# The Effect of Eccentric Exercise on Skeletal Muscle Release of Novel Molecules (Myokines) in Healthy Young Individuals

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## DECLARATION

I declare this thesis to have been solely written by myself in conjunction with being awarded a Masters by Research Degree (MRes) in physiology. Unless stated otherwise in the format of references both in text and figures, the thesis is entirely my own. Participant recruitment was conducted by myself and Dr Kostas Ttintzas, who also helped conduct medical screenings and particular protocols during experimental visits 1 and 2. The intravenous cannula inserted retrograde into the superficial hand vein of one arm for arterialised-venous blood sampling was conducted by Dr Ciaran Doherty and Dr Tariq Taylor, whom also conducted the muscle biopsy procedure. Two further cannulas were inserted into the common femoral vein of the exercised leg and the control (non-exercised) leg (using ultrasound guidance and the Seldinger technique) for deep venous blood sampling which was conducted by Dr Reza Ghasemi. The Doppler ultrasound for blood flow measurements was conducted by Dr Joanne Mallinson. In regards to muscle analysis, this was conducted solely by myself using the western blotting procedure. Prior to completing the analysis, the procedure was learnt through the guidance of Dr Scott Cooper and Mr Robbie Jones. Plasma samples were measured and analysed at Eli Lilly, Indianapolis by Dr Ricardo Samms.

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## ABSTRACT

The aim of this thesis was to test the hypothesis that maximal eccentric contractions would augment the release of novel myokines (including Fibroblast Growth Factor-21) from muscle tissue into systemic circulation due to the greater structural damage and increased inflammatory response normally associated with eccentric exercise. Furthermore, for the first time the active form of FGF-21 was measured in circulation in response to exercise in healthy humans.

Nine physically active young healthy males (age 25 ± 3.8 years, height 177.5 ± 7.24 cm, body mass 73.3 ± 11.8 kg, body mass index (BMI) 23.1 ± 2.78 kg/m$^2$) at baseline completed a series of functional tests to access maximal isometric strength and maximal isokinetic strength. All subjects then completed 3 sets of single-leg maximal eccentric contractions using their non-dominant leg, whilst the dominant leg served as a control. Each of the 3 sets consisted of 25 repetitions with a 5-minute rest period between each set. Subjects thereafter rested in a semi-supine position for 3-hours. Forty-eight hours after the end of the last set of maximal eccentric contractions, subjects returned to the lab and conducted the same muscle function tests. Prior to exercise, an intravenous cannula was inserted retrograde into the superficial hand vein of one arm for arterialised-venous blood sampling for the determination of glucose, lactate, total FGF-21, active FGF-21 and Fibroblast activation protein (FAP). Two further cannulas were inserted into the common femoral vein of the exercised leg and the control leg for deep venous blood sampling. Blood samples from each sampling line were obtained before and after each exercise bout and at regular intervals (every 20 minutes) during the 3-hour recovery period. Blood flow of the superficial femoral artery using Doppler ultrasound was also measured at the same time intervals. Muscle biopsy samples from the vastus lateralis were also taken at baseline (exercise and control legs), immediately post-exercise (exercise leg only), after 3-hours of recovery (exercise leg only) and 48-hours after exercise (exercise and control legs) for the determination of FGF-21 protein content.

The main findings from this study showed there was no significant increase in total or active FGF-21 secreted from skeletal muscle into the systemic circulation in response to exercise. Furthermore, skeletal muscle FGF-21 protein content showed no changes in response to eccentric contractions. However, there was a significant increase in arterialised and venous FAP concentrations, an enzyme that cleaves and inactivates FGF-21 during exercise, which may suggest that the release of FAP may occur via other tissues. In conclusion, the present study shows that single leg maximal eccentric contractions do not augment the release of total or active FGF-21 from skeletal muscle into systemic circulation in humans. It also raises a novel interesting possibility that FAP released into systemic circulation may play a role in the inactivation of FGF-21 during exercise.

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# CHAPTER 1: INTRODUCTION AND A REVIEW OF LITERATURE

## Implications of a sedentary lifestyle on health and disease

A sedentary lifestyle which consists of physical inactivity is now regarded as one of the most common causes of death worldwide (Booth *et al.*, 2012). Numerous chronic diseases for example metabolic conditions, cardiovascular and pulmonary diseases, cancers and immune system disorders are all associated with a sedentary state (Handschin & Spiegelman, 2008). The most common clinical consequences are metabolic conditions which refer to obesity, dyslipidaemia, type 2 diabetes mellitus (T2DM) and hypercholesterolemia (Blair & Church, 2004). Insufficient exercise resulting from a sedentary lifestyle is considered a major risk factor for numerous chronic pathologies. Additionally, it has been suggested that inadequate physical activity many lead to or contribute to the detrimental effects of dementia (Rovio *et al.*, 2005) and neurodegenerative disorders (Booth *et al.*, 2002).

Physical inactivity and obesity are key determinates of chronic systemic inflammation. The inflammation of particular tissues is associated with the development of numerous chronic diseases (**Figure 1, adopted from Handschin & Spiegelman, 2008**). The accumulation of adipocytes, which results in obesity, signals the release of pro-inflammatory cytokines such as tumour-necrosis factor-a (TNF-a) and interleukin 1-β (IL-β) which aid in the development and progression of T2DM and insulin resistance (Hotamisligil & Spiegelman, 1994; Hotamisligil, 2006). Organs such as skeletal muscle and the liver also contribute to the secretion of pro-inflammatory cytokines into the circulation (Esser *et al.*, 2014). The reduced

expression of peroxisome proliferator-activated receptor-γ coactivator-1a (PGC-1a) in skeletal muscle fibres, in the context of a sedentary state, is linked to systemic chronic inflammation and a risk of numerous metabolic diseases (Handschin, 2009). The decreased expression of PGC-1a through inactive muscle may also have a detrimental effect on tissues such as pancreatic β-cells, which involve insulin secretion. PGC-1a suppresses the release of nuclear factor $_{K}$B ($NF_{k}B$), which seems to be a master switch in regulation of pro-inflammatory gene expression (Eisele *et al.*, 2013) .Therefore an inactive muscle leads to the decreased PGC-1a expression, allowing increased release of $NF_{k}B$ which contributes to the increasing pro-inflammatory cytokine production proposing a higher risk of metabolic diseases. Furthermore, there might be adverse effects for distal organs and body homeostasis (Handschin & Spiegelman, 2008).

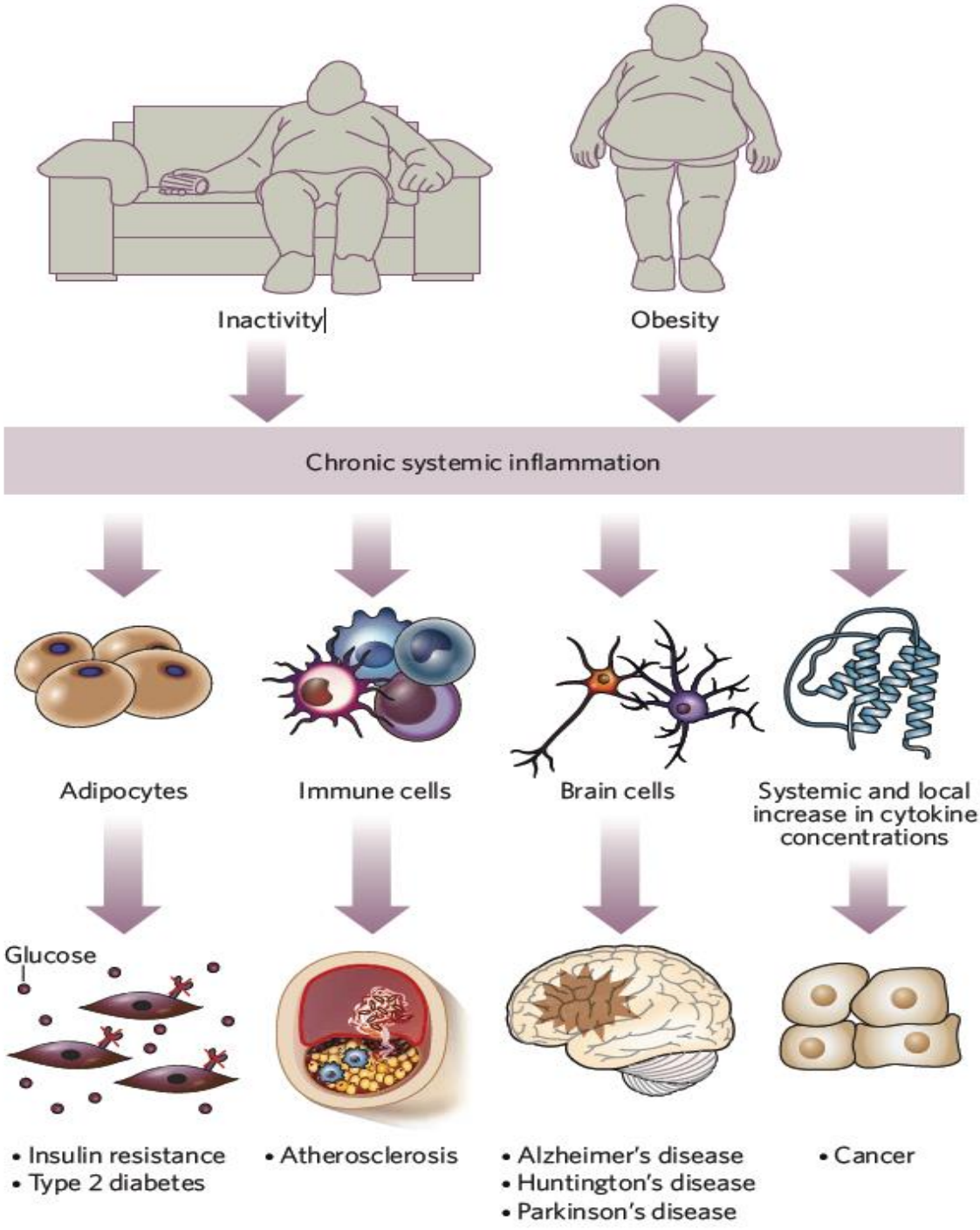



**Figure 1:** *Physical Inactivity, Obesity & Systemic Inflammation.* Physical inactivity and obesity result in chronic systemic inflammation. Furthermore, chronic diseases develop in particular tissues through the development of inflammation. Examples are shown above to the potential risks and consequences of inflammation causes by inactivity and obesity. The accumulation of adipocytes releases inflammatory cytokines which trigger the development of T2DM and insulin resistance. Atherosclerosis and neurodegenerative diseases are associated with an inflammatory immune response, whilst systemic and local increases in cytokine concentration are linked to cancer (Handschin & Spiegelman, 2008).

## The beneficial adaptations of skeletal muscle metabolism through regular exercise

Physical activity has been shown to serve as a great intervention in improving or preventing metabolic disorders in a range of populations (Blair & Church, 2004). In response to exercise, patients diagnosed with T2DM have shown increased insulin sensitivity in muscle and liver (Meex *et al.*, 2010), improved cardiorespiratory fitness and health (Stewart, 2002) and reduced dyslipidaemia (Balducci *et al.*, 2012). Moderate exercise conducted by rats have shown beneficial whole body adaptations, for example reduced hypertension (Kokkinos *et al.*, 2001), hyperglycaemia (Richter *et al.*, 1992) and hypercholesterolemia (Stone, 2006), whilst increasing expected life span (Holloszy *et al.*, 1985).

The effect of exercise therefore seems to transcend further than skeletal muscle adaptation. Although exercise directly affects contractile activity of skeletal muscle, metabolic alterations can also be elicited in distal organs such as the brain, liver, kidney and the heart. Research exploring the cross-talk between skeletal muscle and other organs remains poorly understood. However, possible mechanisms have been studied which could underlie the effect of physical activity and its relation to systemic health. An emerging mechanism is the endocrine ability of skeletal muscle to secrete particular proteins to add to systemic circulation. A recent study highlighted that acute exercise performed on one leg can enhance gene expression in the inactive leg, supporting the cross-talk between active and inactive organs (Catoire *et al.*, 2012). Most recently, it has become evident that these proteins secreted by skeletal muscle are referred to as myokines (Raschke *et al.*, 2013).

Myokines symbolise putative ‘exercise factors’ which have a variety of positive effects on chronic diseases and metabolic disorders (Pedersen & Febbraio, 2008). Accordingly, as physical inactivity causes chronic systemic inflammation, physical activity releases myokines through contracting skeletal muscle which influence metabolism whilst producing an anti-inflammatory response (Pedersen, 2009). Hence, the study of skeletal muscle cross-talk with other tissues such as adipose tissue, liver, gut and pancreas remains a large focus of research.

