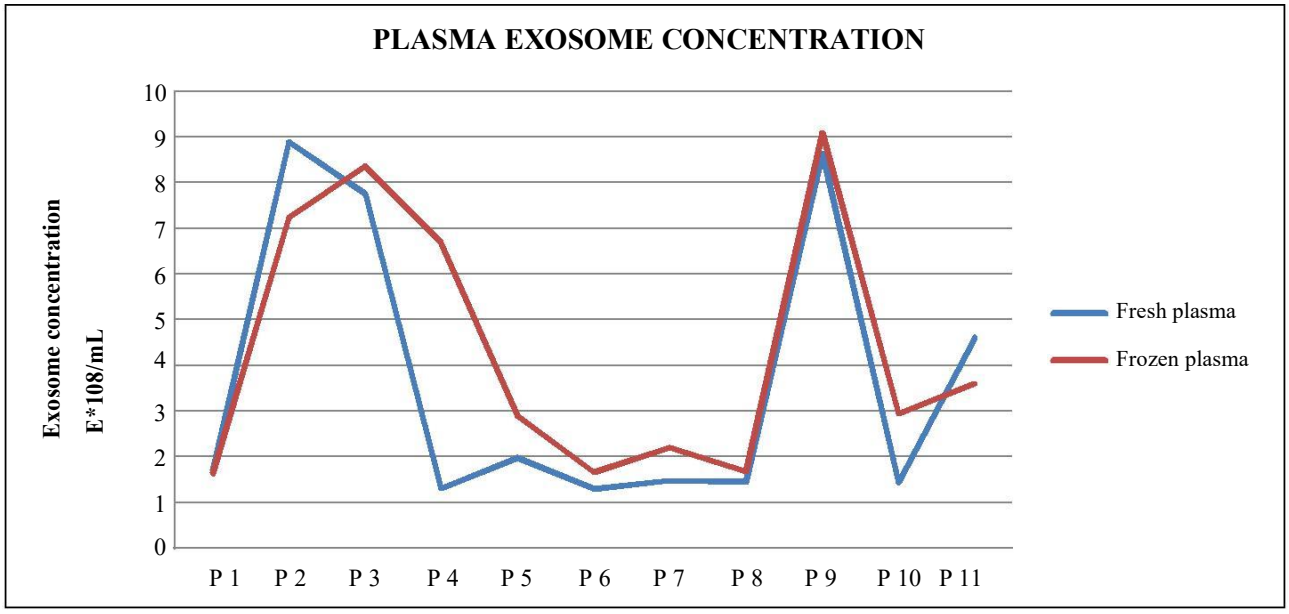



**Fig 2 shows the number of exosomes which were measured under different conditions (fresh and frozen sample).**

**Table 1 Subjects characteristics.**

| Number of subjects | 11 |
|---|---|
| Age (years) | 27.6 ± 4.2 |
| Height (cm) | 177.2 ± 9.4 |
| Weight (kg) | 80.3 ± 12 |
| Blood pressure (mm Hg) | 130/73 ± 7.37 |
| Heart rate (beat/min) | 90 ± 30 |

*Data is reported as mean ± standard deviation (SD).

**Table 2 Within sample, between and within day measures of plasma exosomes concentration.**

| | ANOVA *P VALUE* | ICC | 95% CONFIDENCE INTERVALS (CI) | Coefficient of variation (CV) % |
|---|---|---|---|---|
| Within Sample | 0.67 | 0.88 | 0.24-0.9 | 42 |
| Between Day | 0.42 | 0.84 | 0.827-0.926 | 34 |
| Within Day | 0.95 | 0.75 | 0.457-0.924 | 30 |

CV showed as SD/Mean × 100.