## Myokines

In recent years, over 50 novel myokines (Norheim *et al.*, 2011; Raschke *et al.*, 2013), signalling and inflammatory molecules (cytokines) released from skeletal muscle, have been identified and may be involved in the cross-talk between skeletal muscle, adipose tissue, liver and pancreas. Dysregulation of this cross-talk between tissues may have imperative implications for their metabolic regulation in the development of many chronic diseases (such as T2DM, obesity and dyslipidemia). Skeletal muscle is the largest organ of the body and can be labelled as an endocrine organ (**Figure 2 adopted from Schnyder & Handschin, 2015**); evidence is suggesting a number of myokines [such as Fibroblast Growth Factor-21 (FGF-21), Interleukin- 15 (IL-15) and Interleukin-6 (IL-6)] are secreted in response to exercise (Pedersen & Febbraio, 2012; Kim *et al.*, 2013b; Pierce *et al.*, 2015). In addition, an accumulation of literature provides strong evidence that exercise-induced myokines exert beneficial effects on fat oxidation (Pedersen & Febbraio, 2008), glucose uptake (Barra *et al.*, 2012) and glucose tolerance (Alvarez *et al.*, 2002). Given the beneficial effects of exercise as a treatment in helping to eradicate/or improve chronic diseases, it is not surprising that the autocrine, paracrine and endocrine effects of various myokines released during exercise are a large focus of research (Blair & Church, 2004). However, the molecular mechanisms involved in the secretion of these myokines by human skeletal muscle are poorly understood.

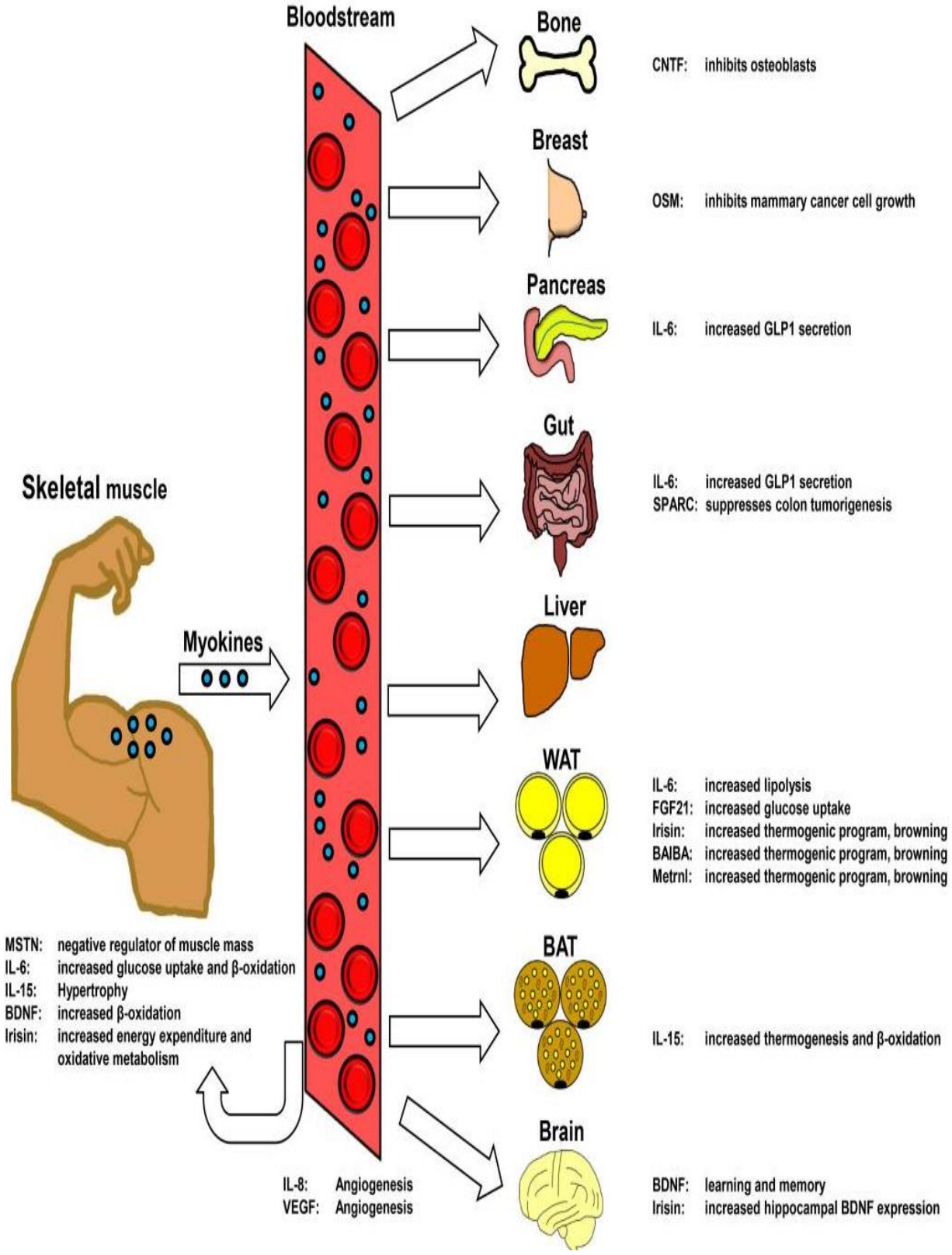



**Figure 2:** *Endocrine Skeletal Muscle Secretion of Myokines.* Accumulating evidence is suggesting that human skeletal muscle secretes myokines into the bloodstream (circulation) which exert autocrine, paracrine or endocrine effects to other tissues. The cross-talk between skeletal muscle and other tissues may have beneficial consequences in preventing diseases such as obesity, T2DM and dyslipidemia. The figure gives examples of the release of particular myokines and their systemic effect on other tissues (Schnyder & Handschin, 2015).

## Fibroblast Growth Factor-21 (FGF-21)

Isolation of cDNA subsequently unearthed the identification of a novel protein derived from the liver (Nishimura *et al.*, 2000). FGF-21 identified as the novel protein has to date been established as a key regulator of energy metabolism and plasma glucose homeostasis (Kharitonenkov *et al.*, 2005). The major site of FGF-21 production occurs through the liver but also by adipose tissue, pancreas and skeletal muscle and functions in a paracrine or hormone like fashion in these tissues (Tacer *et al.*, 2010). FGF-21 binds to the Fibroblast Growth Factor Receptor-βklotho (FGFR—βklotho) complex in turn inducing adipose tissue glucose uptake and fatty acid oxidation in the liver (Kharitonenkov & Larsen, 2011).

Studies involving humans and mice have shown that long-term fasting resulting in energy deficiency, increases levels of FGF-21 in the circulation (Galman *et al.*, 2008; Gaich *et al.*, 2013). In addition, mice genetically modified to overexpress FGF-21 had improved insulin sensitivity and were not affected by diet-induced obesity (Kharitonenkov *et al.*, 2005). Furthermore, pharmacological studies showed that FGF-21 administration serves as a potent treatment for monkeys and rodents with T2DM (Kharitonenkov *et al.*, 2007; Xu *et al.*, 2009a). Given the beneficial impact of FGF-21, research into the therapeutic role in metabolic disorders is gathering considerable interest (Dostalova *et al.*, 2009).

Similar to fasting, during exercise humans undergo a state of energy deficiency. Consequently, metabolic and hormonal signals are very similar in a long-term fasted state and a prolonged exercised state (Longuet *et al.*, 2008). During starvation, lipolysis regulates the release of free fatty acids via adipose tissue, which in turn

provide energy through fatty acid oxidation in peripheral tissues or the liver for ketogenesis. Lipolysis also occurs during exercise, through adipose tissue breakdown, which releases fatty acids which are used as energy fuel (Berglund *et al.*, 2010). FGF-21 seems to play a key role in ketogenesis in the liver, lipolysis and fatty acid oxidation (Badman *et al.*, 2007; Inagaki *et al.*, 2007).

Importantly, research shows acute exercise increases circulating levels of serum FGF-21 in humans (Berglund *et al.*, 2010; Kim *et al.*, 2013b). The reduction of plasma lipids, hepatic steatosis and hyperglycaemia are factors that are influenced by enhanced FGF-21 signalling and exercise (Pedersen, 2009). This augments a likely role of FGF-21 being a key advocate in metabolic improvement through exercise. These metabolic processes are proposed to occur in the liver and adipose tissue. Moreover, skeletal muscle has been proposed to secrete FGF-21 under particular physiological conditions with its expression regulated by the P13-kinase/Akt1 cascade. To support this theory, in Akt-1 transgenic mice, an increase of FGF-21 expression in skeletal muscle prevented diet-induced obesity and insulin resistance, indicating FGF-21 as a beneficial myokine that improves metabolic conditions (Izumiya *et al.*, 2008). Furthermore, skeletal muscle with autophagy deficiency and consequent mitochondrial dysfunction also stimulate FGF-21 production which protect mice from obesity and insulin resistance (Kim *et al.*, 2013a). Thus, the referral of FGF-21 as a myokine leads to the possibility that, human skeletal muscle also has the ability to add to the systemic levels of FGF-21 through exercise (Keipert *et al.*, 2014).

## Fibroblast Activation Protein (FAP)

As mentioned in the previous section, FGF-21 plays an important role in combating diabetes and obesity, with pharmacological doses on animals showing improvements in insulin sensitivity and the reduction of adipose tissue (Markan & Potthoff, 2016). These findings have also been translated into human studies, which show FGF-21 administration to improve metabolic disorders as well as drastically reducing adipose tissue mass in obese individuals (Gaich *et al.*, 2013). Most recently, considerable effort has been made to identify the mechanisms involved in the actions of FGF-21, to provide efficient therapeutic strategies to treat metabolic disorders.

As FGF-21 enters circulation, in order to function, FGF-21 signals to specific tissues which express two common fibroblast growth factor receptors (FGFRs), co-receptor βklotho and fibroblast growth factor receptor 1 (FGFR1) (Tacer *et al.*, 2010). The structure of FGFRs contain an intracellular tyrosine kinase domain, extracellular ligand-binding domain and a single transmembrane domain. In total there are four FGFRs with a splice variable (b and c) for FGFR 1-3 (Owen *et al.*, 2015). FGFR1 is expressed in a wide variety of tissues, while βklotho is expressed in only some metabolic tissues which include the liver, white adipose tissue (WAT) and brown adipose tissue (BAT) presenting more accurate FGF-21 signalling. The interaction between FGF-21 and FGFR1 occur via the N-terminus, whereas βklotho occurs through the C-terminus. Moreover, it has been shown that the C-terminus site of FGF-21 is important in activating the receptor complex in order to trigger signalling (Micanovic *et al.*, 2009).

Importantly, the activity and stability of FGF-21 in circulation and the mechanisms regulating this remain largely scarce. Exogenous administration of FGF-21 has a relatively short half-life, approximately lasting between 0.4-2 hours based on the manner of delivery and the species involved (Kharitonenkov *et al.*, 2007; Xu *et al.*, 2009b; Huang *et al.*, 2013). Studies using animal models have shown that human FGF-21 (hFGF-21) which contain the full length 181 amino acids is cleaved at the C-terminus between $Ser^{172}$ and $Pro^{171}$ (Hecht *et al.*, 2012; Hager *et al.*, 2013). Moreover, studies that have initiated the breakdown of FGF-21 proteins have shown that at the C-terminus the interaction between FGF-21 and βklotho is disrupted, ultimately preventing the ability of FGF-21 to signal to specific tissues (Micanovic *et al.*, 2009; Yie *et al.*, 2009). Notably, a specific mutation occurs around the $Pro^{171}$ region which eradicates the breakdown process of FGF-21 and extends the protein half-life (Hecht *et al.*, 2012). Research indicates a protease triggers the breakdown process and if further established could potentially be supressed to increase endogenous active FGF-21 levels.

A recent paper sought to investigate the specific protease that is responsible for the breakdown and inactivation of FGF-21 in circulation. Fibroblast activation protein (FAP) is described as a serine protease that consists of enzymes that are associated with metabolic disorders. The authors used mass spectrometry to show that hFGF-21 is cleaved at 3 points which include $pro^{2}$, $pro^{4}$ and $pro^{171}$. Importantly, FGF-21 function was impaired only after being cleaved at $pro^{171}$. The study concluded that FAP inactivates active FGF-21 and novel approaches need to be identified in order to target FAP which may prove beneficial in combating metabolic diseases (Zhen *et*

*al.*, 2016). Another research group found similar findings that further amplified the role FAP plays in inactivating FGF-21. Pharmacological administration of FAP cleaved FGF-21 at the $pro^{171}$ region and prevented the release of FGF-21 in systemic circulation in mice. The group extended current knowledge by inhibiting FAP in monkeys which showed a 3-fold increase in endogenous FGF-21 (Dunshee *et al.*, 2016). Collectively, both groups show interesting findings which indicate that FAP is responsible for inactivating FGF-21 in circulation and novel strategies need to be investigated in order to target FAP.

**Skeletal muscle secretion of FGF-21 in response to exercise**

In recent years, serum FGF-21 levels have been investigated in human exercise studies, with divergent outcomes. A study examined 40 non-diabetic obese women and the effect of a 3-month exercise intervention on circulating FGF-21 levels (Yang *et al.*, 2011). Aerobic exercise consisted of 45 minutes at 60-70% of age predicted maximum heart rate and resistance exercise for 20 minutes, both of which were conducted 5 times a week for 3 months. This particular exercise intervention resulted in a 40% decrease in serum FGF-21 levels. Simultaneously, a 2-month endurance study on 11 non-diabetic obese men showed no changes in both FGF-21 serum levels and skeletal muscle mRNA expression (Besse-Patin *et al.*, 2014). Interestingly, 2 weeks of treadmill exercise using the Bruce protocol showed a 1.7-fold increase in circulating FGF-21 levels in sedentary young women. In addition, an acute bout of endurance exercise at 50 and 80% of $VO_{2max}$ enhanced levels of circulating serum FGF-21 by 2 fold in healthy male subjects. Furthermore, the same research group observed no increased expression of FGF-21 mRNA in the

gastrocnemius and soleus muscles in mice, whereas a significant increase was reported in hepatic FGF-21 mRNA production (Cuevas-Ramos *et al.*, 2012). Consequently, the authors proposed that during exercise, the liver is the main source of FGF-21 production, which is probably triggered by exercise-induced lipolysis. The proposed mechanism suggests adipose tissues releases FFAs through exercise-induced lipolysis, which subsequently increase the expression of PPARα in the liver, ultimately resulting in enhanced hepatic FGF-21 production (Kim *et al.*, 2013b). However, the discrepancies between the aforementioned studies may be partly explained by the differences in subject population, age, obesity status, exercise volume and intensity.

It has been suggested FGF-21 is elevated after exercise in both humans and rodents with beneficial metabolic adaptations (Berglund *et al.*, 2010; Cuevas-Ramos *et al.*, 2010). However, the secretion of FGF-21 by human skeletal muscle and the mechanism involved are currently incompletely understood. Furthermore, the majority of studies that have investigated the effects of FGF-21 release from human skeletal muscle have used sub-maximal exercise involving mainly concentric contractions.

A recent study sought to address the origin and regulation of FGF-21 production during exercise. Ten healthy males took part in 2 hours of cycling at 60% $VO_{2max}$. Catheters were placed in their hepatic vein and brachial artery of the non-dominant arm to allow venous and arterial blood sampling during and after exercise. In addition, further 9 males took part in 2 hours of single leg kicking at 50% of their maximum workload. Catheters were placed in the femoral artery and the femoral

vein of the exercising and resting leg. This invasive technique allowed the research group to study the net-flux of FGF-21 secretion in both skeletal muscle and the splanchnic bed using arterio-venous differences in FGF-21. It was shown that at rest and during exercise the splanchnic circulation is the origin of FGF-21 secretion, in contrast skeletal muscle did not contribute to the secretion or circulating levels of FGF-21 during exercise. The study concluded that under normal physiological circumstances, skeletal muscle does not secrete FGF-21 in humans (Hansen *et al.*, 2015). However, it should be highlighted that the study used 50% maximum workload with one legged exercise, which is less demanding and effective on metabolic systemic stress in stimulating the release of FGF-21, compared to two legged exercise with higher maximum workload (Hansen *et al.*, 2015). A recent study sought to investigate if acute exercise resulted in an increase in circulating serum FGF-21 levels and if metabolic organs, specifically skeletal muscle induced the release of FGF-21. 19 sedentary men took part in 60 minutes of cycling at 75% $VO_{2max.}$ Venous blood samples were taken from the antecubital vein of the arm before and after exercise. The second part of the study involved male mice being split into two groups, a sedentary group and an exercise group. After an overnight fast, the exercise group ran on the treadmill for 60 minutes at a speed of 30m $min^{-1}$. Immediately after, mice were killed and skeletal muscle, liver and blood samples were taken. The results demonstrate that acute exercise in both humans and mice showed an increase in serum FGF-21 levels. Moreover, there was an increase in protein expression of FGF-21 in skeletal muscle and the liver following acute exercise in mice. The results showed that acute exercise increases circulating serum FGF-21 levels and FGF-21 expression in metabolic organs. Even though data did

not show that serum FGF-21 is directly induced by skeletal muscle, evidence does suggest that the increase in skeletal muscle protein may contribute to the overall systemic effects of circulating FGF-21 (Tanimura *et al.*, 2016). The study highlighted some encouraging findings, however research still needs clarification on whether skeletal muscle can directly influence circulating FGF-21 levels

**Conclusion of literature review on myokine FGF-21**

In recent years, novel myokines have been investigated with regards to their role in metabolic disorders. Most importantly, researchers have investigated strategies in treating metabolic disorders through identifying stimuli that aid in the secretion of these myokines.

FGF-21, predominately expressed in the liver, is also produced in and secreted from skeletal muscle, and is a key regulator of energy metabolism and plasma glucose homeostasis. A pharmacological dose of FGF-21 in monkeys and rodents has served as a beneficial strategy in treating T2DM. Furthermore, endogenous FGF-21 production improves insulin sensitivity with anti-obesity effects in animals. Muscular contractions have been identified to be a key stimulus in inducing the release of FGF-21, by the liver, in humans. Studies have shown FGF-21 serum levels to be elevated after endurance exercise between 50-80% $VO_{2max.}$ Additionally, even under particularly less demanding muscular contractions, most of the release of FGF-21 into the systemic circulation occurs through the liver rather than skeletal muscle. Collectively, results based on the secretion of FGF-21 through skeletal muscle may suggest that FGF-21 is a stress-induced myokine rather than an exercise-induced myokine.

Interestingly, as most studies have used protocols consisting of sub-maximal exercise involving concentric contractions, it may be possible that increased exercise intensity may influence an alternative outcome. Despite the growing interest in this area, there is a paucity of studies investigating the effects of eccentric exercise on myokine release in humans. Eccentric exercise involves muscle lengthening whilst producing a force (Smith, 1991; Friden & Lieber, 1992) and, when executed by unaccustomed individuals, it is commonly accompanied with augmented (but revocable) impaired muscle strength, ultrastructural disruption to muscle architecture, muscle inflammatory response and delayed onset muscle soreness (DOMS) (Clarkson & Hubal, 2002). Interestingly, maximal eccentric contractions lead to greater muscle force generation, therefore training protocols involving eccentric contractions result in greater gains in muscle strength and mass in comparison to concentric contractions (Vogt & Hoppeler, 2014). Numerous studies have highlighted the metabolic benefits of eccentric training on young healthy individuals which augment muscular hypertrophy, enhanced skeletal muscle performance and a lower demand on the cardiopulmonary system in comparison to concentric training (LaStayo *et al.*, 1999; Perrey *et al.*, 2001). Furthermore these findings translate to the elderly population, which show eccentric exercise results in beneficial muscular adaptations coupled with less cardiopulmonary demands in comparison to concentric exercise (Vallejo *et al.*, 2006). Cytokines are released by the immune system in response to inflammation in the body, likewise myokines are released in response to inflammation in the muscle. Eccentric exercise causes more muscle inflammation compared to concentric exercise, there it can be hypothesised that there will be an increased release of myokines in response to eccentric training

as opposed to sub-maximal concentric training. This may have important metabolic implications for the use of exercise as medicine, in both healthy individuals and those with chronic metabolic disorders. Moreover, it should be noted that all present studies involving FGF-21 have only measured the total amount of FGF-21 in circulation. Data has shown that total FGF-21 in circulation may not represent a true value of the amount of active FGF-21 in circulation (Umberger *et al.*, 2014). The current thesis will be the first to measure FGF-21 in circulation in its activated format in response to exercise.

## Aims and Hypothesis

To date, most questions regarding myokines and their release during exercise have been based on evidence primarily involving sub-maximal exercise. Given the beneficial impact these myokines have on human metabolic disorders it seems imperative to study more demanding forms of exercise, in particular eccentric contractions. It has also been theorised that myokines exert an endocrine ability to affect other bodily organs. Although, thus has been proven in animal models evidence is largely scarce and not entirely consistent in humans. Therefore, the aim of this thesis was to test the hypothesis that performing maximal eccentric muscle contractions will augment the release of novel myokines (including FGF-21) from muscle tissue into the systemic circulation due to greater muscle structural disruption and increased inflammatory response following eccentric exercise.

# CHAPTER 2: MATERIALS AND METHODS

## Ethical Approval

The current study was approved by the University of Nottingham Medical School Research Ethics Committee in observance of the present regulations imposed by the Code of Ethics of World Medical Association (Declaration of Helsinki).

## Subjects

All subjects were young healthy males aged (n=9, age 25 ± 3.8 years, height 177.5 ± 7.24 cm, body mass 73.3 ± 11.8 kg, body mass index (BMI) 23.1 ± 2.78 kg/m$^2$), who were physically active individuals but unaccustomed to maximal eccentric exercise with a BMI of <28 kg/m$^2$. Subjects were recruited from The University of Nottingham (UoN) student/staff cohort and the general public and attended the laboratory on 4 separate occasions.

## Screening Visit

Prior to any participation, all subjects were fully briefed on all aspects of the study and thereafter signed an informed consent. They were also made aware of their right to withdraw during any time point of the study. Subjects arrived at the Greenfield Human Physiology Unit, Queens Medical Centre (QMC), University of Nottingham (UoN) where all subjects took part in an individual medical screening (visit 1). The medical screening consisted of subjects completing health and physical activity questionnaires, having a 12-lead electrocardiogram (ECG) and blood pressure measured, and a blood sample taken for routine screening. A 3-day food diary (2 weekdays and 1 weekend) was asked to be completed prior to the familiarisation

visit (visit 2) in order to provide the subject with a standardised diet that fits with their normal routine. Subject diets were analysed through Macro Diet version 9, which allowed us to provide a 2-day standardised diet providing 50% energy from carbohydrate, 30% from fat and 20% from protein.

**Familiarisation Visit**

All subjects attended a preliminary laboratory session (visit 2) to familiarise themselves with the exercise testing procedure following satisfactory completion of the medical screening. The exercise testing procedure consisted of a series of sub-maximal and maximal eccentric, concentric and isometric knee-extensor contractions with their non-dominant leg using an isokinetic dynamometer (HUMAC NORM, CSMi solutions, MA, US). Subject took part in a total of 5 sets, 1) 10 isokinetic concentric contractions at 60 degrees per second for functional measurements, 2) 10 isokinetic concentric contractions at 60 degrees per second for the assessment of maximum voluntary contractions (MVC), 3) 3 isometric concentric contractions at 60 degrees for 3 seconds, the last two reps used for the assessment of MVC, 4) 10 isokinetic eccentric contractions at 30 degrees per second for functional measurements, 5) 10 isokinetic eccentric contractions at 30 degrees per second for the assessment of MVC.

**Experimental Visit 1**

The outline of the experimental design is shown via **figure 3**. Following the familiarisation visit, all subjects attended the laboratory on 2 further occasions (main experimental visits) separated by 48 hours. On the first occasion (visit 3), subjects arrived at the laboratory at 08.00am following an overnight fast (10-12 hours),

having abstained from heavy exercise and alcohol and having consumed a standardised mixed diet (providing 50% energy from carbohydrate, 30% from fat and 20% from protein) for the previous 24 hours. Subjects were then asked to rest on a bed in a semi-supine position and an intravenous cannula was inserted retrograde into the superficial hand vein of one arm for arterialised-venous blood sampling for the determination of glucose, lactate, FGF-21 and FAP. The hand thereafter remained in a hand-warming unit (50-55$^{o}$C) with the hand and air temperature continuously monitoring the arterialised-venous blood drainage (Gallen & Macdonald, 1990). Two further cannulas were inserted anti-grade into the common femoral vein of the exercised leg and the control (non-exercised) leg (using ultrasound guidance and the Seldinger technique) for deep venous blood sampling. Furthermore, the cannulas were kept patent via a saline drip. Blood samples from each sampling line were obtained for baseline measurements, after each exercise bout and at regular intervals (every 20 minutes) during a 3-hour recovery period.