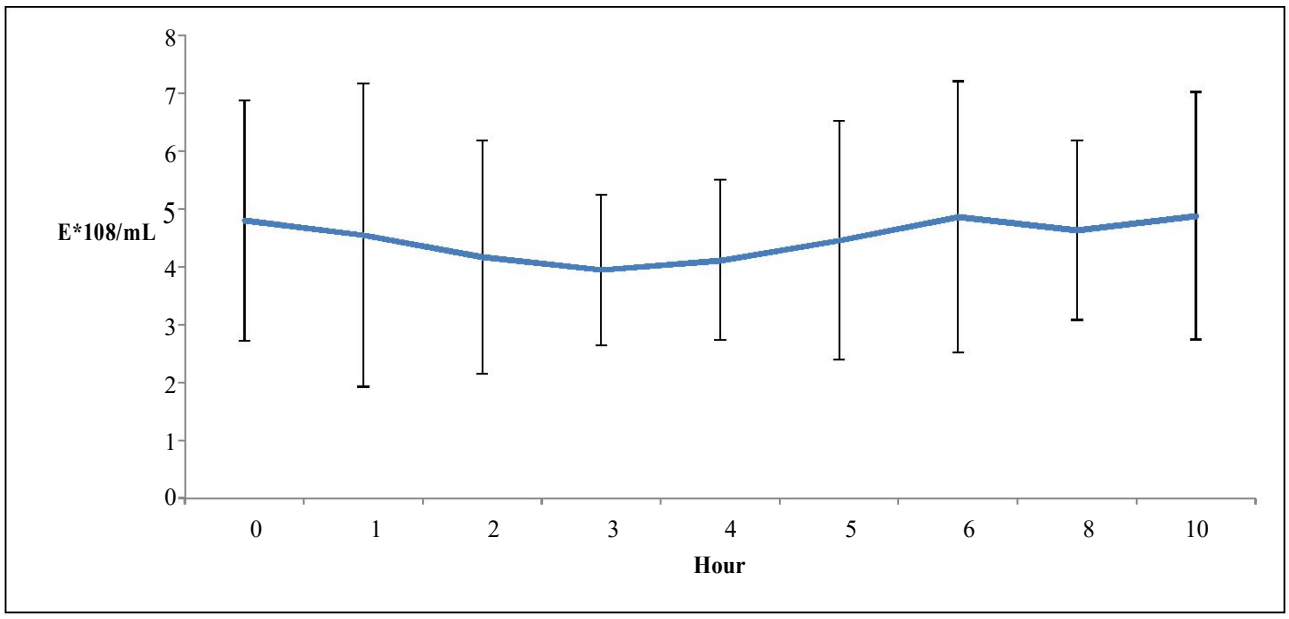



**Fig. 2 Plasma exosome concentration of nine samples within one day over many hours showing the average of eleven participant samples, each replicate was measured five times on the Nanosight ( represented as mean ± standard deviation).**

The results from single level (sample-variation) of a repeated measures ANOVA test was performed in this study show that there was no significant difference between the three different repetitions of blood collection ($P$ = 0.676). The reliability of plasma exosomes measures showed good within samples reliability (ICCs ≥ 0.88) and minimal variability within sample (Coefficient of variation CV = 42) (Table 2). Therefore, our results show good and reliable results in plasma exosomes concentration within consecutive samples.

Reliability and variability of plasma exosome concentration and size within the same subject at multiple time points of the day

To assess within day reliability of exosomes concentration we measured plasma vesicles over 10 hours from each subject. The size distribution for exosomes was also measured within a day at different time points and also between days over five consecutive days in healthy individuals.

A single level repeated measures ANOVA test was performed in this study. The results show that there was no significant differences at any time point ($P$ = 0.95). The average exosomes values were $2.24 \times 10^{\wedge 8}$/mL for all time points in all subjects (represented in Fig. 2). Moreover, the reliability of within day plasma exosomes measures showed a moderate reliability (ICCs ≥ 0.75, CV = 34%). For size distribution, the results shows that there was no change in the average size distribution at any time point of the day ($P$ = 0.014).

Reliability and variability of plasma exosome concentration and size over multiple days

The reliability and variability of plasma exosomes measures can be seen in Table 2, demonstrated good reliable measure of plasma exosome (ICCs ≥ 0.84)

**Table 2 Exosome concentration represented as mean ± standard deviation of averages of eleven subjects per each day over five consecutive days.**

| | Mean ± SD | *P* value |
|---|---|---|
| Number of subjects | 11 | |
| Average D1 | 1.52 ± 0.74 | *P* = 0.64 |
| Average D2 | 1.58 ± 0.96 | *P* = 0.63 |
| Average D3 | 2.53 ± 2.37 | *P* = 0.67 |
| Average D4 | 1.42 ± 0.84 | *P* = 0.57 |
| Average D5 | 1.94 ± 1.88 | *P* =0.50 |

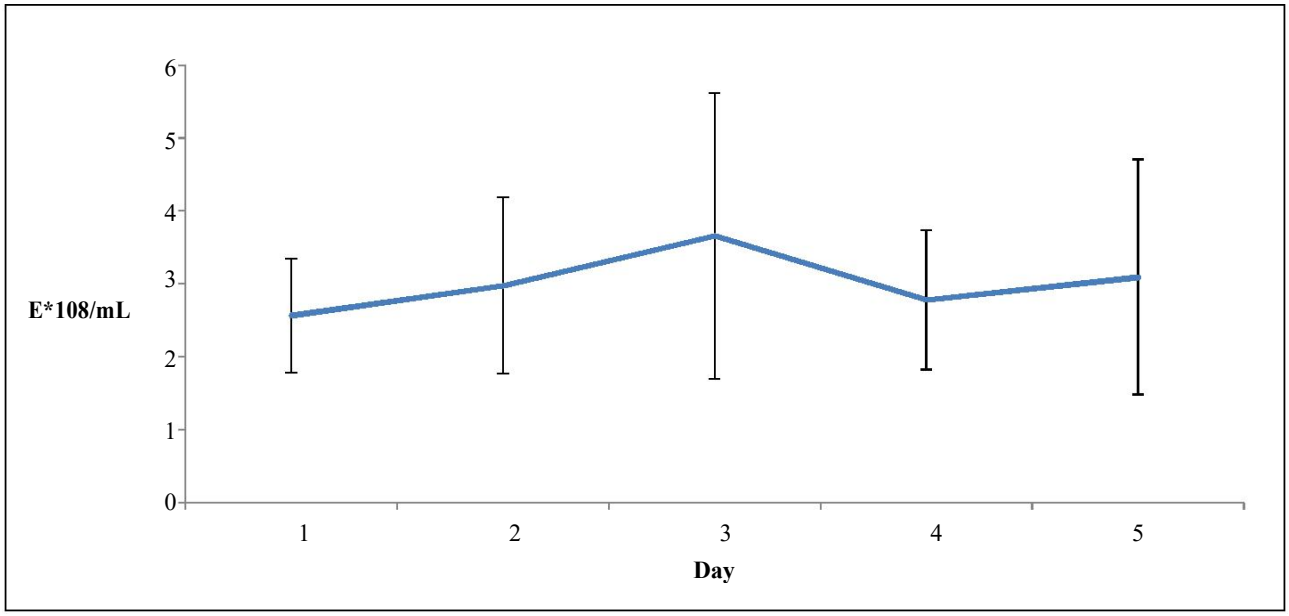



**Fig. 3 Plasma exosome concentrations taken from the average of eleven participant samples over five consecutive days, each replicate was measured five times on the Nanosight (represented as mean ± standard deviation).**

And CV = 30). Over five days the average exosomes values of each sample were 1.505 $\times 10^{\wedge 8}$/mL. There was no significant difference in between day concentrations ($P$ = 0.423). One-way analysis of variance (ANOVA) of the exosomes data also showed no significant effects differences between days (represented by the average value for each of the five days) is represented in Fig. 3. Moreover, there was no change in the average size distribution of all eleven subjects over the five consecutive days. These data represented in Table 2.