Following the collection of baseline blood samples, muscle biopsy samples were obtained from the vastus lateralis of both legs (control and exercised) using the needle biopsy technique for the determination of intramuscular protein levels of FGF-21 using Western blotting, respectively. Muscle biopsy samples were also taken immediately post-exercise and after the 3-hour recovery period from the exercise leg only during which subjects were rested on a bed in a semi-supine position. Blood flow of the superficial femoral artery using Doppler ultrasound (Toshiba, Aplio 300) was also measured at the same frequency. Differences between arterialised venous (from the hand) and deep venous (from both femoral veins)

myokine concentrations (along with regular measurements of blood flow of the superficial femoral artery using Doppler ultrasound) were used to determine the flux of myokines across both upper legs, whilst providing an index of the contribution of local muscle release (from both legs) and/or contraction per se (from exercised leg) to their levels in the general circulation.

Following completion of all for baseline measurements, subjects undertook a series of muscle function tests 10 minutes before the allocated exercise protocol to assess their (1) maximal isometric strength at a fixed (60 degrees from full extension) knee angle (best of 2 contractions) and (2) total work and peak torque attained during 10 maximal isokinetic concentric contractions of the knee extensors performed at 60 degrees per second. All subjects then performed 3 bouts of single-leg maximal eccentric muscle contractions using their non-dominant leg, whilst their dominant leg served as a control. Each exercise bout consisted of 25 contractions performed at a speed of 30 degrees per second with a 5-minute rest interval between each exercise bout. Subjects then rested on a bed in a semi-supine position for 3 hours. Thereafter, they were provided with a standardised carbohydrate-rich lunch (250g of pasta, 100g of tomato sauce and 40g of cheddar cheese) and asked to consume a standardised mixed diet providing 50% energy from carbohydrate, 30% from fat and 20% from protein for the next 48 hours. All subjects were then subsequently allowed to leave the laboratory.

**Experimental Visit 2**

Forty-eight hours after the end of the last bout of maximal eccentric muscle contractions, subjects returned to the laboratory (visit 4) after an overnight fast having abstained from heavy exercise and alcohol in the intervening period between visits 3 and 4. Upon arrival to the laboratory subjects were asked to rest on a bed in a semi-supine position and a single arterialised blood sample was obtained from a superficial hand vein. Following this, a muscle biopsy sample was obtained from the vastus lateralis of both legs (control and exercised). Subjects subsequently performed the same muscle function tests conducted during the experimental visit 3, using the previously exercised leg to assess the extent to which muscle function had been restored, following the performance of the eccentric protocol 48 hours beforehand. Following the completion of the 4th laboratory visit, subjects were provided with lunch and then allowed to leave the laboratory.

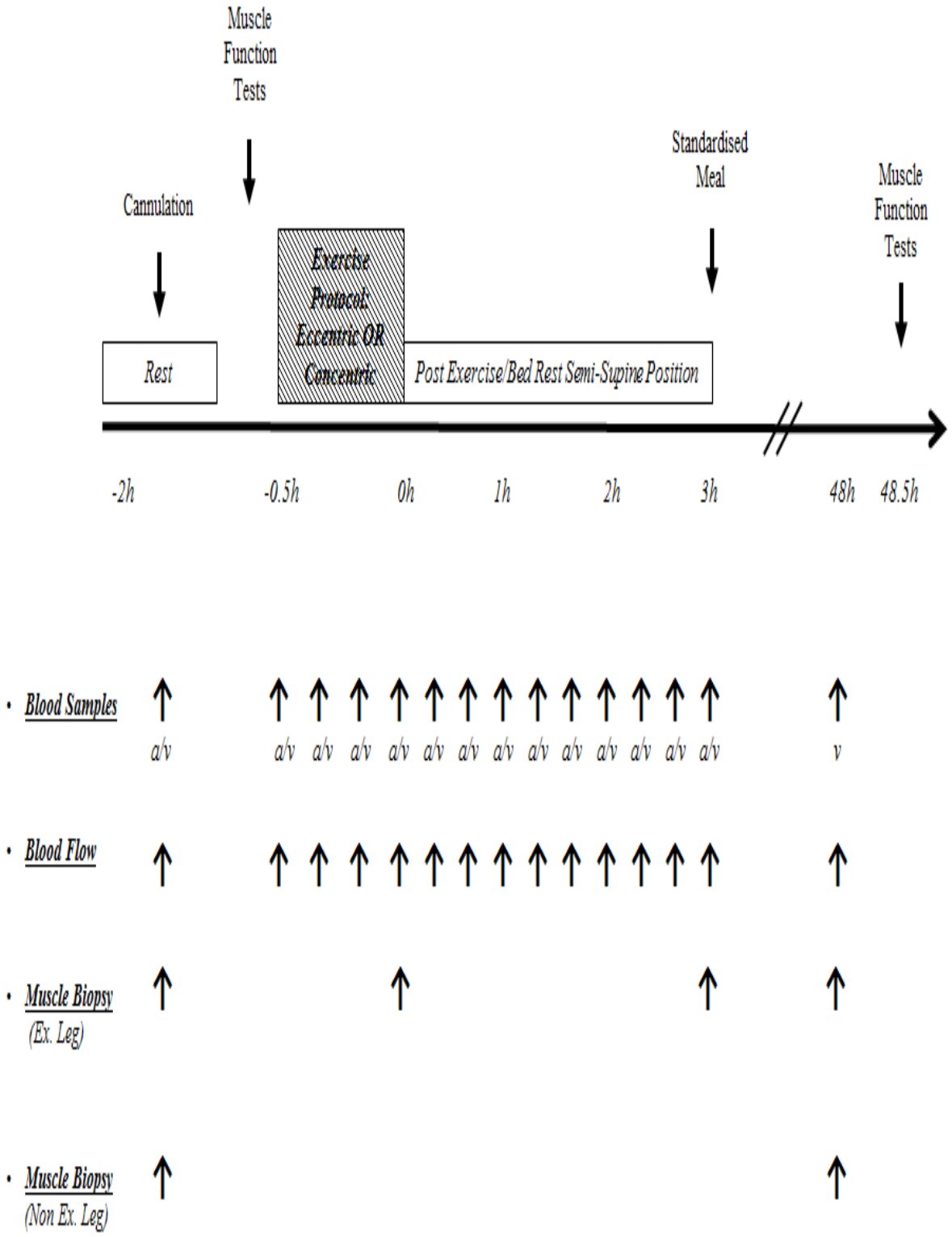



**Figure 3:** Schematic diagram outlining the experimental protocol.

**Analysis of Muscle Samples**

**Protein Extraction**. Protein extraction was conducted by adding 50mg of frozen tissue to 300ul of HEPES homogenisation buffer (SIGMA H4034) and protease inhibitor cocktail (SIGMA P-8340). Samples were then homogenised on a polytron at a medium speed for 30 seconds and left on ice for 20 minutes. Samples were then transferred into an eppendorf tube and centrifuged at 10,000g for 20 minutes at 4$^{o}$C. Supernatant was transferred into clean eppendorfs and SDS was then added. Each sample was added with water for protein quantification which was conducted by using Pierce bovine serum albumin protein assay and stored at -20 $^{o}$C.

**Western Blotting**. Protein separation was carried out using an electrophoresis tank (ATTO AE-6620) using a 14% gel which was thereafter transmitted onto a hydrophobic polyvinylidene difluoride membrane (PVDF) and left overnight. The PVDF membrane was then removed from the western blot, incubated in BSA blocking reagent and then left on the rocker for 1 hour at room temperature. FGF-21 (Eli Lilly, IN) primary antibody was diluted in 3% BSA and TBS-T forming a diluted ratio of 1:4000. The PVDF was then placed in a sealed bag and incubated over night at 4$^{o}$C. Following incubation, the PVDF membrane was soaked in TBS-T 3 times for 10 minutes to wash off non-specific bindings. Thereafter, the membrane was sealed in a bag and probed with a secondary antibody Actin (Cell Signaling) diluted in a 1:2000 dilution with anti-mouse horseradish peroxidase (HRP) and 1% blocking reagent for 1 hour on a shaker at room temperature. The PVDF membrane was then soaked in TBS-T 3 times for 10 minutes. Protein molecular weight bands were thereby made visible by soaking the PVDF membrane in Amersham ECL

prime (GE health care) for 5 minutes. Membranes were then placed in a hypercassette and developed in a dark room in order to identify immunoreactive proteins **(Appendices A)**.

**Plasma Blood Analysis**

The determination of blood lactate arterio-venous differences (a-v diff mmol/l) and blood glucose (mmol/l) a-v diff was analysed using a Yellow Springs Instrument (YSI, 2300, Stats Plus, Glucose & L-Lactate analyser). Plasma blood samples were centrifuged immediately, whereas serum blood was left to clot for 20 minutes after each time point, both were centrifuged at 10,000g for 10 minutes at 4$^{o}$C. Active FGF-21, total FGF-21 and FAP analysis was performed by Eli Lilly using in house antibody based ELISAs.

**Statistical Analysis**

Data analysis was carried out using GraphPad Prism 6 Software (GraphPad Software Inc., San Diego, CA). Baseline, western blot protein content (control leg), 48-hour isometric, isokinetic measurements including fatigue index were analysed using a paired sample t-test with Bonferroni corrections. Lactate, glucose, blood flow, western blot protein content (exercised leg), eccentric peak torque measurements and FGF-21 and FAP (total and active) were analysed by using a one-way repeated measures ANOVA using Bonferroni corrections. All data was reported using means ± SEM and statistical significance was accepted if $P < 0.05$.

# CHAPTER 3: RESULTS

## Exercise Contractions

Maximal isometric peak torque did not show a significant difference between baseline (206.4 ± 17.1 Nm) and 48-hours after exercise (201.2 ± 27.2 Nm) **(Figure 4A)**. Similarly, maximal isometric average torque did not indicate a significant difference between baseline (171.1 ± 13.6 Nm) and 48-hours after exercise (156.6 ± 21.3 Nm) **(Figure 4B)**.

Maximal isokinetic concentric – peak torque showed no significant differences between baseline (139.3 ± 17 Nm) and 48-hours post-exercise (122.2 ± 20 Nm) **(Figure 4C)**. Furthermore, isokinetic concentric total work output showed no significant difference between baseline (1488.8 ± 149.3 Nm) and 48-hours post-exercise (1325.9 ± 194.9 Nm) **(Figure 4D)**.

Maximal eccentric peak torque showed no significant difference between set 1 (86.8 ± 11.5 Nm), set 2 (74.6 ± 10.4 Nm) and set 3 (74.5 ± 10.6 Nm) **(Figure 4E)**. However, maximal eccentric power per repetition showed a significant difference in treatment between set 1 (20.3 ± 2.6 Nm), set 2 (16 ± 2.2 Nm) and set 3 (13.8 ± 1.9 Nm) with the main effect being P=0.0013 **(Figure 4F)**. There was a significant decrease of power between set 1 and set 2 ($P < 0.05$). Furthermore, a significant decrease was observed between set 1 and set 3 ($P < 0.01$). A paired sample t-test revealed no significant differences in the amount of fatigue experienced when conducting maximal isokinetic concentric contractions during baseline and 48-hours post-exercise.