## 4. Discussion

In this study, we examined the within and between subject reliability of plasma exosome concentrations. As stated by Hopkins [26], that reliability refers to the reproducibility of values of a measurement in repeated trials on the same subject. However, Batterham and George [27] state that within subject variation, systemic change in the mean, and re-test correlations are the main measures of reliability. First, we examined the within sample reliability in three different samples withdrawn at the same time from each individual. There was no significant difference between the three different repetitions of blood collection ($P > 0.05$), which suggest that there is no significant variability explained by technical issues with blood sampling as it showed good reliability. Second, we examined the within day reliability through consequent measures of plasma exosomes over different time points (10 h monitoring). We found that there was no significant difference at any

time point, which suggests that exosomes are considerably stable within the day, also there was no significant differences between day measures. We found that average plasma exosomes concentration was ranged between 1.505-2.245 (vesicles × 10^8/mL).

Our results are demonstrated that measurement of plasma exosomes concentration is reliable and repeatable in healthy subjects. The ICCs were ≥ 0.8 for within sample and between day measures, which shows good reliable measures of plasma exosomes concentration. Whereas ICCs of ≥ 0.75 was moderate for within day reliability. We also examined the stability of plasma exosomes under different conditions. Our results showed that freeze condition had no effect on size and number of plasma exosome. These results are consistent with Kalra, Adda [28], they assessed the stability of exosomes in plasma under various storage conditions and over 90 days. In which fresh and frozen plasma samples were obtained from five healthy donors and then blood plasma was pooled and prepared for exosomes isolation. Furthermore, a study conducted by [29] also tested the stability of exosomes during freeze thaw cycles, however plasma samples were collected from healthy pregnant women. Further to exosomes isolation by differential and sucrose density gradient centrifugation, they also measured common proteins identified in exosomes isolated from fresh plasma and after freeze/thaw cycles [29]. Exosomes are stable after a freeze and thaw cycle in healthy women during pregnancy. Recently, several studies investigated the stability of exosomes and also circulating miRNA from exsomes in plasma. It is therefore indicate that exosomes can be good biomarkers according to their stability under various conditions [29]. Finally we analysed plasma exosomes based on particle size. Data were divided into two categories, vesicles size ranges between 30-100 nm terms as exosomes and microvescicles for vesicles size ranged between 101-1,000 nm. There was no change in the number of both extracellular vesicles. Also there was no significant change in exosomes size within the same subject at multiple time points of the day and over multiple days.

There were several limitations which may contribute to increased variability in the measurement of plasma exosomes. First, there was no dietary control and dietary record for what they had in the day before. However, we attempted to control this by asking all the participants to be in a fasted state and by collecting the blood samples at the same time of each day over the five consecutive days. Second, the variability may be due to the number of physiological fluctuations which associated with an impairment of some immune parameter's response [30]. Therefore, stress is one of the physiological factors that fluctuate from day to day which could be a possible cause for the variability in exosomes concentration over days. However, we attempted to minimise this by asking subjects to report to the laboratory in the same time every morning, in which blood collection was in rested state. Third, small sample size could be one of study limitations; however our results showed a good reliability of ICC.

## 5. Conclusions

This study has assessed the within sample, within day and between day reliability of plasma exosomes concentration in healthy male individuals. We showed that exosomes isolated from blood plasma are reliable and can be a good indicator to search for vesicular biomarker. Also, we showed that freeze condition had no effect on size and number of plasma exosome.

## Acknowledgments

I am grateful to my supervisors Professor Helen Dawes and Dr. Ryan Pink whom experience and continuous support helped to accomplish this work. I would like also to thank Dr. Johnny Collett for his continuous support and help in data analysis and study finalising. Also, I would like to thank the Saudi Cultural Bureau in London for their administrative

and financial support. Finally, I thank all participants in this study for their kind cooperation.

## References

[1] Azmi, A.S., B. Bao, and F.H. Sarkar, *Exosomes in cancer development, metastasis, and drug resistance: a comprehensive review.* Cancer and Metastasis Reviews, 2013. 32(3-4): p. 623-42.

[2] Théry, C., L. Zitvogel, and S. Amigorena, *Exosomes: composition, biogenesis and function.* Nature Reviews Immunology, 2002. 2(8): p. 569-79.

[3] Johnstone, R.M., et al., *Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes).* Journal of Biological Chemistry, 1987. 262(19): p. 9412-9420.

[4] Sadallah, S., C. Eken, and J. Schifferli, *Ectosomes as modulators of inflammation and immunity.* Clinical & Experimental Immunology, 2011. 163(1): p. 26-32.

[5] Lässer, C., M. Eldh, and J. Lötvall, *Isolation and characterization of RNA-containing exosomes.* JoVE (Journal of Visualized Experiments), 2012(59): p. e3037-e3037.

[6] Logozzi, M., et al., *High levels of exosomes expressing CD63 and caveolin-1 in plasma of melanoma patients.* PloS one, 2009. 4(4): p. e5219.

[7] Kobayashi, M., et al., *In vitro immunological and biological evaluations of the angiogenic potential of platelet-rich fibrin preparations: a standardized comparison with PRP preparations.* International Journal of Implant Dentistry, 2015. 1(1): p. 1.

[8] Ohno, S.-i., A. Ishikawa, and M. Kuroda, *Roles of exosomes and microvesicles in disease pathogenesis.* Advanced drug delivery reviews, 2013. 65(3): p. 398-401.

[9] Kharaziha, P., et al., *Tumor cell-derived exosomes: a message in a bottle.* Biochimica et Biophysica Acta (BBA)-Reviews on Cancer, 2012. 1826(1): p. 103-111.

[10] Taylor, D.D. and C. Gercel-Taylor, *MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer.* Gynecologic oncology, 2008. 110(1): p. 13-21.

[11] Ren, Y., et al., *Exosomal-like vesicles with immune-modulatory features are present in human plasma and can induce CD4+ T-cell apoptosis in vitro.* Transfusion, 2011. 51(5): p. 1002-1011.

[12] Khan, S., et al., *Plasma-derived exosomal survivin, a plausible biomarker for early detection of prostate cancer.* PloS one, 2012. 7(10): p. e46737.

[13] Raposo, G. and W. Stoorvogel, *Extracellular vesicles: exosomes, microvesicles, and friends.* The Journal of cell biology, 2013. 200(4): p. 373-383.

[14] Bobrie, A., et al., *Exosome secretion: molecular mechanisms and roles in immune responses.* Traffic, 2011. 12(12): p. 1659-1668.

[15] Théry, C., *Exosomes: secreted vesicles and intercellular communications.* F1000 Biol Rep, 2011. 3(15): p. 130.

[16] Stoorvogel, W., *Functional transfer of microRNA by exosomes.* Blood, 2012. 119(3): p. 646-648.

[17] Ündar, L., et al., *Circadian variations in natural coagulation inhibitors protein C, protein S and antithrombin in healthy men: a possible association with interleukin-6.* Thrombosis and haemostasis, 1999. 81(4): p. 571-575.

[18] Picotte, M., C.G. Campbell, and W.G. Thorland, *Day-to-day variation in plasma interleukin-6 concentrations in older adults.* Cytokine, 2009. 47(3): p. 162-165.

[19] Gardiner, C., et al., *Extracellular vesicle sizing and enumeration by nanoparticle tracking analysis.* Journal of extracellular vesicles, 2013. 2.

[20] Bala, S., et al., *Circulating microRNAs in exosomes indicate hepatocyte injury and inflammation in alcoholic, drug-induced, and inflammatory liver diseases.* Hepatology, 2012. 56(5): p. 1946-1957.