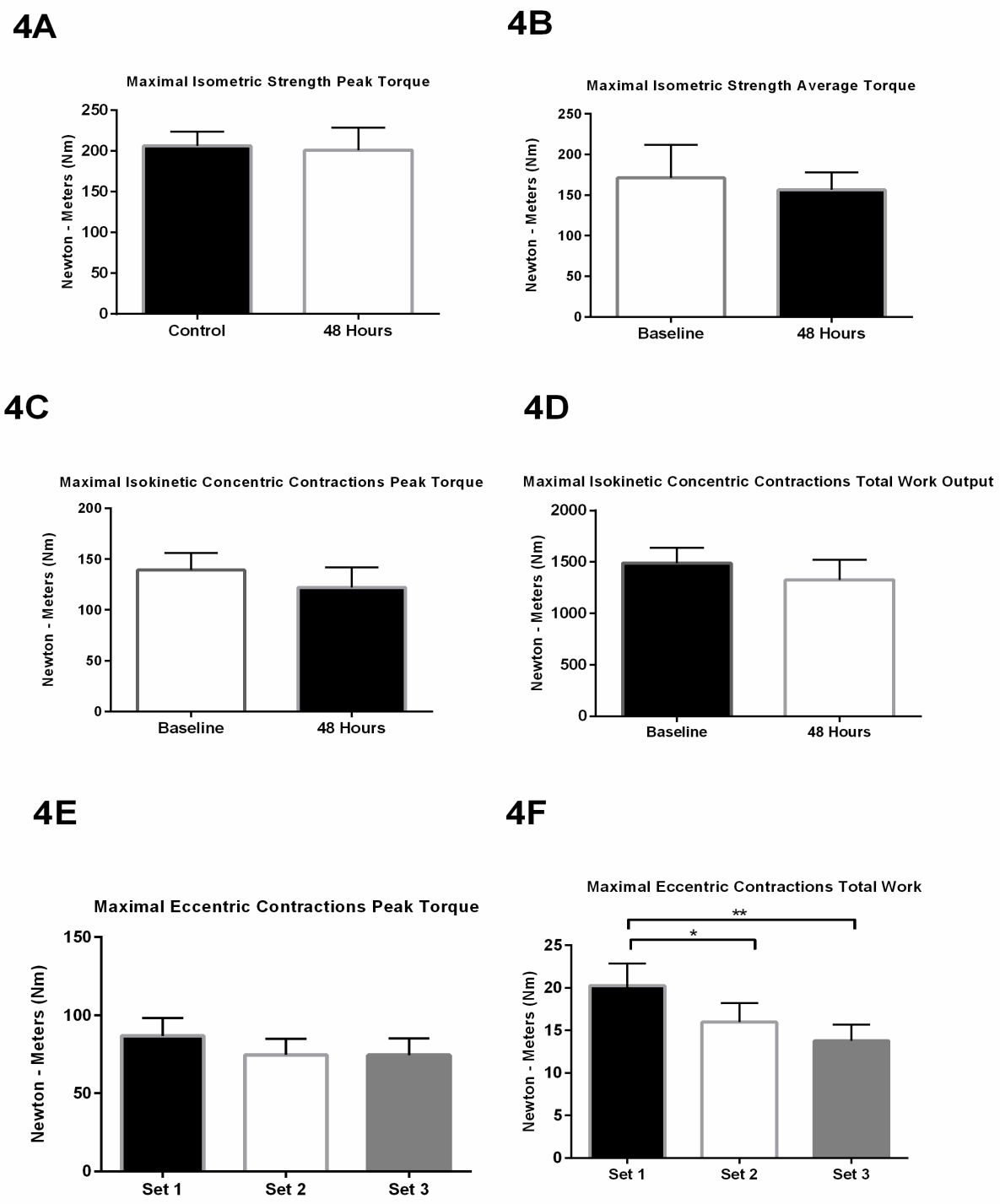



**Figure 4:** Maximal isometric strength peak torque (Nm) **(A)**, Maximal isometric strength average torque (Nm) **(B)**, Maximal isokinetic concentric contractions peak torque (Nm) **(C)**, Maximal isokinetic concentric contractions total work output (Nm) **(D)** reported at baseline, (pre-exercise) and 48-hours into the recovery period. Maximal eccentric contractions peak torque (Nm) **(E)** and maximal eccentric contractions total work output (Nm) after set 1, set 2 and set 3 with each set consisting of 25 repetitions **(F)**. Values represent means ± SEM. N=9, * denotes P <0.05 from baseline, ** denotes P <0.01 from baseline.

**Lactate and Glucose Blood Analysis**

A one-way ANOVA revealed a significant release of lactate from the exercised leg during exercise ($P < 0.001$). However, there was no release of lactate during the recovery period. A multiple comparisons test showed a significant difference between baseline ($0.03 \pm 0.07$ mmol/l, $P < 0.0001$) and set 1 (S1) ($-2.67 \pm 0.23$ mmol/l, $P < 0.0001$), set 2 (S2) ($-2.41 \pm 0.59$ mmol/l, $P < 0.0001$), set 3 (S3) ($-1.91 \pm 0.60$ mmol/l, $P < 0.0001$) **(Figure 5A).**

A-V differences of blood lactate in the control leg showed a statistically significant effect of time ($P < 0.05$). Set 1 values ($-1.87 \pm 0.71$ mmol/l) indicated a release of lactate in comparison to baseline values ($0.04 \pm 0.05$ mmol/l) ($P < 0.05$). However, there was no significant difference seen after set 1 and during the recovery period **(Figure 5B).** A-V differences of blood glucose in the exercised leg showed no significant differences between baseline ($0.15 \pm 0.16$ mmol/l) and all time points post-exercise **(Figure 5C).**

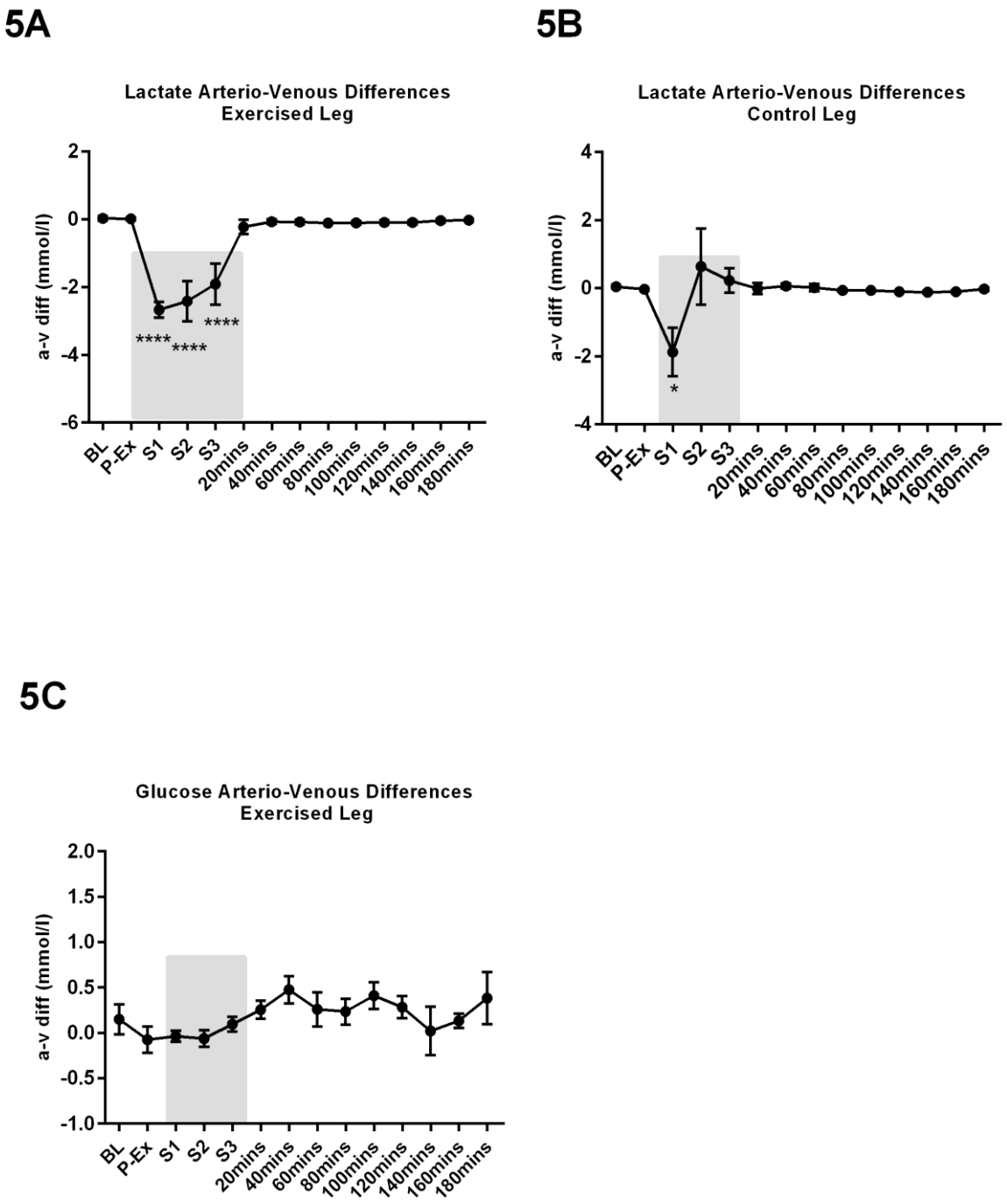



**Figure 5:** Effect of exercise on lactate arterio-venous differences of the exercised leg (mmol/l) **(A)**, and control leg (mmol/l) **(B)**, glucose arterio-venous differences of the exercised leg (mmol/l) **(C)**, at baseline, during exercise (sets 1,2,3) and subsequent recovery (20mins-180mins). Values represent means ± SEM. N=9, denotes * P=<0.05 from baseline, ** denotes P <0.01 from baseline, **** denotes P <0.0001 from baseline. The shaded grey area represents the exercise period (sets 1,2,3).

**Total Plasma FGF-21**

Total FGF-21 in arterialised blood showed no significant differences between baseline (176.9 ± 73.6 pg/ml) and all subsequent time points **(Figure 6A).** Total FGF-21 in venous blood (exercised leg) indicated no significant differences between baseline (74.9 ± 18.1 pg/ml) and all time points **(Figure 6B).** Total FGF-21 in venous blood (control leg) showed no significant differences between baseline (77 ± 27.8 pg/ml) and all time points **(Figure 6C).**

A one-way repeated measures ANOVA revealed no significant difference over time in A-V differences of total FGF-21 between baseline (-4.1 ± 1.4 pg/ml) and all time points **(Figure 6D).**

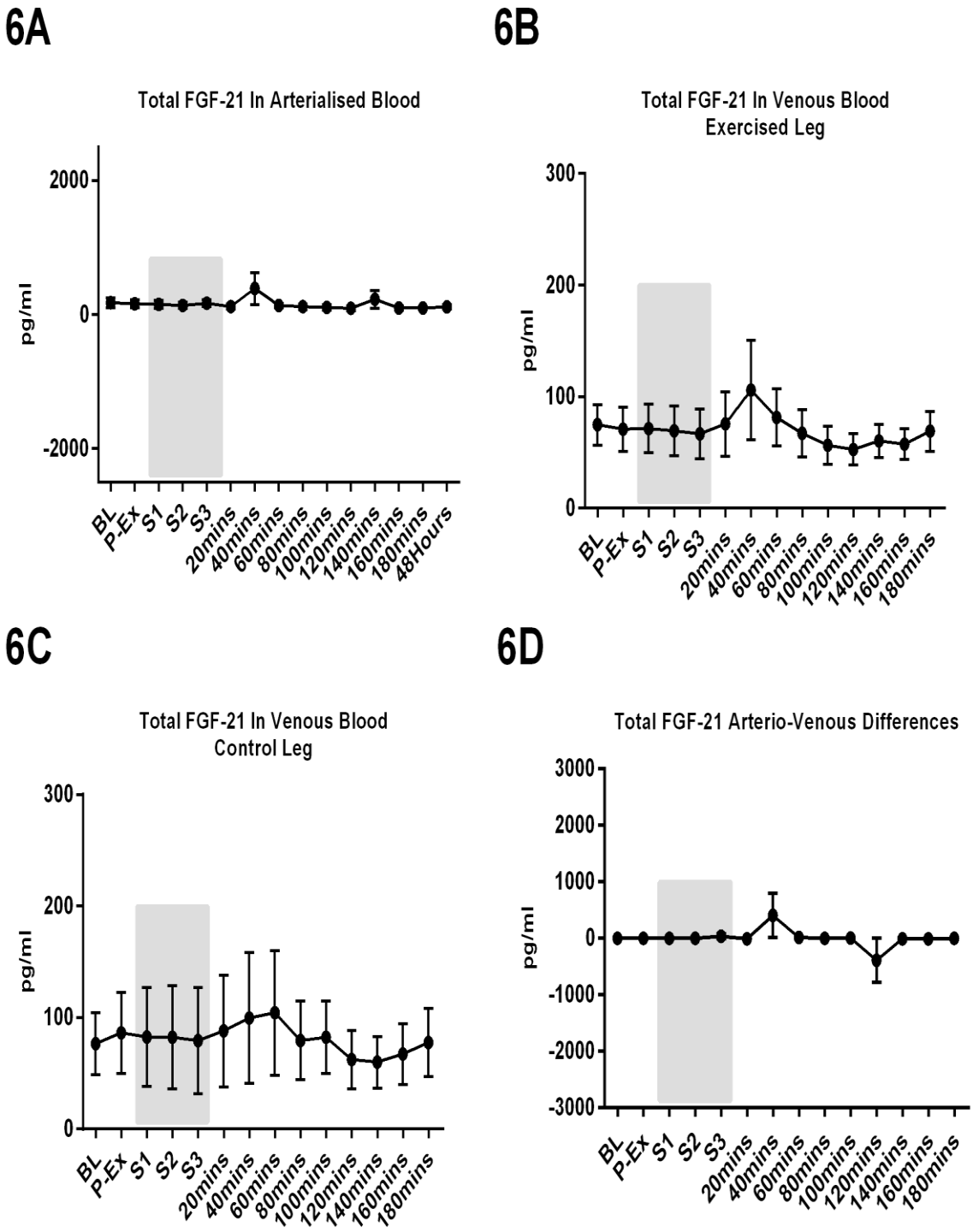



**Figure 6:** Effect of exercise on total FGF-21 concentration at baseline during exercise (sets 1,2,3) and subsequent recovery (20mins-180mins). Total FGF-21 in arterialised blood N=8 (pg/ml) **(A)**, Total FGF-21 in venous blood of the exercised leg N=6 (pg/ml) **(B)**, Total FGF-21 in venous blood of the control leg (pg/ml) N=3 **(C)**, Total FGF-21 arterio-venous differences of the exercised leg N=5 (pg/ml) **(D)** Values represent means ± SEM. No report of statistically significant values. The shaded grey area represents the exercise period (sets 1,2,3).