[21] Ploeger, H.E., et al., *The effects of acute and chronic exercise on inflammatory markers in children and adults with a chronic inflammatory disease: a systematic review.* Exerc Immunol Rev, 2009. 15(1): p. 6-41.

[22] Aoi, W., et al., *Muscle-enriched microRNA miR-486 decreases in circulation in response to exercise in young men.* Front Physiol, 2013. 4: p. 80.

[23] Théry, C., et al., *Isolation and characterization of exosomes from cell culture supernatants and biological fluids.* Current protocols in cell biology, 2006: p. 3.22. 1-3.22. 29.

[24] Rani, S., *MicroRNA profiling of exosomes isolated from biofluids and conditioned media.* RNA Mapping: Methods and Protocols, 2014: p. 131-144.

[25] Shrout, P.E. and J.L. Fleiss, *Intraclass correlations: uses in assessing rater reliability.* Psychol Bull, 1979. 86(2): p. 420-8.

[26] Hopkins, W.G., *Measures of reliability in sports medicine and science.* Sports medicine, 2000. 30(1): p. 1-15.

[27] Batterham, A.M. and K.P. George, *Reliability in evidence-based clinical practice: a primer for allied health professionals ☆.* Physical therapy in Sport, 2003. 4(3): p. 122-128.

[28] Kalra, H., et al., *Comparative proteomics evaluation of*

*plasma exosome isolation techniques and assessment of the stability of exosomes in normal human blood plasma.* Proteomics, 2013. 13(22): p. 3354-3364.

[29] Sarker, S., et al., *Placenta-derived exosomes continuously increase in maternal circulation over the first trimester of pregnancy.* Journal of translational medicine, 2014. 12(1): p. 1.

[30] Marcos, A., E. Nova, and A. Montero, *Changes in the immune system are conditioned by nutrition.* European journal of clinical nutrition, 2003. 57: p. S66-S69.

Founded in 1954

# AMERICAN COLLEGE of SPORTS MEDICINE



**Advanced Team Physician Course**
December 5-8, 2013
Las Vegas, Nevada

**ACSM Team Physician, Course, Part I**
February 19-23, 2014
San Diego, California

**ACSM's 10th Health & Fitness Summit & Exposition**
April 1-4, 2014
Atlanta, Georgia

**ACSM's 61st Annual Meeting, 5th World Congress on Exercise is Medicine® and World Congress on the Role of Inflammation in Exercise, Health and Disease**
May 27-31, 2014
Orlando, Florida

**ACSM Conference on Integrative Physiology of Exercise**
Fall 2014

**Mission Statement:** The American College of Sports Medicine advances and integrates scientific research to provide educational and practical applications of exercise science and sports medicine.


***May 31, 2014***

***TO WHOM IT MAY CONCERN:***

***Please allow this letter to serve as official confirmation that***

**Mrs. Eman Abu Seer**

***attended the 61st Annual Meeting of the American College of Sports Medicine, World Congress on Exercise is Medicine and World Congress on The Role of Inflammation in Exercise, Health and Disease held May 27-31, 2014 at the Orange County Convention Center, Orlando, Florida***

***If I can provide any further information, please contact me.***

***Sincerely,***

*Dawn M. Hamilton*

**Dawn M. Hamilton**
**Registration Coordinator**
**dhamilton@acsm.org**

AMERICAN COLLEGE of SPORTS MEDICINE
LEADING THE WAY

**Street Address:** 401 W. Michigan St.
Indianapolis, IN. 46202-3233 USA

**Mailing Address:** P.O. Box 1440
Indianapolis, Indiana, 46206-1440 USA

**Telephone:** (317) 637-9200
**FAX:** (317) 634-7817
**Web Site:** www.acsm.org
**Federal I.D. Number:** 23-6390952