**Active Plasma FGF-21**

Active FGF-21 in arterialised blood showed no significant increase with time between baseline (68.5 ± 22.5 pg/ml) and all points **(Figure 7A).** Active FGF-21 in venous blood (exercised leg) showed no significant difference between baseline (39.5 ± 8.3 pg/ml) and all time points **(Figure 7B).** Active FGF-21 in venous blood (control leg) presented no significant difference between baseline (44 ± 4.1 pg/ml) and all time points **(Figure 7C).**

A one-way repeated measures ANOVA showed no significant differences over time in A-V differences of active FGF-21 between baseline (-3.6 ± 2.4 pg/ml) and all subsequent time points **(Figure 7D).**

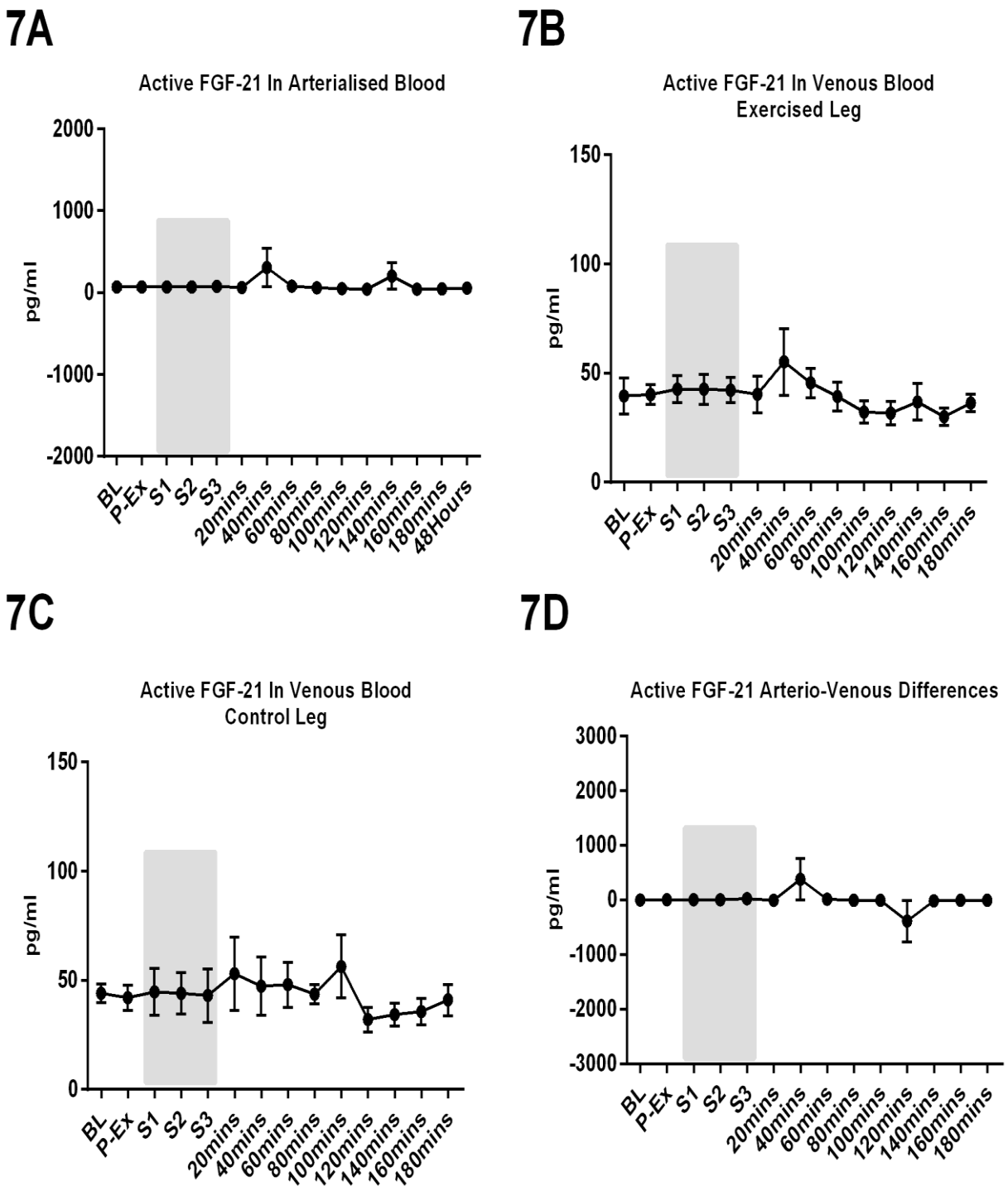



**Figure 7:** Effect of exercise on active FGF-21 concentration at baseline, during exercise (sets 1,2,3) and subsequent recovery (20mins-180mins). Active FGF-21 in arterialised blood N=8 (pg/ml) **(A)**, Active FGF-21 in venous blood of the exercised leg N=6 (pg/ml) **(B)**, Active FGF-21 in venous blood of the control leg N=3 (pg/ml) **(C)**, Active FGF-21 arterio-venous differences of the exercised leg N=5 (pg/ml) **(D)**. Values represent means ± SEM. No report of statistically significant values. The shaded grey area represents the exercise period (sets 1,2,3).

**% Active/Total FGF-21 Plasma**

Arterialised % active/total FGF-21 blood showed no significant differences between baseline (64.9 ± 20.7 %) and all subsequent time points **(Figure 8A).** Similarly, venous % active/total FGF-21 blood in the exercised leg showed no changes between baseline (75.2 ± 25.7 %) and all other time **(Figure 8B).** Furthermore, venous % active/total FGF-21 blood in the control leg showed no significant differences between baseline (81 ± 38 %) and all time points **(Figure 8C).** There were no differences observed in % active/total A-V differences of FGF-21 between baseline (0 ± 2.4 %) and all time points **(Figure 8D).**

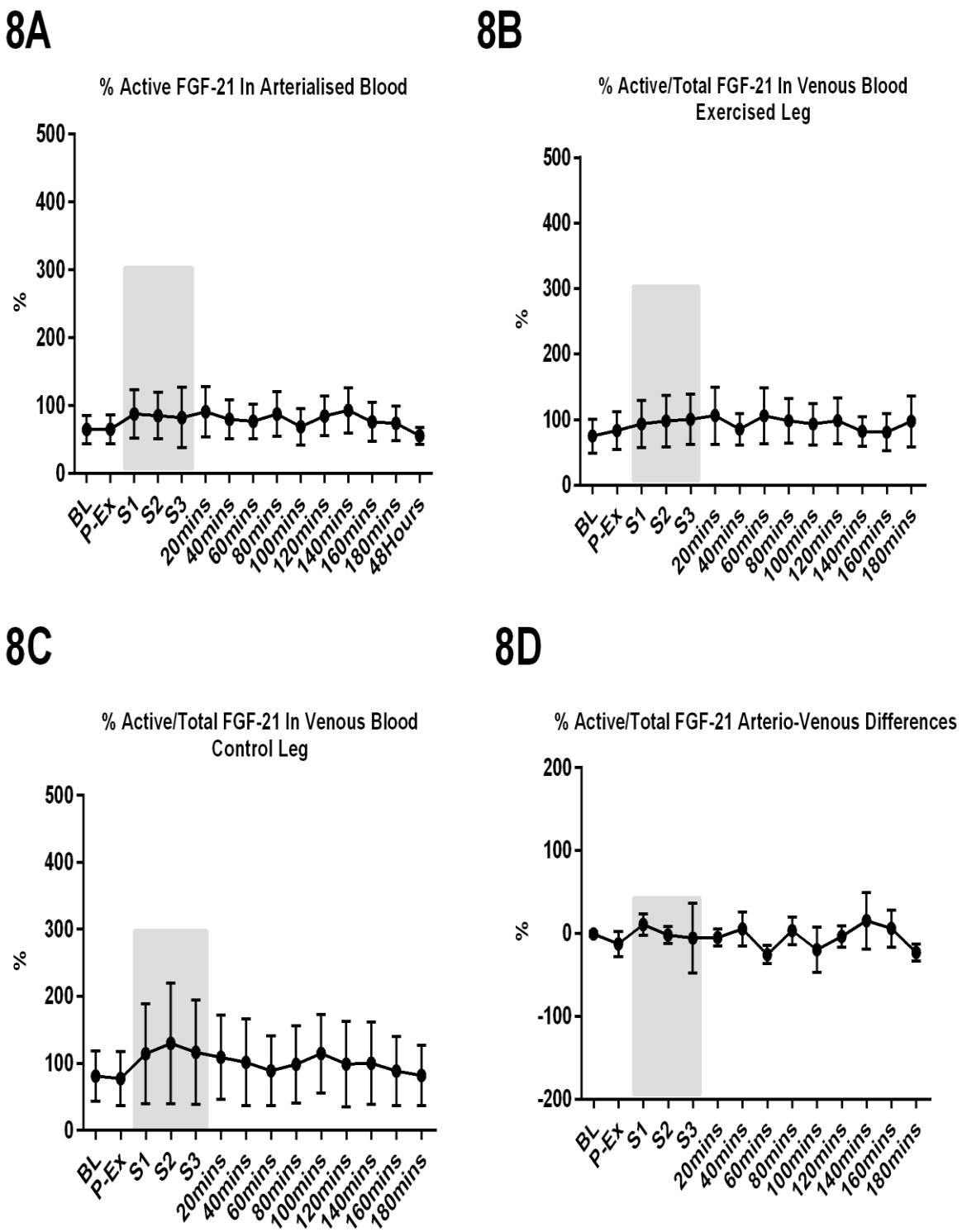



**Figure 8:** Effect of exercise on % Active/Total FGF-21 concentration at baseline, during exercise (sets 1,2,3) and subsequent recovery (20mins-180mins). % Active/Total FGF-21 arterialised blood N=8 %) **(A)**, % Active/Total FGF-21 venous blood of the exercised leg N=6 (%) **(B)**, % Active/Total FGF-21 venous blood of the control leg N=3 (%) **(C)**, % Active/Total FGF-21 arterio-venous differences of the exercised leg N=5 (%) **(D)**. Values represent means ± SEM. No report of statistically significant values. The shaded grey area represents the exercise period (sets 1,2,3).

**Fibroblast Activation Protein (FAP) Blood Analysis**

Arterialised FAP showed a significant increase during the exercise period ($P < 0.05$) **(Figure 9A)**. Similarly, venous FAP in the exercised leg also showed a significant increase during the exercise period ($P < 0.05$) **(Figure 9B)**. On the other hand, venous blood FAP in the control leg showed no significant differences between baseline (188.3 ± 18.4) and all subsequent time points **(Figure 9C).**

A one-way repeated measures ANOVA revealed no significant differences over time in A-V differences of FAP between baseline (7.2 ± 18.6 ng/ml) and all time point **(Figure 9D).** Furthermore, there was no release of FAP between baseline (-1352.04 ± 4699.31) and all subsequent time points in the exercised leg **(Figure 9E).**

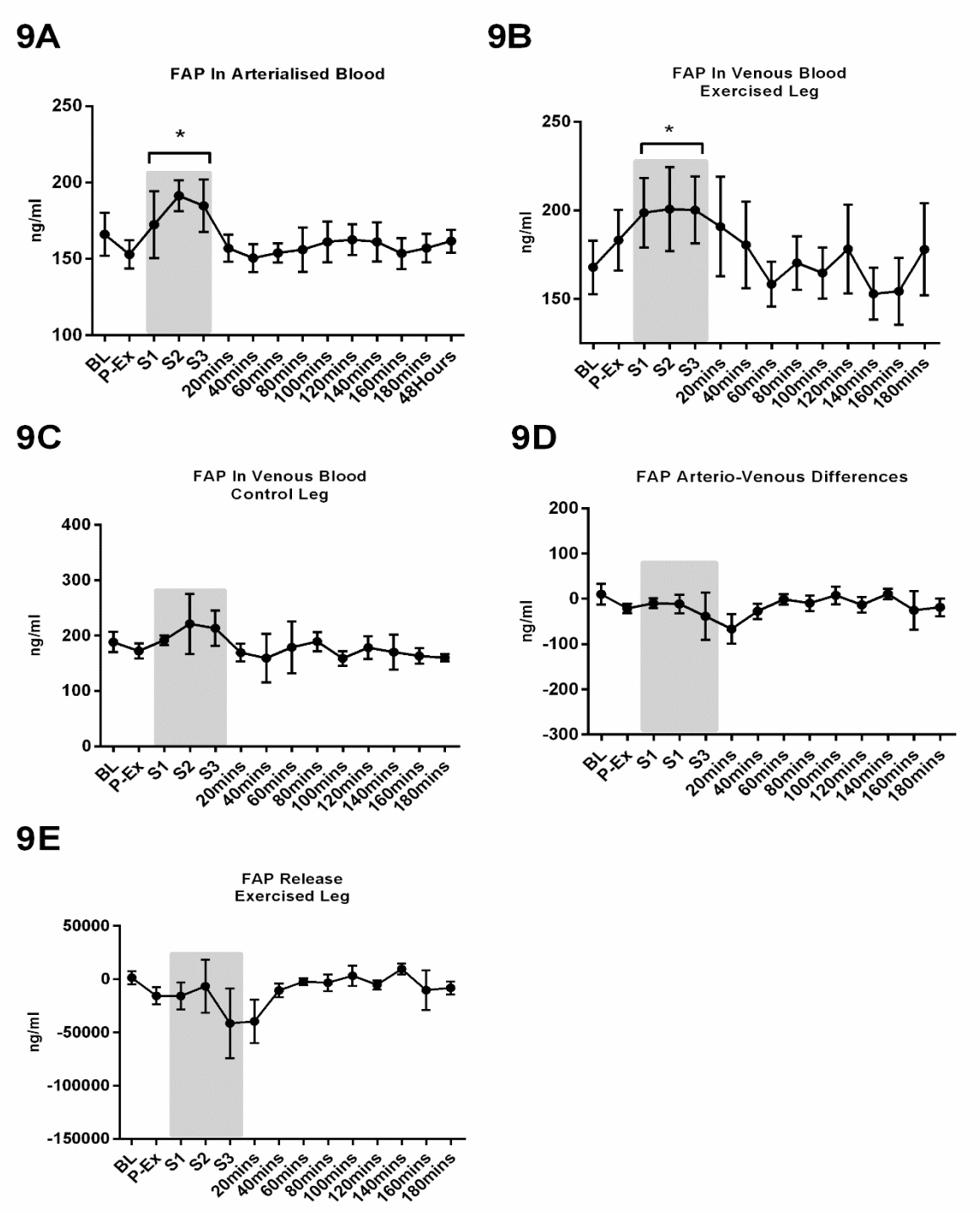



**Figure 9:** Effect of exercise on FAP concentration during at baseline, during exercise (sets 1,2,3) and subsequent recovery (20mins-180mins). FAP in arterialised blood N=8 (ng/ml) **(A)**, FAP in venous blood of the exercised leg N=6 (ng/ml) **(B)**, FAP in venous blood of the control leg N=3 (ng/ml) **(C)**, FAP arterio-venous differences of the exercised leg N=5 (ng/ml) **(D)**. FAP release in the exercised leg **(E)**. Values represent means ± SEM. * denotes P <0.05. The shaded grey area represents the exercise period (sets 1,2,3).

**FGF-21 Muscle Protein Expression**

A one-way repeated measures ANOVA revealed no significant differences in FGF-21 muscle protein expression in the exercised leg between baseline (0.32 ± 0.10 au) and all other time points **(Figure 10A).** A paired t-test showed no significant differences in FGF-21 muscle protein expression in the control leg between baseline (0.32 ± 0.09 au) and 48hours post-exercise P = 0.6577 **(Figure 10B).**

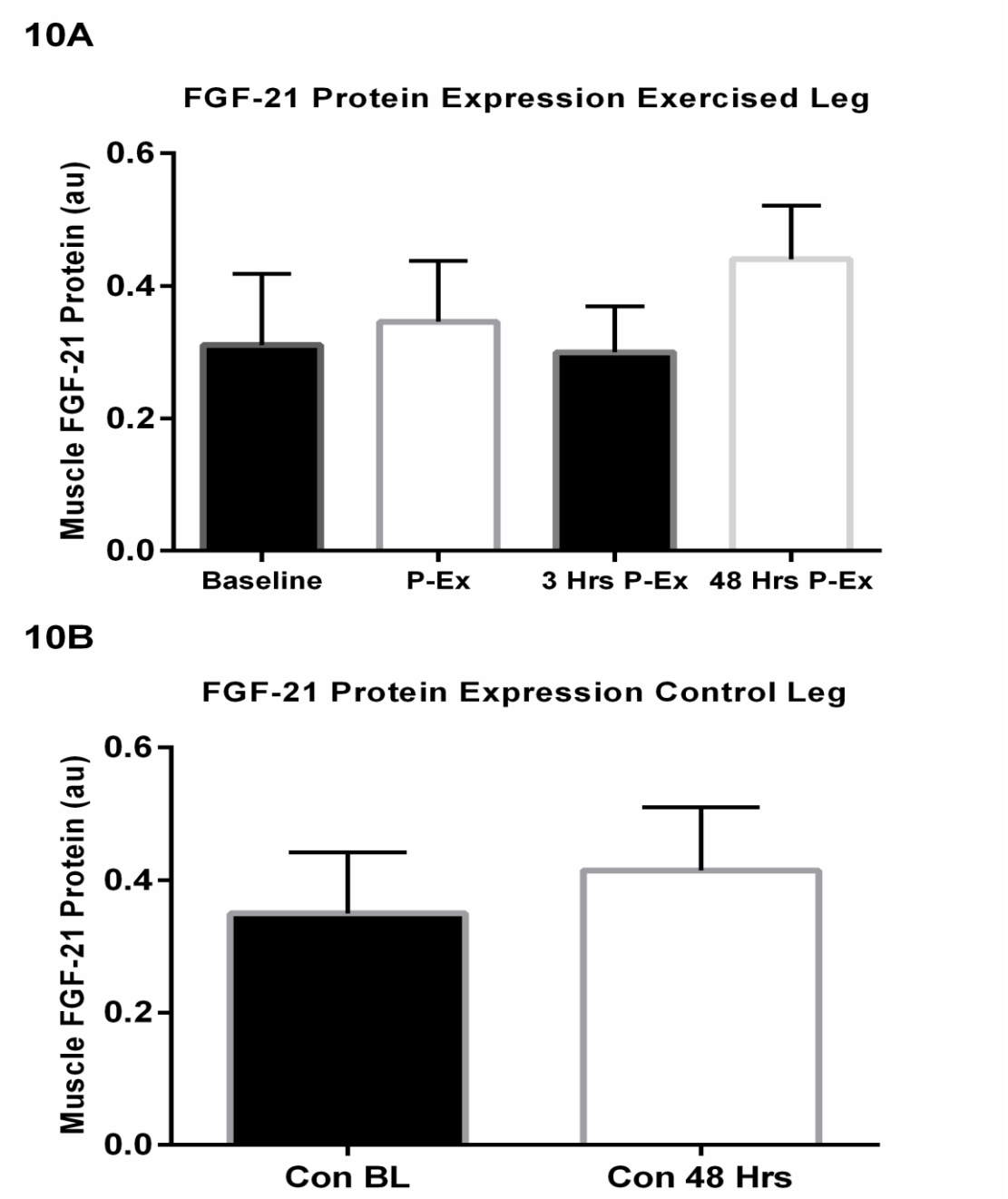



**Figure 10:** Effect of exercise on muscle FGF-21 protein expression in the exercised leg during baseline, post-exercise (P-Ex), 3-hours post-exercise (3 Hrs P-Ex), 48-hours post-exercise (48 Hrs P-Ex) and the control leg during baseline (Con BL) and 48 hours (Con 48 Hrs). FGF-21 protein expression of the exercised leg (au) **(A)** and FGF-21 protein expression of the control leg (au) **(B)**. Values represent means ± SEM. N=8. No report of statistically significant values.

# CHAPTER 4: DISCUSSION

The present study demonstrated that in accordance with previous literature, primarily involving sub-maximal concentric contractions (Kim *et al.*, 2013b; Hansen *et al.*, 2015), more demanding eccentric contractions do not cause the release of FGF-21 from human skeletal muscle tissue into the systemic circulation. Specifically, there was no secretion of total or active FGF-21 from either the exercise leg or the control leg demonstrating insufficient evidence that FGF-21 is an exercised-induced myokine that exerts an endocrine ability in humans. This was further supported by the observation that no increased expression of muscle FGF-21 protein content was observed in the exercised or control leg in response to eccentric contractions. Interestingly, exercise significantly increased levels of arterialised and venous FAP, although it did not appear to be released from skeletal muscle, it may suggest FAP played a role in preventing the release of active FGF-21 in systemic circulation.

The findings of this thesis show that FGF-21 is not released from skeletal muscle in response to maximal exercise, which support the data acquired by Hansen *et al.*, (2015). In that study in response to sub-maximal exercise, skeletal muscle demonstrated no release of FGF-21 from the exercising or resting leg. This was further supported by an increase in FGF-21 mRNA in the liver, whereas no increase was observed in skeletal muscle. In additon, data presented by Kim *et al.*, (2013b) conducted on mice showed that exercise increased hepatic FGF-21 mRNA, while skeletal muscle showed no increases in mRNA. Collectively, the data from the present thesis and the aforementioned studies indicate that in response to both sub-

maximal and maximal contractions, FGF-21 is not released from skeletal muscle. Moreoever, the two latter studies (Kim *et al.*, 2013b; Hansen *et al.*, 2015) indicate that the liver is the main source of FGF-21 secretion into systemic circulation under resting and exercised conditions. It should be noted, in the current thesis, we measured FGF-21 protein content unlike Kim *et al.,* (2013b) and Hansen *et al.,* (2015) who measured mRNA, which allowed us to give a more relevant indication of FGF-21 release from muscle tissue. However, this does not exempt the possibility of FGF-21 as a myokine exerting localised autocrine or paracrine effects. Indeed, mitochondrial dysfunction induced by autophagy deficiency in skeletal muscle tissue results in the secretion of FGF-21 (Kim *et al.*, 2013a). A possible explanation of mitochondrial dysfunction could be associated with lipodystrophy (Lindegaard *et al.*, 2013), UCP-1 deficiency (Keipert *et al.*, 2014) and hyperinsulinemia (Hojman *et al.*, 2009) and the role it plays in the marked increase of FGF-21 mRNA in skeletal muscle tissue. Subsequently, mitochondrial dysfunction induced on transgenic mice showed significant increases in FGF-21 plasma and mRNA levels in skeletal muscle (Keipert *et al.*, 2014). In addition, the sustained expression of mTORC1 in skeletal muscle enhanced metabolism via the production of FGF-21 (Guridi *et al.*, 2015). This may imply under particular conditions such as mitochondrial dysfunction FGF-21 may act as a myokine. Although, the current data shows that under exercising conditions, FGF-21 is not secreted by skeletal muscle in humans.

Previously, in response to acute and long-term aerobic exercise, data shows both serum and plasma FGF-21 to significantly increase in systemic circulation (Cuevas-Ramos *et al.*, 2012; Tanimura *et al.*, 2016). However, the present study showed no

changes in arterialised FGF-21 indicating no organs (including the liver) released FGF-21 into whole body circulation. A possible explanation may be that the current study used contractions involving one-leg which is less demanding on systemic stress than contractions using two-legs. Furthermore, the studies by Cuevas-Ramos *et al.,* (2012) and Tanimura *et al.,* (2016) used a whole body treadmill exercise with a $VO_{2max}$ of 75-85%. This may suggest that in order to augment the release of FGF-21 into circulation, exercise involving substantial muscle mass (more than one localised muscle group) may be needed. Although, surprisingly the present data and the data obtained by Hansen *et al.*, (2015) shows some contrasting findings. Hansen's subjects took part in single-leg kicking at a sub-maximal intensity, whereas our subjects took part in single-leg kicking at a maximal intensity. Hansen's results indicated a release of arterialised FGF-21 in response to concentric contractions, albeit from the liver, our data showed no release even though exercise was at a more demanding intensity in the form of eccentric contractions. This may propose an alternative explanation or mechanism in preventing the release of FGF-21 into systemic circulation.

Exercise is well established as a parameter to improve metabolic conditions and skeletal muscle plays an important role in regulating energy metabolism. A novel aspect of our study allowed us to measure the active form of FGF-21 in response to exercise, whereas all studies to date involving exercise and FGF-21 have only measured total FGF-21. This is important to note, as data has most recently shown that a large percentage of total FGF-21 is not active (Umberger *et al.*, 2014). The majority of ELISA kits used to measure total FGF-21 do not reflect true values of

active FGF-21. The measurement of active FGF-21 in circulation allowed us to present a novel finding that exercise significantly increased arterialised and venous FAP concentrations, although skeletal muscle did not contribute to the release. While no previous studies have investigated the response of FAP during exercise, Zhen *et al.*, (2016) and Dunshee et *al.*, (2016) have shown that FAP plays a key role in preventing the release of active FGF-21 into systemic circulation. As FAP concentrations increased in systemic circulation, this may have been a potential explanation as to why there was no secretion of FGF-21. However, interestingly the ratio between active and total FGF-21 remained unchanged, which may suggest that FAP may not in fact be the reason for FGF-21 inhibition. Future research into the role of FAP released during exercise in humans may provide useful treatments in preventing or eradicating metabolic disorders. However, it should be highlighted that hFGF-21 is cleaved by FAP, whereas FAP in mouse models is resistant to the breakdown process of FGF-21 (Niedermeyer *et al.*, 2001). In fact, this presents a caution in comparisons between human and rodent studies as the regulating mechanisms and the exogenous levels of FGF-21 may present inaccurate findings. Conversely, data presented by Zhen et *al.*, (2016) and Dunshee *et al.*, (2016) provide a foundation into identifying and targeting FAP in order to increase active FGF-21 levels in circulation. Further research needs to be explored in humans in order to investigate the mechanisms involved in the regulation of FAP and active FGF-21.

## Conclusion

In conclusion, the present study provides evidence that single-leg maximal eccentric contractions do not augment the release of either total or active FGF-21 from skeletal muscle into systemic circulation in humans. Furthermore, skeletal muscle FGF-21 protein content did not increase in the exercised or control leg supporting the claim that FGF-21 is not released from skeletal muscle in response to maximal exercise using eccentric contractions. Finally, the data from this thesis showed that there was a significant increase in arterialised and venous FAP levels during maximal eccentric contractions with no apparent contribution to its release from the exercised leg. Thus, these findings raise the interesting possibility that FAP may play a role in the inactivation of FGF-21 during exercise.

## Future Research/Recommendations

The novel aspect of this present thesis showed that FAP concentrations increased in response to exercise. Further research needs to establish the regulatory role of FAP in the inactivation of FGF-21 and if inhibition of FAP could extend the life of exogenous FGF-21 and/or affect its secretion from other tissues. Accordingly, FAP inhibition could provide important novel strategies in the regulation of metabolism and the potential implications it may have in helping to combat metabolic diseases.

**Limitations**

The current study has a few limitations, firstly we studied young healthy males, it may be that overweight or obese individual's express different amounts of FGF-21 in skeletal muscle and circulation. As the majority of overweight or obese individuals suffer from metabolic disorders it may be beneficial to investigate their response to eccentric exercise. Secondly, as subjects performed 3 bouts of 25 maximal eccentric contractions we could not determine if an inflammatory response was achieved. This could be addressed by measuring creatine kinase or heath shock proteins, which are markers of muscle damage. Thirdly, it may have been helpful to use more than one muscle group, as previous literature indicates that FGF-21 is released into circulation via exercises involving substantial muscle mass. One-legged eccentric contractions may have not been demanding enough to cause systemic stress. Furthermore, the common femoral vein of the control leg for deep venous sampling was only inserted into three subjects as muscular architecture varied. A potential of studying more subjects may have allowed us to yield more accurate data of the control leg in response to exercise. Finally, for deep venous sampling, a cannula was inserted anti-grade rather than retrograde for technical reasons. Inserting the cannula retrograde gives more accuracy in the potential release by skeletal muscle rather than other organs, although the release of lactate from skeletal muscle proved that inserting the cannula anti-grade did not present a major issue.

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**APPENDICES A**

Protein Extraction Method for Western blots (when probing for phosphorylated proteins)

**<u>Homogenisation Buffer</u>** (for **500 ml**)

- 50 mM HEPES (SIGMA H4034) 5.95 g
- 10% Glycerol (SIGMA G8773) 50 ml
- 1mM EDTA (SIGMA E4884) 186 mg
- 10 mM NaF (SIGMA S6521) 210 mg
- 1mM $Na_3VO_4$ (SIGMA S6508)
- 5 ml of 100 mM activated solution (or 2.86 ml of 175 mM)
- 150 mM NaCl 4.383 g
- 1% Triton X-100 (SIGMA X100) 5 ml
- pH to 7.5 with NaOH

On day of homogenisation, take required amount of buffer and add 4 ul of protease inhibitors cocktail (SIGMA P-8340) per ml of buffer

Add muscle chunk (~30-50mg wet tissue) to the above buffer (6 times dilution i.e 50 mg of tissue in 300 ul of buffer + protease inhibitors) (**Note**. For small amounts of tissue (~10-20 mg) use 10 times dilution).

Homogenise with polytron at medium speed (2-3) for 30 sec (keep on ice)

Leave on ice for 20 min and then transfer to eppendorf tube and spin at 10,000 g for 20 min at 4$^{o}$C

Transfer supernatant to clean eppendorf and add equal volume of 2xSDS mix***** (blue colour) (6xSDS mix is also OK)

Transfer 5ul of supernatant into a separate eppendorf tube containing 45ul of water for protein quantification.

Add 200 ul of SDS mix to pellets

Store at -20$^{o}$C (stable for weeks)

**<u>Preparing 2xSDS mix (for 50 ml):</u>**

- 1M Tris (pH 6.8) 6.25 g
- 20% Glycerol 10 ml
- SDS 2 g
- 100 mM DTT 0.77 g
- Top up to 50 ml with distilled water (store at -20$^{o}$C)

**Western Blot Protocol**

Preparing Gels

Use 1.5 mm glass plates – wash with soap, dry with tissue and wipe with ethanol.

Insert plates into the electrophoresis tank.

Prepare a fresh 10% acrylamide gel solution (20 ml is enough for 2 plates):

| | for 20 ml |
|---|---|
| • 30% Acrylamide:bis | 9.35 ml |
| • 2M Tris HCl (pH = 8.8) | 3.76 ml |
| • 10% SDS (20g/200ml) | 0.2 ml |
| • TEMED (SIGMA T-9281) | 20 ul |
| • Distilled water | 6.51 ml |
| • 10% Ammonium Persulfate (AMPS) | 160 ul |

(SIGMA A-3678, stored at room temp. Weigh out ~0.6-0.8 g and add 10 times distilled water. Prepare fresh on the day).

Note. Add AMPS last to activate the gel – mix by gentle inversion prior to adding AMPS.

Use glass pipette to insert acrylamide gel in between glass plates (up to ¾ of their height). Add some butanol/water on top of gel to smooth/even the surface.

Leave for ~45 min for gel to set. Rinse with distilled water to remove the butanol solution.

Add a stack gel solution (5% acrylamide) on top of the 10% gel using a glass pipette (up to ~2mm beneath the top edge of the glass plate).

Prepare a 5% acrylamide stacking gel solution (10 ml is enough for 2 plates):

| | for 10 ml |
|---|---|
| • 30% Acrylamide:bis | 850 ul |
| • 1M Tris HCl (pH = 6.8) | 630 ul |
| • 10% SDS (20g/200ml) | 50 ul |
| • TEMED | 5 ul |
| • Distilled water | 3.42 ml |
| • 10% AMPS | 50 ul |

Insert 1.5 mm combs on top of stacking gel and push firmly to seal while making sure that no bubbles are formed.

Leave for ~30 min for stacking gel to set. Then, either run and blot the gel on the same day, or cover with fling and store at 4$^{o}$C (can be stored up to 3 days before running it).

**Running Gels**

Take out the combs and rinse well with distilled water.

Set up the electrophoresis tank (ATTO AE-6220) and fill up with 1x running buffer (either dilute ten times a 10x Tris/Glycine/SDS stock buffer by BIORAD or make up your own 1x buffer: 3g Tris, 14 g glycine and 1g SDS in 1 litre of water).

Heat up protein/SDS samples for 2-3 min at 100$^{o}$C and then load 5-10 □g per lane (including a protein marker ~ 8 ul).

Run at 40 mA for ~2h (until the 50kDa marker has progressed ¾ down the gel). Alternatively, run at 200 V constant (it takes longer ~4h but it keeps it cooler).

Western blotting (must be done on the same day as running the gels)

Chemicals & Materials

Western transfer buffer: for 1 litre: 14g glycine, 3g TRIS and 200 ml methanol (top up with water). Need ~2.5 l to fill up the tank.

Note. Make up buffer fresh on the day of blotting (but can be reused for soaking only)

Nitrocellulose paper or PVDF (in which case you soak it in 100% methanol for 15 secs exactly and soak in water for 2 min)

3MM paper

TBS (1.2 g Tris base and 8.75 g NaCl in 1 litre of water, pH 7.5 with HCl)

TBS-T (1 ml of Tween 20 in litre of TBS)

Ponceau S solution

**Method**

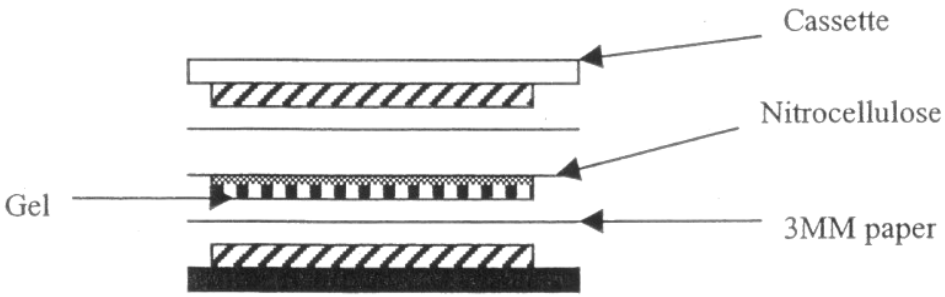
After electrophoresis remove the top plate and cut stack from gel and lip from bottom (using a surgical blade). Pipette some western transfer buffer on gel to keep it wet.

Remove gel from bottom plate (note that the marker protein bands appear on the left hand side of the gel so you must flip it over when making the sandwich ie the marker band is on the right hand side during transfer onto the nitrocellulose paper in order to end up on the left side), place it in a plastic tray and soak it in western blot buffer for 10-20 min. Alternatively, place both glass plates and gel in buffer and they will separate.

Cut a piece of nitrocellulose paper to size of gel (approx. 10cm x 8cm) and hydrate in Milli Q water for about 10 seconds. Then leave to equilibrate in western transfer buffer.

Cut 4 pieces of 3MM paper to size of gel.

Assemble gel sandwich onto black side of cassette as below ensuring sponges and 3MM paper are soaked in western transfer buffer to ensure good transfer. See below. (Note. Cut a small part of the upper left corner of the nitrocellulose paper to identify sides after transfer. In addition, when making sandwich make sure you remove all




bubbles between layers using a glass roller)

Place gel sandwich in the transfer tank (EPS 600) (make sure that the front clear side of cassette faces the red front side of the transfer tank), fill up with western transfer buffer and blot at constant current 200 mA for between 1-2 h depending on strength of gel (1 h is usually sufficient for 8% gels). Alternatively, blot at 100 mA overnight in a cold room (as is the case with Akt).

Remove blot from blot sandwich, flip over so protein marker is on the left hand side, place it in a plastic tray and stain protein bands with Ponceau S solution.

Destain with distilled water in an orbital shaker and mark position of protein lanes and markers using a permanent marker.

- Complete destaining by rinsing with TBS-T for a few minutes.
- Keep membrane in a TBS solution at 4ºC.
- Hazards and precautions (see safety sheets)
- Methanol - Highly flammable
- Blocking and Immunoprobing
- Chemicals & Materials
- Primary antibody
- HRP-conjugated secondary antibody
- Western blocking reagent (BSA)
- TBS (1.2 g Tris base and 8.75 g NaCl in 1 litre of water, pH 7.5 with HCl)
- TBS-T (1 ml of Tween 20 in litre of TBS)
- Marvel-TBS-T 5%: 10g Marvel in 200 ml of TBS-T
- Hypercassette
- Tracker tape
- ECL Hyperfilm (by Amersham)
- ECL solutions
- Developing and fixative reagents

Block membrane with 5% blocking reagent for 1h on shaker at room temperature. (Longer blocking times can be used. Can block overnight at 4°C on shaker).

Probe with primary antibody, diluted in 5% blocking reagent for 1h on shaker at room temperature. (A range of dilutions must be tried to determine the optimum dilution ie for FGF-21 dilution of 1:4000 was used). (Also longer probing times can be used. For FGF-21 probe overnight at 4°C). Incubation can be performed in a sealed bag or container. Using a bag saves considerable amount of antibody.

Wash off non-specific binding with 5 x 10 min washes of TBS-T. Probe with secondary antibody diluted 1:2000 in 1% blocking reagent for 1h on shaker at room temperature. Incubation can be performed in a sealed bag or container. Wash off non-specific binding with 5 X 10 min washes of TBS-T followed by 1 x15 min wash of TBS (or TBS-T)

Develop in a hypercassette using ECL or ECL plus.